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Piwi Is Required to Limit Exhaustion of Aging Somatic Stem Cells

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Report

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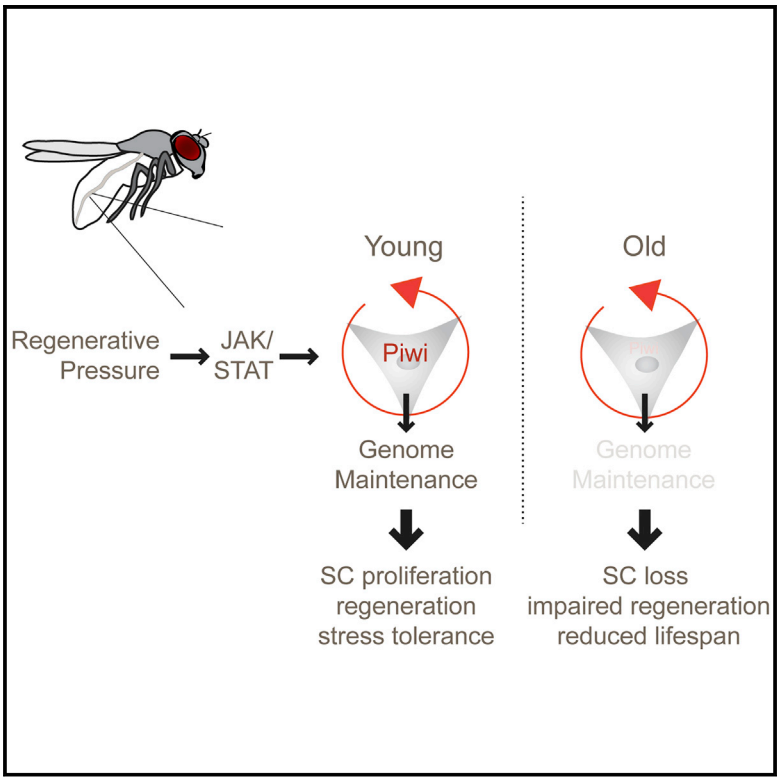
Graphical Abstract



Authors

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In Brief

Stem cell function depends on mechanisms that ensure the maintenance of genome integrity. Using the *Drosophila* intestine as a model, Sousa-Victor et al. identify Piwi as a regulator of somatic stem cell genomic integrity, required for long-term maintenance and function of ISCs, and they find that it can allay age-related stem cell dysfunction.

Highlights

- Piwi is induced in ISCs under regenerative pressure in a STAT-dependent manner
- Piwi is required for long-term maintenance and function of ISCs
- Piwi prevents retrotransposon activity, heterochromatin loss, and apoptosis in ISCs
- Piwi overexpression prevents age-related ISC dysfunction

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Report

Piwi Is Required to Limit Exhaustion of Aging Somatic Stem Cells

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SUMMARY

Sophisticated mechanisms that preserve genome integrity are critical to ensure the maintenance of regenerative capacity while preventing transformation of somatic stem cells (SCs), yet little is known about mechanisms regulating genome maintenance in these cells. Here, we show that intestinal stem cells (ISCs) induce the Argonaute family protein Piwi in response to JAK/STAT signaling during acute proliferative episodes. Piwi function is critical to ensure heterochromatin maintenance, suppress retrotransposon activation, and prevent DNA damage in homeostasis and under regenerative pressure. Accordingly, loss of Piwi results in the loss of actively dividing ISCs and their progenies by apoptosis. We further show that Piwi expression is sufficient to allay age-related retrotransposon expression, DNA damage, apoptosis, and mis-differentiation phenotypes in the ISC lineage, improving epithelial homeostasis. Our data identify a role for Piwi in the regulation of somatic SC function, and they highlight the importance of retrotransposon control in somatic SC maintenance.

INTRODUCTION

Stem cells in high-turnover tissues require high precision in genome maintenance mechanisms to ensure their long-term maintenance. In vertebrate stem cell populations, such mechanisms include effective cell cycle checkpoints and DNA repair machineries (Behrens et al., 2014). Deficiency in these mechanisms can result in stem cell exhaustion, mis-differentiation, and cancers (Adams et al., 2015). Especially during periods of high proliferative pressure, genome integrity becomes quickly compromised, increasing the potential for stem cell exhaustion and cancers. DNA repair pathways play a critical role in limiting stem cell failure and exhaustion during replicative pressure, and their loss contributes to the development of age-associated phenotypes (Murga et al., 2009; Walter et al., 2015). It can be anticipated that dynamically regulated genome maintenance strategies are employed to ensure resilience of proliferating stem cell populations. The identity and regulation of these strategies remain to be established in vivo.

Potential dangers to genome integrity include replication-related DNA damage and telomere dysfunction, but also deficiencies in higher-order chromatin regulation and dysfunction in retrotransposon control. The cell-autonomous activation and integration of transposable elements (TEs) can lead to insertional mutagenesis and genome rearrangements (Burns and Boeke, 2012). Such phenomena have recently been implicated in the aging process of multiple organisms (De Cecco et al., 2013; Maxwell et al., 2011; Wang et al., 2011). In the aging fly brain, somatic transposition increases and exacerbated TE expression results in age-associated impairment of memory and shorter lifespan (Li et al., 2013).

Given the mutagenic potential and the impact at the level of genomic instability of transposition events (Wang et al., 2011), organisms have evolved mechanisms to repress the activity of their endogenous TEs. In the germline, this is primarily achieved by the Piwi-interacting RNA pathway (Siomi et al., 2011), which represses TE activity by promoting post-transcriptional processing of TE mRNAs and mediating transcriptional silencing of TE-rich regions through chromatin modifications. Piwi is the founding member of the evolutionarily conserved Ago/Piwi protein family, and it is required for the self-renewal of germline stem cells (GSCs) in flies and mice (Carmell et al., 2007; Cox et al., 1998; Unhavaithaya et al., 2009). In both species, loss of Piwi is associated with transposon desilencing and increased apoptosis (Juliano et al., 2011). The functions of Piwi outside the germline are only beginning to be explored (Ross et al., 2014). In differentiated somatic tissues, a physiologic function for the Piwi pathway has been reported in the brain and fat body of *Drosophila* (Janic et al., 2010; Jones et al., 2016). Piwi protein orthologs are also expressed in neoblasts of planaria (Reddien et al., 2005) and blastemal cells of salamander (Zhu et al., 2012), where they are required for efficient regeneration. However, the physiological function of the pathway and its relevance for stem cell maintenance and regenerative capacity have not been explored. Intestinal stem cells (ISCs) of the *Drosophila* posterior midgut epithelium constitute an experimentally accessible system to address these questions (Ayyaz and Jasper, 2013).

ISCs are the main mitotically competent cell type in the intestinal epithelium of flies, giving rise to an enteroblast (EB) daughter cell that further differentiates into an enterocyte (EC) or an enteroendocrine cell (EE). A complex network of local and systemic signals regulates ISC maintenance and proliferative homeostasis (Ayyaz and Jasper, 2013). In response to an acute stress signal, such as the infection by a pathogen, ISCs readily increase their proliferative activity to fulfill regenerative demands, and subsequently they shut down the proliferative response to avoid hyperplasia and reinstate epithelial homeostasis (Ayyaz et al., 2015). Damage to the epithelium induces the expression of cytokines of the Unpaired family (Upd 2 and 3) in ECs, which in turn activate JAK/STAT signaling in ISCs to promote the shift from quiescence to proliferation. JAK/STAT signaling cooperates with epidermal growth factor (EGF) receptor (EGFR) activation to promote proliferation of ISCs, and the return to quiescence is associated with the inhibition of JAK/STAT signaling by negative feedback regulators like SOCS36E (Ayyaz and Jasper, 2013).

The control of proliferative activity is de-regulated in old flies, resulting in dysplasia that is reminiscent of hyper-proliferative phenotypes acquired in young animals in response to chronic stress (Ayyaz and Jasper, 2013). This is accompanied by widespread mis-differentiation of ISC daughter cells, caused by ectopic activation of Delta/Notch signaling (Biteau et al., 2008).

Due to the frequent exposure of ISCs to regenerative pressure, mechanisms that control genomic integrity of ISCs are likely to be critical for long-term functional maintenance of the intestinal epithelium. However, how this is achieved remains unclear. Here we show that the dynamic control of Piwi expression in ISCs is one such mechanism. Piwi is transcriptionally activated in ISCs that are induced to proliferate, and it is required for regenerative capacity and maintenance of ISCs during the regenerative process. Our findings suggest that a decline in the ability of Piwi to maintain heterochromatin contributes to the age-associated deregulation of ISC function and loss of tissue homeostasis.

