Immunity, Volume *51*

Supplemental Information

Nephrocytes Remove Microbiota-Derived Peptidoglycan from Systemic Circulation to Maintain Immune Homeostasis

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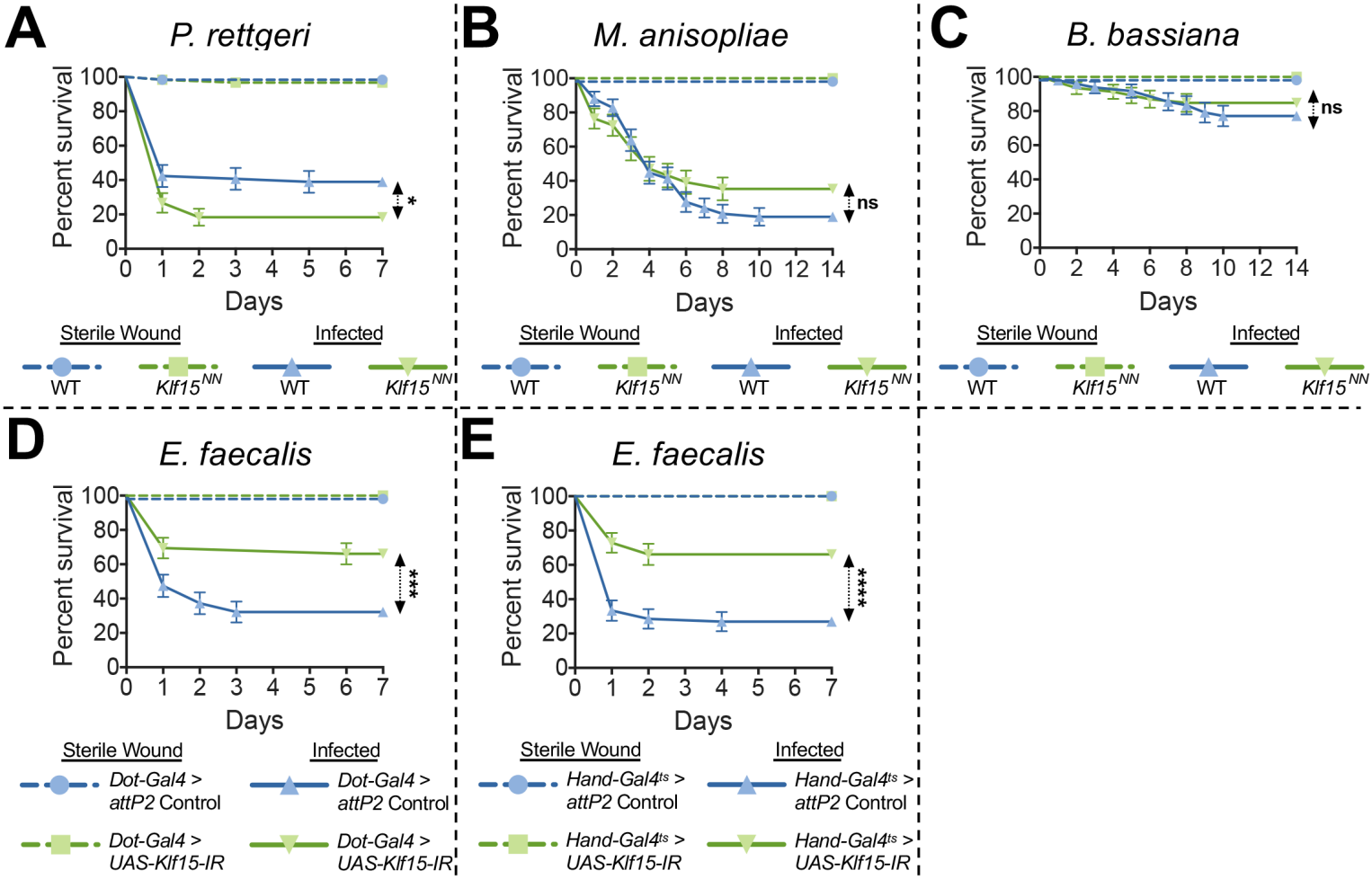
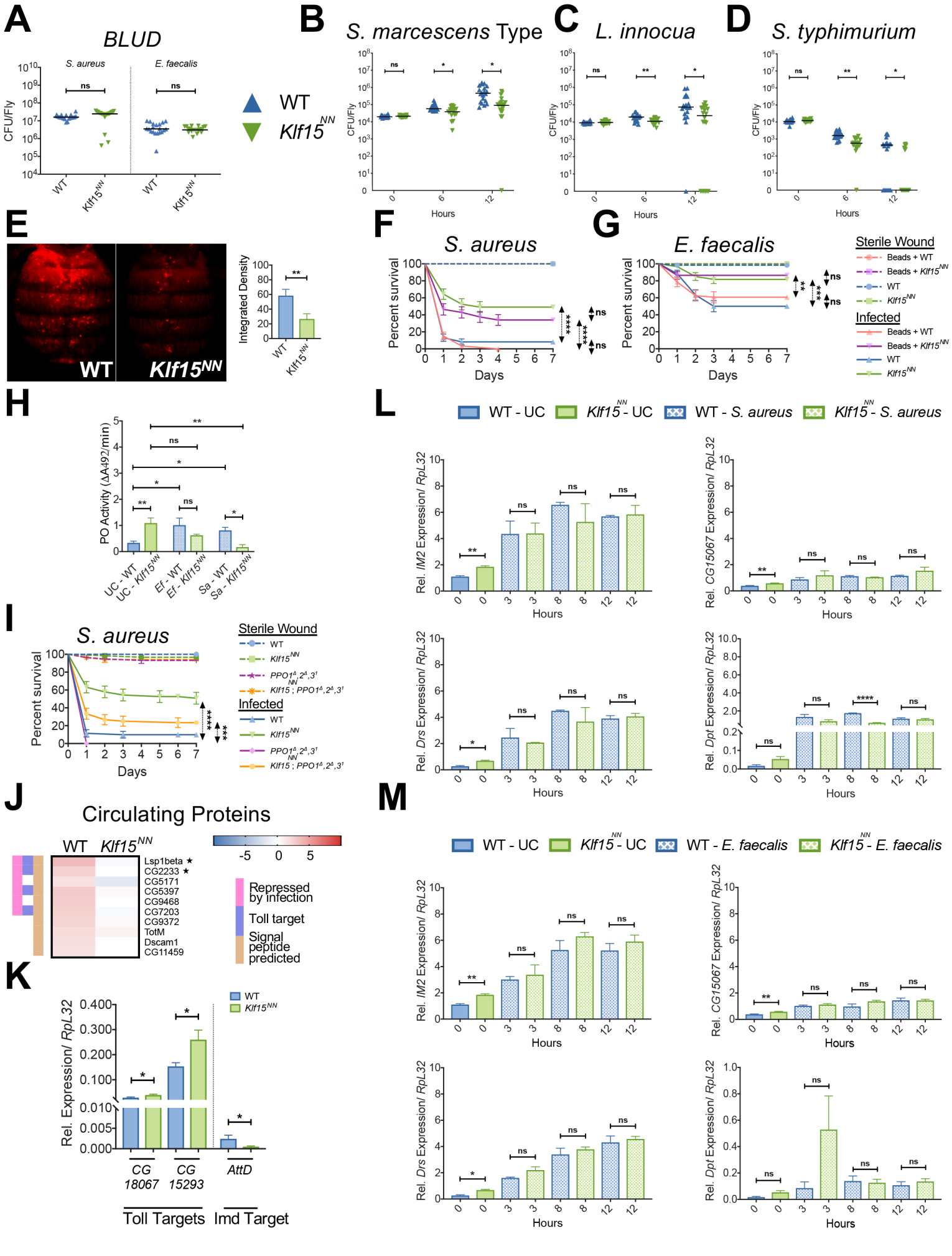


