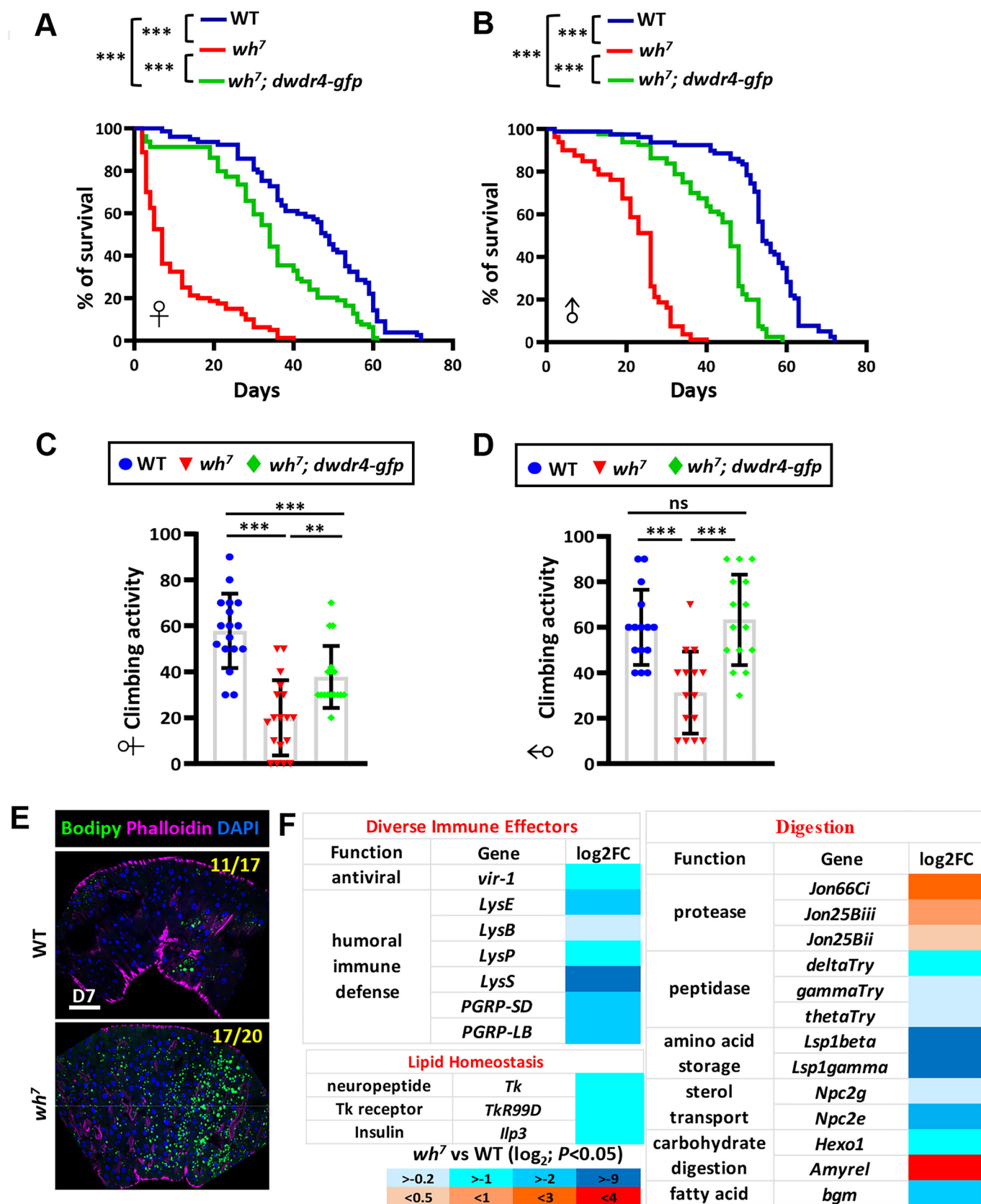


Expanded View Figures

Figure EV1. Loss of dWdr4 shortens lifespan, impairs locomotor activity, and disrupts gut function in flies.

(A, B) *Drosophila* *dwdr4* mutant (*wh⁷*) female (A) and male flies (B) show shortened lifespans compared to wild-type (WT) controls. Lifespan defects were rescued by introducing a copy of *dwdr4-gfp* ($N = 80$ flies). (C, D) Climbing assays reveal reduced locomotor activity in *wh⁷* mutant female (C) and male flies (D) compared to WT control. Climbing performance was partially improved in females ($N = 17$ flies) and completely rescued in males ($N = 15$ flies) by *dwdr4-gfp* expression. (E) dWdr4 loss causes the accumulation of lipid droplets. WT ($N = 7$) and *wh⁷* mutant ($N = 9$) guts with Bodipy (green, oil droplets), Phalloidin (magenta, F-actin), and DAPI (blue, nuclei). The fraction of gut sections with the pattern shown is indicated. (F) Expression differences in genes involved in lipid metabolism and gut digestion between the *wh⁷* and WT female guts. Fold change of $\log_2$ values ($P < 0.05$) is indicated by the color indicator. Each dot in (C, D) represents one fly. Scale bar in (E) is 50 μm . Bars represent the mean; error bars indicate standard deviation (SD). Statistically significant differences in (A, B) were identified by the Log-rank test, and in (C, D) were identified by the unpaired Student's *t* test. Statistical analysis in (F) was performed on two biological replicates. Lifespan analysis in (A, B) showed significant differences between WT and *wh⁷*, *wh⁷* and *wh⁷;dwdr4-gfp*, and between WT and *wh⁷;dwdr4-gfp* ($***P = < 0.0001$). Climbing assay in (C, D) showed significant differences between WT and *wh⁷* ($***P = < 0.0001$), *wh⁷* and *wh⁷;dwdr4-gfp* ($**P = 0.0014$), and between WT and *wh⁷;dwdr4-gfp* ($***P = 0.0004$) in females, but in males, no differences were observed between WT and *wh⁷;dwdr4-gfp* (ns, $P = 0.621$). *N*, number of midguts, unless otherwise stated. Source data are available online for this figure.



