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Supplemental Information

Intestinal Snakeskin Limits Microbial Dysbiosis during Aging and Promotes Longevity

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Supplemental Information

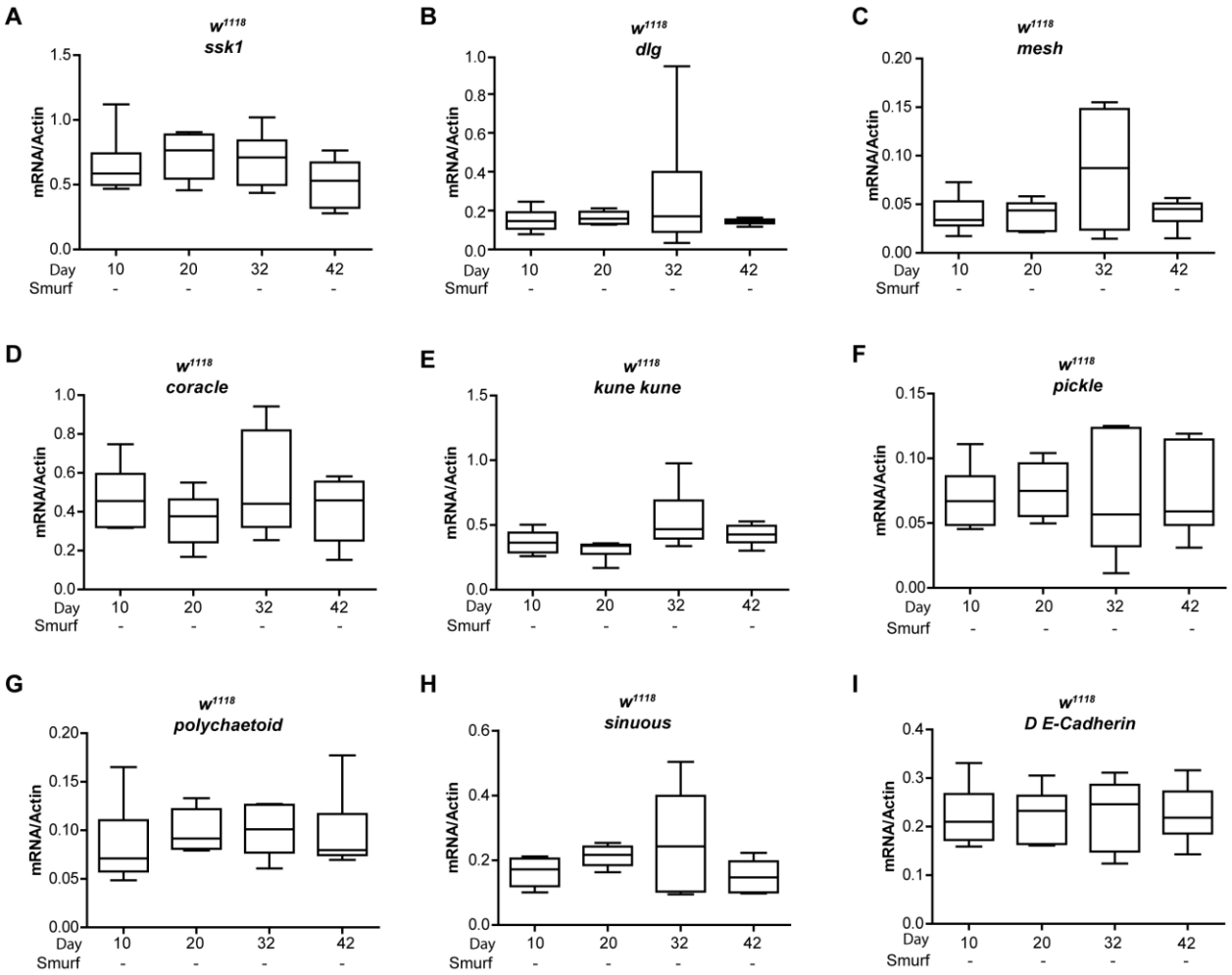
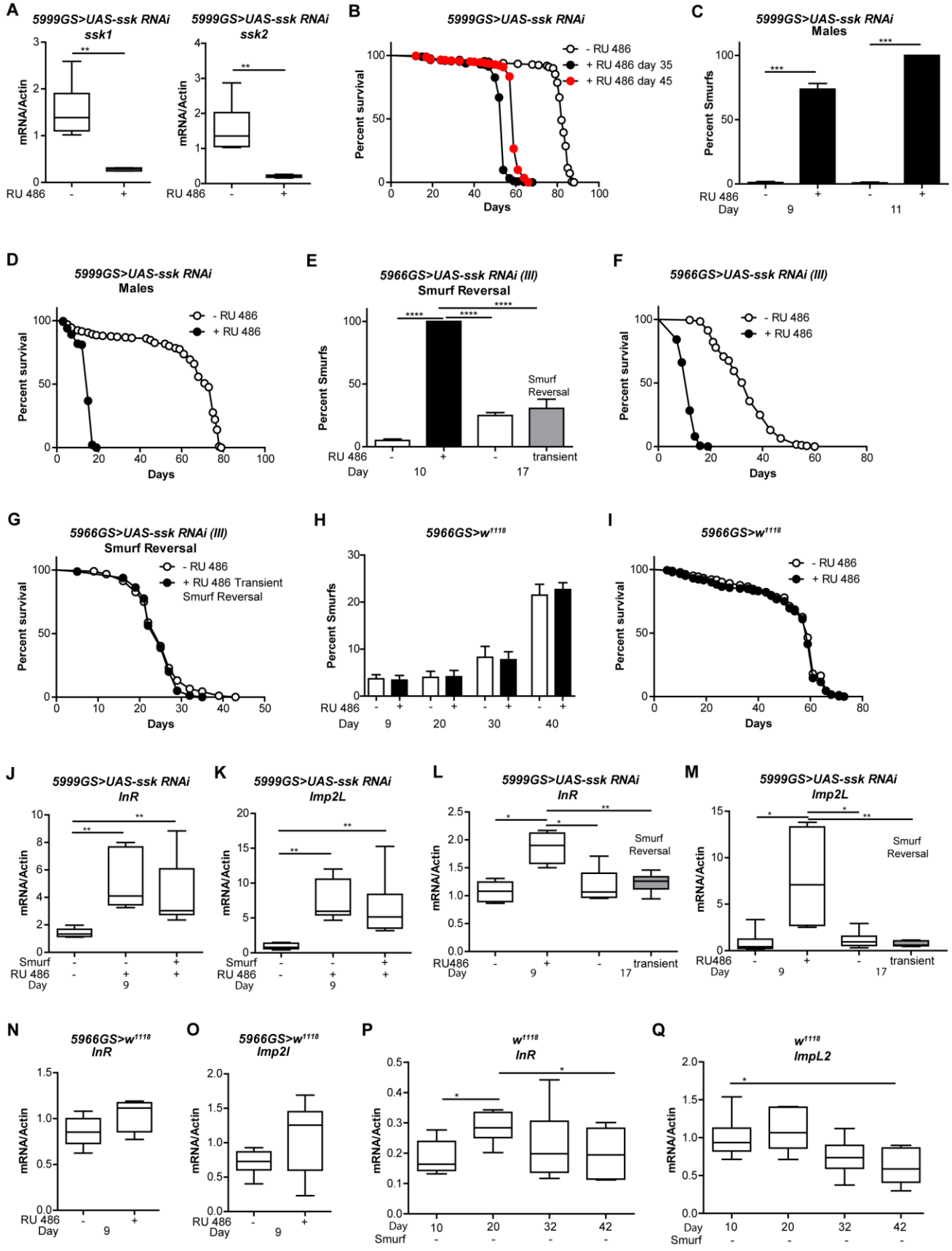


Figure S1. Alterations in SJs in posterior midguts of Smurf and non-Smurf flies. Related to Figure 1

(A-I) Junction protein gene expression assayed by qPCR from dissected intestines of w^{1118} non-Smurf (Smurf -) female flies at 10, 20, 32, and 42 days of age. n = 6 replicates of five intestines. *ssk, Snakeskin; dlg1, discs large 1; mesh; coracle; kune-kune; pickle; polychaetoid; sinuous; D E-Cadherin, Drosophila E-Cadherin.*

Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. No significant difference; Wilcoxon test.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RU 486
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ssk2
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- RU 486
+ RU 486 day 35
+ RU 486 day 45
Percent survival
Days
C
5999GS>UAS-ssk RNAi
Males
Percent Smurfs
Day
D
5999GS>UAS-ssk RNAi
Males
E
5966GS>UAS-ssk RNAi (III)
Smurf Reversal
Smurf Reversal
transient
F
5966GS>UAS-ssk RNAi (III)
G
5966GS>UAS-ssk RNAi (III)
Smurf Reversal
+ RU 486 Transient Smurf Reversal
H
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I
5966GS>w1118
J
5999GS>UAS-ssk RNAi
InR
Smurf
K
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InR
M
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Imp2L
N
5966GS>w1118
InR
O
5966GS>w1118
Imp2l
P
w1118
InR
Q
w1118
ImpL2

Figure S2. Loss of intestinal Ssk leads to rapid and reversible intestinal barrier dysfunction and early-onset mortality. Related to Figure 2

(A) *ssk* gene expression assayed by qPCR utilizing two different sets of primers: *ssk1* and *ssk2,* from dissected intestines of *5966GS > UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of five intestines.

