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Supplemental Methods

Supplemental Data files

Guo et al., Supplemental Text and Figures

Supplemental Figure Legends

Figure S1 (relates to Fig. 1): Age-related changes in intestinal microflora

A) Transmission Electron micrographs of the intestinal lumen of wild-type (OreR) flies of the indicated ages. Sections of the posterior midgut are shown. Note the dramatic increase of microorganisms in the lumen of the gut of conventionally aged flies (middle panel; arrowheads). These are absent in axenically aged flies (right panel). BB: Brushed border of ECs is indicated for orientation. PM: peritrophic matrix.

B) Midgut homogenates from wild type flies at various ages were plated on mannitol-agar or MRS-agar plates for CFU quantitation. Values are normalized against protein amounts of homogenates. Commensal bacteria load in 40 day old axenic flies (sterilized and treated with antibiotics) are similar to those of conv reared 10 day olds.

C) Mitotic figures in intestines of conventionally or axenically aging flies. Here, flies were maintained axenically by culturing them in a laminar flow hood on sterile food not containing antibiotics.

D) Expression trajectories of selected highly expressed 'housekeeping' genes using RNASeq data. Little change is seen in expression over the time of the experiment, confirming the overall health of the tissue.

E) Expression trajectories of cytokine (*Upds, Pvfs*) and JAK/Stat response (*Socs36E*) genes using RNASeq (lines) and qRT-PCR (bars, for Socs36E) data. Cytokine induction and JAK/Stat activation occurs in both axenic and conventionally reared animals.

Figure S2 (relates to Fig. 2): Duox and antimicrobial peptide (AMP) expression

A) Duox and Diptericin (Dpt) expression trajectories determined using qRT-PCR. Averages and SEM from at least three independent samples are shown.

B, C) qRT-PCRs determining transcription of Relish, Dpt, Attacin A (AttA) and Drosomycin (Dros). Averages and SEM from at least three independent samples are shown. P Values from Student's Ttest (*p<0.05; **p<0.01).

D) Survival of young and old wild-type flies (OreR outcrossed into y^1w^1 background) fed S. Marcescens or E. Carotovora c. for 42 or 64 hours.

Figure S3 (relates to Fig. 3): Induction of Foxo target genes and Foxo dependent Rel induction

A, B) qRT-PCR detecting expression of Foxo target genes InR and dLip4 in aging intestines. Foxo target genes are induced with age in both axenic and conventionally reared animals. Averages and SEM from at least three independent samples are shown. P Values from Student's Ttest (*p<0.05; **p<0.01).

C) qRT-PCR detecting expression of Rel in wild-type (OreR) and *foxo*^{W24} mutant animals. Averages and SEM from at least three independent samples are shown. P Values from Student's Ttest.

Figure S4 (relates to Fig. 4): Regulation of PGRP-SCs by Foxo

A) qRT-PCR detecting PGRP-SC2 and PGRP-SC1a expression in aging wild-type flies or in flies expressing Foxo^{RNAi} under the control of NP1::Gal4. Averages and SEM from at least three independent samples are shown. P Values from Student's Ttest.

B) qRT-PCR detecting PGRP-SC1a expression in young wild-type flies or in flies expressing Foxo using NP1::Gal4 in combination with tub::Gal80ts. Foxo expression was induced in ECs by incubating flies at 29ºC for 5 days. Averages and SEM from at least three independent samples are shown. P Values from Student's Ttest.

C) qRT-PCR detecting *hid* expression in the same animals as in B.

Figure S5 (relates to Fig. 5): Verification of efficacy and specificity of Foxo^{RNAi}

A) Commensal numbers assessed using NR plates in age-matched wild-type (NP1::Gal4/+) flies, and in flies expressing dsRNA against PGRP-SC2 under the control of NP1::Gal4. Populations were bred and aged in parallel.

B) Mitotic figures in guts from animals from the same populations as in A.

C, D) Adult-onset inhibition of Foxo (NP1G4,tubG80^{ts}/UAS-Foxo^{RNAi}) blocks age-related increases in commensal bacteria (C) and mitotic figures (pH3 positive cells, D). Inhibiting *nebbish* (NP1G4,tubG80^{ts}/UAS-Nebbish^{RNAi} (VDRC:105891)) does not rescue these phenotypes. Female flies were reared at 18°C to prevent developmental expression of transgenes, and then 2-3 day old flies were shifted to 29°C and aged to 20 days. Midgut homogenates were plated on various plates for CFU quantitation. Averages and SEM from 8 intestines (CFU counts) or at least 15 intestines (pH3+ cells) are shown.