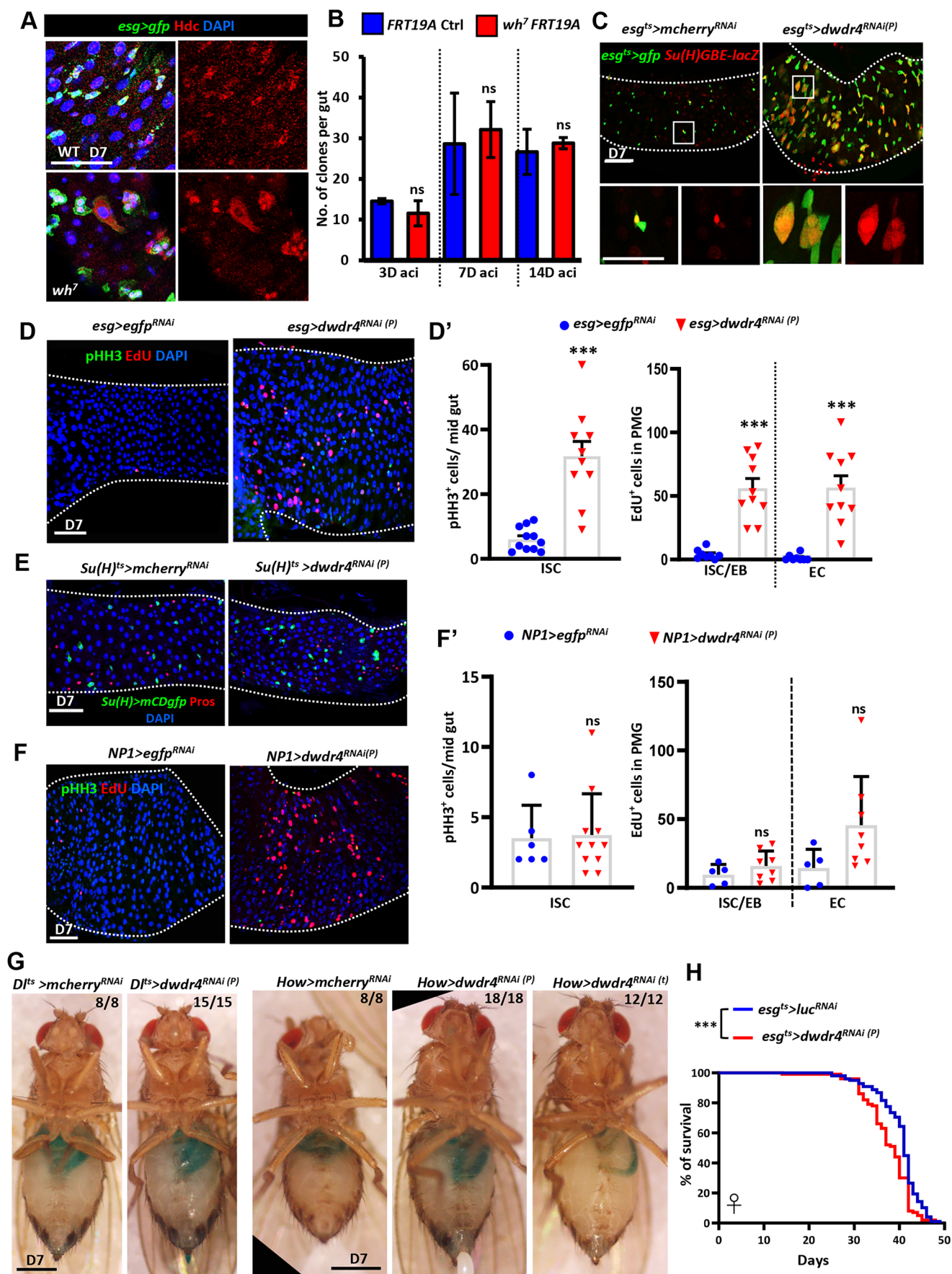


Figure EV2. Loss of *dWdr4* in ISCs promotes their proliferation and shortens lifespan but does not compromise intestinal epithelial integrity.