RESULTS

ISCs Respond to Infection-Induced JAK/STAT Signaling by Increasing Piwi Expression

In response to infection with the mild enteropathogen *Erwinia carotovora carotovora 15* (*Ecc15*), ISCs undergo transient activation followed by a return to quiescence when the infection is cleared (Figures 1A and 1B). To characterize molecular changes that are associated with this transient activation of ISCs, we analyzed transcriptomes of wild-type and STAT-deficient ISCs isolated at various time points after infection by fluorescence-activated cell sorting (FACS; compare Dutta et al., 2015 and Figures 1A and 1C). STAT was knocked down by the expression of a double-stranded RNA (dsRNA) against STAT using the ISC/EB-specific driver escargot::Gal4 (esg::Gal4, UAS::GFP), combined with ubiquitous expression of temperature-sensitive Gal80 (tub::Gal80ts, TARGET system). RNAi was induced in young (3-day-old) adults for 3 days prior to infection (Figure 1A) to knock down STAT expression (Figure S1A).

ISCs were identified in the FACS experiment by their expression of GFP, and they were differentiated from EBs by their smaller size (Figure 1C; note that inhibition of Gal4 in EBs by the expression of Gal80 under the control of a Su(H) promoter [Wang et al., 2014b] selectively inhibits GFP in the larger cell population). This strategy allows faithful purification of ISCs, as confirmed by the expression of the ISC marker Delta (Dl) and enrichment for progenitor-specific genes (Figures 1D and S1B). This cell population is also depleted for genes expressed in ECs (Figure S1B).

Using RNA sequencing (RNA-seq) (Figure S1C), we identified over 655 genes induced in wild-type ISCs at 4 hr after *Ecc15* infection. About one-half of those genes required JAK/STAT activity for their induction, and this subset was highly enriched for genes associated with DNA replication and repair (Figures 1E and 1F; Table S1). *piwi* was found among these genes (Figure 1G), and analysis of independent samples collected in the same conditions, but analyzed by qRT-PCR, confirmed STAT-dependent Piwi induction in ISCs upon regenerative pressure (Figure 1H).

Using in situ hybridization (Figure 2A), immunohistochemistry (Figure 2B), a lacZ enhancer trap line (Figure S2A), and a reporter line expressing an N-terminally EGFP-tagged Piwi from the endogenous *piwi* locus (Figure S2B; Sienski et al., 2012), we found that Piwi was specifically expressed in ISCs and EBs of the posterior midgut, but not in differentiated cell types. In these experiments, ISCs/EBs were detected by GFP expression driven by esg::Gal4 (Figures 2A and 2B) or by co-staining with Dl (Figures S2A and S2B), which co-localized with *piwi* mRNA or protein signal. We confirmed the induction of Piwi protein after *Ecc15* infection (Figures 2B and S2A–S2D) and the dependence on STAT signaling (Figure 2B). Piwi protein detected using immunohistochemistry (Figure 2B) or with the GFP signal of the tagged Piwi protein (Figure S2B) further revealed that Piwi localizes both to the cytoplasm and to the nucleus.

Piwi expression was also induced, in a STAT-dependent manner, in ISCs/EBs of flies exposed to other damaging insults, such as bleomycin (Figure S2E), and in conditions where proliferative pressure is imposed by the overexpression of constitutively active Ras (Ras^{v12}; Figure S2F). Furthermore, activation of STAT signaling, through overexpression of a constitutively active form of the JAK kinase hopscotch (hop^{TumL}; Hanratty and Dearolf, 1993), was sufficient to induce Piwi expression in progenitor cells (Figure S2F). Conversely, blocking EGFR signaling prevented *Ecc15*-dependent Piwi induction (Figure S2F). Thus, Piwi is induced in ISCs/EBs of the *Drosophila* midgut under proliferative pressure, independently of the type of regenerative stimulus.

To test if Piwi activity is required to regulate or maintain ISC function during regeneration, we analyzed the effects of Piwi knockdown (Figure 3A). Expression of an RNAi against Piwi for 3 days in ISCs/EBs of young adult flies was sufficient to significantly reduce its expression in the midgut (Figures S2G and S2H). Loss of Piwi impaired regenerative capacity of the gut (Figure 3A), leading to reduced density of both ECs and progenitor cells in infected midguts containing Piwi-deficient progenitors. This was likely associated with the inability to maintain ISCs rather than with defects in ISC activation, as the number of Dl^+ cells was significantly reduced in midguts containing Piwi-deficient ISCs, while mitotic activity (determined using phosphorylated Histone H3 [pH3]) was similar to wild-type guts at earlier time points (Figures 3A and S3A). Similarly, $piwi^2$ homozygous

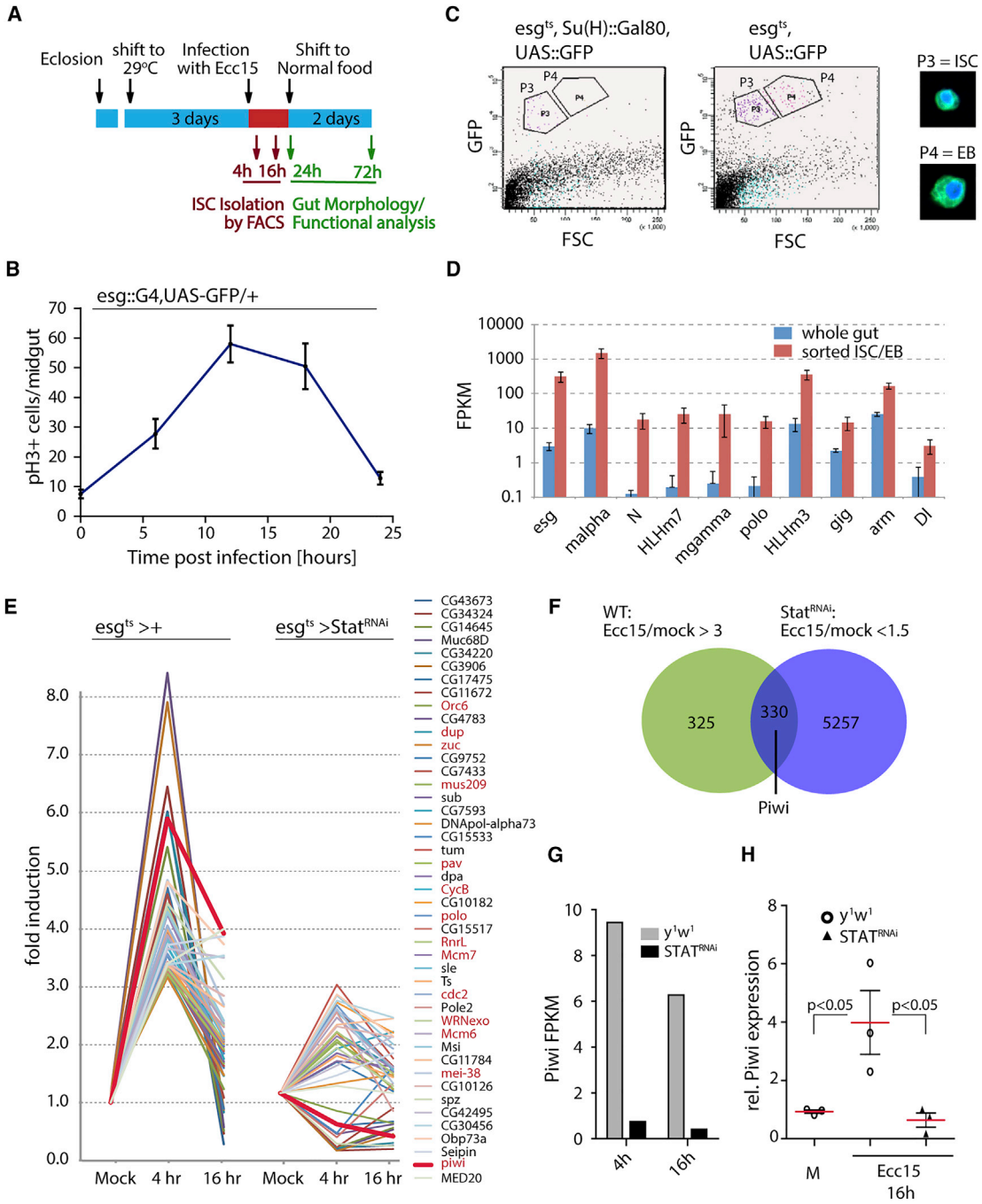


Figure 1. Infection-Induced JAK/STAT Signaling Promotes Piwi Expression in ISCs/EBs

(A) Setup and timeline for *Ecc15* infection experiments.