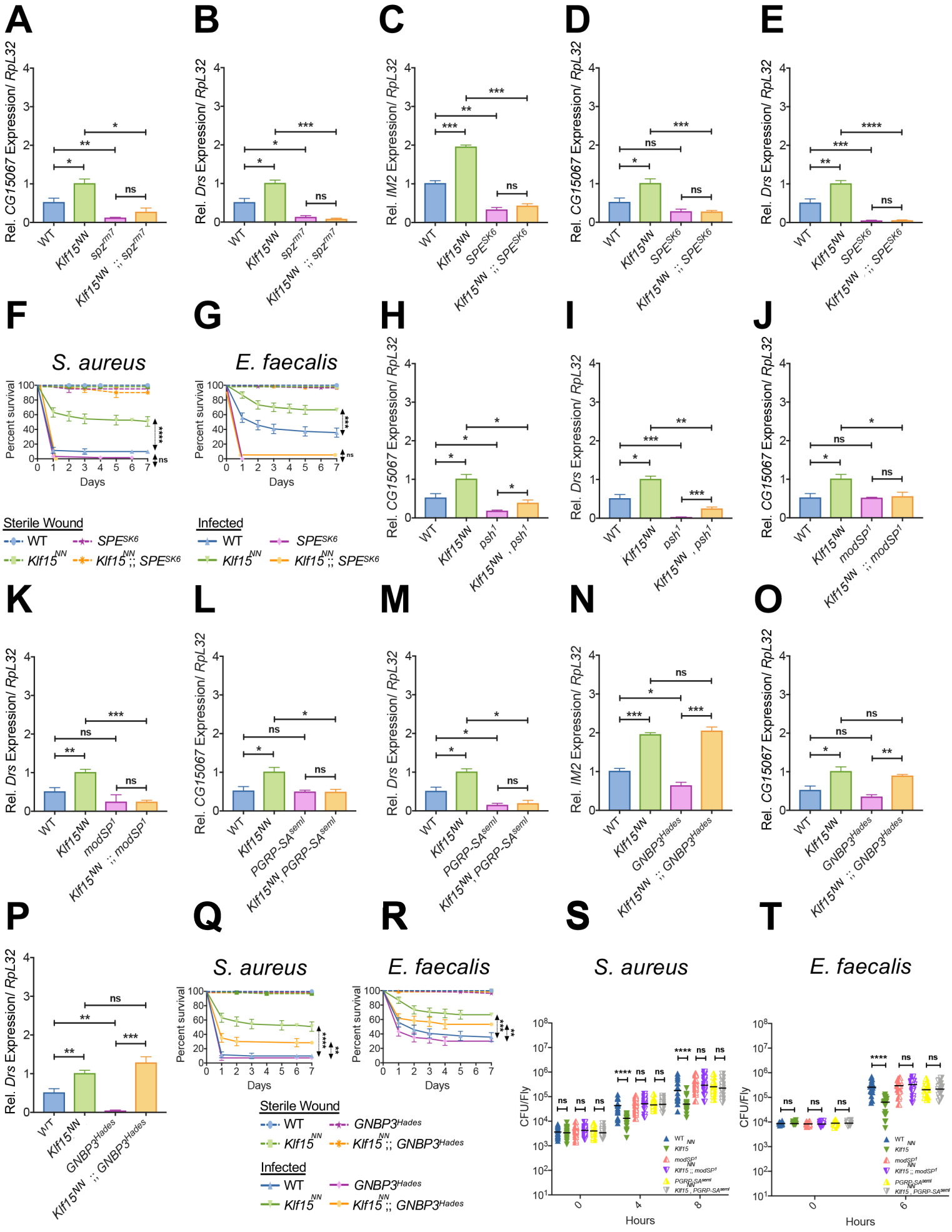
Supplementary Figure 1 (Related to Figure 1). *Klf15^{NN}* flies display improved survival against a variety of microbial infections