(A) Posterior midguts (PMGs) from wild-type (WT) and *dwd4* mutant (*wh⁷*) females expressing *esg>gfp* (green, ISCs, EBs), stained for Hdc (red, ISCs/EBs) and DAPI (blue, nuclei). In the WT control PMG ($N = 10$), the ISC-EB two-cell cluster has both cells expressing Hdc, whereas in the *dwd4* mutant (*wh⁷*) PMG ($N = 13$), Hdc expression was seen in all *esg>gfp* cell clusters as well as in misdifferentiated ECs. (B) Number (no.) of clones per midgut in *FRT19A* control (ctrl) (3D: $N = 27$, 7D: $N = 30$, 14D: $N = 34$) and *wh⁷FRT19A* heterozygous mutant guts (3D: $N = 29$, 7D: $N = 28$, 14D: $N = 28$) at 3, 7, and 14 days after clone induction (aci). (C) *esg^{ts}>dwd4^{RNAi(P)}* PMG ($N = 7$) shows expansion of ISCs/EBs (green, labeled with *esg^{ts}>gfp*) with some ISCs/EBs also expressing *Su(H)-lacZ* (red, a Notch signaling reporter used as an EB marker). In the *esg^{ts}>mcherry^{RNAi}* control PMG ($N = 5$), the ISC-EB two-cell cluster has only one *Su(H)-lacZ*-positive cell. Enlarged views of the white boxed regions are shown below. (D, D') *esg>dwd4^{RNAi(P)}* PMG ($N = 10$) shows increased mitotic ISCs (PHH3⁺, green), proliferating ISCs/EBs (EdU⁺ with the small nuclei, red), and endoreplicating ECs (EdU⁺ with the large nucleus, red), as compared to the control (*esg>egfp^{RNAi}*) ($N = 8$). (D') Number of PHH3⁺ ISCs per midgut (left) and number of EdU⁺ ISCs/EBs and ECs per PMG (right) for the indicated genotypes. (E) Knockdown of *dwd4* in EBs (*Su(H)^{ts}>dwd4^{RNAi(P)}*) ($N = 22$) causes no obvious change in the EB population (*Su(H)^{ts}>mcd8gfp*, green) compared to *Su(H)^{ts}>mcherry^{RNAi}* control PMGs ($N = 20$). Pros, red, pEEs/EEs. (F, F') Knockdown of *dwd4* in ECs (*NPI>dwd4^{RNAi(P)}*) ($N = 11$) does not increase mitotic ISCs (PHH3⁺, green) or proliferating ISCs/EBs (EdU⁺) compared to the control (*NPI>egfp^{RNAi}*) ($N = 6$). Still, it slightly increases endoreplicating ECs (EdU⁺ with the large nucleus, red) compared to the control. DAPI, blue. (F') Number of PHH3⁺ ISCs per midgut (left) and number of EdU⁺ ISCs/EBs and ECs per PMG (right) for the indicated genotypes. (G) *D¹¹⁸>dwd4^{RNAi(P)}*, *how>dwd4^{RNAi(P)}*, and *how>dwd4^{RNAi(1)}* female flies do not show the leaky gut phenotype, as revealed by the smurf assay. Fractions of flies with the pattern shown are indicated. (H) Female flies carrying *esg^{ts}>dwd4^{RNAi(P)}* have a shortened lifespan compared to control (*esg^{ts}>LucRNAi*) ($N = 100$ flies). Each dot in (D', F') represents one midgut. The scale bar in (A) and enlarged views of (C) are shown at 25 μ m; (C, D, E, F) are 50 μ m, and in (G) is 0.5 mm. White dashed lines in (C, D, E, F) outline the edge of the gut. Bars represent the mean; error bars indicate standard deviation (SD). Statistically significant differences in (B) were identified by the unpaired Student's *t* test, in (D', F') by the non-parametric Mann-Whitney *U* test, and in H by the Log-rank test. (B) No differences were observed between *FRT19A* ctrl and *wh⁷ FRT19A* in clones per gut after 3D aci (ns, $P = 0.23$), 7D aci (ns, $P = 0.69$), and 14D aci (ns, $P = 0.57$). (D') PHH3⁺ cells were significantly increased in *esg>dwd4^{RNAi(P)}* compared with *esg>egfp^{RNAi}* ($***P = < 0.0001$); EdU⁺ ISC/EB ($***P = < 0.0001$) and EC cells ($***P = < 0.0001$) showed significant increase. (F') no differences in PHH3⁺ cells were observed in *NPI>dwd4^{RNAi(P)}* compared with *NPI>egfp^{RNAi}* (ns, $P = 0.9$); EdU⁺ ISC/EB (ns, $P = 0.3$) showed no difference; whereas in EC cells (ns, $P = 0.6$) increased slightly. Lifespan analysis in (H) showed a significant reduction in *esg^{ts}>dwd4^{RNAi(P)}* compared with *esg>egfp^{RNAi}* ($***P = 0.0003$). *N*, number of midguts; unless otherwise stated. Source data are available online for this figure.