Figure S6 (relates to Fig. 6): Characterization of 5966GS and NP1::Gal4 expression and lifespan of NP1::Gal4-mediated PGRP-SC2 overexpression

A) qRT-PCR detecting *dpt* transcript in young and old wild-type or animals expressing PGRP-SC2 under the control of NP1::Gal4. Averages and SEM are plotted, p values from Student's Ttest.

B) Confocal images of the intestinal epithelium (top panels) of flies expressing GFP under the control of 5966::GS. GFP is excluded from ISCs (detected using anti-Dl antibody; arrowheads). Lower panels show specificity of this driver compared to ubiquitin-GFP expressing flies. Strong GFP expression is observed only in the abdomen, and, among abdominal tissues, only in the intestine and the salivary glands (m: muscle, fb: fatbody, lg: leg, sg: salivary gland, in: intestine, ov: ovary)

(C) Mortality trajectories of females over-expressing PGRP-SC2 in ECs using the NP1::Gal4 and 5966::GS drivers aged in conventional conditions. UAS::PGRP-SC2 flies were backcrossed 10X into the w^{1118} background.

Left panel shows NP1::Gal4/+ (n= 217) and +/+ (n=160) sibling flies (generated by crossing NP1::Gal4/+ virgin females to +/+ males). Central panel shows NP1::Gal4/UAS::PGRP-SC2(#5) (n=278) and UAS::PGRP-SC2(#5)/+ (n=202) sibling flies (generated by crossing NP1Gal4/+ virgin females to UAS-PCRP-SC2 males).

Right panel shows 5966::GS/UAS::PGRP-SC2(#8) flies maintained on food with (n=113) or without (n=157) RU486.

Table S1 (relates to Fig. 1): Complete RNAseq results from aging intestines.

Raw RPKM values for all genes in axenically and conventionally aging guts at 2, 10, 20, 30, and 40 days of age are listed.

Table S2 (relates to Fig. 1): Analyzed RNAseq results from aging intestines.

Expression values of genes filtered for detectable intestinal expression, and normalized to expression at 2 day timepoints.

Table S3 (relates to Fig. 1): Hierarchical clustering data

Gene tables generated by 'Cluster 3.0' by hierarchical clustering can be visualized using Java TreeView.

Supplemental Experimental Procedures

Fly handling and husbandry

UAS-PGRP-SC2 transgenic lines were generated by cloning the coding sequence of PGRP-SC2 (amplified using the following primers: 5'-AGACTCATGGCAAACAAAGCTCTCATCCTTCTG-3′ and 5′-GGTACCTTAGGCCTTCCAGTTGGACCAGG-3′) into pUAST. Transgenic flies were generated using standard procedures by Genetic Services, Inc.

All flies were maintained on standard molasses/yeast food. Recipe as follows: 1 liter distilled water, 13.8 g agar, 22 g molasses, 80 g malt extract, 18 g Brewer's yeast, 80 g corn flour, 10 g soy flour, 6.25 mL propionic acid, 2 g methyl-p-benzoate, 7.2 mL of Nipagin (20% in EtOH).

Flies were maintained at 25°C and 65% humidity, on a 12 h light/dark cycle, unless otherwise indicated.

For RU486 food supplementation, 100 µl of a 5 mg/ml solution of RU486 or vehicle (ethanol 80%) was deposited on top of the food and dried for at least 16 hours to ensure complete evaporation, resulting in a 0.2 mg/ml concentration of RU486 in the food accessible to flies (determined using a dye control as previously described for drug treatments [Grover, 2009 #2634]).

For all populations, plastic cages (175 ml volume, 5 cm diameter from Greiner bio-one) were used for lifespan experiments. Food, changed every 2-3 days, was provided in vials inserted into a foam plug (4.9 cm in diameter, 3 cm thick from Greiner bio-one). Dead flies were visually identified (flies not moving, not responding to mechanical stimulation and laying on their side or back were deemed dead), and their numbers were recorded. Cages were replaced after 20 days (flies were transferred into new cages without anesthesia).