(B) Survival curves of *5966GS>UAS-ssk RNAi* females with or without RU486-mediated transgene induction from day 35 or 45 onwards. $p < .0001$, log rank test; n > 196 flies/condition. Median lifespan 54, 59, and 82 days, respectively.

(C) Intestinal integrity of *5966GS>UAS-ssk RNAi* males with or without RU486-mediated transgene induction from day 3 onwards. Percentage Smurfs were assessed at 9 and 11 days of age. One-way ANOVA/Bonferroni's multiple comparisons test; n > 188 flies/condition.

(D) Survival curves of *5966GS>UAS-ssk RNAi* males with or without RU486-mediated transgene induction from day 3 onwards. $p < .0001$, log rank test; n > 231 flies/condition. Median lifespan 15 and 73 days, respectively.

(E) Reversal of intestinal integrity of *5966GS>UAS-ssk RNAi (III),* a different *ssk RNAi* line, females with or without RU486-mediated transgene induction from day 3 until day 10, when all of the induced flies become Smurfs, then moved to RU486- food for 7 days and assayed again for barrier integrity on day 17. One-way ANOVA/Bonferroni's multiple comparisons test; n > 177/condition.

(F) Survival curves of *5966GS>UAS-ssk RNAi (III)* females with or without RU486-mediated transgene induction from day 3 onwards. $p < .0001$, log rank test; n > 177 flies/condition. Median lifespan 12 and 35 days, respectively.

(G) Survival curves with reversal of early death of *5966GS>UAS-ssk RNAi (III)* females with or without RU486-mediated transgene induction from day 3 to day 10, then without RU486-mediated transgene induction from day 10 onwards. There is no significant difference, log rank test; n > 80 flies/condition. Median lifespan 25 days for each.

(H) Intestinal integrity of *5966GS>w^{1118}* females with or without RU486-mediated transgene induction from day 3 onwards. Percentage Smurfs were assessed at 9 and 11 days of age. There is no significant difference, one-way ANOVA/Bonferroni's multiple comparisons test; n > 267 flies/condition.

(I) Survival curves of *5966GS>w^{1118}* females with or without RU486-mediated transgene induction from day 3 onwards. There is no significant difference, log rank test; n > 267 flies/condition. Median lifespan 59 and 59 days, respectively.

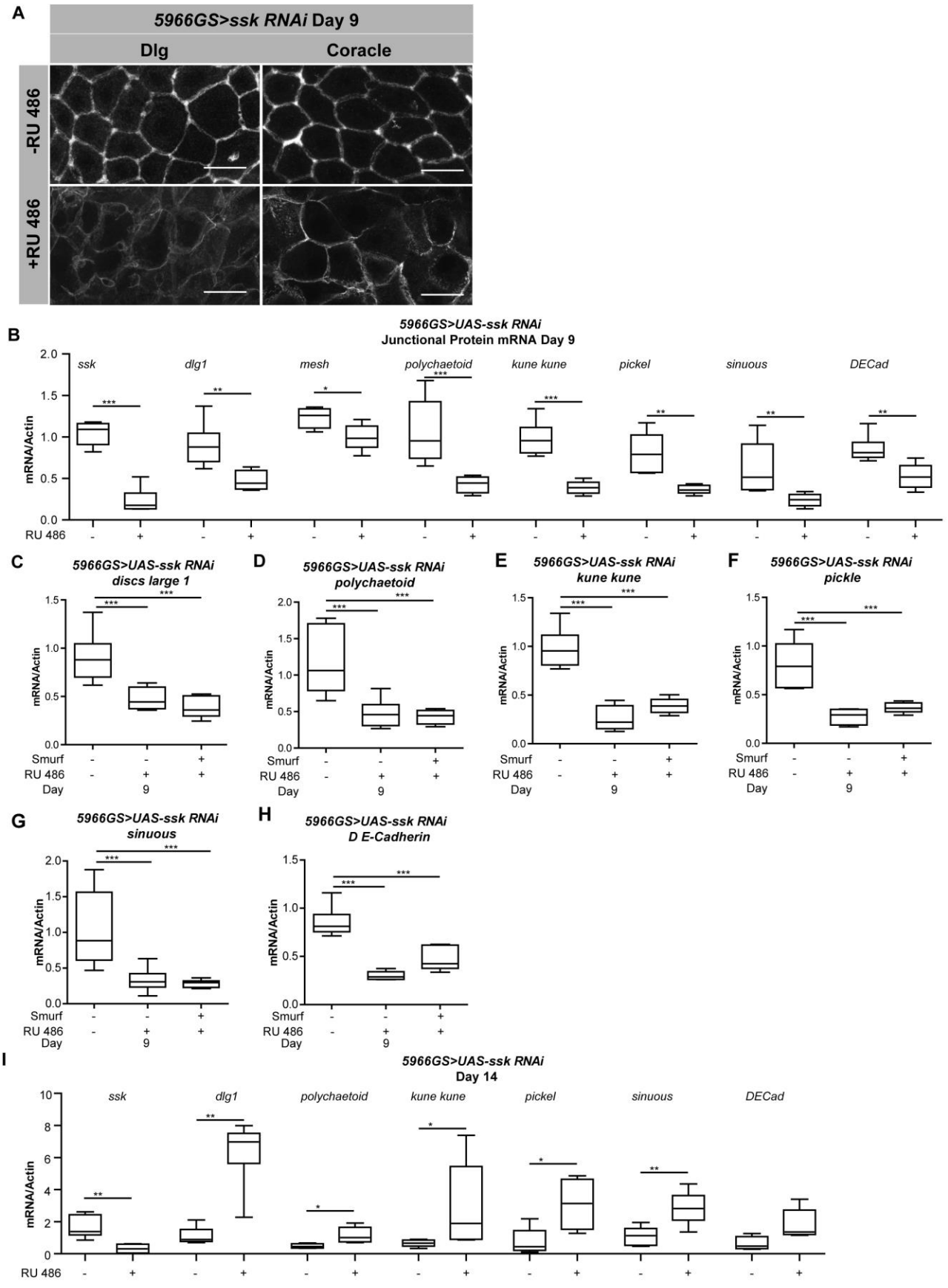
(J and K) FOXO target gene expression assayed by qPCR from dissected intestines in Smurf and non-Smurf *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. *Insulin-like Receptor, InR*; Ecdysone-inducible gene L2, *ImpL2;* n = 6 replicates of five intestines.

(L and M) Reversal of FOXO target gene expression assayed by qPCR from dissected intestines in *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 until day 10, when all of the induced flies become Smurfs, then moved to food lacking RU486 for 7 days and assayed on day 17. *Insulin-like Receptor, InR*; Ecdysone-inducible gene L2, *ImpL2*; n = 6 replicates of five intestines.

(N and O) FOXO target gene expression assayed by qPCR from dissected intestines in *5966GS>w^{1118}* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. *Insulin-like Receptor, InR*; Ecdysone-inducible gene L2, *ImpL2*; n = 6 replicates of five intestines.

(P and Q) FOXO target gene expression assayed by qPCR from dissected intestines in *w^{1118}* non-Smurf (Smurf -) female flies at 10, 20, 32, and 42 days of age. *Insulin-like Receptor, InR*; Ecdysone-inducible gene L2, *ImpL2*; n = 6 replicates of five intestines.

Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. Error bars on bar graphs depict mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ represent a statistically significant difference. Wilcoxon test unless otherwise stated.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-RU 486
+RU 486
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kune kune
pickel
sinuous
DECad
mRNA/Actin
RU 486
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D
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polychaetoid
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pickle
Smurf
RU 486
Day
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Day 14

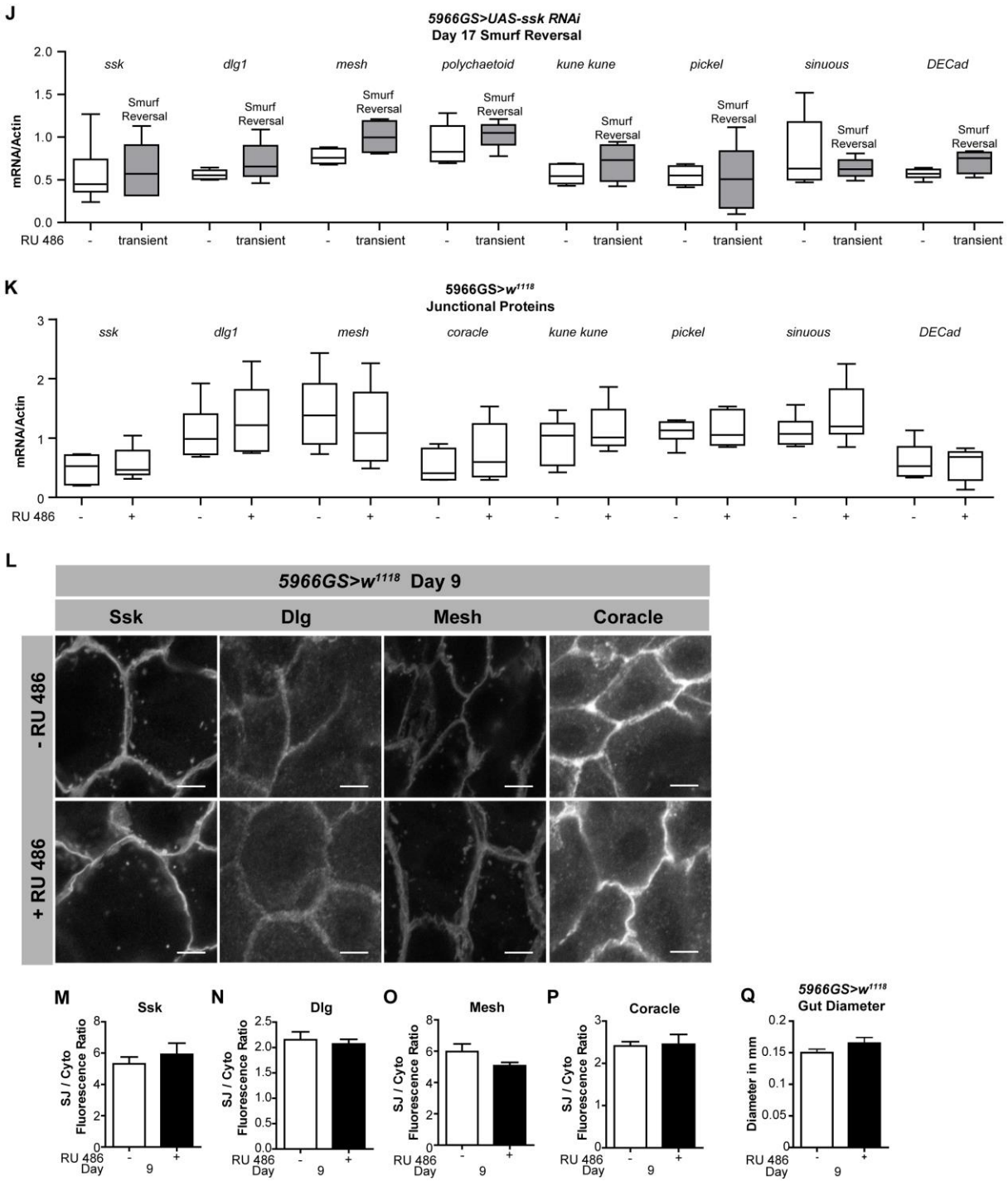


Figure S3. Loss of intestinal Ssk leads to gut distention and altered SJs. Related to Figure 3

(A) SJ protein localization in *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. Representative images for Dlg and Coracle. SJ protein mislocalization is observed in the presence of RU486. *Scale bar* is 20 μm.

(B) Junction protein gene expression assayed by qPCR from dissected intestines of *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of five intestines. *ssk, snakeskin; dlg1, discs large 1; mesh; polychaetoid; kune-kune; pickle; sinuous, sinu; DeCad, Drosophila E-Cadherin.*

(C-H) Junction protein gene expression assayed by qPCR from dissected intestines in Smurf and non-Smurf *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of five intestines. *dlg1, discs large 1; polychaetoid; kune-kune; pickle; sinuous, sinu; DeCad, Drosophila E-Cadherin.*

(I) Junction protein gene expression assayed by qPCR from dissected intestines of *5966GS>UAS-ssk RNAi* female flies on day 14 with or without RU486-mediated transgene induction from day 3 to day 14. n = 6 replicates of five intestines. *ssk, snakeskin; dlg1, discs large 1; polychaetoid; kune-kune; pickle; sinuous, sinu; DeCad, Drosophila E-Cadherin.*

(J) Reversal of junction protein gene expression assayed by qPCR from dissected intestines of *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 until day 10, when all of the induced flies become Smurfs, then moved to food lacking RU486 for 7 days and assayed on day 17. n = 6 replicates of five intestines. *ssk, snakeskin; dlg1, discs large 1; mesh; polychaetoid; kune-kune; pickle; sinuous, sinu; DeCad, Drosophila E-Cadherin.*

(K) Junction protein gene expression assayed by qPCR from dissected intestines of *5966GS>w^{1118}* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of five intestines. *ssk, Snakeskin; dlg1, discs large 1; mesh; coracle; kune-kune; pickle; sinuous, sinu; DeCad, Drosophila E-Cadherin.*

(L) SJ protein localization for Ssk, Dlg, Mesh and Coracle in ECs from dissected intestines of *5966GS>w^{1118}* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. No difference in SJ protein mis-localization is observed. n>13 midguts per condition; n=10 ECs were observed per midgut; s*cale bar* is 5 μm.

(M-P) SJ/Cytoplasm fluorescence ratios for Ssk, Dlg, Mesh and Coracle. There is no significant difference; two-tailed unpaired Student's t-test.

(Q) Posterior midgut diameter in mm from dissected intestines of *5966GS>w^{1118}* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. No difference in midgut diameters are observed, two-tailed unpaired Student's t-test; n>13 midguts per condition.

Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. Error bars on bar graphs depict mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Wilcoxon test unless otherwise stated.