(A) Survival curve of *Klf15^{NN}* and WT flies infected with *P. rettgeri*.
(B-C) Survival curves over 14 days following natural infection of WT and *Klf15^{NN}* flies with the fungal pathogens *M. anisopliae* (B) and *B. bassiana* (C).
(D-E) Survival of flies expressing nephrocyte-specific RNAi against *Klf15* throughout development (*Dot-Gal4 > UAS-Klf15-IR*) (D) or only during the adult stage (*Hand-Gal4^{ts} > UAS-Klf15-IR*) (E) after infection with *E. faecalis*.
The curves represent the average percent survival ±SE of three biological replicates. *p<0.05 ***p<0.001 ****p<0.0001 in a Log-rank test.


A
BLUD
S. aureus
E. faecalis
CFU/Fly
ns
WT
Klf15NN
B
S. marcescens Type
C
L. innocua
D
S. typhimurium
Hours
E
WT
Klf15NN
Integrated Density
F
S. aureus
Percent survival
Days
G
E. faecalis
Sterile Wound
Beads + WT
Beads + Klf15NN
WT
Klf15NN
Infected
H
PO Activity (ΔA492/min)
UC - WT
UC - Klf15NN
Ef - WT
Ef - Klf15NN
Sa - WT
Sa - Klf15NN
I
S. aureus
Sterile Wound
WT
Klf15NN
PPO1Δ,2Δ,3t NN
Klf15 ; PPO1Δ,2Δ,3t
Infected
J
Circulating Proteins
WT
Klf15NN
Lsp1beta ★
CG2233 ★
CG5171
CG5397
CG9468
CG7203
CG9372
TotM
Dscam1
CG11459
Repressed by infection
Toll target
Signal peptide predicted
K
Rel. Expression/ RpL32
CG 18067
CG 15293
AttD
Toll Targets
Imd Target
L
WT - UC
Klf15NN - UC
WT - S. aureus
Klf15NN - S. aureus
Rel. IM2 Expression/ RpL32
Rel. CG15067 Expression/ RpL32
Rel. Drs Expression/ RpL32
Rel. Dpt Expression/ RpL32
M
WT - UC
Klf15NN - UC
WT - E. faecalis
Klf15NN - E. faecalis

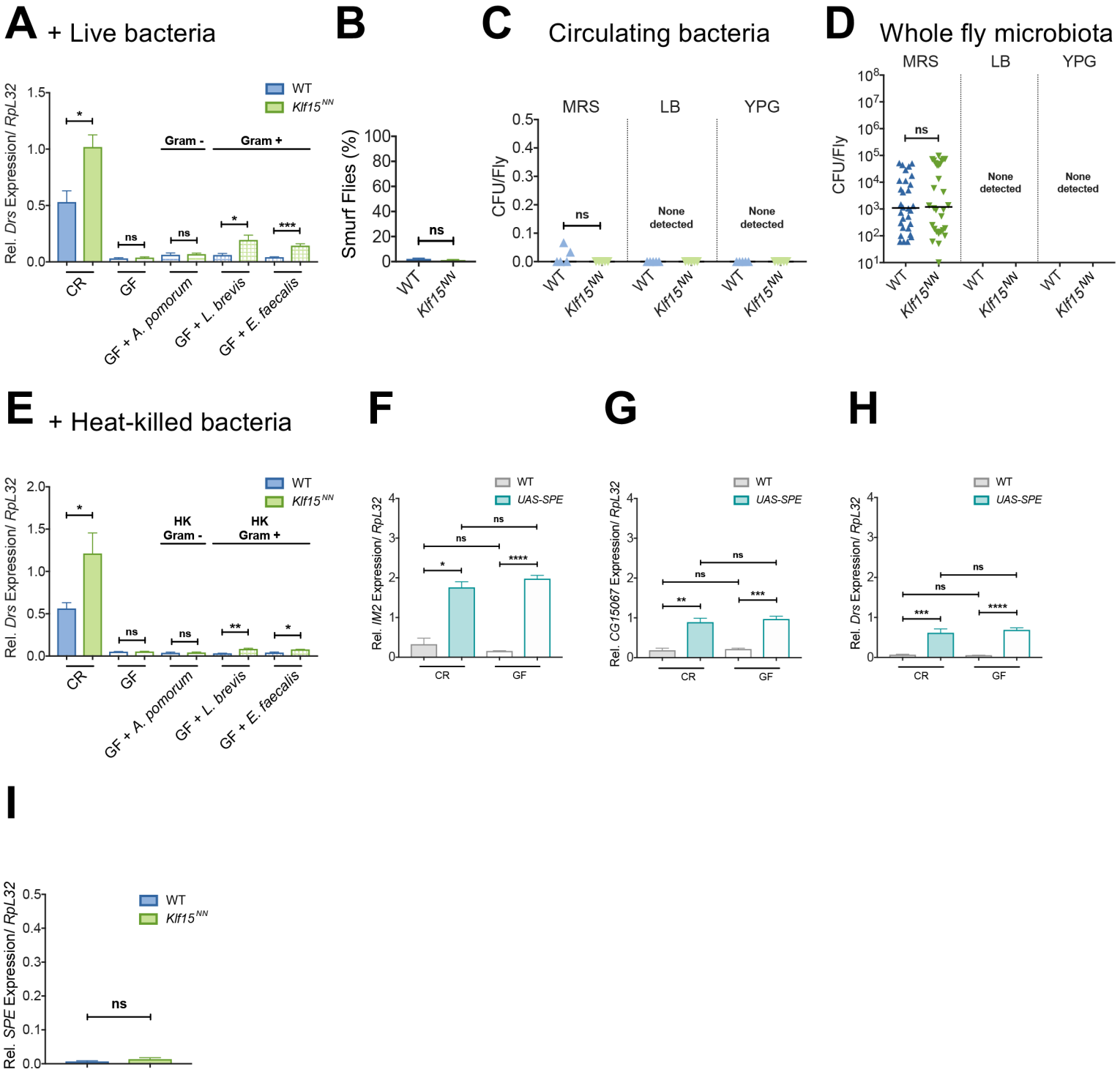
Supplementary Figure 2 (Related to Figure 2). The Toll pathway is turned on in *Klf15^{NN}* flies