Axenic fly cultures

12 hour collections of embryos were dechorionated for 3 min in 2.7% sodium hypochlorite [2-fold diluted bleach (Kem Tech, St. Ixonia, WI)] and then washed

twice with sterile, distilled water for 1 min. These embryos were transferred in a tissue culture hood into sterile food bottles. Then 70% sterile glycerol was laid over the embryos in the food. Axenia of embryos was confirmed by plating homogenates onto LB agar plates. Flies were maintained in a laminar flow hood and transferred every 2-3 days into new, sterile vials.

Sterile food bottles and vials were generated by autoclaving at 121^0C for 30 min. Food was then allowed to cool in a tissue culture hood before Nipagin was added. To generate control populations for axenic flies, 12 hour collections of embryos were collected and flies were cultured using standard procedures.

Alternatively, flies were sterilized as embryos as described above, then reared on food containing antibiotics (100 µg/ml ampicillin, 50 µg/ml vancomycin, 100 µg/ml neomycin, 100 µg/ml metronidazole, and 50 µg/ml Tetracyclin).

Conditional expression of UAS-linked transgenes

The TARGET system was used in combination with NP1::Gal4 to conditionally express UAS-linked transgenes in ECs (McGuire et al., 2004). Flies were developed at room temperature (20ºC), then shifted to 29°C to induce transgene expression.

qRT-PCR Analysis of gene expression

Total RNA was extracted from dissected guts (7 per sample) using Trizol and cDNA was synthesized using Superscript II (Invitrogen). Real time PCR was performed in triplicate for each sample using SYBR Green on a Biorad IQ5 system.

Expression values were calculated using the ΔΔCt method and normalized to *actin5C* expression levels. Results are shown as Average +/− SEM of at least 3 independent biological samples.

Primer sequences for RT-PCR

The following primers pairs were used:

PGRP-SC2:	5′-AACTACCTGAGCTACGCCGTGAT-3′	5′-AGCAGAGGTGAGGGTGTTGGTATT-3′
PGRP-SC1a:	5′-CTATGTCGTCTCCAAGGCGGAGT-3′	5′-CGATCAGGAAGTTGTAGCCGATGT-3′
FOXO:	5′-TGTCGCTGCACAACCGCTTTATGA-3′	5′-TTGCCGGAAATCGGGCGATAATTG-3′
Actin5C:	5′-CTCGCCACTTGCGTTTACAGT-3′	5′-TCCATATCGTCCCAGTTGGTC-3′;
DUOX:	5′-GTCGCACGCCAACCACAAGAGACT-3′	5′-CACGCGCAGCAGGATGTAAGGTTT-3′
DPT:	5′-GGCTTATCCGATGCCCGACG -3′	5′-TCTGTAGGTGTAGGTGCTTCCC -3′
PGRP-LC:	5′-TTGAACCAAAGTAAGATCAGAGAT-3′	5′-GTCCAGATATATTGTTGAATT-3′
Drosomycin:	5′-CGTGAGAACCTTTTCCAATATGAT-3′	5′-TCCCAGGACCACCAGCAT-3′
Rel:	5′-ACAGCTACAGGAACTGCATCAGGAA-3′	5′-TCATCCTCCTCGAAGAACCTCACT -3′
InR:	5′-CATCGGAAGGGAGGCGTAA -3′	5′-CGTTTGCCTAATCGTCGAACA-3′
AttA:	5′-TCGTTTGGATCTGACCAAGGGCAT-3′	5′-TTCCGCTGGAACTCGAAACCATTG-3′
Thor:	5′-CACTTGCGGAAGGGAGTACG-3′	5′-TAGCGAACAGCCAACGGTG-3′

Selective plates for bacterial cultures

Selective plates were generated according to the following recipes:

Acetobacteriaceae: 25 g/l D-mannitol, 5 g/l yeast extract, 3 g/l peptone, and 15 g/l agar.

Enterobacteriaceae: 10 g/l Tryptone, 1.5 g/l yeast extract, 10 g/l glucose, 5 g/l sodium chloride, 12 g/l agar.

Lactobacilli MRS agar: 70 g/l BD Difco Lactobacilli MRS agar.