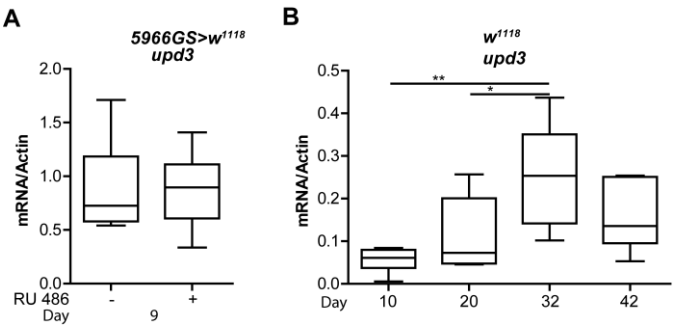
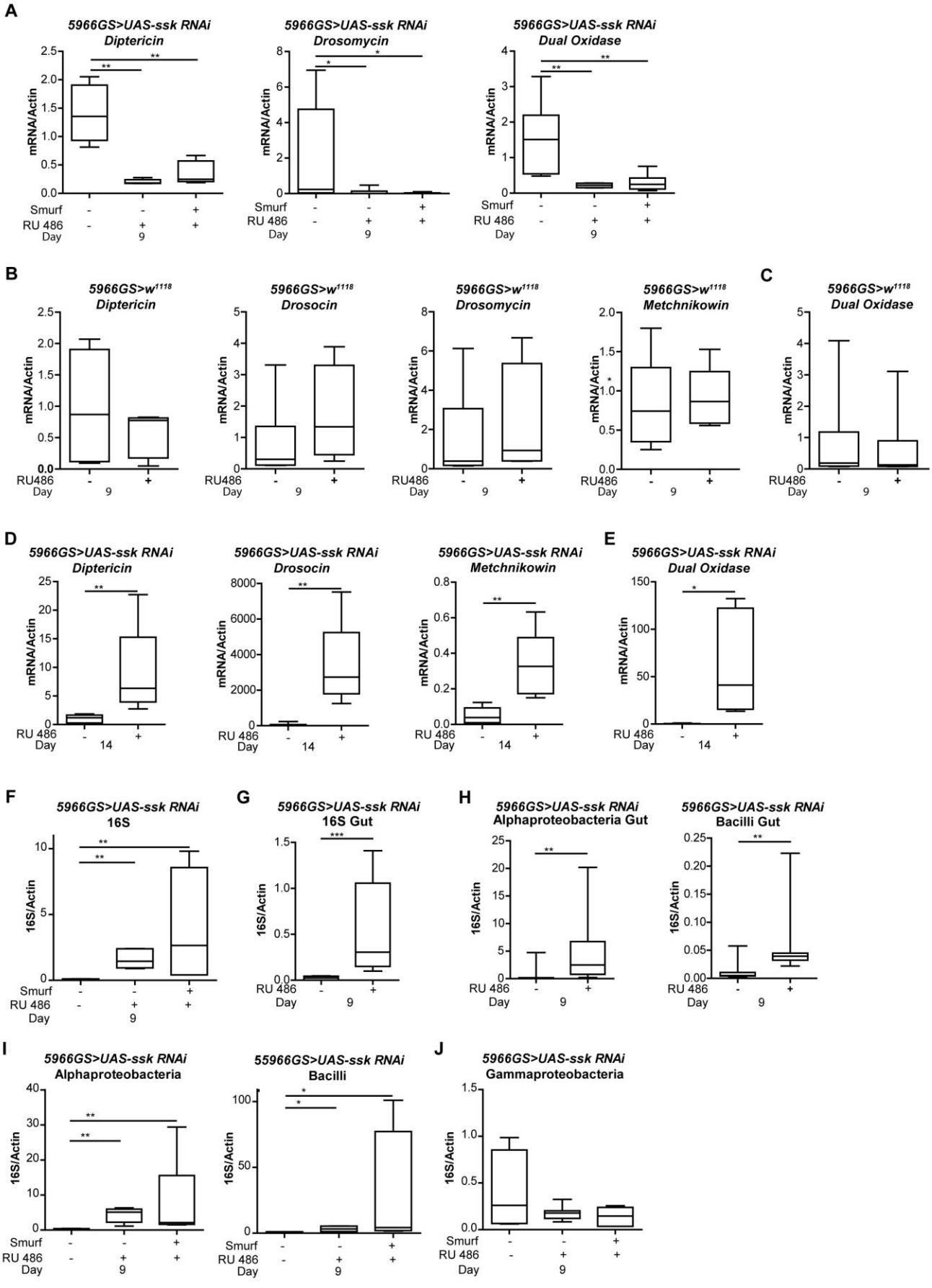


Figure S4. Loss of intestinal Ssk leads to intestinal stem cell dysfunction. Related to Figure 4

(A) *upd3* gene expression assayed by qPCR from dissected intestines in *5966GS> w^{1118}* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of five intestines.

(B) *upd3* gene expression assayed by qPCR from dissected intestines of w^{1118} non-Smurf female flies at 10, 20, 32, and 42 days of age. n = 6 replicates of five intestines.

Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. *p < 0.05; **p < 0.01; Wilcoxon test.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Smurf
RU 486
Day
B
5966GS>w1118
Diptericin
5966GS>w1118
Drosocin
5966GS>w1118
Drosomycin
5966GS>w1118
Metchnikowin
C
5966GS>w1118
Dual Oxidase
D
5966GS>UAS-ssk RNAi
Diptericin
5966GS>UAS-ssk RNAi
Drosocin
5966GS>UAS-ssk RNAi
Metchnikowin
E
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Dual Oxidase
F
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16S
16S/Actin
G
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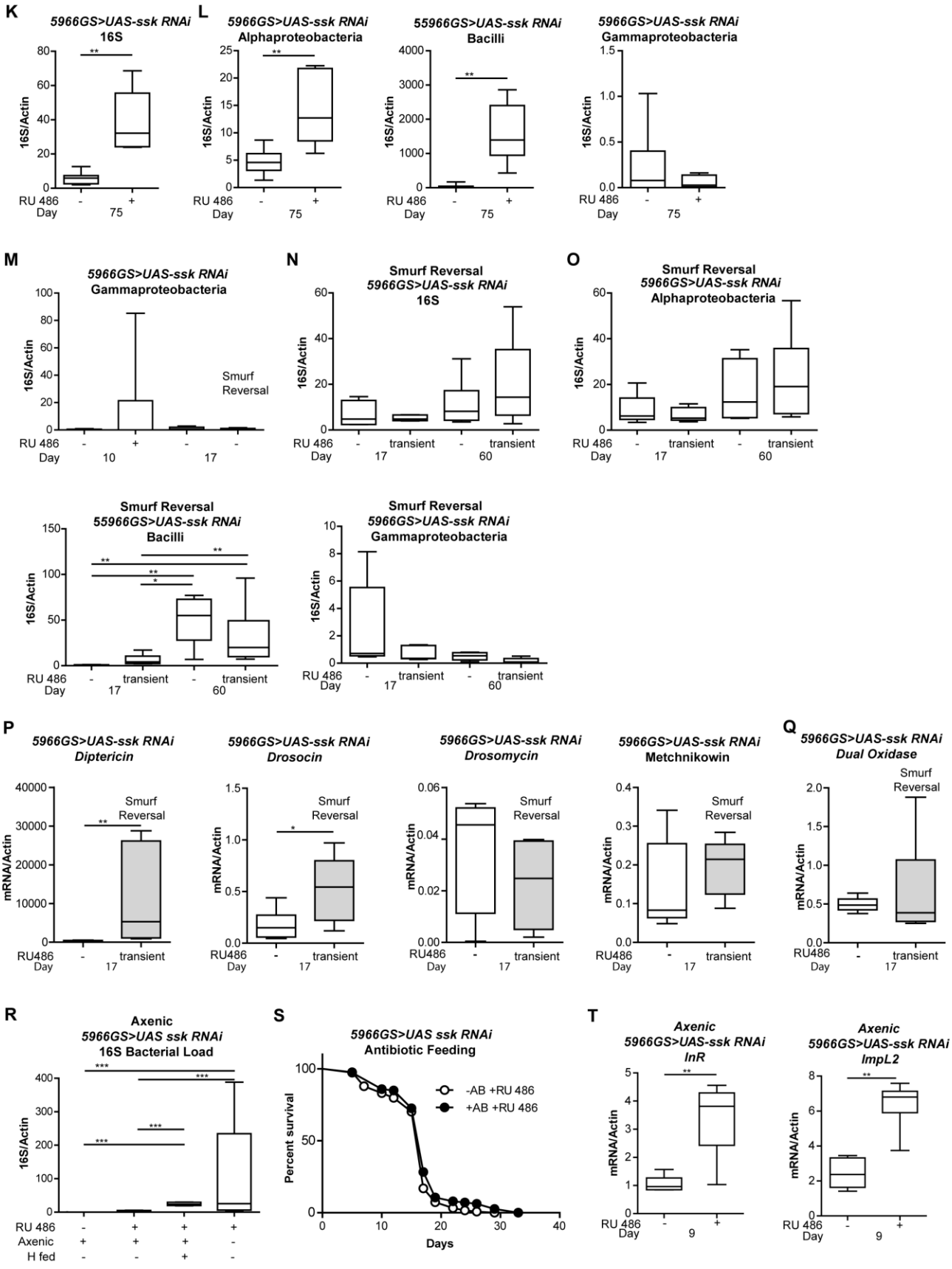
Gammaproteobacteria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Gammaproteobacteria
M
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Gammaproteobacteria
Smurf
Reversal
10
17
N
Smurf Reversal
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16S
transient
60
O
Smurf Reversal
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Smurf Reversal
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Diptericin
Drosocin
Drosomycin
Metchnikowin
mRNA/Actin
RU486
Q
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Dual Oxidase
R
Axenic
5966GS>UAS ssk RNAi
16S Bacterial Load
Axenic
H fed
S
5966GS>UAS ssk RNAi
Antibiotic Feeding
-AB +RU 486
+AB +RU 486
Percent survival
Days
T
Axenic
5966GS>UAS-ssk RNAi
InR
ImpL2
9