(A) Bacterial load upon death (*BLUD*) of wildtype and *Klf15^{NN}* flies infected with *S. aureus* and *E. faecalis.*
(B-D) Bacterial load data of control and *Klf15^{NN}* flies following infection with *S. marcescens* Type strain, *L. innocua*, and *S. typhimurium*. Three repeats are graphed together, with each symbol representing an individual fly's number of colony forming units (CFU). Horizontal lines represent median values for each time point. Results were analyzed using a two-way ANOVA followed by Sidak's post-test for specific comparisons (*p<0.05 **p<0.01).
(E) Representative fluorescence images of the abdomens of control and *Klf15^{NN}* flies 3 h post-injection with pHrodo bacteria. Fluorescence was quantified and the average plotted ±SE. **p<0.01 in a Student's t-test.
(F-G) Survival curves over 7 days of WT and *Klf15^{NN}* flies that were pre-injected with latex beads 24 h prior to infection with the pathogens *S. aureus* (D) and *E. faecalis* I. **p<0.01 ***p<0.001 ****p<0.0001 in a Log-rank test.
(H) Phenoloxidase activity was measured using the L-DOPA assay. WT and *Klf15^{NN}* samples were measured in unchallenged conditions as well as following infection with *E. faecalis* and *S. aureus*. *p<0.05 **p<0.01 in a Student's t-test.
(I) Comparison of *Klf15^{NN}* ; *PPO1$^{\Delta}$, 2$^{\Delta}$,3^{1}* quadruple mutants to WT, *Klf15^{NN}*, and *PPO1$^{\Delta}$, 2$^{\Delta}$,3^{1}* mutants in experiments measuring survival against *S. aureus*. ***p<0.001 ****p<0.0001 in a Log-rank test.
(J) Heat map showing a list of circulating proteins depleted (≥ 1.5-fold) in the hemolymph (insect blood) of *Klf15^{NN}* flies relative to WT. A color scale on the left side of the heat map denotes whether the gene that encodes each protein is transcriptionally decreased by infection (pink), a target of the Toll pathway (blue), or predicted to possess a signal peptide (beige). Core genes are highlighted with a ★ symbol (Troha et al., 2018).
(K) Whole fly RT-qPCR of Toll target genes *CG18067* and *CG15293* and Imd target gene *AttD* using unchallenged WT and *Klf15^{NN}* samples. *p<0.05 in a Student's t-test.
(L-M) Whole fly RT-qPCR of Toll target genes *IM2*, *CG15067*, and *Drs* and Imd target gene *Dpt* following infection with *S. aureus* (J) and *E. faecalis* (K). *p<0.05 **p<0.01 ****p<0.0001 in a Student's t-test.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
Rel. CG15067 Expression/ RpL32
Rel. Drs Expression/ RpL32
Rel. IM2 Expression/ RpL32
WT
Klf15NN
spzrm7
Klf15NN ;; spzrm7
SPESK6
Klf15NN ;; SPESK6
psh1
Klf15NN , psh1
modSP1
Klf15NN ;; modSP1
PGRP-SAseml
Klf15NN,PGRP-SAseml
GNBP3Hades
Klf15NN ;; GNBP3Hades
S. aureus
E. faecalis
Percent survival
Days
Sterile Wound
Infected
Klf15NN ;; SPESK6
Klf15NN ;; GNBP3Hades
CFU/Fly
Hours