Nutrient Rich Broth: 23g/l BD Difco Nutrient agar.

All media were autoclaved at 121 degree for 15 minutes.

Immunostaining and microscopy

Intact guts were fixed at room temperature for 45 minutes in 100 mM glutamic acid, 25 mM KCl, 20 mM MgSO4, 4 mM Sodium Phosphate, 1 mM MgCl2, 4% formaldehyde. All subsequent incubations were done in PBS, 0.5% BSA, 0.1% TritonX-100 at 4°C. The following primary antibodies were used: rabbit anti-pH3 (Upstate) 1:1000. Fluorescent secondary antibodies were obtained from Jackson Immunoresearch. Hoechst was used to stain DNA.

Detection of β-galactosidase activity

Intact guts were dissected in PBS + 2mM $MgCl_2$ and fixed for 10 minutes in 0.5% glutaraldehyde. Detection of β-galactosidase activity was carried out at room temperature in staining buffer (PBS, 2 mM MgCl2, 5 mM K4(Fe[CN]6), 5 mM K3(Fe[CN]6), 0.1% X-gal).

Pharmacological inhibition of NF-kB

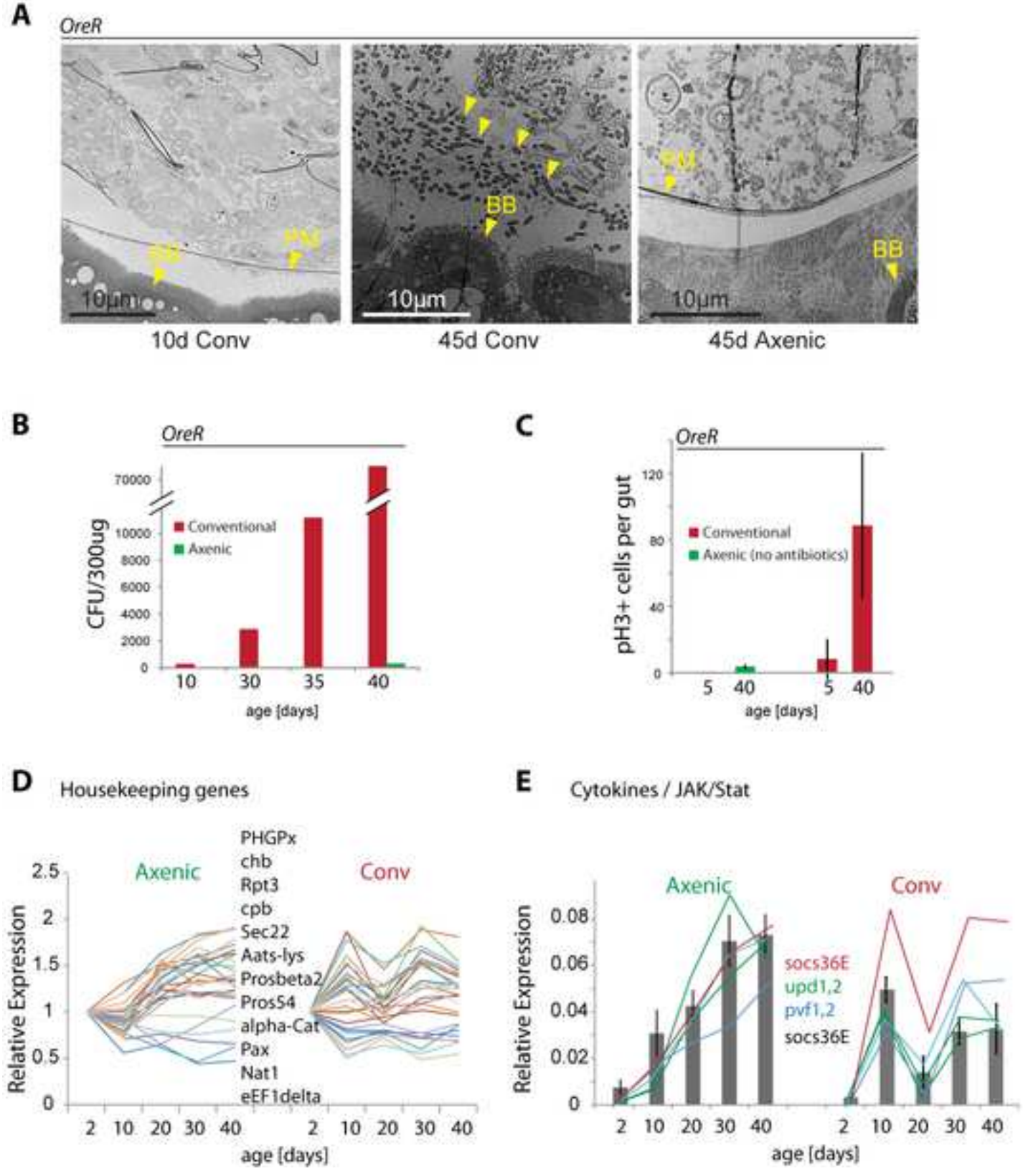
Pyrrolidine dithiocarbamate (PDTC, Sigma) was used as a selective inhibitor of the NF-kB transcription factor. Flies were fed 5% sucrose solution with or without PDTC [0.02 mg/ml] for 24 hours before feeding 700 µl *Ecc* (OD=100) suspended in 5% sucrose with or without PDTC for another 24 hours. Flies were then dissected to harvest RNA from guts.