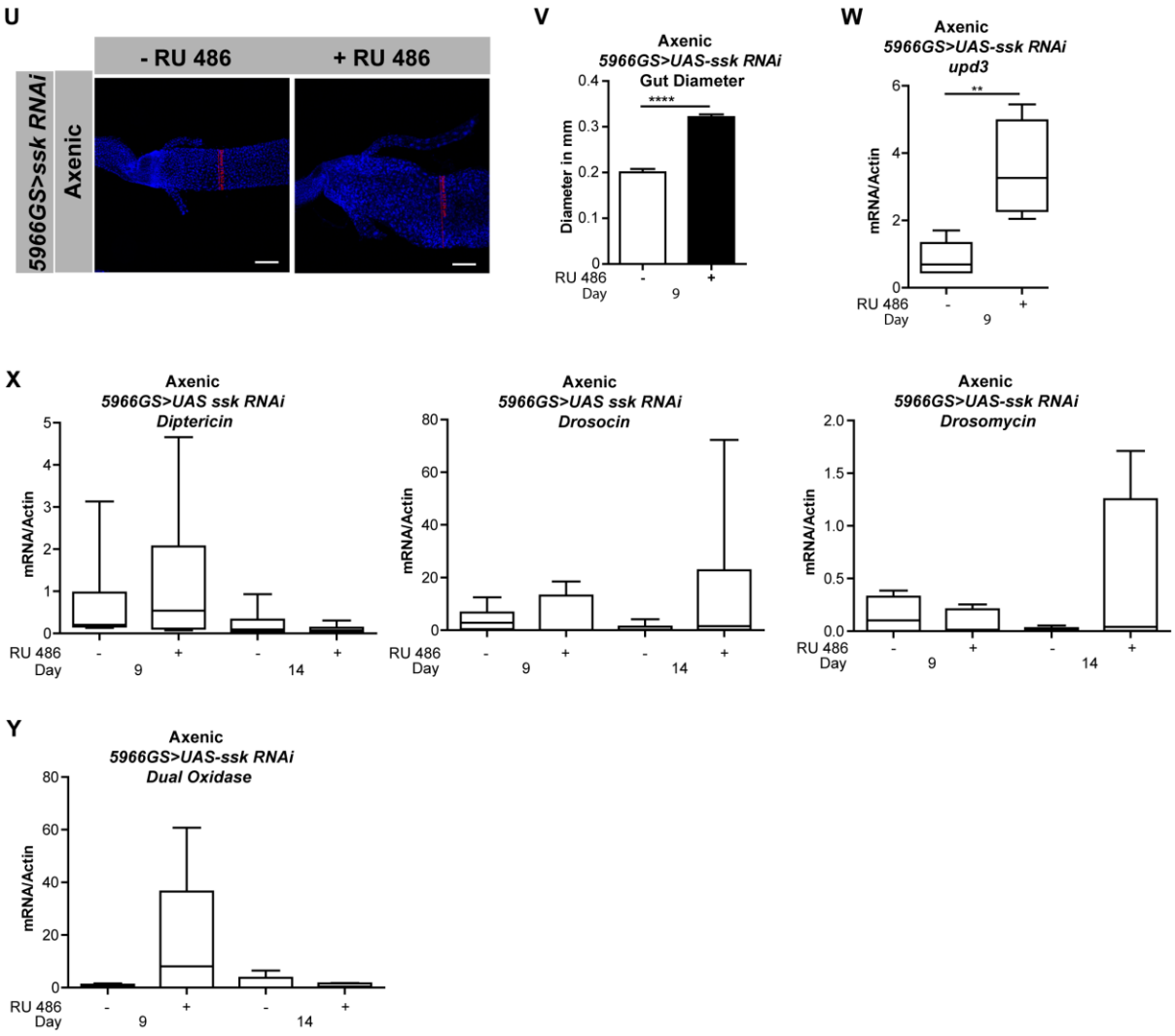

Figure S5. Loss of intestinal Ssk leads to reversible microbial dysbiosis. Related to Figure 5

(A) Antimicrobial peptide and *Dual Oxidase* gene expression assayed by qPCR from dissected intestines in Smurf and non-Smurf *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. *Diptericin, Drosomycin*; n = 6 replicates of five intestines.

(B and C) Antimicrobial peptide and *Dual Oxidase* gene expression assayed by qPCR from dissected intestines in *5966GS>w^{1118}* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. There is no significant difference. *Diptericin, Drosocin, Drosomycin, Metchnikowin*; n = 6 replicates of five intestines.

(D and E) Antimicrobial peptide and *Dual Oxidase* gene expression assayed by qPCR from dissected intestines in *5966GS>UAS-ssk RNAi* female flies on day 14 with or without RU486-mediated transgene induction from day 3 to day 14. *Diptericin, Drosocin, Metchnikowin*; n = 6 replicates of five intestines.

(F) Bacterial levels assayed by qPCR of the 16S rRNA gene with universal primers in Smurf and non-Smurf *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of 5 flies.

(G) Gut bacterial levels assayed by qPCR of the 16S rRNA gene from dissected intestines with universal primers in *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of 5 flies.

(H) Gut bacterial levels assayed by qPCR of the 16S rRNA gene from dissected intestines by taxon-specific qPCR of the 16S rRNA gene in *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 to day 9. Alphaproteobacteria, Bacilli; n = 6 replicates of 5 flies.

(I and J) Bacterial levels assayed by taxon-specific qPCR of the 16S rRNA gene in Smurf and non-Smurf *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 to day 9. Alphaproteobacteria, Bacilli, Gammaproteobacteria; n = 6 replicates of 5 flies.

(K) Bacterial levels assayed by qPCR of 16S with universal primers in *5966GS>UAS-ssk RNAi* female flies at 75 days of age with or without RU486-mediated transgene induction from day 69 to day 75. n = 6 replicates of 5 flies.

(L) Bacterial levels assayed by taxon-specific qPCR of the 16S rRNA gene in *5966GS>UAS-ssk RNAi* female flies at 75 days of age with or without RU486-mediated transgene induction from day 69 to day 75. Alphaproteobacteria, Bacilli, Gammaproteobacteria; n = 6 replicates of 5 flies.

(M) Reversal of bacterial levels assayed by taxon-specific qPCR of the 16S rRNA gene in *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 to day 10, when all induced flies become Smurfs, then removed from RU486-mediated induction for 7 more days until day 17, where flies were assayed to now be non-Smurfs. Gammaproteobacteria; n = 6 replicates of 5 flies per replicate.

(N) Maintenance of reversal of bacterial levels assayed by qPCR of the 16S rRNA gene with universal primers in *5966GS>UAS-ssk RNAi* female flies at 17 and 60 days of age with or without RU486-mediated transgene induction from day 3 to day 10, when all induced flies become Smurf, then removed from RU486-mediated induction for 7 more days until day 17, where flies were assayed to now be non-Smurfs, then were aged until day 60 on food lacking RU486. n = 6 replicates of 5 flies per replicate.

(O) Maintenance of reversal of bacterial levels assayed by taxon-specific qPCR of the 16S rRNA gene in *5966GS>UAS-ssk RNAi* female flies at 17 and 60 days of age with or without RU486-mediated transgene induction from day 3 to day 10, when all induced flies become Smurfs, then removed from RU486-mediated induction for 7 more days until day 17, where flies were assayed to now be non-Smurfs, then were aged until day 60 on food lacking RU486. Alphaproteobacteria, Bacilli, Gammaproteobacteria; n = 6 replicates of 5 flies per replicate.

(P and Q) Reversal of antimicrobial peptide and *Dual Oxidase* gene expression assayed by qPCR from dissected intestines of *5966GS>UAS-ssk RNAi* female flies at 9 days of age with or without RU486-mediated transgene induction from day 3 until day 10, when all of the induced flies become Smurfs, then moved to food lacking RU486 for 7 days and assayed on day 17. *Diptericin, Drosocin, Drosomycin, Metchnikowin;* n = 6 replicates of five intestines.

(R) Bacterial levels assayed by qPCR of the 16S rRNA gene with universal primers in Smurf and non-Smurf *5966GS>UAS-ssk RNAi* female flies at 14 days of age with or without RU486-mediated transgene induction from day 3 to day 14 under Axenic conditions, standard conditions, or after embryonic homogenate feeding. n = 6 replicates of 5 flies.

(S) Survival curves of *5966GS>UAS-ssk RNAi* females with or without antibiotics during RU486-mediated transgene induction from day 3 onwards. There is no significant difference, log rank test; n > 173 flies/condition. Median lifespan 17 and 17 days, respectively.

(T) FOXO target gene expression assayed by qPCR from dissected intestines in Axenic *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. *Insulin-like Receptor, InR*; Ecdysone-inducible gene L2, *ImpL2*; n = 6 replicates of five intestines.

(U and V) Representative images (U) and quantification (V) of posterior midgut diameters in mm from dissected intestines of Axenic *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. A significant increase in the midgut diameter is observed, $p < .0001$; two-tailed unpaired Student's t-test; n>14 midguts per condition; *scale bar* is 50 μm.

(W) *upd3* gene expression assayed by qPCR from dissected intestines in Axenic *5966GS>UAS-ssk RNAi* female flies on day 9 with or without RU486-mediated transgene induction from day 3 to day 9. n = 6 replicates of five intestines.