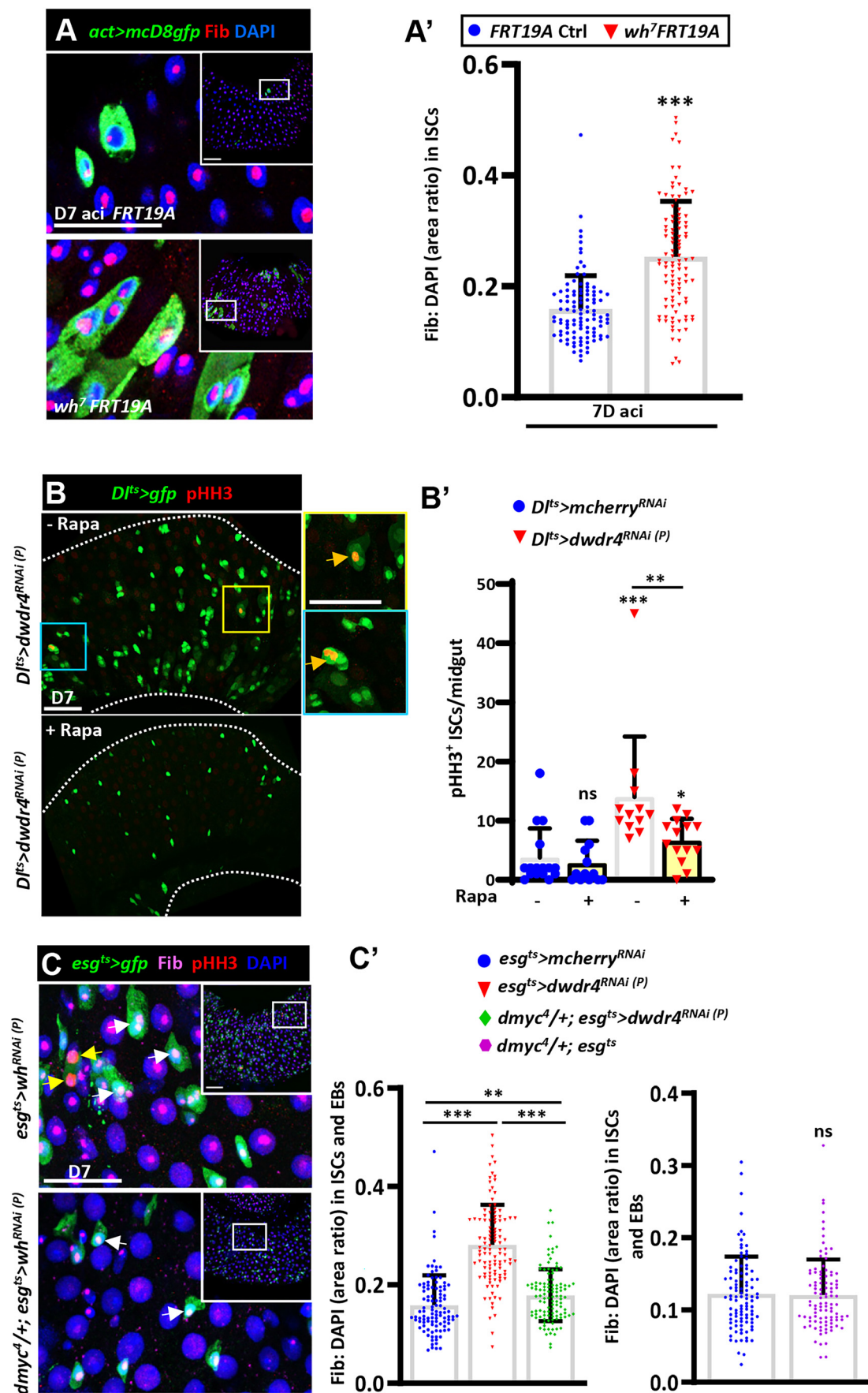


Figure EV3. Wdr4 regulates ISC proliferation via TOR and dMyc.

(A, A') dWdr4 cell-autonomously controls ribosome biogenesis in ISCs/EBs. (A) Mosaic *FRT19A* control ($N = 11$) and *wh⁷FRT19A* heterozygous mutant posterior midguts (PMGs) ($N = 10$) ($n = 103$ ISC/EB cells) with *act>mCD8gfp* (green, mutant cells), Fibrillarin (Fib) (red, nucleoli), and DAPI (blue, nuclei) labels. (A') Quantification of nucleolar (Fib) to nuclear (DAPI) area ratio in GFP-positive cells with small nuclei in the indicated genotypes, at 7 days after clone induction (*aci*). (B, B') Rapamycin treatment suppresses ISC expansion and overproliferation caused by dWdr4 depletion. (B) PMGs from *Dl^{ts}>dwd4^{RNAi(P)}* flies expressing *Dl^{ts}>gfp* (green, ISCs) with or without rapamycin (*rapa*) treatment, stained for PHH3 (red, mitosis marker). Enlarged views of the boxed regions are shown on the right. (B') Quantification of PHH3⁺ ISCs per midgut for the *Dl^{ts}>dwd4^{RNAi(P)}* (dH_2O : $N = 12$ midguts; *Rapa*: $N = 14$) and *Dl^{ts}>mcherry^{RNAi}* (dH_2O : $N = 15$; *Rapa*: $N = 13$). (C, C') Removal of a copy of dMyc suppresses elevated ribosome biogenesis induced by dWdr4 depletion. (C) PMGs from *esg^{ts}>dwd4^{RNAi(P)}* and *dmyc^d/+*; *esg^{ts}>dwd4^{RNAi(P)}* females bearing *esg^{ts}>gfp* (green, ISCs/EBs), stained for PHH3 (red, mitosis marker) and Fibrillarin (Fib) (magenta, nucleoli) and DAPI (blue, nuclei). These images are also shown in Fig. 5F. (C') Quantification of nucleolar (Fib) to nuclear (DAPI) area ratio in ISCs/EBs in the *esg^{ts}>mcherry^{RNAi(P)}* ($N = 14$), *esg^{ts}>dwd4^{RNAi(P)}* ($N = 8$), *esg^{ts}>dwd4^{RNAi(P)}* with *dmyc^d* mutant ($N = 10$), and *dmyc^d* mutant alone ($N = 16$) (111 ISC/EB cells) flies. Yellow arrows in (B, C) indicate representative mitotic ISCs, and white arrows mark representative ISC or EB nucleoli. Insets in (B) show enlargements of boxed regions; (A, C) show enlargements of boxed regions within insets. Each dot in (A', C') represents a single ISC/EBs, and each dot in (B') represents one midgut. The low magnification and enlarged views of (A, C) are shown at 50 and 25 μ m; (B) is 50 μ m. White dashed lines in (B) outline the edge of the gut. Bars represent the mean; error bars indicate standard deviation (SD). Statistically significant differences in (A', B', C') were identified by the non-parametric Mann-Whitney *U* test. (A') The nucleolar size differed in *FRT19A* and *wh⁷FRT19A* ($***P = < 0.0001$). (B') PHH3⁺ cells were unchanged by rapamycin in *Dl^{ts}>mcherry^{RNAi}* (ns, $P = 0.32$), but differed between *Dl^{ts}>mcherry^{RNAi}* and *Dl^{ts}>dwd4^{RNAi(P)}* with ($*P = 0.03$) and without rapamycin ($***P = < 0.0001$); in *Dl^{ts}>dwd4^{RNAi(P)}*, rapamycin significantly reduced PHH3⁺ cells ($**P = 0.002$). (C', left graph) The nucleolar size showed differences in *esg^{ts}>dwd4^{RNAi(P)}* compared to *esg^{ts}>mcherry^{RNAi(P)}* without *dmyc^d* ($***P = < 0.0001$) and with *dmyc^d* ($**P = 0.0012$); nucleolar size differed significantly between *esg^{ts}>dwd4^{RNAi(P)}* with and without *dmyc^d* ($***P = < 0.0001$); whereas *dmyc^d* alone had no effects (ns, $P = 0.75$) (right graph). *N*, number of midguts; *n*, number of ISCs or ISC/EBs analyzed, unless otherwise stated. Source data are available online for this figure.

