Piwi Is Required in Multiple Cell Types to Control Germline Stem Cell Lineage Development in the Drosophila Ovary

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Abstract

The piRNA pathway plays an important role in maintaining genome stability in the germ line by silencing transposable elements (TEs) from fly to mammals. As a highly conserved piRNA pathway component, Piwi is widely expressed in both germ cells and somatic cells in the Drosophila ovary and is required for piRNA production in both cell types. In addition to its known role in somatic cap cells to maintain germline stem cells (GSCs), this study has demonstrated that Piwi has novel functions in somatic cells and germ cells of the Drosophila ovary to promote germ cell differentiation. Piwi knockdown in escort cells causes a reduction in escort cell (EC) number and accumulation of undifferentiated germ cells, some of which show active BMP signaling, indicating that Piwi is required to maintain ECs and promote germ cell differentiation. Simultaneous knockdown of dpp, encoding a BMP, in ECs can partially rescue the germ cell differentiation defect, indicating that Piwi is required in ECs to repress dpp. Consistent with its key role in piRNA production, TE transcripts increase significantly and DNA damage is also elevated in the piwi knockdown somatic cells. Germ cell-specific knockdown of piwi surprisingly causes depletion of germ cells before adulthood, suggesting that Piwi might control primordial germ cell maintenance or GSC establishment. Finally, Piwi inactivation in the germ line of the adult ovary leads to gradual GSC loss and germ cell differentiation defects, indicating the intrinsic role of Piwi in adult GSC maintenance and differentiation. This study has revealed new germline requirement of Piwi in controlling GSC maintenance and lineage differentiation as well as its new somatic function in promoting germ cell differentiation. Therefore, Piwi is required in multiple cell types to control GSC lineage development in the Drosophila ovary.

Introduction

Small RNAs have received much attention in recent years because of their important and diverse roles in the regulation of various biological processes \[1,2,3,4,5\]. In contrast to other small RNAs, Piwi-associated small RNAs, also known as piRNAs, are abundantly expressed in germ cells of organisms ranging from C. elegans to human, and have emerged as an important class of small RNAs for maintaining genome stability in germ cells \[6,7,8,9\]. Recent studies have shown that piRNAs also function in somatic cells to regulate gene expression and repress TEs \[10,11,12,13,14,15\]. However, biological functions of piRNAs still remain poorly defined.

The Drosophila ovary is an attractive system for studying stem cell lineage development \[16\]. Two types of stem cells, germline stem cells (GSCs) and follicular stem cells (FSCs), are responsible for continuously producing differentiated germ cell cysts and follicle cells, respectively, which are assembled into egg chambers that eventually develop into mature oocytes. Two or three GSCs are situated in the tip of each ovariole, known as the germarium, and can be easily identified by their direct contact with cap cells and presence of an anteriorly localized spectrosome (Fig. 1A). Immediate GSC daughters, also known as cystoblasts (CBs), move away from cap cells and undergo four rounds of synchronized cell division to form 2-cell, 4-cell, 8-cell and 16-cell cysts. CBs and cysts are tightly encased by cellular processes of escort cells (ECs), also known as inner germarial sheath cells (Fig. 1A). Genetic and cell biological studies have shown that terminal filament (TF)/cap cells and anterior ECs form the self-renewing niche for GSCs, which provides the essential BMP signal for repressing GSC differentiation and thereby maintaining their self-renewal \[16\].
Based on recent studies from us and others [17,18], we have recently proposed that posterior ECs function as the microenvironment or niche for promoting germ cell differentiation [18]. One of the key functions of ECs is to prevent BMP signaling via two distinct strategies. First, EGFR-MAPK signaling has been proposed to directly repress expression of *dally*, encoding a proteoglycan facilitating BMP signal transduction and diffusion [17]. Rho signaling and Eggless have been shown to repress *dally* expression in ECs, thus promoting germ cell differentiation, but it remains unclear how they might regulate *dally* expression [18,19].

**Figure 1. Piwi is required in ECs to promote germ cell differentiation and maintain EC survival.** Ovals indicate cap cells, whereas brackets denote the germarial region covered by ECs. (A) c587 drives GFP expression specifically in ECs (arrow). (B) c587-mediated piwi knockdown (piwiKD) leads to an accumulation of many single germ cells (SGCs) carrying a spectrosome (arrowhead) mixed with differentiated cysts containing a branched fusome (arrow) in the germarium and its associated egg chamber. (C) Piwi protein is expressed in PZ1444-positive cap cells and ECs (arrow) as well as in follicle cells and germ cells. (D–F) c587-mediated piwiKD by three independent RNAi lines efficiently eliminates Piwi protein expression in PZ1444-positive cap cells and ECs (arrows), whereas Piwi protein expression in germ cells remains normal. (G–I) c587-mediated piwiKD by THU and HMS lines leads to the accumulation of SGCs (arrowhead) mixed with differentiated cysts (arrow). I shows the quantitative results on the numbers of SGCs and agametic germaria (n indicates total germaria examined). (J–L) c587-mediated piwiKD causes the formation of the germaria (arrows) containing no germ cells. (M–P) c587-mediated piwiKD (O, P) results in a significant reduction in EC numbers in comparison with the control (M). N shows the quantitative results on EC numbers (n indicates total germaria examined). Scale bars: 25 μm.