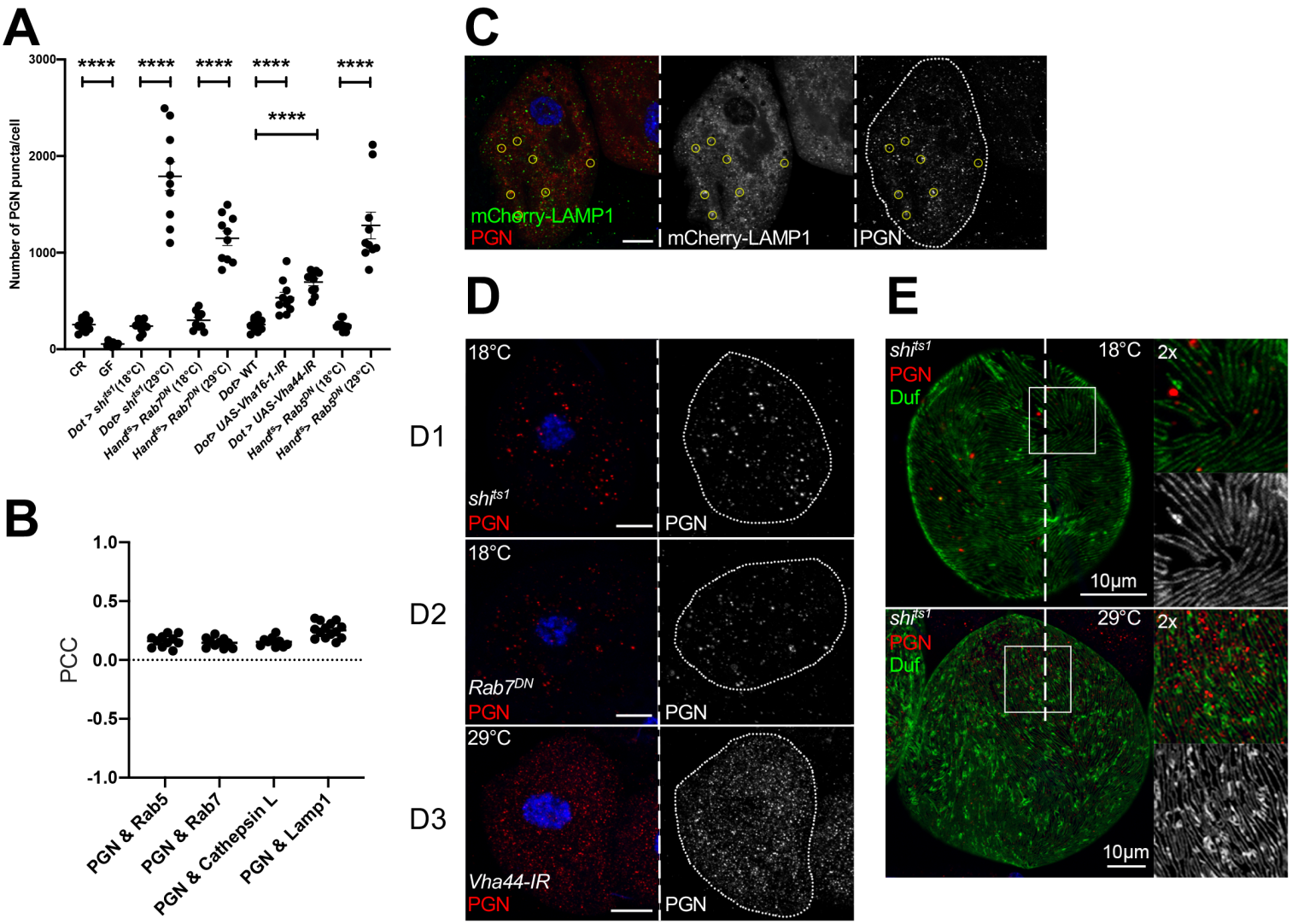
Supplementary Figure 3 (Related to Figure 3). Increased resistance to infection in *Klf15^{NN}* flies is Toll-dependent