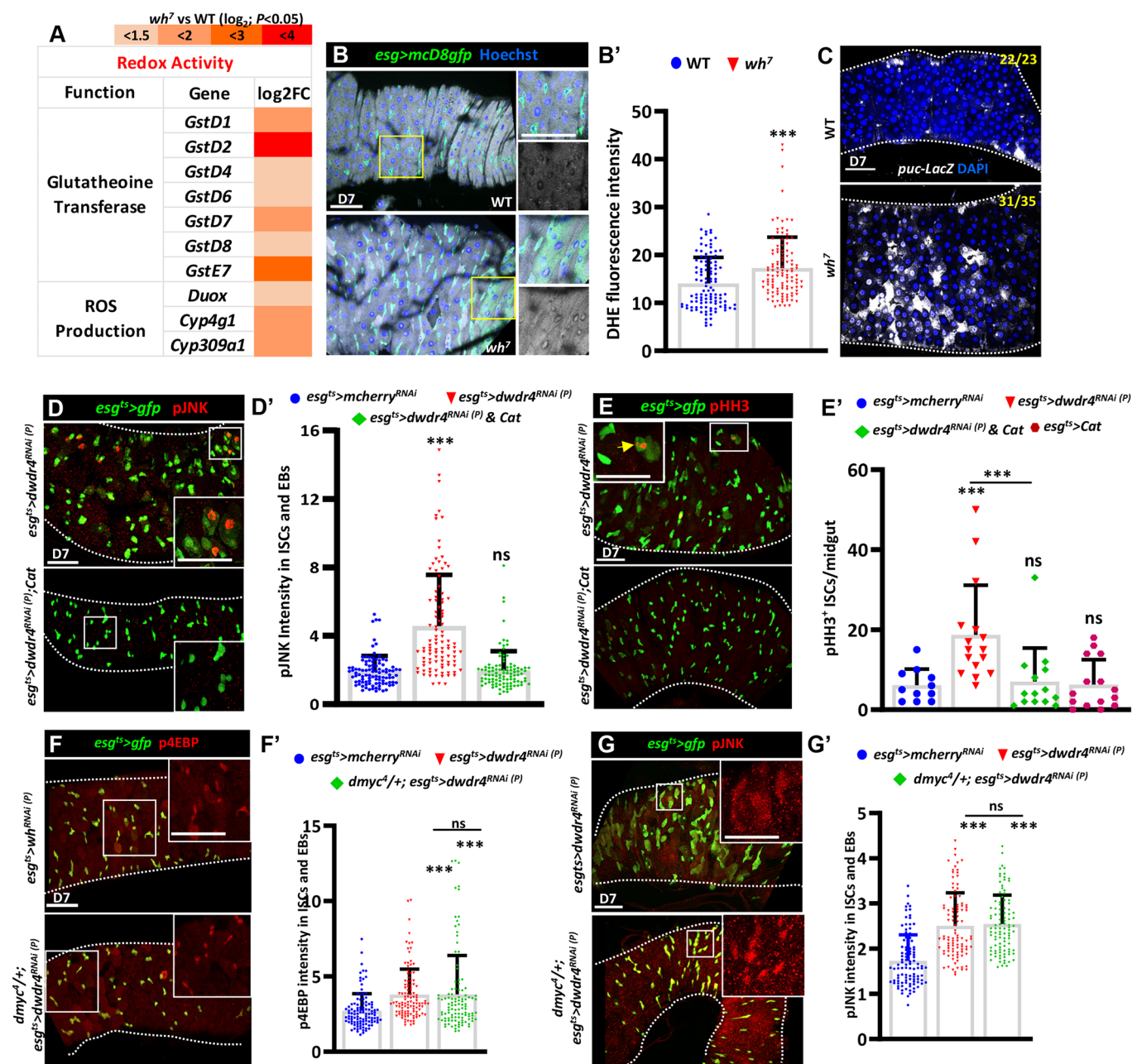


Figure EV4. Loss of Wdr4 in the gut increases expression of genes involved in stress and redox responses as well as JNK signaling.