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The second strategy is direct repression of transcription of dpp, which encodes a BMP ligand essential for GSC self-renewal in Drosophila. Histone lysine-specific demethylase 1 (Lsd1, a chromatin regulator) and Rho signaling have been shown to repress dpp transcription in ECs [18,20]. dpp knockdown can partially rescue the germ cell differentiation defects caused by inactivation of Lsd1 and Rho signaling in ECs, indicating that dpp upregulation contributes to the germ cell differentiation defects. Therefore, ECs have so far been demonstrated to promote germ cell differentiation by preventing the spreading of BMP signaling.

It is the Drosophila ovary in which the first piRNA regulator, piwi, was identified for its critical role in maintaining GSCs [21,22]. Although it is expressed in all germ cells and somatic cells of the Drosophila ovary, it has been suggested to function in TF/cap cells for maintaining GSCs [22,23]. In addition, Piwi is also required intrinsically to promote GSC division and primordial germ cell formation [24,25]. In Drosophila ovarian somatic cells, Yb works with Piwi to control primary piRNA biogenesis [12,14,26], and is also suggested to work in TF/cap cells to maintain GSC self-renewal [27]. In addition, recent studies have shown that Armitage (Armi), Vreteto (Vret) and Tdrd12 are also required in somatic cells to control primary piRNA biogenesis [28,29,30]. Inactivation of histone H3K9 trimethylase Eggless function in ECs leads to defective piRNA biogenesis, upregulation of transposons and germ cell differentiation defects, indicating that piRNAs are important for maintaining EC function by repressing transposons [19,31]. Consistently, vret mutants also have a germ cell differentiation defect, which can be rescued by somatic cell-specific expression of vret [28,29]. In this study, we show that piwi is required in ECs and germline to control germ cell differentiation.

Results

Piwi is required in ECs to control germ cell differentiation and EC survival

To identify the genes that are required in ECs for controlling germ cell differentiation, we carried out a genetic screen using an EC-expressing gal4 driver c587 and transgenic UAS-RNAi lines from the Vienna Drosophila RNAi Center (VDRC). These VDRC RNAi transgenic lines were designed based on the production of a long double-stranded RNA structure that can be further processed into small double-stranded RNAs degrading target mRNAs, and have been used to carry out genetic screens in various Drosophila tissue types [32,33,34]. c587 is expressed specifically in ECs and early follicle cell progenitors based on the expression of UAS-GFP [35] (Fig. 1A). In our screen, piwi was identified for its requirement in ECs to control germ cell differentiation as c587-mediated knockdown of piwi (piwiKD) causes the accumulation of spectroosome-containing ill-differentiated single germ cells (SGCs) located distantly from cap cells in the knockout germaria, which is in great contrast with the control germaria (Fig. 1B). Although GSCs and cystoblasts (CBs, immediate GSC progeny) contain a spherical spectroosome, only GSCs directly contact cap cells. Differentiated 2-cell, 4-cell, 8-cell and 16-cell cysts contain a branched fusome, and can be easily distinguished from GSCs and CBs. The spectroosome and fusome are the same germ cell-specific intracellular organelle with different morphologies, which can be reliably labeled with antibodies against their components such as Hu li-tai shao (Hts) [36] (Fig. 1A and 1B). To verify the RNAi-mediated Piwi knockdown efficiency, we used polyclonal anti-Piwi antibodies to examine Piwi protein expression in the control and piwiKD germaria, in which cap cells and ECs are marked by the PZ1444 enhancer trap line [37]. Two additional microRNA-based UAS-RNAi transgenic strains were also used in this study: one RNAi strain was generated by the Perrimon Laboratory at Harvard Medical School, HMS [38,39], and the other RNAi line, THU, was generated to target a different piwi sequence by the Ni laboratory at Tsinghua University. The PZ1444 enhancer trap line expresses nuclear β-galactosidase protein in cap cells and ECs, which can be reliably distinguished by their location and morphology [37,40] (Fig. 1C). As previously reported [23,41], Piwi is generally expressed in both somatic and germ cells of the control germaria, but ECs express higher levels of Piwi than cap cells (Fig. 1C). Indeed, all the RNAi lines can efficiently eliminate Piwi expression in PZ1444-positive ECs and cap cells, while Piwi expression in germ cells including GSCs remains unchanged in the piwiKD germaria (Fig. 1D–1F). c587-mediated expression of the HSM and THU RNAi lines can also cause the accumulation of ill-differentiated SGCs outside the GSC niche, similar to the VDRC line (Fig. 1G and 1H). Because a wild-type germarium normally contains one or two CBs, a germarium containing three or more SGCs is considered to exhibit the germ cell differentiation defect [42]. To determine the severity of the germ cell differentiation defects, we classified the piwi knockdown germaria into three categories: normal (SGCs ≤2), moderate differentiation defect (3 ≤ SGCs ≤ 10) and severe differentiation defect (SGCs ≥11). Among the germaria in which piwi is knocked down in ECs by three independent RNAi lines, 40–60% of them have 3 or more SGCs, and approximately 20% of them have 11 or more SGCs (Fig. 1I). In addition, egg chambers are also often filled with undifferentiated spectroosome-containing SGCs (Fig. 1B and 1H). Quantitatively, the three RNAi lines produce similar degrees of germ cell differentiation defects (Fig. 1I). As shown in Fig. 1H, there are some variations on SGC numbers in the knockdown germaria by different RNAi strains, and Fig. 1B, 1G and 1H reflect the variations among the three RNAi strains. In addition, we have observed that 10–50% of the piwiKD germaria by the three independent RNAi lines completely lose GSCs and become agamic, suggesting that Piwi is required in somatic cells for maintaining GSCs (Fig. 1I–1L). These results indicate that Piwi is indeed required in ECs to promote germ cell differentiation and in ECs, cap cells or both to maintain GSCs.