(A-B) Comparison of *Klf15^{NN}* ;; *spz^{rm7}* double mutants to WT, *Klf15^{NN}*, and *spz^{rm7}* single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (A) and *Drs* (B) via RT-qPCR.
(C-G) Comparison of *Klf15^{NN}* ;; *SPE^{SK6}* double mutants to WT, *Klf15^{NN}*, and *SPE^{SK6}* single mutants in experiments measuring mRNA expression of Toll target genes *IM2* (C), *CG15067* (D), and *Drs* (E) via RT-qPCR as well as survival against *S. aureus* (F) and *E. faecalis* (G).
(H-I) Comparison of *Klf15^{NN}* , *psh^{1}* double mutants to WT, *Klf15^{NN}*, and *psh^{1}* single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (H) and *Drs* (I) via RT-qPCR.
(J-K) Comparison of *Klf15^{NN}* ;; *modSP^{1}* double mutants to WT, *Klf15^{NN}*, and *modSP^{1}* single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (J) and *Drs* (K) via RT-qPCR.
(L-M) Comparison of *Klf15^{NN}* , *PGRP-SA^{seml}* double mutants to WT, *Klf15^{NN}*, and *PGRP-SA^{seml}* single mutants in experiments measuring mRNA expression of Toll target genes *CG15067* (L) and *Drs* (M) via RT-qPCR.
(N-R) Comparison of *Klf15^{NN}* ;; *GNBP3^{Hades}* double mutants to WT, *Klf15^{NN}*, and *GNBP3^{Hades}* single mutants in experiments measuring mRNA expression of Toll target genes *IM2* (N), *CG15067* (O), and *Drs* (P) via RT-qPCR as well as survival against *S. aureus* (Q) and *E. faecalis* (R).
For RT-qPCR experiments, mean values of three or more repeats are given ±SE (*p<0.05 **p<0.01 ***p<0.001****p<0.0001 in a Student's t-test). Survival curves show the average percent survival ±SE of three biological replicates (**p<0.01 ***p<0.001 ****p<0.0001 in a Log-rank test).
(S-T) Bacterial load data of WT, *Klf15^{NN}*, *modSP^{1}*, *Klf15^{NN}* ;; *modSP^{1}*, *PGRP-SA^{seml}*, and *Klf15^{NN}* , *PGRP-SA^{seml}* flies following infection with *S. aureus* and *E. faecalis*. Three repeats are graphed together, with each symbol representing an individual fly's number of colony forming units (CFU). Horizontal lines represent median values for each time point. Results were analyzed using a two-way ANOVA followed by Sidak's post-test for specific comparisons (****p<0.0001).


A + Live bacteria
B
C Circulating bacteria
D Whole fly microbiota
E + Heat-killed bacteria
F
G
H
I
WT
Klf15NN
Rel. Drs Expression/ RpL32
Gram -
Gram +
CR
GF
GF + A. pomorum
GF + L. brevis
GF + E. faecalis
Smurf Flies (%)
CFU/Fly
MRS
LB
YPG
None detected
HK Gram -
HK Gram +
UAS-SPE
Rel. IM2 Expression/ RpL32
Rel. CG15067 Expression/ RpL32
Rel. SPE Expression/ RpL32
ns

Supplementary Figure 4 (Related to Figure 4). Nephrocytes prevent overactive immune responses to gut microbes