(A) RNA-seq analysis results show that the expression levels of genes involved in redox activity and stress are increased in the guts of *dwdr4* (*wh⁷*) mutant female guts. Fold change of $\log_2$ values ($P < 0.05$) is indicated by color. Statistical analysis was performed on two biological replicates. (B, B') *wh⁷* mutant PMG bearing *esg>mCD8gfp* ($N = 15$) (green, ISCs/EBs) show increased ROS levels compared to WT controls ($N = 16$), ($n = 110$ ISC/EB cells), as detected by DHE staining (gray). (B') Quantification of DHE fluorescence intensity. DAPI (blue) label nuclei. (C) *wh⁷* mutant PMG shows increased JNK signaling compared to controls ($N = 7$), as revealed by *puc-lacZ* (gray, a JNK signaling reporter). DAPI, blue. Fractions of gut sections with the shown pattern are indicated. (D, E') Overexpression of Catalases (Cat) suppresses elevated pJNK and ISC expansion and overproliferation induced by *dWdr4* depletion. (D) PMGs from the indicated genotypes bearing *esg^{ts}>gfp* (green, ISCs/EBs), stained for pJNK (Red, JNK signaling activity). (D') Quantification of pJNK intensity in the *esg^{ts}>mcherry^{RNAi(P)}* ($N = 11$), *esg^{ts}>dwdr4^{RNAi(P)}* ($N = 16$), and *esg^{ts}>dwdr4^{RNAi(P)}* with *cat* overexpression ($N = 16$) ($n = 100$ ISC/EB cells) flies. (E) PMGs from the indicated genotypes bearing *esg^{ts}>gfp* (green), stained for PHH3 (red, mitotic marker). (E') Quantification of PHH3⁺ ISCs per midgut for the *esg^{ts}>mcherry^{RNAi(P)}* ($N = 11$), *esg^{ts}>dwdr4^{RNAi(P)}* ($N = 16$), *esg^{ts}>dwdr4^{RNAi(P)}* with *cat^{OE}* ($N = 14$), and *cat^{OE}* alone ($N = 15$) flies. (F, G) dMyc acts downstream of TOR and JNK signaling in ISCs for ISC homeostasis. (F, F') Loss of one *dmyc* copy in *esg^{ts}>dwdr4^{RNAi(P)}* posterior midguts (PMGs) fails to suppress ISC/EB expansion (*esg^{ts}>gfp*, green) or the increased TOR signaling (Red, TOR signaling activity). (F') Quantification of average p4EBP intensity in the *esg^{ts}>mcherry^{RNAi(P)}* ($N = 11$), *esg^{ts}>dwdr4^{RNAi(P)}* ($N = 12$), *esg^{ts}>dwdr4^{RNAi(P)}* with *dmyc^d* mutant ($N = 10$) ($n = 110$ ISC/EB cells) flies. (G, G') Removal of one copy of *dmyc* in *esg^{ts}>dwdr4^{RNAi(P)}* PMGs does not suppress the increased JNK signaling (Red, JNK signaling activity). (G') Quantification of average pJNK intensity in the *esg^{ts}>mcherry^{RNAi(P)}* ($N = 9$), *esg^{ts}>dwdr4^{RNAi(P)}* ($N = 16$), *esg^{ts}>dwdr4^{RNAi(P)}* with *dmyc^d* mutant ($N = 8$) ($n = 110$ ISC/EB cells) flies. Insets in (B, D, E, F, G) show enlarged views of the boxed regions. Each dot in (B', D', F', G') represents a single ISC or EB, and each dot in (E') represents one midgut. The image and insets of (B, D-G) are shown at 50 and 25 μm . White dashed lines in (C-G) outline the edge of the gut. Bars represent the mean; error bars indicate standard deviation (SD). Statistically significant differences in (B'-G') were identified by the non-parametric Mann-Whitney *U* test. (B') The DHE signals increased in *wh⁷* compared to WT ($***P = < 0.0001$). (D') The pJNK intensity showed differences in *esg^{ts}>dwdr4^{RNAi(P)}* compared to *esg^{ts}>mcherry^{RNAi(P)}* without *cat^{OE}* ($***P = < 0.0001$), but not with *cat^{OE}* (ns, $P = 0.7$). (E') The PHH3⁺ cells showed differences in *esg^{ts}>dwdr4^{RNAi(P)}* compared to *esg^{ts}>mcherry^{RNAi(P)}* without *cat^{OE}* ($***P = 0.0002$), but not with *cat^{OE}* (ns, $P = 0.67$); PHH3⁺ cells differed between *esg^{ts}>dwdr4^{RNAi(P)}* with and without *cat^{OE}* ($***P = 0.0003$); whereas *cat^{OE}* alone had no effects (ns, $P = 0.58$). (F', G') p4EBP and pJNK levels showed differences in *esg^{ts}>dwdr4^{RNAi(P)}* compared to *esg^{ts}>mcherry^{RNAi(P)}* without *dmyc^d* and with *dmyc^d* ($***P = < 0.0001$); p4EBP and pJNK levels did not differ between *esg^{ts}>dwdr4^{RNAi(P)}* with and without *dmyc^d* (ns, $P = 0.52$ in F', 0.42 in G'). *N*, number of midguts; *n*, number of ISCs or ISC/EBs analyzed, unless otherwise stated. Source data are available online for this figure.

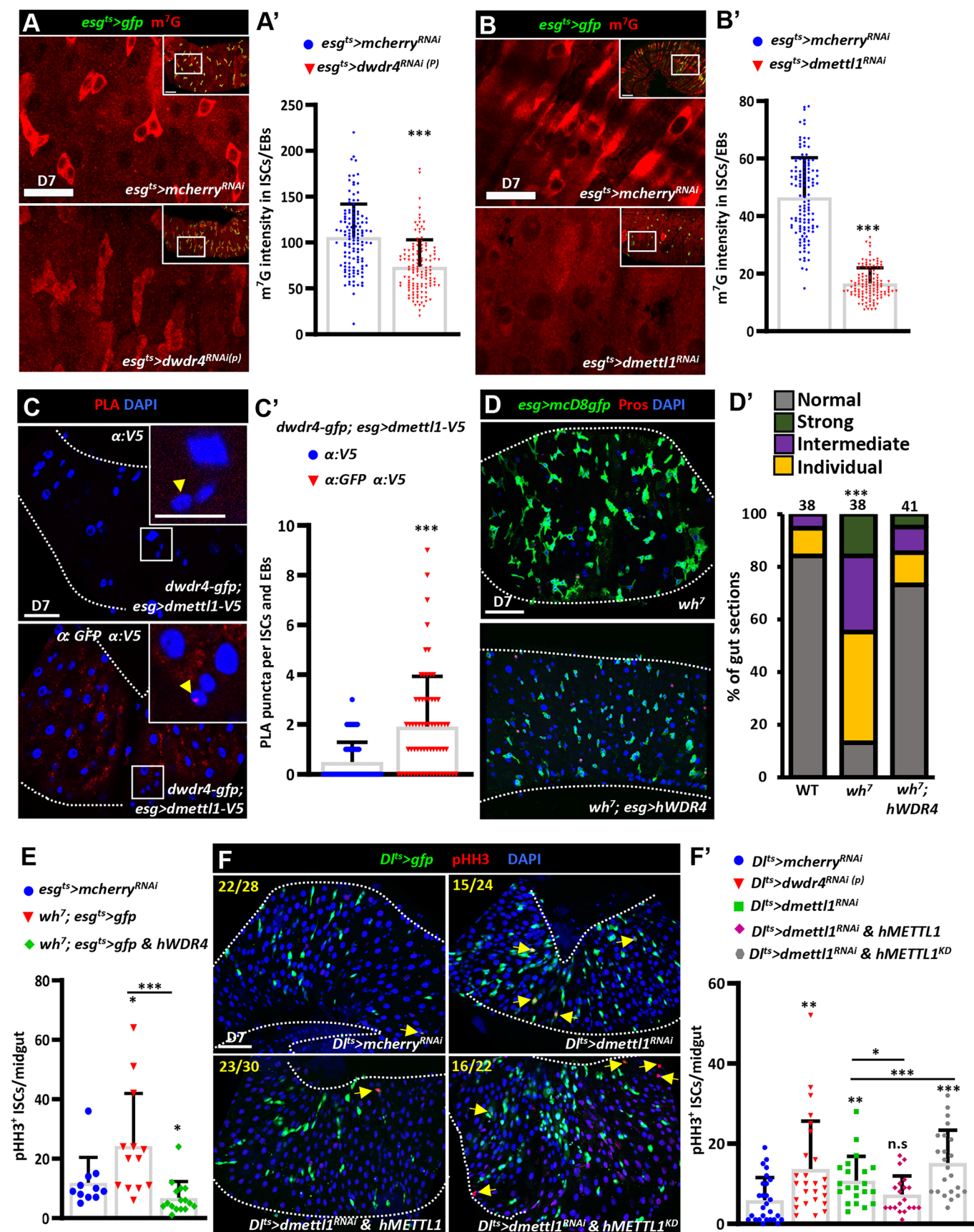


Figure EV5. The *Drosophila* Wdr4-Mettl1 complex mediates m⁷G modification to maintain intestinal homeostasis in a conserved manner.