(B) Proliferative activity of ISCs after *Ecc15* infection, evaluated by the number of pH3^{+} cells/midgut (n = 8/time point).

(C) Scatterplots with GFP intensity (vertical axis) and cell size (horizontal axis) show a GFP^{high} population of smaller cells (P3, ISCs) and a GFP^{high} population of larger cells (P4, EBs).

(D) FPKM values of progenitor-specific genes in ISCs sorted via FACS.

(E) Relative expression of a set of genes induced by *Ecc15* infection in a STAT-dependent manner measured in ISCs sorted via FACS. Genes labeled in red are associated with DNA replication and repair (n = 7 for mock condition).

(F) Venn diagram showing the proportion of genes induced by *Ecc15* infection in ISCs that require STAT function for the induction. *Piwi* is one of these genes.

(G and H) FPKM values (G) and qRT-PCR (H) showing *Piwi* expression in ISCs sorted via FACS, 4 hr (G) and 16 hr (G and H) after *Ecc15* infection in the presence or absence of STAT RNAi (for qRT-PCR, n = 3 sorted samples/condition, 50–100 midguts/sorting).

Error bars indicate SEM and p values are from Student's t test. See also Figure S1 and Table S1.

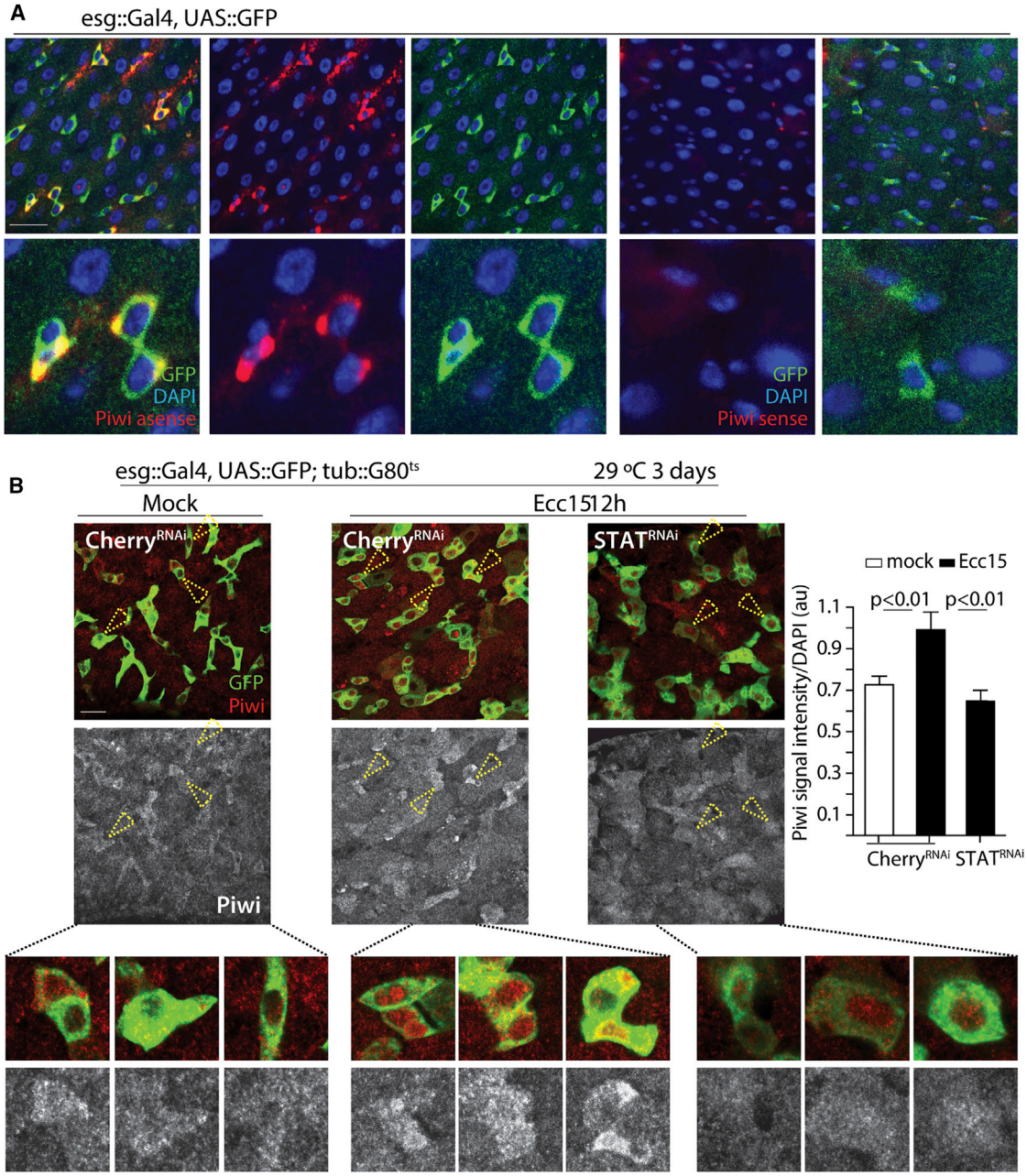


Figure 2. Piwi Expression in ISCs/EBs of the *Drosophila* Midgut

(A and B) Piwi expression in ISCs/EBs of the *Drosophila* midgut, detected by in situ hybridization using an anti-sense probe (A, left panel) or by immunohistochemistry (B). mRNA signal specificity was confirmed by the lack of signal using a sense probe (A, right panel). ISCs/EBs were identified by esg::GFP expression. STAT-dependent induction of Piwi protein in ISCs/EBs after *Ecc15* infection is shown and quantified (B, n = 25 cells quantified/condition using ImageJ; quantification is of the mean intensity in the Piwi channel normalized to the DAPI channel for each ISC/EB area defined by the GFP channel). Arrowheads indicate Piwi nuclear signal. Error bars indicate SEM and p values are from Student's t test. Scale bars, 20 μm. See also Figure S2.

flies had significantly lower numbers of Dl^+ ISCs already 48 hr after *Ecc15* infection, and a large number of these ISCs also expressed the EC marker PDM1, indicating mis-differentiation (Figure S3B).

The requirement of Piwi for ISC function under regenerative pressure was further confirmed by lineage-tracing ISCs homozygous for the *piwi*^{3} loss-of-function allele (Lin and Spradling, 1997), using mosaic analysis with a repressible cell marker (MARCM) during regeneration. Consistent with the previous results, *piwi*^{3} mutant ISCs generated smaller MARCM clones than wild-type controls (Figure 3B). *piwi*-deficient MARCM clones were generally composed of 2–3 cells rather than single