(X and Y) Antimicrobial peptide and *Dual Oxidase* gene expression assayed by qPCR from *5966GS>UAS-ssk RNAi* female flies grown under Axenic conditions on day 9 or day 14 with or without RU486-mediated transgene induction from day 3 to day 14. There is no significant difference. *Diptericin, Drosocin, Drosomycin;* n = 6 replicates of five intestines.

Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. Error bars on bar graphs depict mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Wilcoxon test unless otherwise stated.

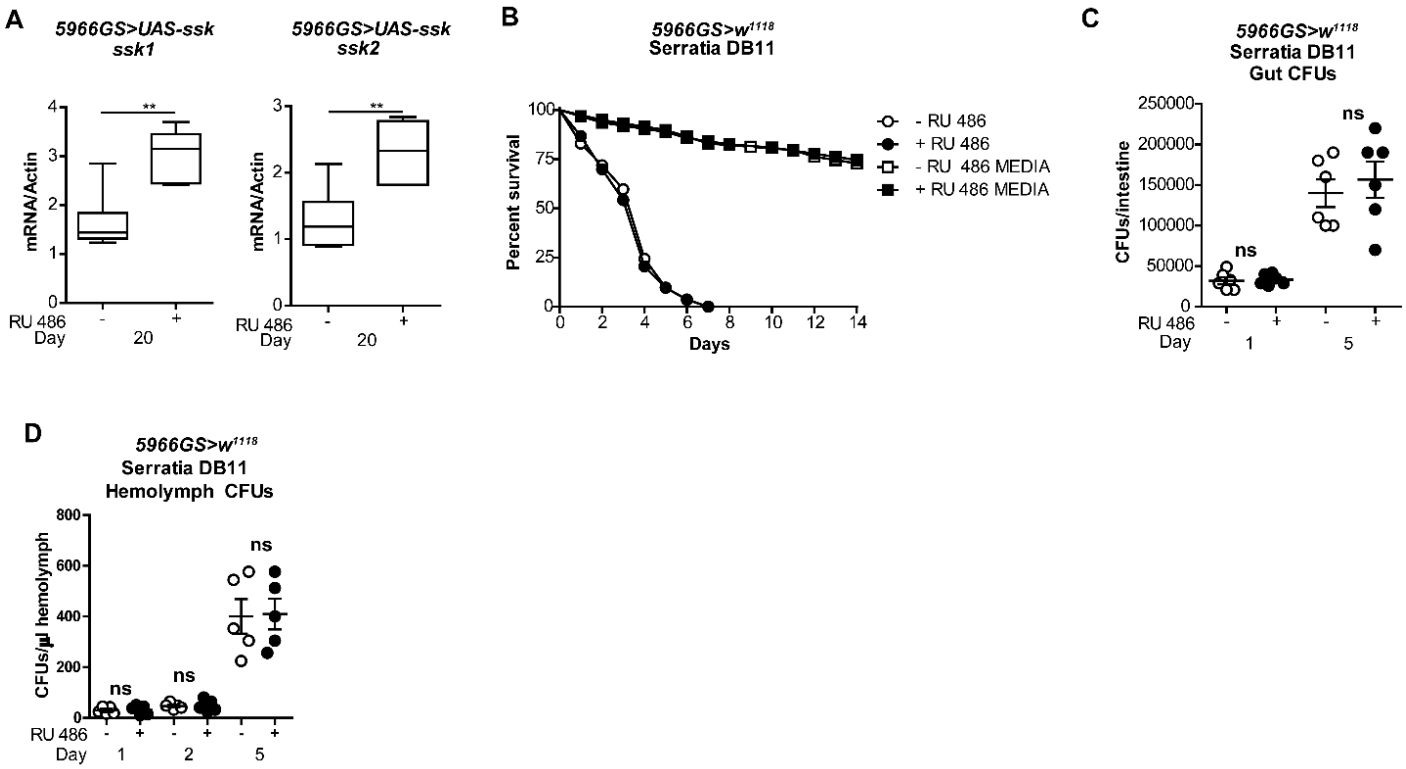

Figure S6. Intestinal Ssk reduces bacterial translocation and improves survival upon oral infection with pathogenic bacteria. Related to Figure 6

(A) *ssk* gene expression assayed by qPCR utilizing two different sets of primers: *ssk1* and ssk2, from dissected intestines of *5966GS > UAS-ssk* female flies at 20 days of age with or without RU486-mediated transgene induction from day 3 to day 20. Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. **p < 0.01; Wilcoxon test; n = 6 replicates of five intestines.

(B) Survival curves of *5966GS>w^{1118}* females with or without RU486 from day 3 until day 20, then Db11 bacteria were fed to the flies and survival plotted. There is no significant difference, log rank test; n > 82 flies/condition. Median survival 4 days.

(C) Colony-forming Units (CFUs) from dissected midguts of *5966GS>w^{1118}* females with or without RU486-mediated transgene induction from day 3 until day 20. Db11 bacteria were fed to the flies and the guts of infected flies were dissected and crushed, and dilutions of the extracts were plated on LB agar containing the appropriate antibiotics, on days specified, following Db11 infection. There is no significant difference; one-way ANOVA/Tukey's multiple comparisons test. Error bars depict mean ± SEM.

(D) Colony-forming Units (CFUs) from the hemolymph of *5966GS>w^{1118}* females with or without RU486-mediated transgene induction from day 3 until day 20. Db11 bacteria were fed to the flies and the hemolymph from infected flies were collected and plated on LB agar containing the appropriate antibiotics, on days specified, following Db11 infection. There is no significant difference; one-way ANOVA/Tukey's multiple comparisons test. Error bars depict mean ± SEM.

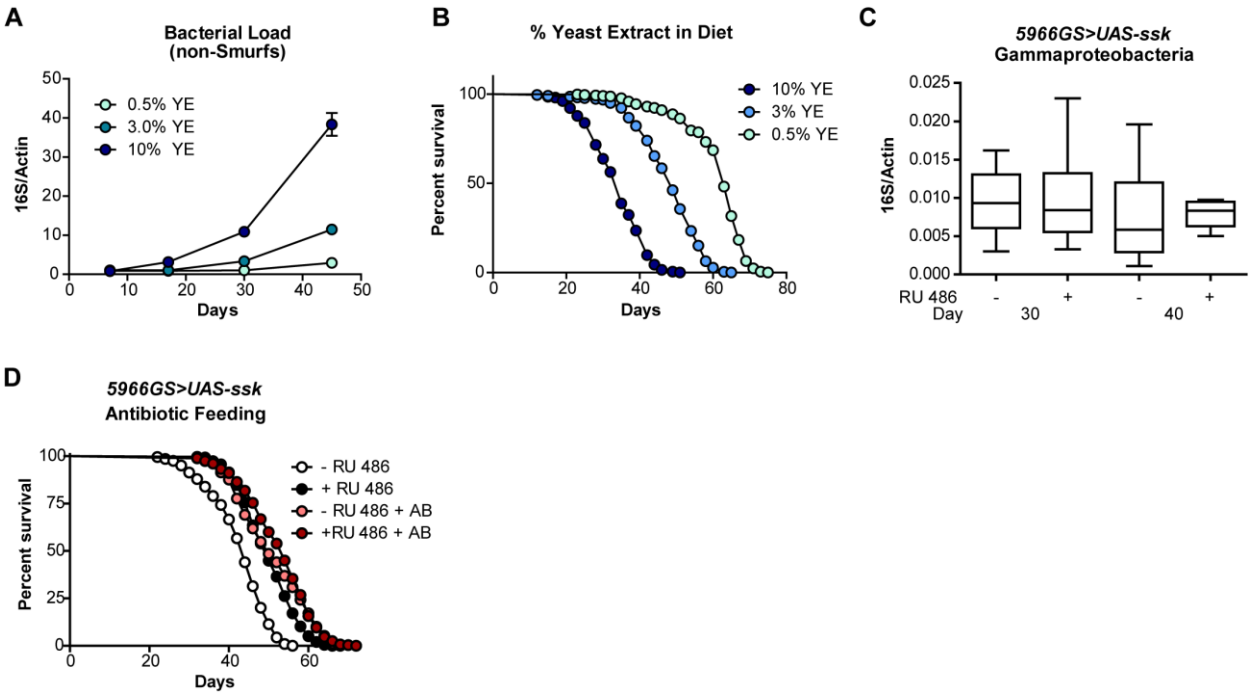

Figure S7. Ssk improves intestinal integrity during aging and prolongs lifespan. Related to Figure 7

A) Bacterial levels assayed by qPCR of the 16S rRNA gene in *Canton S* female flies at 7, 17, 30 and 45 days of age on media containing .5%, 3% or 10% yeast extract. $p < .001$ at day 45 between 10% YE and the other two YE concentrations; one-way ANOVA/Tukey's multiple comparisons test; n = 3 replicates of 30 flies.