(A, B') dWdr4 and dMettl1 control m⁷G levels in ISCs/EBs. (A, B) Posterior midguts (PMGs) from *esg^{ts}>dwdr4^{RNAi(P)}* (A) and *esg^{ts}>dmettl1^{RNAi}* (N = 16) (B) females show decreased m⁷G modification (red) in ISCs/EBs (*esg^{ts}>gfp*, green) as compared to control (*esg^{ts}>mcherry^{RNAi}*) (N = 12) (n = 125 ISC/EB cells). (A', B') Quantification of average m⁷G fluorescence intensity in ISCs/EBs in the indicated genotypes. (C) dWdr4 interacts with dMettl1 in ISCs/EBs, as revealed by PLA assay (red punctate) using anti-GFP and anti-V5 antibodies in PMGs from *dwdr4-gfp; esg>dmettl1-V5* females. *dmettl1-V5* is expressed in ISCs/EBs under the control of *esg-GAL4*, while *dwdr4-gfp* is expressed in all intestinal cells. PMGs of the same genotype exposed only to anti-V5 antibody served as a negative control. The arrowhead in C indicates the PLA puncta. DAPI (blue) labels nuclei. (C') Quantification of PLA puncta number in the *dwdr4-gfp; esg>dmettl1-V5* females with and without anti-GFP antibody (N = 8, n = 65 ISC/EB cells). (D) Overexpression of human WDR4 in ISCs/EBs of *dwdr4* mutant (*wh⁷*) ISCs/EBs (labeled by *esg>mCD8gfp*, green) suppresses ISC/EB expansion. Pros, red. DAPI, blue. The *wh⁷* mutant image was obtained together with Fig. 2A and is shown here for comparison with the human gene rescue. (D') Quantification of ISC/EB expansion phenotypes categorized and color-coded as Fig. 2A. The number of gut sections analyzed (N = 9) per genotype is shown above each bar. (E) Overexpression of human WDR4 in *dwdr4* mutant ISCs/EBs (*esg^{ts}>gfp*) decreases mitotic ISCs (PHH3⁺) in midguts. Number of PHH3⁺ cells per midgut in the *esg^{ts}>mcherry^{RNAi(P)}* (N = 11), *wh⁷* mutant with *esg>gfp* (N = 13), *wh⁷* mutant with *esg>gfp* and human WDR4 overexpression (N = 16) flies. (F) Overexpression of human METTL1, but not the catalytic-dead mutant (hMETTL1^{KD}) in ISCs (labeled by *Dl^{ts}>gfp*, green) of *dmettl1^{RNAi}* knockdown PMGs suppresses the increased ISC expansion and proliferation. Yellow arrows in E indicate the representative mitotic ISCs. Fractions of gut sections with the ISC/EB pattern shown are indicated. (F') Quantification of PHH3⁺ ISC number per midgut in the *Dl^{ts}>mcherry^{RNAi(P)}* (N = 27), *Dl^{ts}>dwdr4^{RNAi(P)}* (N = 26), *Dl^{ts}>dmettl1^{RNAi}* (N = 21), with human METTL1 (N = 21), and hMETTL1^{KD} (N = 23) flies. DAPI, blue. Insets in (C) show enlarged views of the white square in each panel; (A, B) show enlarged views of the square regions in the insets. Each dot in (A', B', C') represents a single ISC or EB, and each dot in (E', F') represents one midgut. The low magnification and enlarged views of (A, B, C) are shown at 50 and 25 μm; (D, F) are 50 μm. White dashed lines in (C, D, F) outline the edge of the gut. Bars represent the mean; error bars indicate standard deviation (SD). Statistically significant differences in (A', B') were identified by unpaired Student's *t* test, in (C', E, F') were identified by non-parametric Mann-Whitney *U* test, and in (D') were analyzed by Chi-square. (A', B') m⁷G intensity decreased in *esg^{ts}>dwdr4^{RNAi(P)}* and *esg^{ts}>dmettl1^{RNAi}* compared to *esg^{ts}>mcherry^{RNAi}* (***P < 0.0001). (C') The ***P < 0.0001. (D') The ***P = 0.001. (E) The PHH3⁺ cells showed differences in *wh⁷* compared to WT without hWDR4 (*P = 0.017) and with hWDR4 (*P = 0.013); PHH3⁺ cells differed between *esg^{ts}>dwdr4^{RNAi(P)}* with and without hWDR4 (***P < 0.0001). (F') The PHH3⁺ cells showed differences in *Dl^{ts}>dwdr4^{RNAi(P)}* (**P = 0.002), *Dl^{ts}>dmettl1^{RNAi}* (**P = 0.004), *Dl^{ts}>dmettl1^{RNAi}* with hMETTL1^{KD} (***P < 0.0001) compared to *Dl^{ts}>mcherry^{RNAi(P)}*, but no difference in *Dl^{ts}>dmettl1^{RNAi}* with hMETTL1 (ns, P = 0.16). PHH3⁺ cells differed in *Dl^{ts}>dmettl1^{RNAi}* with and without hMETTL1 (*P = 0.04), but not with hMETTL1^{KD} (*P = 0.0006). N, number of midguts; n, number of ISCs or ISC/EBs analyzed, unless otherwise stated. Source data are available online for this figure.