The germarial region of the piwiKD ovaries appears to be reduced in size, suggesting that the EC number may also be reduced as well (Fig. 1B and 1H). Our previous studies have suggested that the severity of EC loss is positively correlated with the severity of the germ cell differentiation defects [18,19]. We then quantified EC numbers in the control and piwiKD germaria. In contrast with the control germarium containing an average of 35 ECs (Fig. 1M and 1N), the piwiKD germarium contains significantly fewer ECs (Fig. 1N–P). Because ECs rarely proliferate, the reduction in EC number could be due to apoptosis. To directly test this idea, we used TUNEL labeling to detect dying ECs identified by PZ1444 expression. Indeed, there is a consistent increase in apoptotic piwiKD ECs by the three independent RNAi lines (Fig. S1). These results demonstrate that Piwi is also required for maintaining EC survival.

Piwi functions in adult ECs to promote germ cell differentiation

Since c587 is known to be expressed by most, if not all, somatic precursor cells in the female gonad [43], the differentiation defects and the GSC loss phenotype caused by piwi knockout could be due to its early requirement in somatic gonadal precursors. To definitively determine if Piwi is required in adult ECs to control germ cell differentiation, we carried out temperature shift experiments to inactivate Piwi function specifically in adult ECs. When the genetic crosses were carried out at 18°C, which lowers
Piwi RNAi expression and thus its knockdown effect, the germlia show almost normal germ cell differentiation and GSC maintenance because all the piwiKD germlia have normal SGCs numbers and still retain two or three GSCs (Fig. 2A–D). After the adult females emerged at 18°C, they were cultured at 29°C for a week to increase RNAi expression and piwi knockdown efficiency and thus inactivate Piwi function in adult ECs. Interestingly, the number of the germlia carrying three or more SGCs drastically increases, indicating that Piwi is indeed required in adult ECs to promote germ cell differentiation (Fig. 2E–H). Similarly, the numbers of the germlia containing no GSCs also increase following Piwi knockdown by the three RNAi lines (Fig. 2I–K). In addition, Piwi expression is still reduced in cap cells, suggesting that piwi is likely expressed at low levels in cap cells (Fig. S2). Since the previous findings have shown that ECs also contribute to GSC maintenance [18,19,41], the GSC loss phenotype could come either from Piwi knockdown in cap cells, ECs or both. Taken together, these results indicate that Piwi is required in adult ECs to promote germ cell differentiation as well as in adult ECs, adult cap cells or both for GSC maintenance.

Piwi is required in ECs to prevent BMP signaling in differentiated germ cells

Previous studies have shown that the germ cell differentiation defects caused by defective EC function result from elevated BMP signaling [17,18,19]. To determine if BMP signaling activity is augmented in the germ cells of the piwiKD germlia, we examined the expression of pMad, Dad-lacZ and bam-GFP, three BMP signaling activity reporters in Drosophila, in the control and piwiKD germlia. Activation of BMP receptors (Tkv and Sax) upon BMP ligand binding leads to production of phosphorylated Mad (pMad), which translocates into the nucleus with Medea, a SMAD4 homolog, to activate Dad expression and repress bam expression in GSCs [35,45,46]. In contrast with the control germlia in which pMad accumulates primarily in GSCs (Fig. 3A), pMad is also expressed in some, but not all, SGCs outside the GSC niche of the piwiKD germlia, indicating that BMP signaling activity indeed spreads outside the GSC niche (Fig. 3B, 3C and S3A). bam-GFP and Dad-lacZ can recapitulate the expression patterns of bam and Dad in the control germlia: bam-GFP is normally expressed in differentiated germ cells but is absent from SGCs [47], while Dad-lacZ is normally expressed in GSCs but is absent in differentiated germ cells [35,45,46] (Fig. 3D and Fig. S3C). Although it is still expressed in GSCs of the piwiKD germlia (Fig. 3E, 3F and S3B), Dad-lacZ reduces its expression by about 25% based on quantification results (Fig. 3G). Although bam-GFP remains repressed in the GSCs of the piwiKD germlia, it fails to be upregulated in some SGCs outside the GSC niche in the piwiKD germlia as in control CBs (Fig. S3C–F). These results indicate that Piwi is required in ECs to prevent BMP signaling activity in differentiated germ cells.

Previous studies have revealed that the elevated transcription of dpp, which encodes a BMP ligand, in ECs can contribute to increased BMP signaling in differentiated germ cells [18,19,20]. In Drosophila, another BMP-encoding gene, gbb, is also expressed in the germlia and is required for maintaining GSCs [35]. In addition, daily upregulation in ECs has also been shown to be responsible for BMP signaling activity elevation [17,48,49]. To determine if the elevated BMP signaling activity in SGCs is due to upregulation of dpp, gbb or daily in ECs, we sequenced the mRNAs isolated from the purified GFP-labeled control and piwiKD ECs. Based on RNA sequencing and qRT-PCR results, dpp is significantly upregulated in the piwiKD ECs compared to the control ECs (Fig. 3H and 3I). Although RNA sequencing results show that gbb and daily are slightly upregulated in the piwiKD ECs (Fig. 3I), qRT-PCR results fail to confirm the finding (Fig. 3I). These results suggest that dpp upregulation in the piwiKD ECs might be responsible for germ cell differentiation defects.

To determine if dpp upregulation in the piwiKD ECs contributes to germ cell differentiation defects, we quantified SGCs outside the GSC niche in the germlia in which piwi and dpp are simultaneously knocked down in ECs. Here we used two different piwi (VDRC and HMS) and dpp RNAi (TRP and HMS) lines to knockdown piwi and dpp expression in ECs, respectively. Based on the numbers of the germlia carrying 3 or more SGCs, c587-driven dpp knockdown (TRP) can partially rescue the germ cell differentiation defects caused by piwi knockdown (VDRC) (Fig. 3J–L). c587-driven piwi knockdown by the HMS line yields stronger germ cell differentiation defects, which can be slightly and moderately repressed by c587-driven expression of TRP and HMS dpp RNAi lines, respectively (Fig. 3L and Fig. S4). c587-driven expression of the dpp HMS line causes partial GSC loss, but the expression of the dpp TRP line does not suggest that the HMS line might be stronger than the TRP line in knocking down dpp expression (Fig. S4). Based on the finding that the germ cell differentiation defects caused by piwi knockdown can only be partially repressed by c587-mediated dpp knockdown, the germ cell differentiation defects cannot be solely attributed to upregulated dpp expression in ECs (Fig. S4). Taken together, we propose that dpp upregulation in piwiKD ECs contributes to, but is not one of the major causing factors, for germ cell differentiation defects.

Defective EGFR-MAPK signaling in ECs causes germ cell differentiation defects by upregulating dally expression and thus increasing BMP signaling, and also prevents the formation of long cellular processes [17,50]. Although our results show that Piwi knockdown does not lead to daily upregulation (Fig. 3H and 3I), we wanted to confirm if Piwi is required to maintain EGFR-MAPK signaling in ECs by examining the expression of pERK, a phosphorylated and active form of MAPK, in the piwiKD ECs. In the control, pERK is strongly and specifically expressed in all ECs, but not in cap cells and follicle cells (Fig. S5A). pERK is expressed at low levels in the remaining piwiKD ECs (Fig. S5A–E). Although pERK immunofluorescence intensity in the piwiKD ECs decreases by 25–65% in comparison with the control ECs (Fig. S5E), overall pERK levels might increase instead because the nuclear translocation of pERK is not prevented by piwi knockdown (Fig. S5A–D). To determine if increasing MAPK activity affects GSC maintenance and differentiation, we used c587 to drive the expression of a kinase-active rolled (rl, encoding MAPK in Drosophila) mutant, rlPKM, in ECs [51]. Increasing MAPK activity does not have any obvious effect on GSC maintenance and CB differentiation (Fig. S5). Interestingly, following c587-mediated piwi knockdown by the three RNAi lines, ECs lose their long cellular processes (Fig. S6). These results align well with our earlier finding of no expression changes for dally in the piwiKD ECs, and also suggest that EGFR signaling is not the only pathway for maintaining EC cellular processes.

Piwi is required in ECs for repressing TE activity and preventing DNA damage

piRNAs have been shown to be required for silencing TE activity in both germ cells and somatic cells [6,7,9,52]. One of the outcomes for elevated TE activity is DNA damage. Thus, we examined the expression of phosphorylated H2Av (γ-H2Av), a Drosophila equivalent of mammalian H2AX [53], in the control and piwiKD ECs, and quantified γ-H2Av-positive ECs. γ-H2Av has been shown to be associated with DNA double-strand breaks in Drosophila cells [53]. In the control germlia, less than 5% of the ECs are positive for γ-H2Av (Fig. 4A and 4C). In contrast,
8%–25% of the piwiKD ECs are positive for c-H2Av depending on the RNAi lines (Fig. 4B and 4C). These results indicate that Piwi is required in ECs to prevent DNA damage.

To further determine if Piwi is required in ECs for silencing TE activity, we sequenced the RNAs from the purified GFP-labeled control and piwiKD ECs by fluorescence-activated cell sorting (FACS). In this study, we chose to examine two common somatic cell-specific TEs, gypsy and zam, and a germline-specific TE, tart [54]. Both gypsy and zam transcripts are drastically and significantly upregulated in the piwiKD germaria in comparison with the control (Fig. 4D and 4E). As expected, the germline-specific tart transcripts are not changed dramatically in the piwiKD germaria in comparison with the control (Fig. 4F). In addition, we also used the gypsy-lacZ reporter to verify the qRT-PCR results. In the control germaria, gypsy-lacZ is not expressed (Fig. 4G). In contrast, it is dramatically upregulated in the piwiKD ECs by the three RNAi lines (Fig. 4H–J). These results further support the idea that Piwi is required in ECs to repress TE activity and prevent DNA damage.

Yb has been shown to regulate Piwi expression in TF and cap cells [27]. Indeed, in the c587-mediated YbKD germaria, Piwi protein expression in ECs and follicle cells is consistently downregulated (Fig. S7A–C). However, Yb protein expression in somatic cells, including cap cells, ECs and early follicle cells, remains unchanged in the c587-mediated piwiKD germaria (Fig. S7D–G). To further determine if Yb is also required in ECs to repress TE activity, we examined the expression of gypsy-lacZ in the YbKD germaria. As previously reported, Yb is also expressed in all ovarian somatic cells, including cap cells, ECs and early follicle cells (Fig. 4L and 4M). Interestingly, gypsy-lacZ expression is upregulated in the YbKD cap cells and ECs, indicating that Yb is also required in somatic cells to silence TEs (Fig. 4N and 4O).
However, gypsy-lacZ expression appears to be lower in the piwiKD ECs than in the piwiKD ECs (Fig. 4B–F). Although most of the piwiKD germaria contain normal numbers of GSCs and SGCs (Fig. 4C and 4G), approximately 25% of the piwiKD germaria carry 3 or more SGCs (Fig. S7H–K). These results suggest that Yb is also required in ECs to repress TEs and promote germ cell differentiation.

Piwi is required intrinsically to maintain germ cells before adulthood

Since Piwi is expressed in all the germ cells, including GSCs, we then used nanos-gal4VP16 (nos-gal4) to specifically knock down piwi in germ cells to determine if Piwi is also required intrinsically for GSC maintenance. The nos-gal4 driver is expressed specifically in germ cells from primordial germ cells (PGCs) to adult germ cells, including GSCs [54]. In contrast with the control third-instar female gonad (Fig. 5A), nos-gal4 driven expression of the two independent piwi RNAi strains, HMS and THU, leads to a reduction in PGC numbers in the female gonads, indicating that Piwi is required for PGC proliferation, maintenance or both (Fig. 5B–D). Furthermore, germ cell-specific piwi knockdown germaria in newly emerged adults show a complete loss of all germ cells, including GSCs (Fig. 5E and 5F). The GSC establishment takes place at the transitional period from the third instar-larval stage to the pupal stage. These results indicate that Piwi is required intrinsically to control PGC maintenance and/or GSC establishment.

To further explore whether other piRNA components are also required for GSC maintenance before adulthood, we used nos-gal4 driven expression of RNAi against armi and aub to inactivate their function throughout germ cell development. Germ cell-specific armi or aub knockdown by two independent RNAi lines for each gene leads to a dramatic reduction in nuclear Piwi protein expression in germ cells, but does not affect nuclear Piwi expression in somatic cells (Fig. 5G–I). In addition, germline-specific armi or aub knockdown also causes the full penetrance of female sterility. These results suggest that both of them are efficiently knocked down in the germine because they are required for Piwi nuclear localization and to prevent the activation of meiotic cell cycle checkpoints caused by transposon-induced DNA damage [56]. However, the armi or aub knockdown germaria from newly eclosed females still contain two or three GSCs in their germaria, indicating that they are not required intrinsically for early germ cell development and GSC formation (Fig. 5G–I). Consistently, newly eclosed armi232/armi1 and aubH148/aubQ42 mutant females also maintain two or three GSCs in their germaria, and dramatically decrease nuclear Piwi expression in GSCs and their progeny (Fig. 5J–L). Since these mutants carry strong loss-of-function mutations in armi or aub [56,57], these results further support that Arm and Aub are dispensable for germ cell development before adulthood. Taken together, our results indicate that Piwi controls early germ cell development, GSC formation or both independently of Arm and Aub.
Piwi is required intrinsically to control GSC maintenance and germ cell differentiation in the adult ovary

To determine if Piwi is required in the adult germline to maintain GSCs, we used the flip-out system, in which a transcriptional stop sequence flanked by two FRT sites is inserted between the nanos promoter and gal4VP16, to activate the expression of RNAi lines along with the GFP reporter specifically in germ cells after heatshock treatments of adult females (Fig. 6A).
In the control ovaries, GFP-positive GSCs detected 1 day after heatshock (1 d AHS) remain in the niche for additional three weeks (Fig. 6B and 6C). The GFP-marked piwiKD GSCs can be readily detected in the germaria 1 d AHS (Fig. 6D and 6E). In contrast, most of the GFP-marked piwiKD GSCs are lost three weeks AHS, and consequently over 30% of the piwiKD germaria have completely lost GSCs (Fig. 6F–H). In addition, more undifferentiated SGCs also accumulate in the piwiKD germaria three weeks AHS, indicative of germ cell differentiation defects (Fig. 6I and 6J). Interestingly, some SGCs outside the GSC niche are GFP-negative and also Piwi-negative, which is caused by the failure in nos-gal4-driven GFP expression due to an unknown reason (Fig. 6I and 6J). These results demonstrate that Piwi is required in adult germline for GSC maintenance and germ cell differentiation.

**Discussion**

Although the primary piRNA pathway is known to operate in *Drosophila* ovarian somatic cells to repress TE activity, its biological importance in *Drosophila* oogenesis is not well understood. Piwi, one of the key components in the primary piRNA pathway, has been shown to function in TF/cap cells to control GSC maintenance [22,23,24]. In this study, we have revealed a novel
role of Piwi in ECs to control GSC lineage differentiation and additional roles in the germline for PGC and GSC maintenance (Fig. 6K). \textit{piwi} knockdown in somatic cells results in defective GSC maintenance, defective germ cell differentiation as well as increased TE activity and DNA damage. \textit{dpp} upregulation contributes to the germ cell differentiation defects caused by somatic \textit{piwi} knockdown. A recent study has also come to a similar conclusion that Piwi is required in somatic cells for promoting germ cell differentiation by repressing BMP signaling [58]. In addition, we have also shown that Piwi is required in PGCs to control PGC maintenance, GSC formation or both, and is also required in adult germline to maintain GSCs and promote germ cell differentiation (Fig. 6K). Therefore, our genetic results argue strongly that Piwi functions in germline to maintain PGCs and GSCs as well as to promote germ cell differentiation. Therefore, we have revealed new functions of Piwi in multiple cell types to maintain GSCs and promote germ cell differentiation (Fig. 6K).

Piwi is required in ECs and germ cells to promote germ cell differentiation

Recent studies have shown that ECs play an important role in promoting germ cell differentiation by repressing BMP signaling [16]. Thus far, genes identified to be important in ECs for germ cell differentiation repress the expression of either \textit{daily} or \textit{dpp}, thereby preventing BMP signaling in ECs. EGFR signaling has been proposed to be responsible for directly repressing \textit{daily} expression in ECs, but is dispensable for EC survival [17]. In addition, recent studies have also shown that Rho signaling and Eggless are also required in ECs for repression of \textit{dally} expression, and are also required for EC survival and the maintenance of long EC cellular processes [18,19]. Lsd1, Rho signaling and Eggless have been shown to be required to repress \textit{dpp} transcription [18,19,20]. In this study, we have also shown that Piwi is required in ECs for \textit{dpp} repression but is dispensable for \textit{daily} repression. In addition, it is also required in ECs for maintaining their survival and long cellular processes. Our genetic results suggest that \textit{dpp} upregulation partially contributes to the germ cell differentiation defects caused by Piwi knockdown in ECs. In contrast with the
Piwi is required in both somatic cells and germ cells to maintain GSC lineage

Although Piwi is generally expressed in almost all somatic cells and germ cells of the Drosophila ovary, the previous studies proposed that Piwi acts in TF/cap cells to control GSC self-renewal [22,23,24]. In this study, we have confirmed the somatic role of Piwi in GSC maintenance, and have also revealed new roles of Piwi to maintain PGCs before adulthood and GSCs after adulthood. In addition, our temperature shift experiments have shown that Piwi is also required in adult somatic cells, TF/cap cells, ECs or both, to maintain GSCs. Interestingly, RNAi-mediated knockdown of Piwi function in adult somatic cells only produces a moderate GSC loss phenotype in comparison with the severe GSC loss phenotype of piwi mutants, suggesting that Piwi might also function in other cell types to maintain GSCs. In the future, it will be important to determine if the piRNA pathway, epigenetics or both play a role in the regulation of germ cell differentiation.

Materials and Methods

Drosophila strains and culture

The Drosophila stocks used in this study include: c587 [35], P2144 [37], UAS-dcr2, UAS-dppRNAi lines (TR00047P1; HMS00011), UAS-piwiRNAi lines (NDR101658, HMS00666 and THU00412), UAS-armiRNAi lines (GL00254; HMS00998), UAS-aubRNAi lines (GL00076; HMS00611) and UAS-YBRNAi (GL00053; GL00204). Drosophila strains were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified. To maximize the RNAi-mediated knockdown effect, newly eclosed flies were cultured at 29°C for a week before the analysis of ovarian phenotypes.

Construction of UAS-RNAi and nos->mCherry SV40 polyA->gal4VP16 Strains

The new THU UAS-RNAi line targeting piwi was constructed using the pVALIUM20 vector according to the published procedure [60]. The targeting sequence for piwi is CCCCGTATGCTGACAGAGAA, which was designed based on the algorithm of DSIR.

To construct pnos-FRT-mCherry-SV40 polyA-FRT-gal4VP16-nos 3' UTR, different components were assembled together by five steps. First, the coding region of mCherry was amplified from a mCherry-containing vector using 5'-agcttcaatagtcagacagggagagg-3' and 5'-gaacctgtaagattcgagctc-3' primers (Nde I and Xho I sites underlined), and was cloned into Nhe I site of the pattern targeting piwi. Then, the FRT-mCherry fragment amplified using 5'-atcagttgagctcagagct-3' and 5'-atgctgctcagctcagc-3' primers (Nde I and Xho I sites underlined) from the pFRT-mCherry-SV40 polyA-FRT vector was cloned into the pGEM-T vector (Promega) to generate pFRT-mCherry. The second, SV40 polyA-FRT fragment amplified from the pFRT-SV40 polyA-FRT vector using 5'-gaacctgtaagattcgagctcagagct-3' and 5'-atgctgctcagctcagc-3' primers (Nde I and Xho I sites underlined) from the pFRT-mCherry-SV40 polyA-FRT was cloned into the pGEM-T vector (Promega) to generate pFRT-mCherry.

Although Piwi has been shown to be required to control PGC formation and GSC division [25,27], it remains unclear if Piwi is required intrinsically to maintain PGCs and GSCs. In this study, we have revealed critical roles of Piwi in different developmental stages of germ cells. First, Piwi is required in the developing female gonad to control PGC proliferation, survival or both because germline-specific Piwi knockdown leads to a complete elimination of germ cells including GSCs in newly eclosed adult females, suggesting that Piwi controls PGC proliferation and survival or GSC formation possibly independently of Arm and Aub, possibly piRNAs. Piwi has been shown to physically interact with HIP1a to epigenetically control gene expression in somatic tissues [41]. In addition, piwi genetically interacts with certa, encoding a chromodomain-containing protein, to control GSC maintenance [59]. Our findings are consistent with the notion that Piwi controls early germ cell development perhaps via epigenetics. Third, Piwi is required in adult GSCs for their maintenance because germline-specific knockdown in the adult ovary also causes a moderate GSC loss phenotype. Therefore, we propose that Piwi functions in multiple stages of germline development to control PGC proliferation and survival, and GSC maintenance (Fig. 6K).
to generate *plox-FRT-mCherry-SV40* polyA-FRT-gal4VP16-nos 3′UTR, which was introduced into an *attP* site-containing fly strain [BL#24482] using PhiC31 integrase-mediated transgenesis by BestGene, Inc.

**Immunohistochemistry**

Immunohistochemistry was performed according to our previously published procedures [62,63]. The following antibodies were used in this study: rabbit polyclonal anti-β-galactosidase antibody (1:100, Cappel), Guinea pig polyclonal anti-Piwi antibodies (1:100; produced by H. Lin), chicken polyclonal anti-GFP antibody (1:200, Jax), mouse monoclonal anti-His antibody (1:50, DSHB), mouse monoclonal anti-Yb antibody (1:200; kindly provided by Dr. H. Siomi), rabbit polyclonal anti-pS137 H2Av antibody (1:100, Rockland), rabbit monoclonal anti-pS423/425 Smad3 antibody (1:100, Epitomics), rabbit polyclonal anti-pERK antibodies (1:25, Cell Signaling) and rat monoclonal anti-Vasa antibody (1:50, DSHB). All images were taken with a Leica TCS SP5 confocal microscope.

**Cell sorting and RNA sequencing**

*Drosophila* ovaries were dissected and placed in Grace’s medium (Sigma-Aldrich; G9771) and then washed twice by adding 1× DPBS and centrifuged at 700 × g for 1 minute. The ovaries were incubated with prewarmed Collagenase (Worthington, Collagenase Type II, Lot# 50D11833) in 15 ml conical tube at 37°C water bath for 3 minutes with gentle shaking. Enzyme reaction was stopped after 3 minutes by adding cold 1× DPBS+2% FBS. Dissociated sample was washed by adding 1× DPBS and centrifuged at 700 × g for 5 minutes. The cell pellet was resuspended in 1× DPBS and centrifuged with 70 um Falcon (BD; 340605) in to 5 ml flow tubes. Cells were centrifuged and then resuspended in 200 ul of 1× DPBS for sorting at 45 psi with 70 um tip (BD: InFlux) immediately in to TRIZol (life technologies; 15596-018); Total RNAs were extracted with Trizol and purified by organic extraction followed by isopropanol precipitation.

Following manufacturer’s directions and using the Illumina TruSeq library construction kits (Illumina, Cat. No. RS-122-2001/2), mRNA was isolated from 150 ng of total RNA per sample and short fragment libraries were constructed. The resulting libraries were purified using Agencourt AMPure XP system (Backman Coulter, Cat. No. A63880), and were then quantified using a Bioanalyzer (Agilent Technologies) and a Qubit fluorometer (Life Technologies). All libraries were pooled, re-quantified and run as 50 bp single-end lanes on an Illumina HiSeq 2000 instrument, using HiSeq Control Software 1.5.15.1 and Real-Time Analysis (RTA) version 1.13.48.0. Secondary Analysis version CASAVA-1.8.2 was run to demultiplex reads and generate FASTQ files.

For qRT-PCR, total RNAs were first treated by DNase I, and then used for synthesis of cDNAs using mixed oligo dT and random primers and SuperScript III Reverse Transcriptase (Life Technologies). Fluorescence-based quantitative real-time PCR (qPCR) was performed to quantify *gypsy*, *zan*, *TART*, *gbb*, *dpp* and *daily* with *dpp*, *gapdh* and *rp32* as internal controls using primers shown in Table S1. After cDNAs were diluted at 1:100, 2 μl aliquots of each cDNA sample were added to 5 ul of 2× power SYBR Green PCR Master Mix (Applied Biosystems part No.: 4367659, Lot No.: 1305403), 0.5 μl each of 10 nm Forward & Reverse primer and 2 ul of water in a 384-well plate. The resulting reactions were run on an ABI 7900HT according to the instruments standard protocol. Analysis of the fluorescence curves was done using ABI’s SDS2.4 software. The Ct values were analyzed using the Biogazzelle qBase plus version 2.4 software to generate normalized relative quantities using assays for endogenous controls.

**Supporting Information**

Figure S1  *Piwi* knockdown increases apoptosis in ECs. (A) PZ1444-positive control ECs are negative for TUNEL labeling. (B, C) Apoptotic PZ1444-positive ECs (arrows) are detected in the *piwi* KD germaria by VDRC (B) and HMS (C) RNAi lines. The dying ECs appear to show low PZ1444 expression. (D) Quantification results of TUNEL-positive ECs in control and *piwi* KD germaria. Scale bars: 25 μm.

(TIF)

Figure S2  *c587* drives expression of *piwi* RNAi in adult cap cells. (A, A′) Piwi is expressed in cap cells (broken lines) at low levels. (B–D′) *c587*-driven expression of VDRC (B, B′), HMS (C, C′) and THU (D, D′) *piwi* RNAi lines reduces Piwi protein expression in adult cap cells as well as in ECs. Scale bars: 25 μm.

(JPG)

Figure S3  *Piwi* is required in ECs to prevent BMP signaling in differentiated germ cells. Cap cells are highlighted by asterisks. (A, B) *c587*-mediated *piwi* KD by the THU line results in upregulated *pMad* (A) and *Dad-lacZ* (B) expression in SGCs a few cells away from cap cells. (C) *bam-GFP* is repressed in GSCs and upregulated in differentiated germ cell cysts (arrow) of the control germarium. (D–F) *c587*-mediated *piwi* KD by three *piwi* RNAi lines causes repression of *bam-GFP* expression in some SGCs (arrowheads) outside the GSC niche. Differentiated cysts (arrows) still maintain high *bam-GFP* expression. G shows quantification results of *bam-GFP*-negative CBs. Scale bars: 25 μm.

(JPG)

Figure S4  *dpp* upregulation in *piwi* KD ECs might not be the major factor causing germ cell differentiation defects. Asterisks indicate the GSC niche. (A–C) *c587*-mediated *dpp* knockdown by TRP (A) and HMS (B, C) lines does not affect GSC maintenance and differentiation because the knockdown germaria still maintain two GSCs (arrows). However, some *dpp* KD germaria (C) by the HMS line, but not by the TRP line, completely lose their germ cells including GSCs. (D–H) *c587*-mediated *dpp* knockdown suppresses the germ cell differentiation defects in some *piwi* KD germaria (E, G) but not in the other germaria (F, H) in comparison with the germ cell differentiation defects in the *piwi* KD germaria (D). Arrows in D, F and H point to spectrosomes, whereas those in E and G indicate branched fusions. Scale bars: 25 μm.

(TIF)

Figure S5  *pERK* activity in *piwi* KD ECs. (A) *pERK* is specifically expressed in ECs (one by arrowhead) of the control germarium. (B E) *c587*-mediated *piwi* KD ECs (arrowheads) are often larger and show lower *pERK* fluorescence intensity. E shows quantification results on *pERK* intensity. (F G) *c587*-mediated *p*STK5 expression does not affect GSC and CB numbers (arrows indicate GSCs). H shows that there are no significant differences in GSCs and CBs between control and *p*STK5-expressing germaria. Scale bars: 25 μm.

(TIF)

Figure S6  Piwi knockdown in ECs disrupts the formation of their long cellular processes. (A) *c587*-mediated
CD8GFP expression highlights long EC cellular processes (arrows) wrapping CBs, mitotic cysts and 16-cell cysts in the control germarium. (B–D) In the c587-mediated piwiKD germaria by three RNAi lines, HMS (B), THU (C) and VDRC (D), there are no long-GFP-positive cellular processes wrapping differentiated germ cells. Scale bars: 25 μm. (TIF)

Figure S7 Yb is required in ECs to promote germ cell differentiation. The GSC niche is highlighted by broken lines (A’ C’) or the asterisk (H J). (A’ C’) c587-mediated YbKD by two RNAi lines, GL3 (B B’) and GL2 (C C’), leads to a Piwi protein expression reduction in cap cells (broken lines), ECs (arrowheads) and early follicle cells in comparison with the control (A). (D–G) c587-mediated piwiKD by three RNAi lines, VDRC (E), HMS (F) and THU (G), has no effect on YB protein expression in cap cells, ECs and early follicle cells in comparison with the control (D). (H) The control germarium contains three GSCs and differentiated cysts (arrow). (I–K) c587-mediated piwiKD causes an accumulation of excess SGCs (arrowheads) in the germarium. K represents the quantitative results on the germaria carrying three or more SGCs. Scale bars: 25 μm. (TIF)

Table S1 This table contains the nucleotide sequences of all the primers used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: XM TX. Performed the experiments: XM SW TD XS YN LL YG YM Hui Li KM A. Peak A. Perera Hua Li WM JP KG JH JN. Analyzed the data: Hua Li XM TX. Contributed reagents/materials/analysis tools: MI YY Haifan Lin. Wrote the paper: XM TX.

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