Bacteria Clearance

For experiments reported in Fig. 2, axenic young flies (5d) and old flies (45d) were fed with bacteria or 5% sucrose for 24 hours. Flies were then shifted to normal food and allowed to recover for 3 days before analysis.

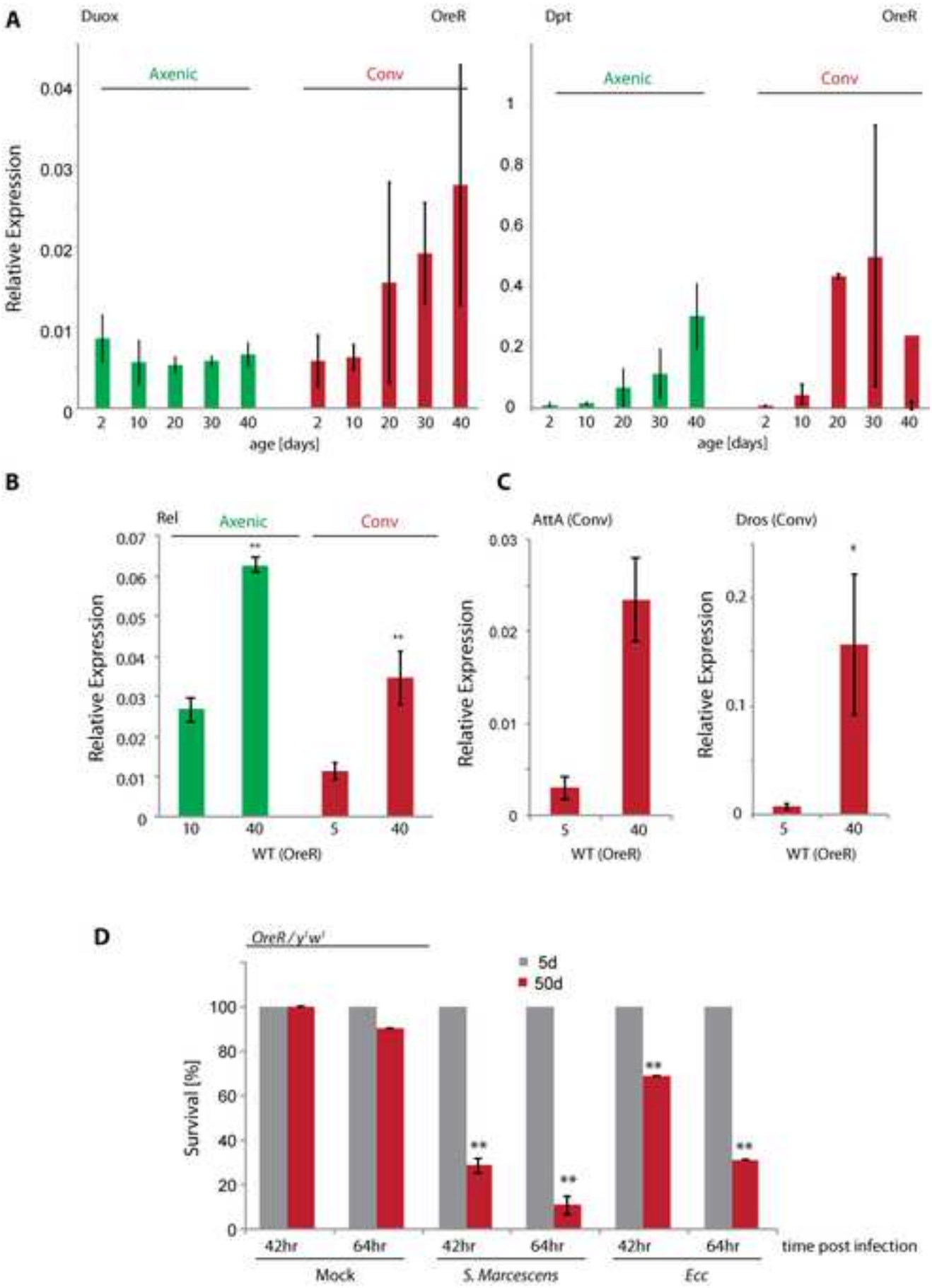
For experiments reported in Fig. 4, flies were kept at 29ºC for 4 days on food containing antibiotics (100 µg/ml ampicillin, 50 µg/ml vancomycin, 100 µg/ml neomycin, 100 µg/ml metronidazole, and 50 µg/ml Tetracyclin) to induce Foxo expression. Flies were then fed 700 µl of *S.Marcescens* or *Ecc* (OD=100) in 5% sucrose on filter paper for 24 hours. Control flies were fed 5% sucrose solution. Flies were then shifted to normal food for 4 days before the gut was dissected and CFUs were determined.