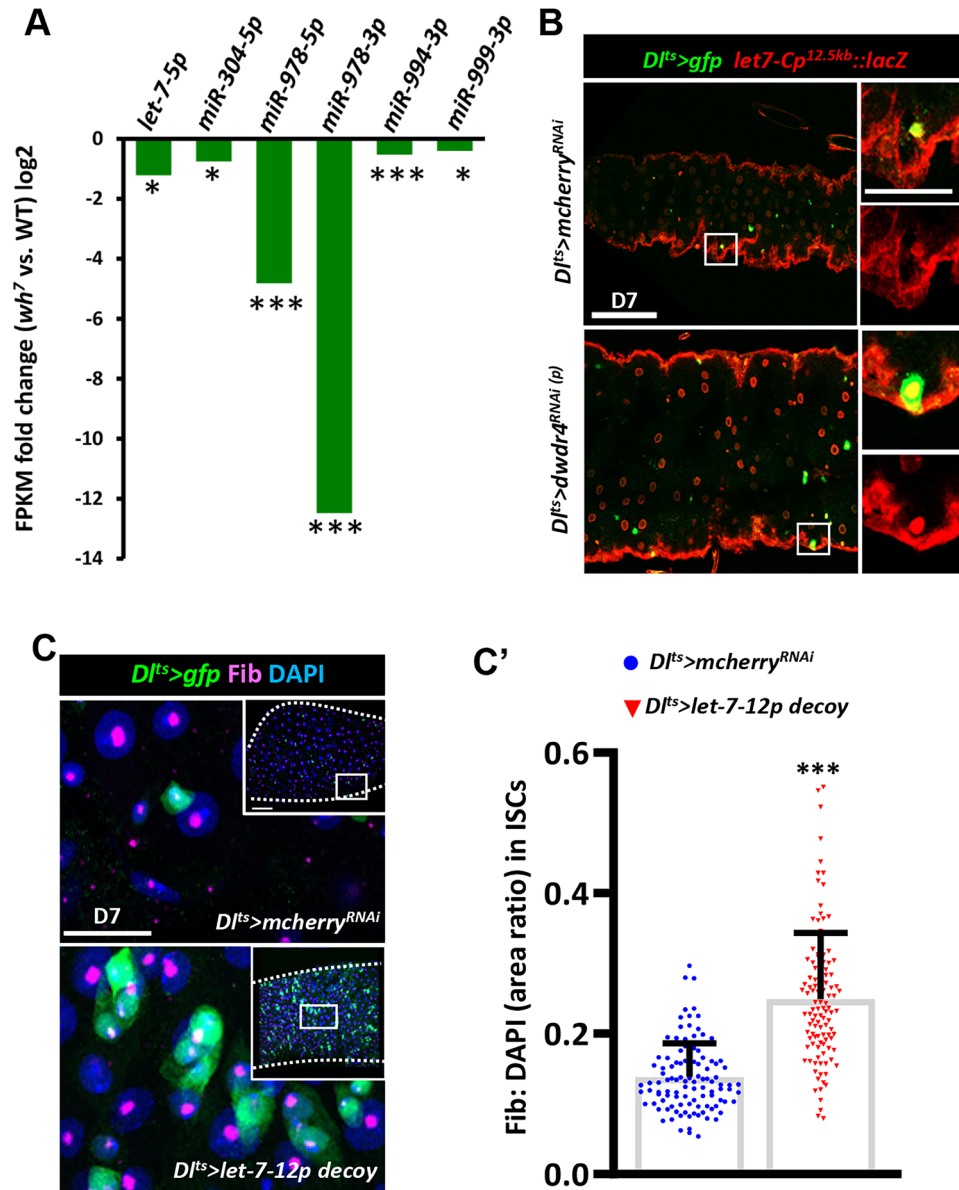


Figure EV6. dWdr4 does not control *let-7* transcription and *let-7* disruption phenocopies *dwd4* mutant PMGs.

(A) Fold changes in gene expression according to RNA-seq (fragments per kilobase of transcript per million mapped reads [FPKM]) in *wdr4* mutant (*wh7*) vs. wild-type (WT) ovaries; log₂ values are shown. Statistical analysis was performed on two biological replicates. **P* < 0.05; ****P* < 0.001. (B) Posterior midgut (PMG) from *DI^{ts}>dwd4^{RNAi} (p)* (*N* = 7), females bearing *DI^{ts}>gfp* (ISCs, green) show no obvious change in the expression of *let7-Cp^{12.5kb}::lacZ* (red, transcriptional reporter of *let-7*) as compared to control (*DI^{ts}>mcherry^{RNAi}*) (*N* = 6). (C) Disruption of *let-7* function (*DI^{ts}>let-7-12p decoy*) (*N* = 11) increases the size of Fibrillarin (Fib) (magenta, nucleoli) in ISCs (labeled by *DI^{ts}>gfp*, green) as compared to control (*DI^{ts}>mcherry^{RNAi}*) (*N* = 9) (*n* = 110 ISC cells). DAPI (blue) marks nuclei. (C') Quantification of nucleolar (Fib) to nuclear (DAPI) area ratio in ISCs in the indicated genotypes. Insets in (B) show enlarged views of the boxed region in each panel; (C) shows enlarged views of the boxed region in the insets. Each dot in (C') represents a single ISC. The low magnification and enlarged views of (B, C) are shown at 50 and 25 μm. White dashed lines in (C) outline the edge of the gut. Bars represent the mean; error bars indicate standard deviation (SD). Statistically significant differences in (A) were identified by unpaired Student's *t* test, and in (C') were by non-parametric Mann-Whitney *U* test. (C') the nucleolar size is increased in *DI^{ts}>let-7-12p decoy* compared to *DI^{ts}>mcherry^{RNAi}* (****P* = < 0.0001). *N*, number of midguts; *n*, number of ISCs or ISC/EBs analyzed, unless otherwise stated. Source data are available online for this figure.