(A) Quantification of mRNA expression in conventional (CR), germ-free (GF), and germ-free flies recolonized with either live *A. pomorum* (DAP-type PGN), live *L. brevis* (Lys-type PGN), or live *E. faecalis* (Lys-type PGN). RT-qPCR measurements of Toll target gene *Drs* are shown.
(B) Percent SMURF flies found after feeding a diet containing 2.5% Blue #1 Dye for both WT and $Klf15^{NN}$ flies.
(C) Comparison of circulating (hemolymph) bacteria between $Klf15^{NN}$ and control flies. Samples were plated on three separate media: De Man, Rogosa, and Sharpe (MRS), Luria-Bertani (LB), and yeast-peptone-glucose (YPG) agar.
(D) Comparison of whole fly microbiota between $Klf15^{NN}$ and control flies. Samples were plated on three separate media: De Man, Rogosa, and Sharpe (MRS), Luria-Bertani (LB), and yeast-peptone-glucose (YPG) agar.
(E) Quantification of mRNA expression in conventional (CR), germ-free (GF), and germ-free flies fed either heat-killed *A. pomorum* (DAP-type PGN), heat-killed *L. brevis* (Lys-type PGN), or heat-killed *E. faecalis* (Lys-type PGN). RT-qPCR measurements of Toll target gene *Drs* are presented. For RT-qPCR experiments, mean values of three or more repeats are given ±SE. *$p<0.05$ **$p<0.01$ in a Student's t-test.
(F-H) Quantification of mRNA expression in flies overexpressing *SPE* (*c564-Gal4^{ts} > UAS-SPE*) in both conventional (CR) and germ-free (GF) conditions. RT-qPCR measurements of Toll target genes *IM2* (F), *CG15067* (G), and *Drs* (H) are shown.
(I) Whole fly RT-qPCR of *SPE* using unchallenged wildtype and $Klf15^{NN}$ samples.



Supplementary Figure 5 (Related to Figure 5). Nephrocytes uptake peptidoglycan from the hemolymph

(A) Quantification and comparison of PGN puncta per cell for CR and GF flies and flies expressing *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*), *Rab5^{DN}* (*Hand-Gal4^{ts} > UAS-Rab5^{DN}*), *Rab7^{DN}* (*Hand-Gal4^{ts} > UAS-Rab7^{DN}*), *Vha16-1-IR* (*Dot-Gal4 > UAS-Vha16-1-IR*), and *Vha44-IR* (*Dot-Gal4 > UAS-Vha44-IR*) in a nephrocyte-specific manner. ****p<0.0001 in a Student's t-test.

(B) Pearson correlation coefficients (PCC) from co-localization experiments.

(C) Immunostaining against PGN reveals colocalization (yellow circles) with the lysosomal marker Lamp1. Scalebar: 10μm

(D1-D2) Control images (18°C) for *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*) and *Rab7^{DN}* (*Hand-Gal4^{ts} > UAS-Rab7^{DN}*) experiments.

(D3) Nephrocyte-specific expression of *Vha44-IR* (*Dot-Gal4 > UAS-Vha44-IR*) led to accumulation of PGN in nephrocytes when compared to control. Scalebars: 10μm

(E) Surface view: Immunostaining against Duf (labels the lacunae) shows expansion of the lacunae in *shibire^{ts1}* (*Dot-Gal4 > UAS-shi^{ts1}*) flies. Immunostaining with PGN antibody reveals that PGN is accumulating in the lacunae.

Gene	Primer Sequences	
	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
AttC	TGCCCGATTGGACCTAAGC	GCGTATGGGTTTTGGTCAGTTC
Dpt	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG
Drs	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT
RpL32	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
IM2	ACCGTCTTTGTGTTCGGTCT	TGCAGTCCCCGTTGATTACC
CG15067	GAGCCTGACGTTATTGGCG	CCTTTTCCACTTGTTGGCTTGT
CG18067	ATGGGCTCGAACACTGGAG	TCCTCATTTCGCTGTATATTCGC
CG15293	TCCTATCTACTGGCAATCTGTGT	CGGCGGATAAAGAAGTGGCA
AttD	AAGGGAGTTTATGGAGCGGTC	GCTCTGGAAGAGATTGGCTTG
TotA	CCCAGTTTGACCCCTGAG	GCCCTTCACACCTGGAGA

Supplementary Table 1 (Related to STAR Methods). List of oligonucleotides