Guo Figure S1:



Supplemental Figure2

Guo Figure S2:



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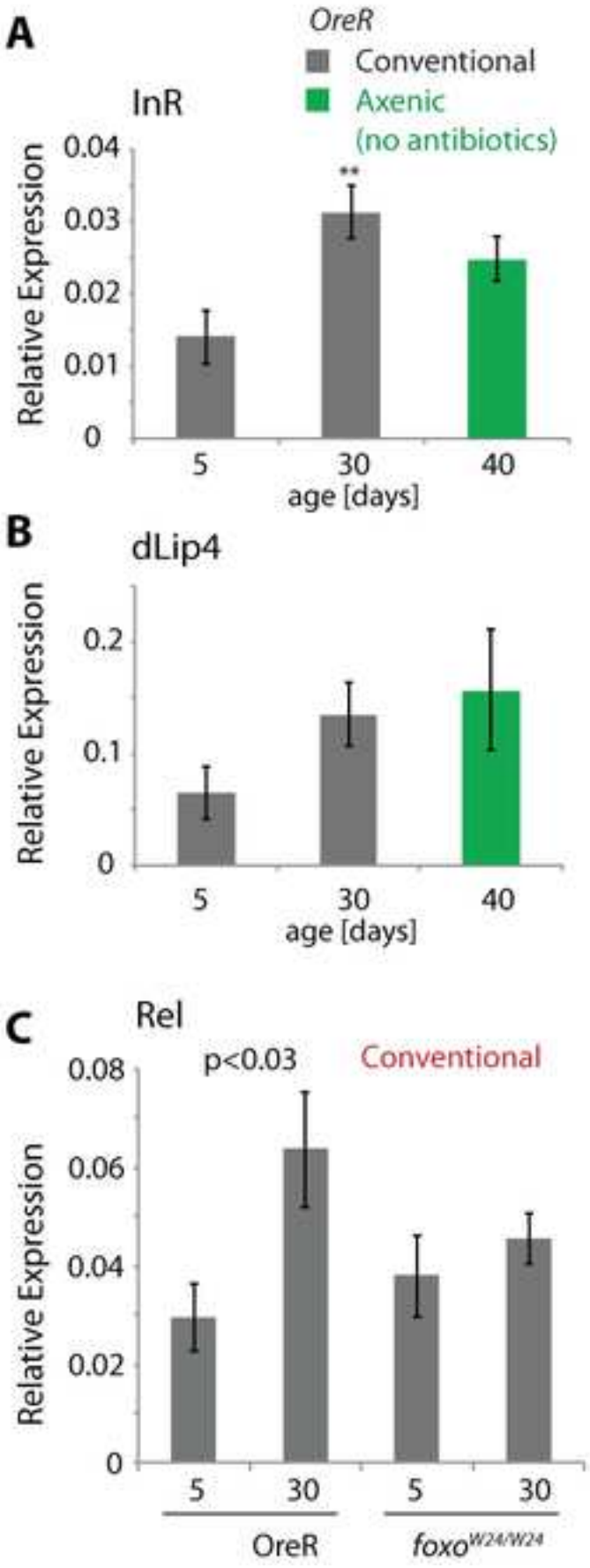