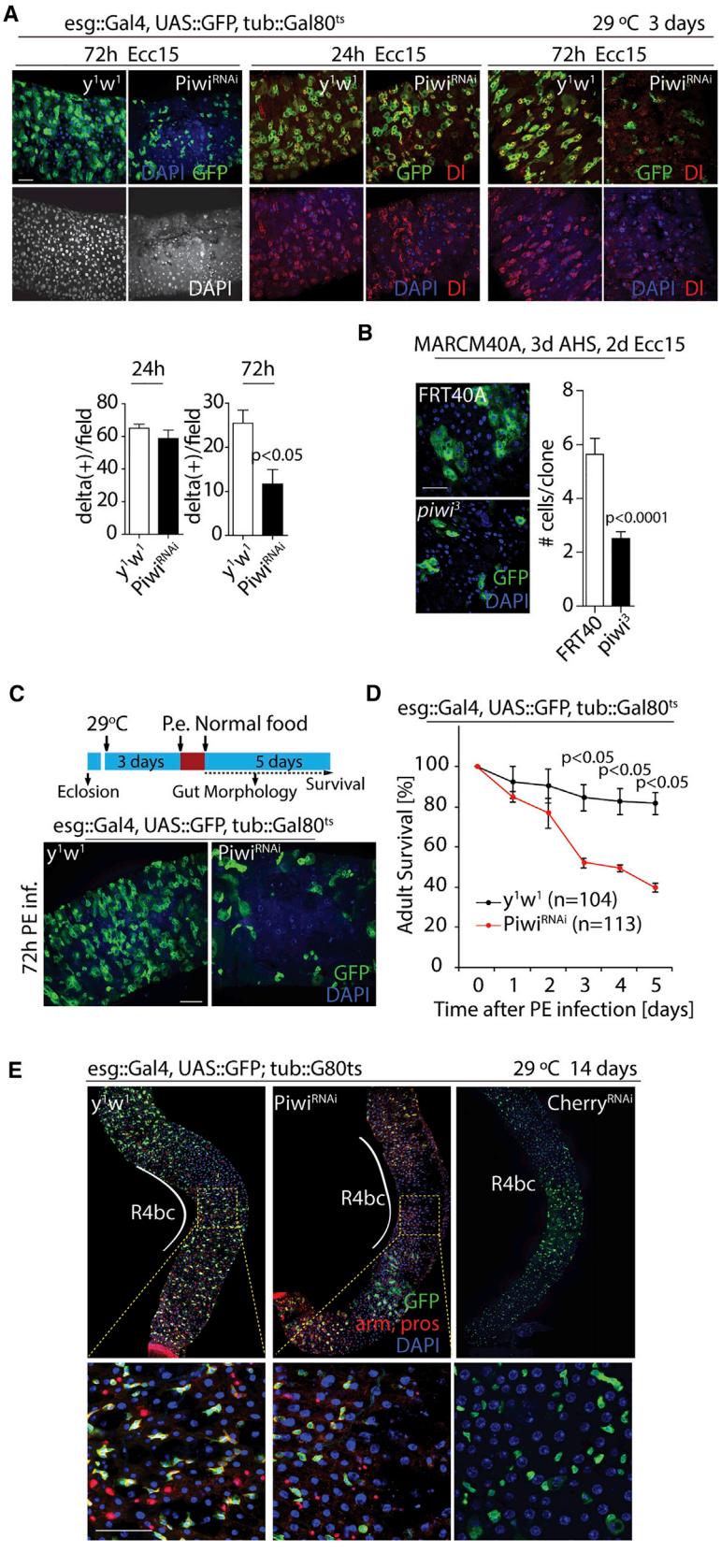


Figure 3. Piwi Is Required for the Maintenance of ISC Function

(A) Representative images of *Drosophila* posterior midguts isolated from wild-type (WT) animals or animals expressing Piwi-RNAi in the ISCs/EBs, after *Ecc15* infection, showing ISC density by Dl staining and posterior midgut morphology by DAPI staining. Quantifications of average number of Dl^+ cells/field are shown (n = 8–10/condition).

(B) Representative images of *Drosophila* posterior midguts infected with *Ecc15*, showing WT (FRT40A) and Piwi-null (*piwi*^{3}) clones. Quantifications of average number of cells/clone are shown (n = 8/condition, 6–10 clones/gut).

(C) Representative images of *Drosophila* posterior midguts isolated from WT animals or animals expressing Piwi-RNAi in the ISCs/EBs, 72 hr after *P.e.* infection. DAPI staining shows the altered posterior midgut morphology in Piwi-deficient animals.

(D) Percentage animal survival after *P.e.* infection.

(E) Representative images of *Drosophila* posterior midguts isolated from WT animals (or animals expressing mCherry-RNAi) and animals expressing Piwi-RNAi in the ISCs/EBs for 14 days. ISCs are identified by esg::GFP. In the two left panels, cell boundaries are labeled by immunostaining against Armadillo (membrane red), and EE cells are labeled by nuclear pros staining (nuclear red).

Error bars indicate SEM and p values are from Student's t test. Scale bars, 50 μm. See also Figures S2 and S3.

ISCs, indicating that, before arresting, ISCs underwent a few rounds of division after the induction of *piwi*^{3} homozygosity.

Survival after enteropathogen infection is an indicator of effective regeneration, as the inability to restore epithelial integrity can result in an animal's death. Thus, we monitored survival following infection with the strong enteropathogen *Pseudomonas entomophila* (*P.e.*). Flies with Piwi-deficient ISCs and EBs died faster after infection with *P.e.* (Figures 3C and 3D), consistent with an inability to efficiently regenerate the intestinal epithelium (Figure 3C).

The role of Piwi in maintaining ISC function during proliferative pressure was not limited to infection conditions, as loss of Piwi limited the growth of ISC tumors generated by Notch deficiency (Figure S3C). Our results suggest that, even in these conditions, Piwi-deficient ISCs cannot sustain a high rate of proliferative activity.

Nuclear Piwi Is Required for Long-Term Maintenance of ISC Function

We asked if Piwi was also required for long-term maintenance of ISCs and midgut homeostasis. We knocked down Piwi in ISCs of young adults and analyzed the effects 7 and 14 days later. While Piwi-deficient intestines did not exhibit major defects at 7 days after Piwi knockdown (data not shown), loss of epithelial homeostasis became apparent at 14 days after Piwi depletion, as evidenced by a reduction in the density of ECs (Figure 3E, large polyploid nuclei stained with DAPI) and progenitor cells (Figure 3E). These effects of Piwi loss are region specific, with most of ISC/EB loss occurring in the R4bc morphological subdomain (Buchon et al., 2013) of the midgut (Figure 3E).

To determine the cell type specificity of this effect, we used ISC-specific and EB-specific drivers to knock down Piwi. ISC-specific knockdown of Piwi resulted in a significant loss of ISCs (Figure S3D), while EB-specific knockdown of Piwi had no effect on ISC or EB numbers (Figure S3E).

Altogether, these data suggested that Piwi acts in an ISC-autonomous fashion to maintain ISC function and that, accordingly, loss of Piwi is accompanied by a progressive loss of ISC function in the posterior midgut. This observation was reproduced in *piwi*^{2} mutant flies (Figure S3F) and by lineage-tracing *piwi* mutant ISCs: clones derived from *piwi*^{3} homozygous ISCs were significantly smaller than clones derived from wild-type ISCs (Figures S3G–S3I). This phenotype was region specific (Figure S3H; also after *Ecc15* infection, Figure S3J) and recapitulated the loss of ISCs observed after Piwi knockdown, as there was a significantly higher proportion of clones derived from *piwi*^{3} homozygous ISCs that were completely depleted of Dl^{+} ISCs (Figure S3I).

Piwi proteins have both cytoplasmic and nuclear functions, having been implicated in the maintenance of the chromatin state in the nucleus (Rozhkov et al., 2013). To assess if Piwi nuclear function is critical for ISC maintenance, we lineage-traced ISCs that were homozygous for a *piwi* allele in which the 26 N-terminal amino acids containing the nuclear localization signal are absent (*piwi*^{NT}). This mutant Piwi lacks the ability to translocate to the nucleus, thus preventing its ability to regulate chromatin structure, while retaining its cytoplasmic function (Klenov et al., 2011). *piwi*^{NT} MARCM clones phenocopied the *piwi*^{3} loss-of-function mutant, suggesting that the nuclear function of Piwi is required for the maintenance of ISC function (Figure S3K).

Piwi Regulates Heterochromatin Maintenance in ISCs

The silencing of transposons in the *Drosophila* germline relies in part on the formation and maintenance of heterochromatic regions by Piwi family proteins, through interaction with heterochromatin-forming pathways (Sienski et al., 2012). To test this function of nuclear Piwi in ISCs, we assessed the extent of heterochromatinization globally using a Position-Effect Variegation (PEV) reporter line (Lu et al., 1996). The *In(3L)BL1* line contains a heat shock-inducible HS-lacZ gene insertion juxtaposed to pericentric heterochromatin, resulting in variegated expression of lacZ. Thus, lacZ expression can be evaluated at the level of single cells as an indicator of heterochromatin status (Figure 4A). *piwi*^{3} heterozygous mutants showed an increase in the number of lacZ-expressing progenitor cells in the posterior midgut at 20 days of age (Figure 4B) and in conditions of acute regenerative pressure caused by infection with *Ecc15* (Figure S4A), associated with a significant increase in LacZ transcript abundance (Figures 4B and S4A). This indicates that a significant loss of constitutive heterochromatin occurred in those animals and that the nuclear function of Piwi that is required for ISC maintenance may involve the regulation of heterochromatin.

Supporting this view, loss of Piwi was associated with derepression of TE expression and with TE mobilization. Knockdown of Piwi, but not of Aub or AGO3, was accompanied by an accumulation of several TE transcripts (Figure 4C). The requirement of Piwi for TE repression was further confirmed in the *piwi*^{3} heterozygous background, where a significant induction of Gypsy could be detected, in homeostasis and after *Ecc15* infection (Figure S4B). Furthermore, the number of integration events in *piwi*^{3} heterozygous flies, assessed using a Gypsy-TRAP reporter system (Li et al., 2013), was higher than in wild-type controls (Figures 4D and 4E). The Gypsy-TRAP reporter consists of a GAL80 (Gal4 inhibitor) transgene expressed under the control of a tubulin promoter separated by a gypsy target site (Tub::OvoSite::GAL80), so that insertions of gypsy prevent GAL80 expression. Combined with esg::Gal4, UAS::GFP, this system allows the detection of de novo gypsy integration events in ISCs and EBs (Figure 4D).

Piwi Is Required to Prevent Apoptosis of ISCs

To further characterize the consequences of Piwi loss in ISCs, we analyzed the transcriptome of Piwi-deficient ISCs (Table S1) isolated by FACS. Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification analysis of genes upregulated after Piwi knockdown indicated enrichment for components involved in proteasome-mediated degradation, DNA damage response (DDR) pathways, and apoptosis (Figures S4C–S4E). Interestingly, we found several subunits of the proteasome complex overexpressed, in agreement with previously reported effects of *piwi* loss in the germline (Le Thomas et al., 2013). The upregulated DDR components and molecules associated with apoptosis suggested an increase in DNA damage, which may result in checkpoint activation and the triggering of apoptotic pathways. Using qRT-PCR in an independent set of samples of ISCs isolated by FACS, we confirmed

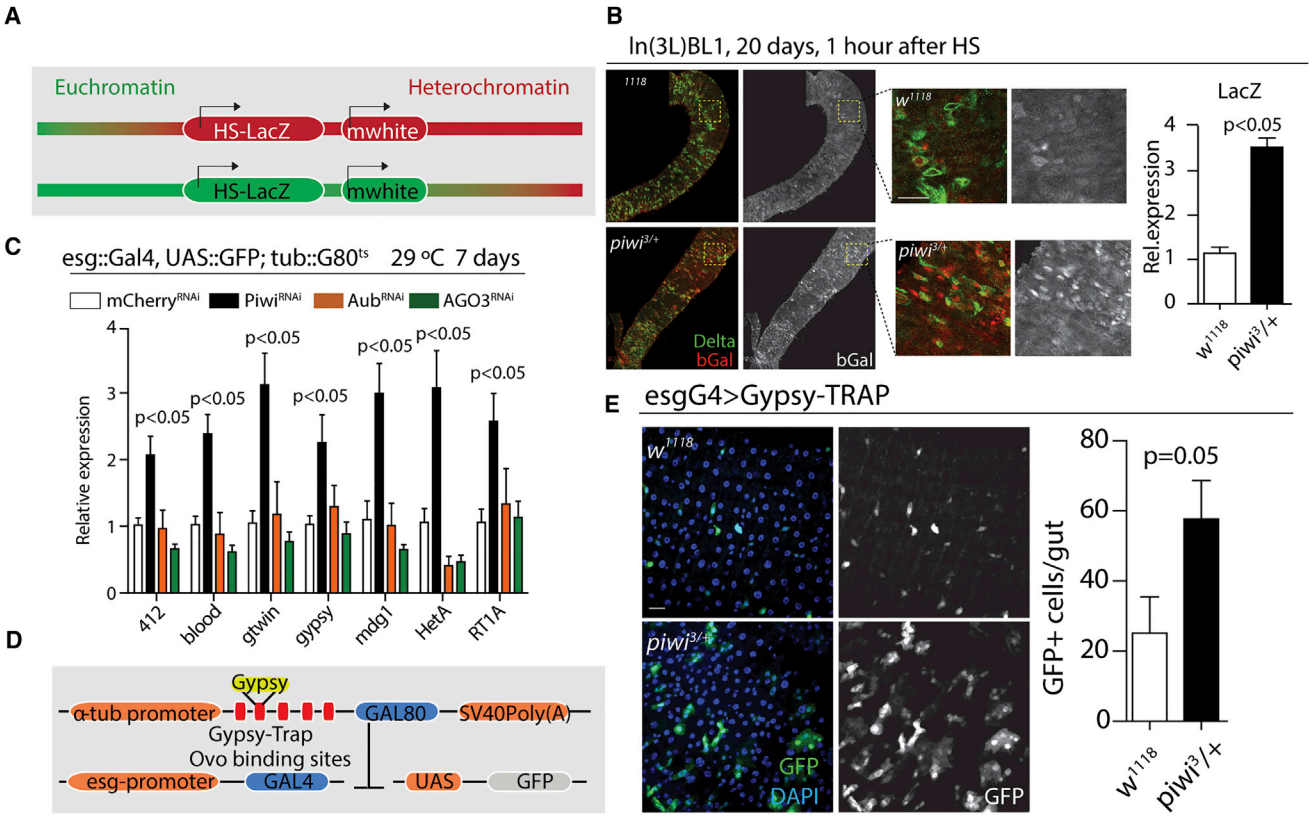


Figure 4. Piwi Is Required for Chromatin Maintenance and Transposon Silencing

(A) Reporter locus in the In(3L)BL1 line used to detect relative levels of heterochromatin (red).

(B) Representative images and relative levels of LacZ transcripts quantified by qRT-PCR (n = 3, 8 guts/sample) of *Drosophila* midguts isolated from 20-day-old WT animals (w^{1118}) or *piwi* heterozygous animals ($piwi^{3/+}$) carrying the reporter locus in (A) and isolated 1 hr after heat shock. Guts were stained for Dl to identify ISCs and β-galactosidase (β-gal) to identify LacZ-expressing cells.

(C) Relative levels of TE transcripts quantified by qRT-PCR in midguts isolated from animals expressing RNAi against Piwi, Aub, or Ago3 in the ISCs/EBs for 7 days, compared to animals expressing a control hairpin (n = 4, 8 guts/sample).

(D) Gypsy-TRAP line used to detect Gypsy integration events.

(E) Representative images of *Drosophila* posterior midguts isolated from 20-day-old WT animals (w^{1118}) or piwi heterozygous animals ($piwi^{3/+}$) carrying the reporter in (D). Quantification of average number of GFP^+ cells/midgut (n = 8/condition) is shown.

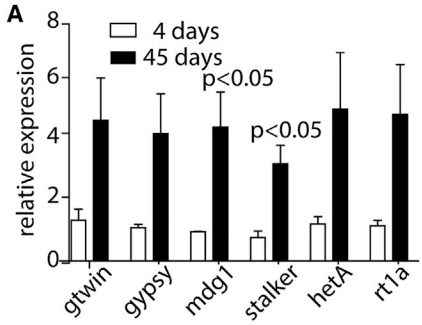
Error bars indicate SEM and p values are from Student's t test. Scale bars, 20 μm. See also Figure S4 and Table S1.

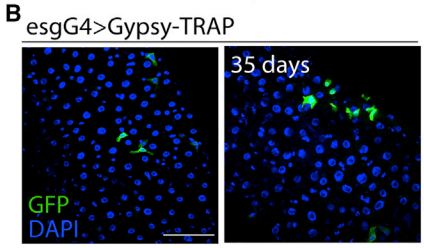
the upregulation of several of the genes listed above, as well as of TEs in Piwi-deficient ISCs (Figures S4F and S4G).

As predicted by the transcriptome analysis, Piwi deficiency led to an increase in ISC apoptosis. Apoptotic cells were detected using either an antibody against the cleaved form of the *Drosophila* effector caspase, also known as death caspase 1 (cDcp1), or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Sarkissian et al., 2014). We observed a significant increase in the percentage of $cDcp1^+/Dl^+$ ISCs expressing an RNAi against Piwi during *Ecc15*-induced regeneration and in 5-day-old $piwi^2$ homozygous flies (Figures S4H and S4I). Similarly, we observed a significant increase of $TUNEL^+$ ISCs/EBs after *piwi* knockdown (Figure S4J). In addition, we used a GFP-based caspase activity reporter (Apoliner; Bardet et al., 2008) comprising two fluorophores, mRFP and EGFP, linked by a specific caspase-sensitive site. *piwi* knockdown resulted in increased caspase activity in esg^+ progenitor cells, as evaluated by the nuclear translocation of GFP in apoliner flies (Figure S4K).

TE Expression and Transposition Increases with Aging in the *Drosophila* Midgut

Consistent with the de-repression of TE loci with aging in other species and fly tissues (De Cecco et al., 2013; Li et al., 2013; Wang et al., 2011), the transcripts of different classes of TEs were significantly increased in midguts from old flies relative to young animals (Figure 5A). Because genomic instability and DNA damage are expected to be linked with insertions of TEs into the genome, we examined whether the increase in TE transcription in older flies was associated with physical transposition and increased DNA damage. Using the gypsy-TRAP system (Figure 4D), we found a progressive accumulation of GFP^+ cells in the intestine of aging wild-type flies (Figures 5B and 5C). These effects were specific for TE integration events and not due to age-associated inactivation of Gal80 due to loss of heterozygosity, as a similar system composed of mutated Ovo sites did not result in increased GFP expression (Figure 5C).





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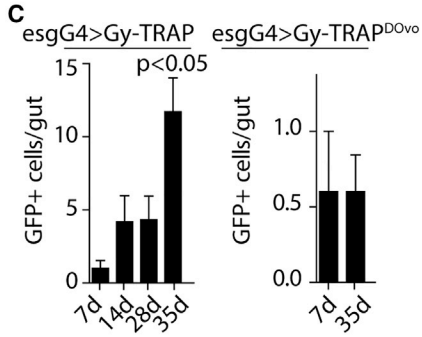
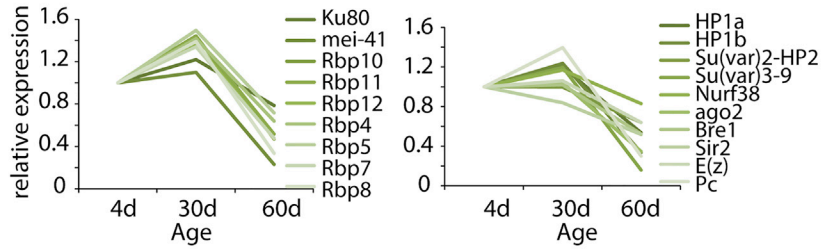
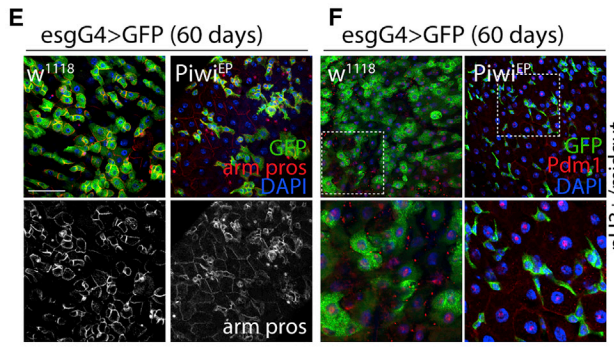
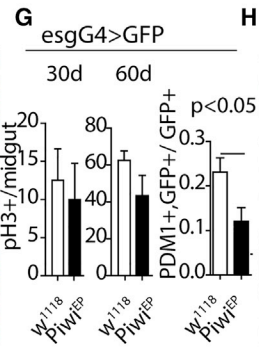
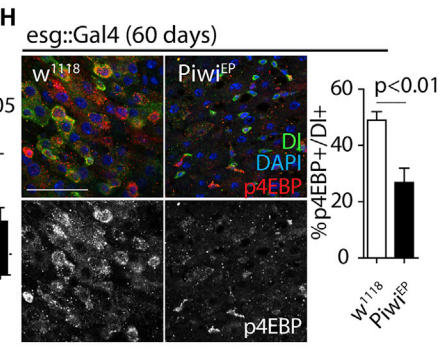
GO	LogP
cellular metabolic process	2.2 E-92
mitotic cell cycle	8.7 E-62
cell differentiation	2.4 E-57
histone modification	7.2 E-17
chromatin remodeling	8.1 E-16
ncRNA processing	1.5 E-13
DNA repair	1.6 E-07
programmed cell death	2.7 E-07
DNA integrity checkpoint	3.2 E-06
heterochromatin organization	7.9 E-06
Wnt signaling pathway	2.3 E-05
Notch signaling pathway	1.1 E-03
stem cell division	1.4 E-03
stem cell differentiation	3.8 E-03
heterochromatin assembly	3.8 E-03
aging	4.5 E-03
JNK cascade	8.8 E-03

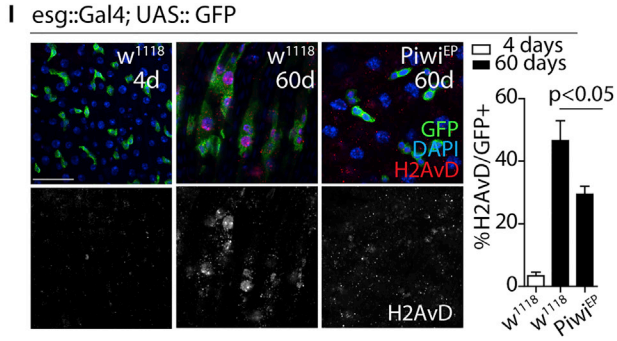


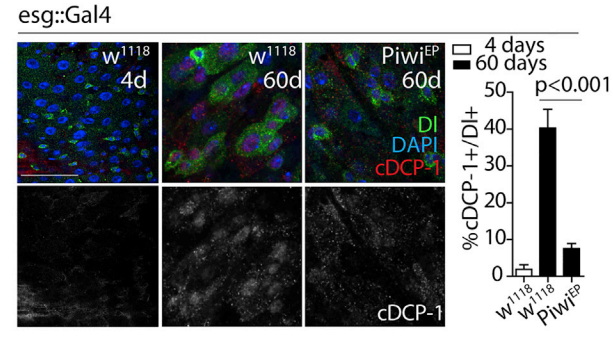












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Staining for the phosphorylated form of the *Drosophila* histone H2AX variant (γ-H2Av; Madigan et al., 2002), which accumulates near chromosomal break sites, indicated that DNA damage was elevated in intestinal cells of 60-day-old flies (Figure S5A; note that the epithelial dysplasia that develops in old intestines precludes clear classification of these cells as ISCs or EBs; Biteau et al., 2008).

The increase in TE expression in old intestines is thus accompanied by an increase in actively integrating transposons and by higher rates of DNA damage, suggesting that a high level of genomic rearrangements and instability could lead to a decline in ISC maintenance and/or function.

Transcriptome Analysis of 60-Day-Old ISCs Reveals Defects in the Maintenance of Heterochromatin Structure and DNA Repair Capacity

To further detail intrinsic changes associated with ISC aging, we sorted via FACS the progenitor cells from midguts of flies at different ages (isolating small, GFP^+ cell populations to enrich for ISCs), and we analyzed the global gene expression alterations occurring during physiological aging by RNA-seq (Table S1). Strikingly, the transcriptomes of ISCs sorted from young flies (4 days old) and mid-aged flies (30 days old) were almost identical, while significant differences were observed between ISCs from 30- and 60-day-old animals (Figure S5B). Based on GO term classification analysis, we discovered that pathways responsible for heterochromatin maintenance and DNA repair were significantly reduced in the 60-day-old animals compared to the 30-day-old animals (Figure 5D). These findings are consistent with our earlier observations that old ISCs accumulate DNA damage and are subject to elevated TE activity. These data suggest that ISC aging is accompanied by robust changes in the highly inter-connected network of genes responsible for chromatin maintenance and repair.

Interestingly, among the GO terms significantly altered in 60-day-old ISCs, we also found pathways known to be involved in the control of ISC function, including Wingless and Notch signaling, as well as genes involved in the regulation of stem cell differentiation and cell cycle (Figures 5D and S5C). GO terms upregulated in aged ISCs included genes associated with proteolytic processes (Figure S5D, green lines), similar to what is observed in Piwi loss-of-function conditions and genes associated with EC differentiation, such as Pdm1 (Figure S5D, red lines), in agreement with observed mis-differentiation phenotypes (see below).

Piwi Overexpression Prevents Age-Associated Decline in ISC Function

In the *Drosophila* midgut, aging is associated with intestinal dysplasia and a disruption of epithelial function, caused by a combination of increased ISC proliferation and mis-differentiation (Ayyaz and Jasper, 2013; Biteau et al., 2008). Thus, given that Piwi expression does not increase in old ISCs, as it does during acute regenerative pressure (see Figure 1 and Table S1), we explored the extent to which we could impact ISC aging by increasing Piwi levels in old progenitor cells. We used esg::Gal4 to drive the expression of a fully functional Piwi protein (using the $piwi^{EP}$ allele, which allows Gal4-mediated overexpression of endogenous Piwi; Cox et al., 2000) in ISCs/EBs of aging flies (Figure S5E). The dysplastic phenotype observed in old flies was reduced in the midgut of animals with elevated expression of Piwi (Figures 5E and 5F). However, analysis of the number of $pH3^+$ cells detected at 30 and 60 days revealed that Piwi overexpression did not affect the capacity of ISCs to proliferate (Figure 5G), suggesting that the rescue of epithelial dysplasia was primarily a consequence of reduced mis-differentiation. Consistently, the percentage of cells co-expressing both progenitor and differentiation markers ($esg^+/PDM1^+$; Figures 5F and 5G) or ISC and EB markers simultaneously ($Dl1^+/p4EBP^+$; Figure 5H) was high at 60 days, but it was significantly rescued in midguts where Piwi was overexpressed in progenitor cells (Figures 5F–5H). In agreement, ISCs sorted from 60-day-old flies overexpressing Piwi in progenitor cells showed significantly lower levels of Pdm1 mRNA compared to wild-type ISCs at the same age (Figure S5F). This was accompanied by a significant reduction in TE expression (Figure S5G). Piwi overexpression could also significantly reduce DNA damage in ISCs, quantified by the presence of γ-H2Av in Dl^+ cells (Figure 5I) and ISC apoptosis, quantified by cDCP1 staining (Figure 5J).

DISCUSSION

Our study identifies Piwi as a critical regulator of somatic stem cell function that is induced in ISCs in periods of regenerative pressure in which JAK/STAT is active. We propose that Piwi is

Figure 5. Piwi Overexpression Prevents Age-Associated Decline in ISC Function

(A) Relative levels of TE transcripts quantified by qRT-PCR in midguts isolated from young (4 days) and old (45 days) wild-type animals (n = 4–6, 8 guts/sample).

(B) Representative images of *Drosophila* posterior midguts isolated from young (7 days) and old (35 days) WT animals carrying the Gypsy-TRAP reporter.

(C) Quantification of the average number of GFP^+ cells per midgut at different ages in flies carrying the Gypsy-TRAP reporter (left, n = 8/condition). The right graph is a similar quantification but in flies carrying the Gypsy-TRAP reporter with mutated ovo sites, where Gypsy cannot integrate (right, n = 8/condition).

(D) GO analysis of the dataset of genes downregulated in ISCs from 60-day-old flies. Graphs on the bottom show the relative levels of expression of some of these genes in ISCs at different ages.

(E and F) Representative images of *Drosophila* posterior midguts isolated from 60-day-old flies overexpressing Piwi in ISCs/EBs and corresponding WT controls, showing esg::GFP, armadillo, and prospero (E) or Pdm1 (F).

(G) Quantification of the average number of $pH3^+$ cells per midgut (left, n = 7/condition) and the average fraction of $Pdm1^+/GFP^+$ cells (right, n = 17/condition) for the different ages and genotypes.

(H–J) Representative images of *Drosophila* posterior midguts isolated from 60-day-old flies overexpressing Piwi in ISCs/EBs and corresponding 60-day-old and young WT controls (I and J), showing the expression of esg::GFP (I) or Dl (H and J) in green to identify progenitor cells/ISCs, respectively. Co-staining with p4EBP (H), H2AvD (I), and cDCP-1 (J) in red is shown. Quantifications on the right are for the percentage of mis-differentiated ISCs (H, n = 4–7/condition), progenitor cells with signs of DNA damage (I, n = 5–7/condition), and apoptotic ISCs (J, n = 5–6/condition).

Error bars indicate SEM and p values are from Student's t test. Scale bars, 50 μm. See also Figure S5 and Table S1.

a critical component of the ISC safeguard machinery that acts throughout life to ensure the maintenance of stem cell function (Figure S5H). Piwi homologs have also been reported to be induced and necessary during regenerative events in other organisms (Reddien et al., 2005; Rizzo et al., 2014; Zhu et al., 2012). Furthermore, other mechanisms controlling genomic integrity, such as telomerase activity, have also been associated with stem cell proliferation and found to be required for stem cell maintenance (Behrens et al., 2014; Flores et al., 2006; Wang et al., 2014a). JAK/STAT-mediated regulation of regenerative responses and their de-regulation during aging are conserved in mammals (Neves et al., 2015), highlighting the importance of its downstream effectors in the proper regulation of tissue homeostasis. Interestingly, hTert expression is also activated downstream of STAT signaling in human cancers (Chung et al., 2013; Konnikova et al., 2005), suggesting that JAK/STAT activation leads to the coordinated activation of pathways safeguarding genomic integrity (see also the Supplemental Discussion).

However, analysis of the Piwi locus does not suggest that Piwi is a direct STAT target. Piwi is induced in proliferating ISCs independently of the activating stimulus, and its induction is also dependent on other pathways that regulate ISC activation. Piwi induction may thus be a general feature of ISCs under proliferative pressure, strengthening the idea that Piwi plays a key function in dividing ISCs. The molecular mechanism of Piwi induction in ISCs during regenerative events will be an interesting area of future study.

Although our data clearly establish a role for Piwi in the prevention of TE expression and transposition in ISCs and in the maintenance of somatic stem cell function during the organism's lifespan, it remains to be determined if there is a causal relation between the two observations. Moreover, it remains to be determined if, as in the germline, Piwi function relies on PIWI-interacting RNA (piRNA)-mediated mechanisms to regulate TE silencing and heterochromatin maintenance. piRNA-independent roles for Piwi in somatic stem cells would be unexpected but of significant interest for further study.

In mouse models, genomic damage and instability are major inducers of the DDR, and defects of the DNA repair machinery can cause phenotypes resembling premature aging (Wong et al., 2003). Our study suggests that targeting Piwi and other chromatin-remodeling pathways may be an effective way to delay or prevent age-associated loss of stem cell function. Interestingly, recent reports support the idea that Piwi expression and TE control in other somatic tissues influence organismal homeostasis and lifespan (Jones et al., 2016). Further studies comparing the relative effects of *piwi* loss of function on lifespan in a tissue-specific manner will contribute to our understanding of the different mechanisms through which the Piwi pathway contributes to tissue homeostasis.

EXPERIMENTAL PROCEDURES

Fly Lines and Husbandry

Flies were cultured on yeast/molasses-based food at 25°C with a 12-hr light/dark cycle, and female animals were used in all experiments. For details and the use of the TARGET and MARCM systems, see the Supplemental Experimental Procedures and Table S2.

FACS and RNA-Seq

For ISC isolation by FACS, 80–100 adult female *Drosophila* guts were dissected in 1× PBS with 1% bovine serum albumin (PBS-BSA), dissociated for 30 min with 500 μL 0.5% Trypsin-EDTA twice, and then passed through the fine mesh of Polystyrene tubes with Cell-strainer cap (Falcon 352235). Cells were sorted using a FACS Aria II.

Bacterial Infection and Survival Assay

Previously described procedures (Ayyaz et al., 2015) were followed for oral bacterial challenge. For details see the Supplemental Experimental Procedures.

qRT-PCR Analysis

cDNA was synthesized using an oligo-dT primer. Real-time PCR was performed on a Bio-Rad CFX96 detection system. Relative expression was normalized to Actin5C. For details on the primers used, see the Supplemental Experimental Procedures.

Statistical Analysis

Statistical significance of differences between groups was analyzed by Student's unpaired two-tailed t test using GraphPad Prism 5 software. Data are represented as mean ± SEM of the "n" designated for each experiment.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Discussion, Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.059.

AUTHOR CONTRIBUTIONS

Conceptualization, P.S.-V., V.V.L., and H.J.; Methodology, Validation, and Formal Analysis, P.S.-V. and H.J.; Investigation, P.S.-V., A.A., R.H., Y.Q., and D.T.M.; Writing – Original Draft, P.S.-V. and H.J.; Writing – Review & Editing, P.S.-V. and H.J.; Visualization, P.S.-V. and H. J.; Supervision, H.J.; Project Administration, P.S.-V. and H.J.; Funding Acquisition, H.J.

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REFERENCES

Adams, P.D., Jasper, H., and Rudolph, K.L. (2015). Aging-induced stem cell mutations as drivers for disease and cancer. Cell Stem Cell *16*, 601–612.

Ayyaz, A., and Jasper, H. (2013). Intestinal inflammation and stem cell homeostasis in aging *Drosophila* melanogaster. Front. Cell. Infect. Microbiol. *3*, 98.

Ayyaz, A., Li, H., and Jasper, H. (2015). Haemocytes control stem cell activity in the *Drosophila* intestine. Nat. Cell Biol. *17*, 736–748.

Bardet, P.L., Kolahgar, G., Mynett, A., Miguel-Aliaga, I., Briscoe, J., Meier, P., and Vincent, J.P. (2008). A fluorescent reporter of caspase activity for live imaging. Proc. Natl. Acad. Sci. USA *105*, 13901–13905.

Behrens, A., van Deursen, J.M., Rudolph, K.L., and Schumacher, B. (2014). Impact of genomic damage and ageing on stem cell function. Nat. Cell Biol. *16*, 201–207.

Biteau, B., Hochmuth, C.E., and Jasper, H. (2008). JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging *Drosophila* gut. Cell Stem Cell *3*, 442–455.

Buchon, N., Osman, D., David, F.P., Fang, H.Y., Boquete, J.P., Deplancke, B., and Lemaitre, B. (2013). Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. Cell Rep. *3*, 1725–1738.

Burns, K.H., and Boeke, J.D. (2012). Human transposon tectonics. Cell *149*, 740–752.

Carmell, M.A., Girard, A., van de Kant, H.J., Bourc'his, D., Bestor, T.H., de Rooij, D.G., and Hannon, G.J. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev. Cell *12*, 503–514.

Chung, S.S., Aroh, C., and Vadgama, J.V. (2013). Constitutive activation of STAT3 signaling regulates hTERT and promotes stem cell-like traits in human breast cancer cells. PLoS ONE *8*, e83971.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., and Lin, H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. *12*, 3715–3727.

Cox, D.N., Chao, A., and Lin, H. (2000). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development *127*, 503–514.

De Cecco, M., Criscione, S.W., Peterson, A.L., Neretti, N., Sedivy, J.M., and Kreiling, J.A. (2013). Transposable elements become active and mobile in the genomes of aging mammalian somatic tissues. Aging (Albany NY) *5*, 867–883.

Dutta, D., Dobson, A.J., Houtz, P.L., Gläßer, C., Revah, J., Korzelius, J., Patel, P.H., Edgar, B.A., and Buchon, N. (2015). Regional cell-specific transcriptome mapping reveals regulatory complexity in the adult *Drosophila* midgut. Cell Rep. *12*, 346–358.

Flores, I., Benetti, R., and Blasco, M.A. (2006). Telomerase regulation and stem cell behaviour. Curr. Opin. Cell Biol. *18*, 254–260.

Hanratty, W.P., and Dearolf, C.R. (1993). The *Drosophila* Tumorous-lethal hematopoietic oncogene is a dominant mutation in the hopscotch locus. Mol. Gen. Genet. *238*, 33–37.

Janic, A., Mendizabal, L., Llamazares, S., Rossell, D., and Gonzalez, C. (2010). Ectopic expression of germline genes drives malignant brain tumor growth in *Drosophila*. Science *330*, 1824–1827.

Jones, B.C., Wood, J.G., Chang, C., Tam, A.D., Franklin, M.J., Siegel, E.R., and Helfand, S.L. (2016). A somatic piRNA pathway in the *Drosophila* fat body ensures metabolic homeostasis and normal lifespan. Nat. Commun. *7*, 13856.

Juliano, C., Wang, J., and Lin, H. (2011). Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms. Annu. Rev. Genet. *45*, 447–469.

Klenov, M.S., Sokolova, O.A., Yakushev, E.Y., Stolyarenko, A.D., Mikhaleva, E.A., Lavrov, S.A., and Gvozdev, V.A. (2011). Separation of stem cell maintenance and transposon silencing functions of Piwi protein. Proc. Natl. Acad. Sci. USA *108*, 18760–18765.

Konnikova, L., Simeone, M.C., Kruger, M.M., Kotecki, M., and Cochran, B.H. (2005). Signal transducer and activator of transcription 3 (STAT3) regulates human telomerase reverse transcriptase (hTERT) expression in human cancer and primary cells. Cancer Res. *65*, 6516–6520.

Le Thomas, A., Rogers, A.K., Webster, A., Marinov, G.K., Liao, S.E., Perkins, E.M., Hur, J.K., Aravin, A.A., and Tóth, K.F. (2013). Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state. Genes Dev. *27*, 390–399.

Li, W., Prazak, L., Chatterjee, N., Grüninger, S., Krug, L., Theodorou, D., and Dubnau, J. (2013). Activation of transposable elements during aging and neuronal decline in *Drosophila*. Nat. Neurosci. *16*, 529–531.

Lin, H., and Spradling, A.C. (1997). A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. Development *124*, 2463–2476.

Lu, B.Y., Bishop, C.P., and Eissenberg, J.C. (1996). Developmental timing and tissue specificity of heterochromatin-mediated silencing. EMBO J. *15*, 1323–1332.

Madigan, J.P., Chotkowski, H.L., and Glaser, R.L. (2002). DNA double-strand break-induced phosphorylation of *Drosophila* histone variant H2Av helps prevent radiation-induced apoptosis. Nucleic Acids Res. *30*, 3698–3705.

Maxwell, P.H., Burhans, W.C., and Curcio, M.J. (2011). Retrotransposition is associated with genome instability during chronological aging. Proc. Natl. Acad. Sci. USA *108*, 20376–20381.

Murga, M., Bunting, S., Montaña, M.F., Soria, R., Mulero, F., Cañamero, M., Lee, Y., McKinnon, P.J., Nussenzweig, A., and Fernandez-Capetillo, O. (2009). A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nat. Genet. *41*, 891–898.

Neves, J., Demaria, M., Campisi, J., and Jasper, H. (2015). Of flies, mice, and men: evolutionarily conserved tissue damage responses and aging. Dev. Cell *32*, 9–18.

Reddien, P.W., Oviedo, N.J., Jennings, J.R., Jenkin, J.C., and Sánchez Alvarado, A. (2005). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. Science *310*, 1327–1330.

Rizzo, F., Hashim, A., Marchese, G., Ravo, M., Tarallo, R., Nassa, G., Giurato, G., Rinaldi, A., Cordella, A., Persico, M., et al. (2014). Timed regulation of P-element-induced wimpy testis-interacting RNA expression during rat liver regeneration. Hepatology *60*, 798–806.

Ross, R.J., Weiner, M.M., and Lin, H. (2014). PIWI proteins and PIWI-interacting RNAs in the soma. Nature *505*, 353–359.

Rozhkov, N.V., Hammell, M., and Hannon, G.J. (2013). Multiple roles for Piwi in silencing *Drosophila* transposons. Genes Dev. *27*, 400–412.

Sarkissian, T., Timmons, A., Arya, R., Abdelwahid, E., and White, K. (2014). Detecting apoptosis in *Drosophila* tissues and cells. Methods *68*, 89–96.

Sienski, G., Dönertas, D., and Brennecke, J. (2012). Transcriptional silencing of transposons by Piwi and maelstrom and its impact on chromatin state and gene expression. Cell *151*, 964–980.

Siomi, M.C., Sato, K., Pezic, D., and Aravin, A.A. (2011). PIWI-interacting small RNAs: the vanguard of genome defence. Nat. Rev. Mol. Cell Biol. *12*, 246–258.

Unhavaithaya, Y., Hao, Y., Beyret, E., Yin, H., Kuramochi-Miyagawa, S., Nakano, T., and Lin, H. (2009). MILI, a PIWI-interacting RNA-binding protein, is required for germ line stem cell self-renewal and appears to positively regulate translation. J. Biol. Chem. *284*, 6507–6519.

Walter, D., Lier, A., Geiselhart, A., Thalheimer, F.B., Huntscha, S., Sobotta, M.C., Moehrle, B., Brocks, D., Bayindir, I., Kaschutnig, P., et al. (2015). Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. Nature *520*, 549–552.

Wang, J., Geesman, G.J., Hostikka, S.L., Atallah, M., Blackwell, B., Lee, E., Cook, P.J., Pasaniuc, B., Shariat, G., Halperin, E., et al. (2011). Inhibition of activated pericentromeric SINE/Alu repeat transcription in senescent human adult stem cells reinstates self-renewal. Cell Cycle *10*, 3016–3030.

Wang, J., Lu, X., Sakk, V., Klein, C.A., and Rudolph, K.L. (2014a). Senescence and apoptosis block hematopoietic activation of quiescent hematopoietic stem cells with short telomeres. Blood *124*, 3237–3240.

Wang, L., Zeng, X., Ryoo, H.D., and Jasper, H. (2014b). Integration of UPRER and oxidative stress signaling in the control of intestinal stem cell proliferation. PLoS Genet. *10*, e1004568.

Wong, K.K., Maser, R.S., Bachoo, R.M., Menon, J., Carrasco, D.R., Gu, Y., Alt, F.W., and DePinho, R.A. (2003). Telomere dysfunction and Atm deficiency compromises organ homeostasis and accelerates ageing. Nature *421*, 643–648.

Zhu, W., Pao, G.M., Satoh, A., Cummings, G., Monaghan, J.R., Harkins, T.T., Bryant, S.V., Randal Voss, S., Gardiner, D.M., and Hunter, T. (2012). Activation of germline-specific genes is required for limb regeneration in the Mexican axolotl. Dev. Biol. *370*, 42–51.