(B) Survival curves of *Canton S* females on media containing .5%, 3% or 10% yeast extract. $p < .0001$ between each YE concentration, log rank test; n > 204 flies/condition. Median lifespan 35, 49, and 63 days, respectively.

(C) Bacterial levels assayed by taxon-specific qPCR of the 16S Gammaproteobacteria rRNA gene in *5966GS>UAS-ssk* female flies at 30 and 40 days of age with or without RU486-mediated transgene induction from day 3 onward on rich media. n = 6 replicates of 5 flies.

(D) Survival curves of *5966GS>UAS-ssk* females with or without RU486-mediated transgene induction with or without antibiotics from day 3 onwards on rich media. $p < .0001$, log rank test; n > 268 flies/condition. Median lifespan 44, 50, 50, and 54 days, respectively.

Boxplots display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box. Wilcoxon test unless otherwise stated.

Transparent Methods

Fly culture and Lifespan
Genotypes used were the standard laboratory strain *w^{1118}*, *5966 GeneSwitch* and *Su(H)lacZ; esg:GFP,5966GAL4^{GS}*, and *UAS-ssk* and *UAS-ssk RNAi* provided by M Furuse. We also obtained a second RNAi-mediated knock down line from the VDRC: *UAS-ssk RNAi* (11906GD). Flies were cultured in a humidified, temperature-controlled incubator with 12h on/off light cycle at 25 °C, in vials containing standard cornmeal medium (1% agar, 3% brewer's yeast, 1.9% sucrose, 3.8% dextrose and 9.1% cornmeal; all concentrations given in wt/vol). Overexpression studies were also carried out using an additional diet (1% agar, 3% yeast extract, 1.9% sucrose, 3.8% dextrose and 9.1% cornmeal; all concentrations given in wt/vol), designated as rich media in figures because of the higher nutritional content. Three concentrations of yeast extract were utilized to compare changes in bacterial load with diet: (1% agar; .5%, 3%, or 10% yeast extract; 1.9% sucrose; 3.8% dextrose; and 9.1% cornmeal; all concentrations given in wt/vol). Adult animals were collected under light nitrogen induced anesthesia, housed at a density of 27-32 flies per vial and flipped to fresh vials and scored for death every 2-3 days throughout adult life. RU486 (Cayman Chemical Company) was dissolved in ethanol and mixed into the media when preparing food vials.

RU486 doses used were 25 ug/ml final concentration and control food had ethanol alone, the volume of ethanol in each case was kept the same. Antibiotic treatment was conducted as described previously (Brummel et al., 2004). In every experiment, regardless of the conditions used, control and experimental animals are always transferred to fresh food at the same time-points. This provides an important control for bacterial growth in the food throughout these experiments.

Smurf and Smurf Reversal Assays
The Smurf assay was conducted as previously described (Rera et al., 2012), except that flies were kept on the blue food for a 24 hour period before being scored. New Smurfs were collected and removed from the blue dye. For reversal assays, after 7 days, flies were once again placed in vials with blue dye for 24 hours and the number of Smurf flies determined again.

Generation of axenic and re-associated flies
To generate axenic (germ-free) flies, embryos were treated by bleach and ethanol as described previously (Brummel et al., 2004). Briefly, <12-h-old embryos were dechorionated in 3% sodium hypochlorite (50% v/v regular bleach) for 20 min, rinsed in 70% ethanol for 5 min, and then washed three times with 1× PBS + 0.01% Triton X-100. Axenic embryos were transferred to autoclaved medium (50 embryos/vial) in a laminar flow cabinet. Axenic conditions were confirmed by quantitative PCR of the 16S rRNA gene from whole fly samples (see below) or plating the fly homogenate on MRS agar. To generate flies associated with microbes as embryos, whole fly homogenate (10 fly equivalent: 600 μL of conventionally reared fly homogenate glycerol stock/bottle) was added to medium containing axenic embryos.

Preparation of fly homogenate for re-association
Conventionally reared adult flies were surface sterilized by 70% ethanol prior to homogenization to ensure only internal microbes were present in the homogenate. Surface sterile flies were homogenized with a motor pestle in 1.5mL tube with 200 μL of sterile PBS (50 flies/tube). Homogenates were then pooled and sterile PBS added to adjust to one fly equivalent in 50 μL PBS. For storage 1/5 volume of 80% sterile glycerol was added and aliquots were stored at -80º C until use.

Starvation Resistance
Starvation assays were performed with mated female UAS-ssk RNAi knockdown flies crossed to 5966 Geneswitch-Gal4. 3-day-old adult flies were fed on vials containing 25 ug/mL of RU486 for 5 days at a density of 27-32 flies per vial. For agar starvation, flies were maintained on water only medium (1% agar in ddH2O wt/wt) that did not contain RU486 in a 25°C incubator with 12-hour light-dark cycles. Survival was scored multiple times per day.

Triacylglyceride Assay
Lipids were extracted from whole flies in a chloroform:methanol:water solution (2:5:1 by volumes) and nonpolar lipids were separated on thin layer chromatography plates (Analtech, Newark, DE, USA) with a n-hexane:diethylether:glacial acetic acid solution (70:30:1 by volumes). Plates were air-dried, stained (0.2% Amido

Black 10B in 1M NaCl), and lipid bands were quantified by photo densitometry using ImageJ as described (Rera et al., 2011).

Fluorescence Microscopy and Antibody Staining
Mated female flies of the appropriate age and background were sorted for Smurf status. Posterior midguts were dissected into ice-cold PBS/4% formaldehyde and incubated for 1hr in fixative at room temperature. Samples were then washed three times, for 10 min each, in PBT (PBS containing 0.5%Triton X-100), 10 min in Na-deoxycholate (0.3%) in PBT (PBS with 0.3%Triton X-100), and incubated in block (PBT-0.5% bovine serum albumin) for 30 min. Samples were immunostained with primary antibodies overnight at 4°C, washed 4 × 5 min at RT in PBT, incubated with secondary antibodies at RT for 2 h, washed three times with PBT and mounted in Vecta-Shield/DAPI (Vector Laboratories, H-1200).

The following antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Discs large (mouse, 1:20, 4F3) and Coracle (mouse, 1:20, C615.16). GFP (rabbit, 1:3,000, Molecular Probes A-11122); GFP (mouse, 1:200, Molecular Probes A-11120); GFP (chicken, 1:500, Aves Labs GFP-1010); Phospho-histone3 (rabbit, 1:200, Millipore 06-570). Snakeskin (rabbit, 1:1000) and Mesh (rabbit, 1:1000) (gifts from M. Furuse).

Images were acquired on a Zeiss LSM710 inverted confocal microscope, and on a Zeiss Axio Observer Z1 and processed with Fiji/ImageJ and Zen from Zeiss.

Septate Junction Fluorescence Quantification
To measure and quantify the possible differences, SJ fluorescence intensity in posterior midgut ECs, ×100 + ×3 of digital magnification confocal *z*-stack maximum projections at the level of the SJ were generated using Zen 2 pro Blue software edition (Zeiss). SJ fluorescence intensity were measured using a mask of 25.5 pixels in diameter. Then cytoplasm fluorescence intensity was calculated using the same mask. Average fluorescence intensity at the membrane was divided by the cytoplasmic average intensity. Between 3 to 7 measurements were taken per picture and a minimum of 20 posterior midguts were analysed per experimental condition.

Gut Diameter Calculation
To measure variations in gut diameter single z-stack images were taken at 20x magnification in a Zeiss Axio Observer Z1 microscope, and measured using Zen 2 pro Blue software edition (Zeiss). Diameter was calculated for each midgut at the same distance from the pylorus.

Cell Division Quantification Using CellProfiler
For statistical significance four images were taken as *z*-stacks with a typical slice thickness of 750 nm per posterior midgut; two on each side (top and bottom); from contiguous field of view (fov), starting at 1 fov from the pylorus (using a minimum of 20 guts). The images were then processed using the CellProfiler pipeline (Carpenter et al., 2006) developed in the Jones laboratory. ISC number and mitotic events were obtained from esg:GFP/total cell and PH3/total cell ratios respectively. Average ratios from the four images corresponding to a single gut were used in subsequent statistical analyses.

Genomic DNA Isolation to Assess Bacterial Levels
The reported effects of husbandry practices on *Drosophila* bacterial loads (Blum et al., 2013) were controlled for by keeping bacterial sample collection consistent relative to new food transfer and by the collection of non-Smurf controls from the same vials as Smurf individuals. Genomic DNA was extracted using the PowerSoil DNA isolation kit (MoBio). All flies were surface sterilized prior to sample preparation. To ensure consistent homogenization, whole fly samples were pre-homogenized in 150µL of solution from the PowerSoil bead tube using a motor pestle. This homogenate was then returned to the bead tube and the manufacturers protocol was followed. For intestinal samples, flies were surface sterilized in small groups and then dissected over ice, in sterile PBS and with sterile equipment. The dissection surface was swabbed with 75% ethanol between each sample. Intestinal dissections included all but the anterior foregut, from the point at which the crop diverges, and including the crop, to the rectal papilla. Care was taken to keep the full length of the gut intact to prevent loss of lumen contents. Dissected intestines were stored in sterile eppendorf tubes at -80ºC prior to DNA extraction and were then prehomogenized as described above.

Quantitative PCR
DNA samples for qPCR of the 16S ribosomal RNA gene were prepared as described above.
RNA extractions, for gene expression analysis, were carried out in TRIzol (Invitrogen) following the manufacturer's directions. Intestinal dissections for RNA extraction were as described for DNA isolation but without the sterilization steps. cDNA synthesis was carried out using the First Strand cDNA Synthesis Kit from Fermentas. PCR was performed with Power SYBR Green master mix (Applied Biosystems) on an Applied Biosystems 7300 Real Time PCR system. Cycling conditions were as follows: 95ºC for10 minutes; 95ºC for 15s then 60ºC for 60s, cycled 40 times. All calculated gene expression values were normalized to the value of the loading control gene, Actin5C.

The primer sequences used to assess gene expression in this study were as follows:
Act5C_L –TTGTCTGGGCAAGAGGATCAG, Act5C_ R – ACCACTCGCACTTGCACTTTC;
Dro_L –CCATCGAGGATCACCTGACT, Dro_R – CTTTAGGCGGGCAGAATG;
Drs_L –GTACTTGTTCGCCCTCTTCG, Drs_R – CTTGCACACACGACGACAG;
Dpt_L –ACCGCAGTACCCACTCAATC, Dpt_R – CCCAAGTGCTGTCCATATCC;
Mtk_L –TCTTGGAGCGATTTTTCTGG, Mtk_R – TCTGCCAGCACTGATGTAGC;
Duox_L –GGGAGTCTTATGGACTGAAAC, Duox_ R – GTACGCCTCCTTCAGCATGT;
upd3_L –GCAAGAAACGCCAAAGGA, upd3_R – CTTGTCCGCATTGGTGGT;
DEcad_L –GACGAATCCATGTCGGAAAA, DEcad_R – TCACTGGCGCTGATAGTCAT;
delta_L -AGTGGGGTGGGTGTAGCTTT, delta_R – GCTGTTGCTGCCAGTTTTG;
Notch_L –GAATTTGCCAAACACCGTTC, Notch_R – ACCGACACTTGTGCAGGAA;
pck_L –GCTCTCGCTTACCATCATCC, pck_R – TACGGCCAAAAACATGAACA;
Kune_L –AGGTTGTGGGCTCTGTTTTC, Kune_R – ATCCCGAGAATCTCCTTTGG;
sinu_L –CATTGAATTGCATAAACTTCAGCTA, sinu_R – GCGGAGTTTCGCTTACCTT;
pyd_L –TGAATCGAGAGGCAACTTCTT, pyd_R – TTCTCGCGGGACAGACTC;
dlg1_L –AGAGTCGCGATGAGAAGAATG, dlg1_R – GCTGGTGCTGCTCACAACT.
Mesh_L–AGCCCGATCAATACTCAGGA, Mesh_R – CCATATACCAGGCCAGAGGA
Ssk1_L– CACTGGATGCCACACCATT, Ssk1_R–TGGTGTCGCACAGCTCTC
Ssk2_L–TCAAGGCCCTGAAGCTGA, Ssk2_R–GCTCTTCTCCTCGTTTAAGTTCC
InR_L–GCACCATTATAACCGGAACC, InR_R–TTAATTCATCCATGAGGTGAG
Imp2L_L–GCCGATACCTTCGTGTATCC, Imp2L_R– TTTCCGTCGTCAATCCAATAG

Universal primers for the 16S ribosomal RNA gene were against variable regions 1 (V1F) and 2 (V2R) (Claesson et al., 2010), as previously published (Clark et al., 2015). Taxon-specific 16S primers (Clark et al., 2015) were as follows:
Bacilli_F – CGACCTGAGAGGGTAATCGGC, Bacilli_R – GTAGTTAGCCGTGGCTTTCTGG;
Alpha_F – CCAGGGCTTGAATGTAGAGGC, Alpha_R – CCTTGCGGTTCGCTCACCGGC;
Gamma_F – GGTAGCTAATACCGCATAACG, Gamma_ R – TCTCAGTTCCAGTGTGGCTGG.

Intestinal Infection
Oral infection experiments were performed similarly to (Nehme et al., 2007) with some modifications. *Serratia marcescens* strain DB11 was grown in BHI (brain heart infusion) medium containing 100µg/ml of streptomycin at 37˚C to an optical density (OD) at 600nm of 1. Bacterial cultures were centrifuged and resuspended at a final concentration of OD600 0.3 in sterile *Drosophila* infection medium (3% yeast extract, 5% sucrose, and either 25µg/ml of RU486 or the same volume of ethanol vehicle for controls). Approximately 25 *Drosophila* of indicated age were placed in sterile vials containing Kim Wipes © saturated with bacterial solution, or infection medium only controls. Infections were maintained in an incubator at 29˚C at 60% humidity in a 12 hour Light:Dark cycle. Vials were scored for death daily, and vials were changed every two days.

Colony Forming Units Assay (CFUS)
Orally infected *Drosophila* of indicated age and time of infection were anesthetized by cold shock 4˚C. Flies were then washed three times in 70% ethanol. Followed by two washes in sterile PBS to remove bacteria present on the cuticle. For hemolymph CFUS, head hemolymph was extracted from flies using a 10µl pipette tip. Approximately 10 heads of the indicated condition were used per replicate to yield approximate 2 µl of hemolymph. Hemolymph was then serial diluted in PBS + 0.5% EDTA and plated on Luria Broth (LB) plates containing 100µg/ml of streptomycin. Plates were grown overnight at 37˚C and colonies were counted. For intestinal CFUS, five intestines

from flies of indicated condition were dissected in sterile PBS. Intestines lysates were ground up using a plastic pestle then serial diluted in sterile PBS and plated in Luria Broth (LB) plates containing 100µg/ml of streptomycin, followed by overnight growth and colony counting.

Electron Microscopy

Standard procedures for electron microscopic (EM) analysis were carried out as described in (Walker et al., 2006) with slight modifications. Dissected guts were fixed in 2% glutaraldehyde and 2% formaldehyde in 0.1 M sodium phosphate buffer (PB) containing 0.9% NaCl overnight at 4 °C. Guts were then post-fixed in 1% osmium tetroxide in PB, treated with 0.5% uranyl acetate, and dehydrated through a graded series of ethanol concentrations. After infiltration with Eponate 12 resin, the samples were embedded in fresh Eponate and polymerized overnight. Semi-thin sections (1.5 µm) were cut on an ultramicrotome and stained with toluidine blue. The midgut area of interest was identified from these sections. For EM, sections of 50 nm thickness were prepared from the identified area, placed on formvar coated copper grids and stained with uranyl acetate and Reynolds' lead citrate. The grids were examined using a JEOL 100CX transmission electron microscope at 60 kV (Electron Microscopy Facility, UCLA Brain Research Institute).

Statistics

The comparison of survival curves was done using the log-rank test as implemented in the Graphpad Prism software. Comparison of Smurf proportions per time point were carried out using one-way ANOVA/Bonferroni's multiple comparisons test with the error bars representing the standard error of the mean (SEM). SJ/cytoplasm fluorescence ratios for different SJ components were done using a Student's t-test with the error bars representing the SEM. The comparisons of gut diameters and infection CFUs analyzed with one-way ANOVA/Tukey's multiple comparisons test with the error bars representing the SEM range of those averages. All other data comparisons were tested for significant differences using the Wilcoxon-Mann-Whitney U test where sample sizes were greater than five, and a Student's t-test where sample sizes were fewer than five. The number of biological replicate samples is given in each figure legend. All statistical tests were implemented in Graph Pad Prism. All statistical tests are two-sided.

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