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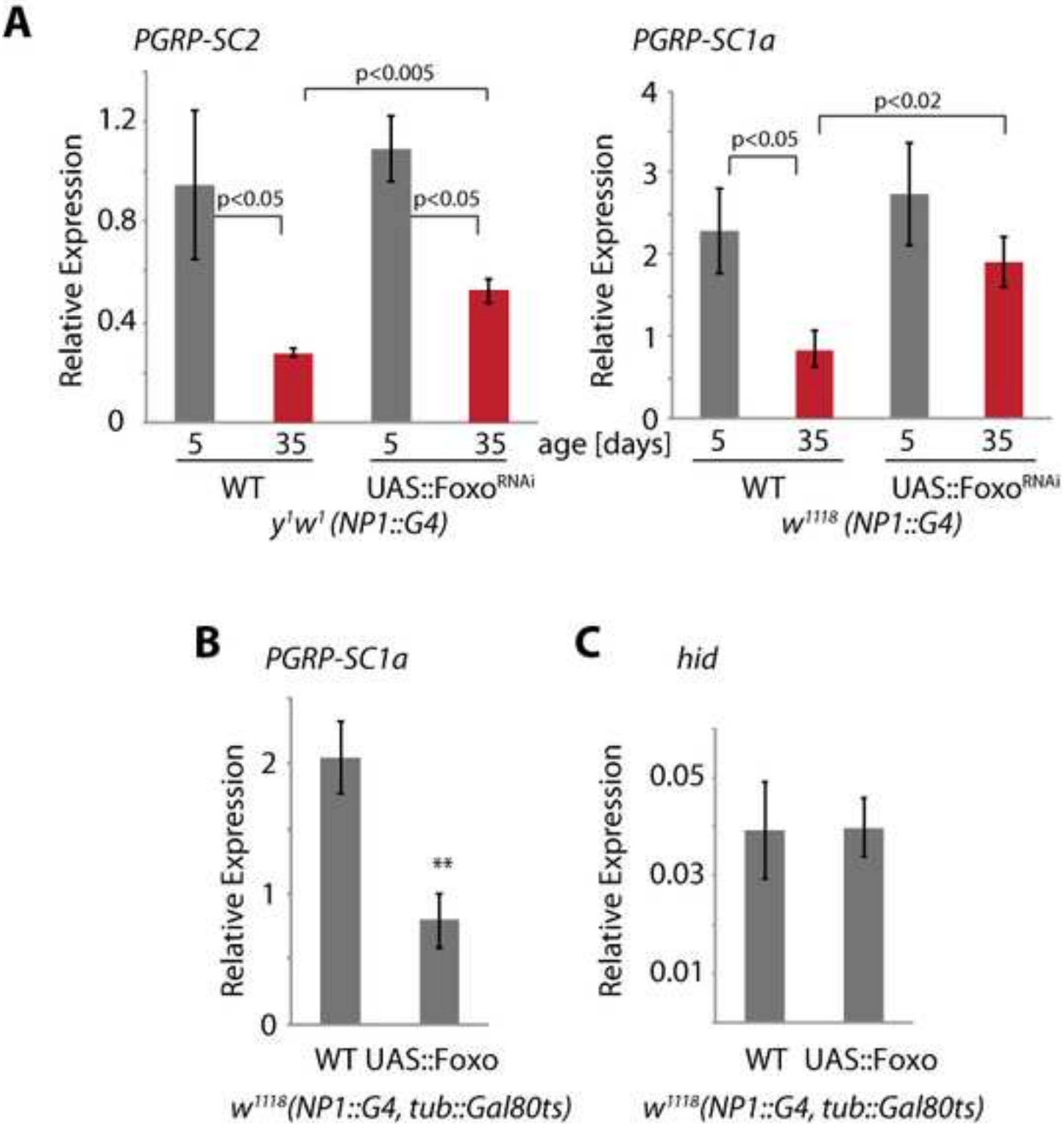
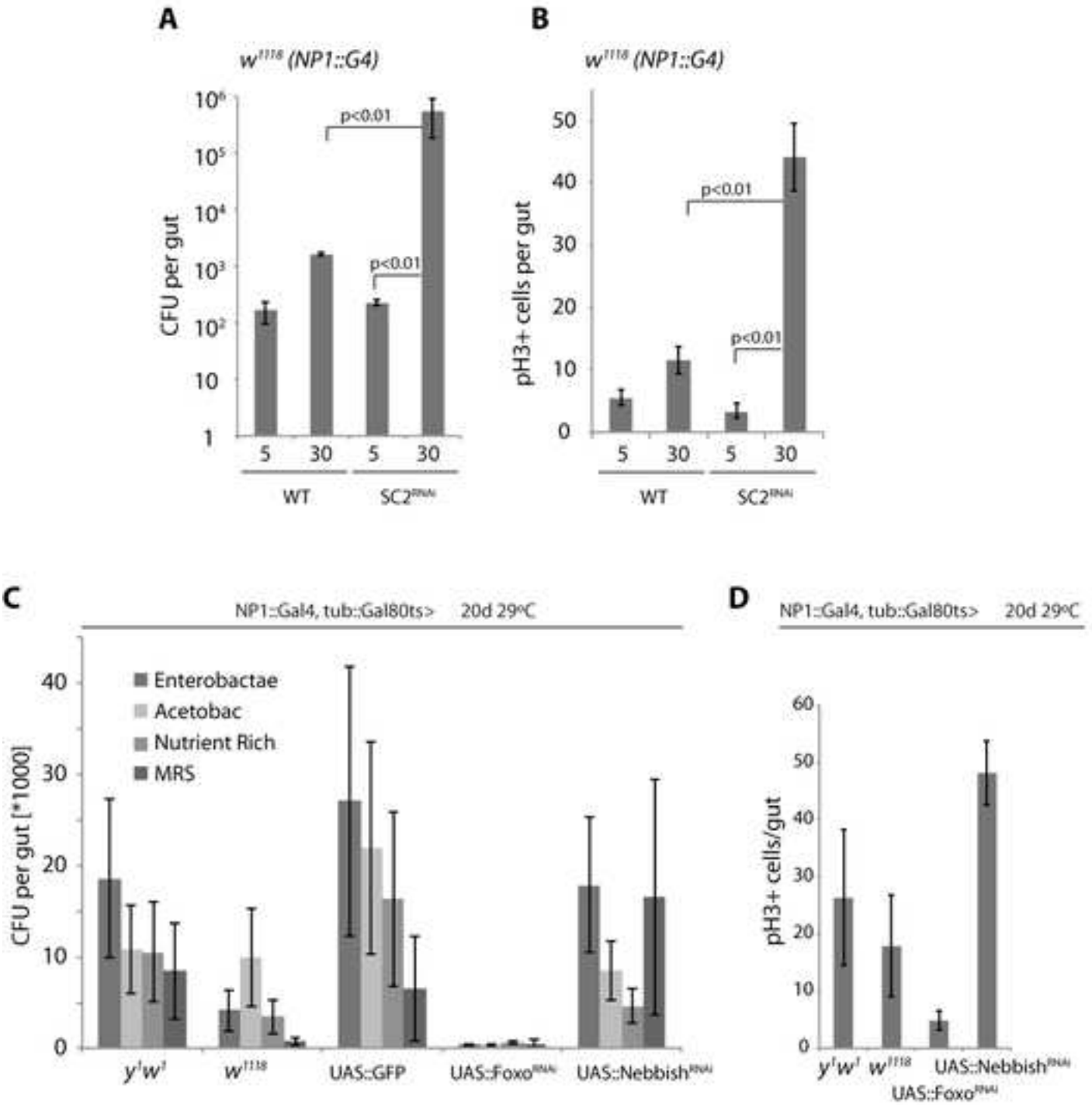


Guo Figure S5:



Guo Figure S6: