RESEARCH ARTICLE

Transposon Dysregulation Modulates dWnt4 Signaling to Control Germline Stem Cell Differentiation in Drosophila

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Abstract

Germline stem cell (GSC) self-renewal and differentiation are required for the sustained production of gametes. GSC differentiation in Drosophila oogenesis requires expression of the histone methyltransferase dSETDB1 by the somatic niche, however its function in this process is unknown. Here, we show that dSETDB1 is required for the expression of a Wnt ligand, Drosophila Wingless type mouse mammary virus integration site number 4 (dWnt4) in the somatic niche. dWnt4 signaling acts on the somatic niche cells to facilitate their encapsulation of the GSC daughter, which serves as a differentiation cue. dSETDB1 is known to repress transposable elements (TEs) to maintain genome integrity. Unexpectedly, we found that independent upregulation of TEs also downregulated dWnt4, leading to GSC differentiation defects. This suggests that dWnt4 expression is sensitive to the presence of TEs. Together our results reveal a chromatin-transposon-Wnt signaling axis that regulates stem cell fate.

Author Summary

Every multicellular organism is made up of tissues that are maintained by stem cells, due to their capacity to both self-renew and differentiate into terminal cell types. Loss of either of these processes can lead to aging, progression towards degenerative diseases and cancers. Insight into how self-renewal and differentiation are regulated will have tremendous therapeutic impact. Drosophila is an excellent model system for stem cell study due to the availability of various mutants, markers and RNAi technology. We study the ovaries of the female Drosophila, whose stem cell population gives rise to gametes. The tissue from which these gametes arise is surrounded by somatic cells, which provide signaling cues required for proper self-renewal and differentiation. While the mechanisms by which

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these somatic signals regulate stem cell self-renewal is known, how somatic cues regulate differentiation remains unclear. Here, we have identified that transposons, selfish genetic elements, modulate dWnt4 expression in the somatic cells. We demonstrate that dWnt4 promotes the somatic encapsulation of the stem cell daughter by regulating adherens junction proteins, thereby promoting differentiation. Transposons have been linked to cancers, and therefore establishing how transposons regulate genes essential for differentiation can provide new perspectives on their role in cancer.

Introduction

Drosophila female germline stem cells (GSCs) are an excellent tractable model system to study the mechanisms that regulate stem cell division and differentiation [1–3]. GSCs reside at the anterior end of the ovaries in a structure called the germarium. GSCs divide to give rise to a stem cell daughter or a cystoblast (CB). The CB then turns on a differentiation factor, bag of marbles (bam) that is both necessary and sufficient to drive differentiation into a sixteen-cell cyst (Fig 1A) [4,5]. One of these sixteen cells becomes an oocyte, while the other fifteen cells become nurse cells.

Both intrinsic and extrinsic factors regulate GSC self-renewal and differentiation into an oocyte [1,2,6]. Two extrinsic factors regulating GSC self-renewal are structural support and Decapentaplegic (Dpp) signaling provided by the terminal filament, cap cells and escort cells located proximally in the somatic niche (Fig 1A) [6,7]. Within the niche, the terminal filament and cap cells provide signaling for GSC self-renewal, while the escort cells physically enclose CBs, allowing for their proper differentiation (Fig 1A) [8,9]. However, signaling pathways that regulate escort cell encapsulation, thereby promoting GSC differentiation, have not been fully elucidated.

dSETDB1 (also known as Eggless [Egg]), a histone methyltransferase, trimethylates histone 3 lysine 9 (H3K9me3) to initiate heterochromatin formation [10]. It activates the transcription of piwi interacting RNAs (piRNAs), which are critical for controlling transposable elements (TEs) to protect genome integrity [11,12]. These piRNAs, with their bound Argonaute proteins, such as Piwi, Aubergine (Aub) and Argonaute 3 (Ago3), target TEs for transcriptional and post-transcriptional silencing [13–15]. dSETDB1 is also an extrinsic factor required in the escort cells to promote GSC differentiation through an undetermined mechanism [11]. Intriguingly, like the loss of dSETDB1, somatic loss of piwi or mutations in the somatic piRNA clusters, such as flamenco, also results in GSC differentiation defects [11,16,17]. It was proposed that the loss of GSC differentiation observed in piwi mutants is due to Dpp over expression [18]. However, it was recently demonstrated that although Dpp upregulation in piwi mutants contributes to GSC differentiation, it is not one of the major controlling factors [16]. Therefore, we hypothesized that up-regulation of TEs in somatic cells could modulate an as yet unidentified signaling cue that promotes GSC differentiation.

Wnt signaling is critical for maintaining various stem cell systems [19]. Wnts are secreted lipid-modified proteins that mostly act over short distances [19]. Secreted Wnts bind to receptors such as Frizzled 2 (Fz2), activating downstream signaling [20]. The binding of Wnt to these receptors results in stabilization of a downstream effector called β-catenin (Armadillo in Drosophila) [20]. β-catenin then translocates to the nucleus, where it binds to the TCF/LEF family of transcription factors, activating transcription of downstream targets [20]. There are two Wnt ligands that have been shown to play an important role in Drosophila oogenesis, wingless (wg) and dWnt4. wg acts later in the differentiation process to regulate follicle stem
cells that envelope the developing cysts [21]. dWnt4 function is required for successful somatic cell migration in the developing gonad [22]. dWnt4 mutants exhibit ovariole ensheathment defects and have been shown to be female sterile [22].

In line with our hypothesis, we have identified the Wnt ligand dWnt4, downstream of dSETDB1, to control GSC differentiation. We find that dWnt4 acts in an autocrine manner in the escort cells of the stem cell niche to promote somatic encapsulation of CB. We have identified the gap junction protein Innexin 2 (inx2), and proteins of the adherens junction (AJ) complex as downstream targets of this dWnt4 signaling. These AJ proteins regulate the encapsulation of the CB by the escort cells. Additionally, piRNA pathway mutations that specifically upregulate TEs in the soma also downregulate dWnt4 expression and lose CB encapsulation, thereby causing differentiation defects. This suggests that the presence of TEs modulates dWnt4 signaling and somatic encapsulation, thereby affecting GSC differentiation.
Results

d\textit{Wnt4} acts downstream of d\textit{SETDB1} to promote CB differentiation

To test the hypothesis that a signaling pathway from the soma controls GSC differentiation, and to identify candidate genes involved in signaling from the niche, we performed microarray analysis comparing ovaries from \textit{dSETDB1} mutants with those from \textit{bam} mutants. Both mutants are arrested at the same developmental stage, resulting in the accumulation of CBs [10,11,23]. While \textit{bam} mutants contain heterochromatic marks in both the somatic cells and the germ line, \textit{dSETDB1} mutants lack heterochromatic marks in either cell type [11]. As predicted by our hypothesis, we found that transposons were upregulated (Fig 1B). Intriguingly, we also observed that the \textit{Drosophila} Wingless type mouse mammary virus integration site number 4 (\textit{dWnt4}) was strongly downregulated, indicating a potential inhibition of the Wnt signaling pathway (Fig 1B and S1A Fig).

\textit{dWnt4} is a conserved Wnt ligand critical for \textit{Drosophila} oogenesis [22]. In mice, Wnts have been shown to mediate encapsulation of the germ line by the surrounding somatic cells, although upstream regulators have yet to be identified [24]. If \textit{dSETDB1} controls GSC differentiation through \textit{dWnt4}, then loss of \textit{dWnt4} should result in GSC differentiation defects. To test this, we analyzed two markers of differentiation, Vasa and 1B1. Vasa stains all germ cells, while 1B1 stains the endoplasmic reticulum-rich organelles known as spectrosomes in the undifferentiated GSCs and CBs, and the branched structures called the fusomes in differentiating cysts (Fig 1A) [25,26]. \textit{dWnt4} mutants accumulated undifferentiated cells, identified by their number of spectrosomes (Fig 1C–1G) (Table 1). In addition, we found pleiotropic differentiation defects in later stages that included fused egg chambers and oocyte specification defects (S1B–S1E Fig). Thus, \textit{dWnt4} is essential for normal GSC differentiation.

If \textit{dSETDB1} and \textit{dWnt4} act together, then they should perturb differentiation at the same step. Loss of \textit{dSETDB1} results in the accumulation of undifferentiated CBs, which can be identified by both the diminished expression of the GSC marker phosphorylated Mothers against dpp (pMad), and a lack of expression of the differentiating CB marker, Bam [11,23,27]. Similarly in \textit{dWnt4} mutants, we found an accumulation of cells expressing neither pMad, nor high

### Table 1. Quantitation of undifferentiated cells in mutants compared to control germaria.

| Genotype | Spectrosomes | n | P-value |
|----------|--------------|---|---------|
| Wild type | 2.5 ± 1.4 | 50 | 2.85381E-20 (compared to \textit{dWnt4}EMS23/CyO) 2.94448E-16 (compared to \textit{dWnt4}C1/CyO) |
| \textit{dWnt4}EMS23/CyO | 2.9 ± 1.1 | 90 | |
| \textit{dWnt4}C1/CyO | 2.2 ± 1.3 | 40 | |
| \textit{egg}1473/CyO | 2.3 ± 1.3 | 50 | |
| \textit{lnx}2\textsuperscript{G0035}/Fm7c | 2.5 ± 1.0 | 57 | |
| \textit{dWnt4}EMS23/d\textit{Wnt4}C1 | 5.2 ± 1.8 | 90 | |
| \textit{dWnt4}C1/\textit{egg}1473 | 5.4 ± 1.7 | 52 | |
| \textit{dWnt4}EMS23/\textit{egg}1473 | 4.7±1.5 | 34 | |
| \textit{dWnt4}C1/\textit{lnx}2\textsuperscript{G0035} | 5.2 ± 2.3 | 57 | |
| c587 | 2.1 ± 1.1 | 50 | |
| c587>\textit{dWnt4}RNAi | 7.7 ± 2.9 | 56 | 1.80E-27 |
| c587>Fz2RNAi | 5.7 ± 1.9 | 55 | 6.36033E-21 |
| c587>DE-CadherinRNAi | 5.9 ± 2.5 | 53 | 1.7529E-14 |
| c587>β-cateninRNAi | 9.7 ± 8.8 | 56 | 2.79694E-08 |

Mean number of cells containing spectrosomes ± standard deviation for the indicated genotypes determined in Z-stacks by confocal microscopy. P-values for the same were determined by two-tailed equal variance t test by comparing the respective mutants vs. wild type strains.

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levels of Bam, suggesting that dWnt4 mutants also accumulate early CBs (Fig 1H and 1I and S1F and S1G Fig). This result was not due to an increase in the niche size, as indicated by staining for cap cell marker, Lamin C, nor by an increased rate of GSC division, as measured by staining for the mitotic marker, PH3 (17% in dWnt4 heterozygous germaria, n = 66, compared to 23% in dWnt4 germaria n = 60, P-value = 0.99) (S1H–S1K Fig) [28]. To determine if these accumulated, undifferentiated CBs were capable of differentiating, we induced the expression of the differentiation factor, Bam, under the control of a heat shock promoter. After heat shock, like in dSETDB1 mutants [11], we observed the development of cysts, as revealed by the formation of fusomes (80% in dWnt4 heterozygous germaria were found to form fusomes, n = 40, compared to 85% in dWnt4 germaria n = 34 P-value = 0.5485) (Fig 1K and 1L) [5]. These results taken together suggest that dWnt4, like dSETDB1, is required for proper Bam-mediated CB differentiation.

To determine whether dSETDB1 and dWnt4 act through the same pathway to promote CB differentiation, we generated flies that were heterozygous for both dWnt4 and dSETDB1 and stained for Vasa and 1B1. 60% (n = 77) of germaria accumulated greater than 3 undifferentiated cells in the trans-heterozygote germaria compared to 4% (n = 43) for dWnt4 heterozygote and 16% (n = 50) in dSETDB1 heterozygotes, suggesting that dSETDB1 and dWnt4 do indeed act through the same pathway to control differentiation (Fig 1M and 1N and S1L and S1M Fig) (Table 1). If dWnt4 is downstream of dSETDB1 and heterochromatin formation, then the loss of dWnt4 should not cause a decrease in dSETDB1-dependent heterochromatin formation, nor an increase in the number of double-strand breaks due to TE upregulation [11,29]. Indeed, we observed no differences in heterochromatic marks using H4K20me3, nor increase of TE levels using qRT-PCR, nor increase of double-strand breaks using H2Av staining in dWnt4 mutants compared to the control (S2A–S2J Fig). Furthermore, dSETDB1 expression did not change in these mutants as measured with a Hemagglutinin (HA) tag knocked into the endogenous dSETDB1 locus (dSETDB1-HA) (S2K–S2L1 Fig) [30]. Thus, we conclude that dWnt4 acts downstream of dSETDB1 to promote CB differentiation.

**dWnt4 is expressed in the escort cells and acts in an autocrine manner to promote CB differentiation**

If dSETDB1 is regulating CB differentiation from the somatic cells via dWnt4 then: 1) dWnt4 should be expressed in the somatic cells, 2) Loss of dSETDB1 in the somatic cells should eliminate dWnt4 expression, and 3) Loss of dWnt4 in the somatic cells should prevent CB differentiation. Using fluorescent in situ hybridization (FISH) in wild type germaria, we found that dWnt4 is primarily expressed in the somatic cells that surround the germ line (Fig 2A). To independently verify this result, we used a GFP-containing Minos element inserted into the dWnt4 promoter as a reporter. We observed GFP expression in the escort cells of the somatic niche (Fig 2B and 2B1). To determine if dSETDB1 is required in the escort cells of the germarium to permit dWnt4 expression, we utilized RNA interference (RNAi) driven by c587-GAL4, which is expressed in the escort cells, to knock down dSETDB1 [31]. dSETDB1 knockdown caused a significant reduction in dWnt4 mRNA levels as measured using qRT-PCR (Fig 2C).

Next, we determined if dWnt4 is required in the escort cells for differentiation by depleting dWnt4 using c587-GAL4 to drive RNAi (Fig 2C). Loss of dWnt4 specifically in the escort cells of the germaria resulted in the loss of CB differentiation (60%, n = 86) compared to the control (4%, n = 77) (Fig 2D and 2E) (Table 1). Depletion of dWnt4 in the germ line using nos-GAL4 or in the terminal filament and cap cells using hedgehog (hh)-GAL4, did not cause any loss of differentiation (S3A–S3E Fig) [32–34]. Thus, dSETDB1 regulates dWnt4 in the escort cells of germaria to promote CB differentiation.
**Fig 2. dWnt4 is expressed in and acts from the escort cells.** (A) Fluorescent in situ hybridization (FISH) for dWnt4 mRNA (green) showing its expression in the somatic niche cells. (B-B1) Germarium of a dWnt4 reporter stained for Zfh1 (red), GFP (green) and DAPI (blue) showing the expression of GFP in the escort cells (white arrows). (C) A qRT-PCR analysis showing dWnt4 mRNA levels are downregulated in escort cell knockdowns (KD) of dsEtD81 and dWnt4. (D-E) Control and dWnt4 KD stained with 1B1 (red) and Vasa (green) showing an accumulation of >3 undifferentiated cells in dWnt4 KD (white line). (F) A schematic of the dWnt4 pathway. dWnt4 binds to its receptor, Frizzled2 (Fz2) and activates a signaling cascade. This leads to stabilization of a transcription factor, β-catenin (Armadillo) and leads to activation of downstream target genes, such as fz3. (G-G1) Germarium of a fly carrying frizzled3 RFP (fz3-RFP) stained with RFP (red), 1B1 (blue) and Vasa (green) showing expression of Fz3RFP in the escort cells (white arrow). (H-I1) dWnt4 mutants carrying the same transgene stained with RFP (red), 1B1 (blue) and Vasa (green) showing downregulation of Fz3-RFP expression (white arrow). (J) Quantification (n = 6) of RFP in the escort cells showing a downregulation in dWnt4 mutants. (K-L) Control and Frizzled2 KD, stained with 1B1 (red), Vasa (green) and DAPI (blue) showing an accumulation of >3 undifferentiated cells in Frizzled2 KD (white line).

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The dWnt4 ligand is known to bind to the Fz2 receptor to induce a signaling cascade, but in which cell type does the dWnt4 from the escort cell cause a response? (Fig 2F) [19,22,35]. We considered two possibilities: 1) dWnt4 from the escort cells directly promotes CB differentiation by activating the receptors on the germline cell surface, or 2) dWnt4 from the escort cells indirectly causes CB differentiation by activating the receptors on the escort cell surface. To distinguish between these two possibilities, we monitored the expression of a downstream transcriptional reporter activated in response to Wnt signaling, namely the Frizzled3 (Fz3) promoter fused to RFP (Fz3RFP) [36]. Fz3RFP was primarily expressed in the escort cells but not in the germ line, and this expression was significantly reduced in dWnt4 mutants (Fig 2G–2I). To further test if dWnt4 signals act on the escort cells, we selectively depleted the Fz2 receptor by RNAi. We found that somatic escort cell depletion of Fz2 results in an accumulation of undifferentiated cells (Fig 2K and 2L) (Table 1). Thus, dWnt4 acts in an autocrine loop, being released from and acting on the escort cells to indirectly promote CB differentiation.

**dWnt4 regulates escort cell encapsulation of CB to promote differentiation**

The observation that dWnt4 acts in the escort cells was intriguing, as dWnt4 signaling is known to regulate cell adhesion [37,38], a critical function for encapsulating Cbs and promoting their differentiation [9,39]. The gap junction protein Inx2 is required in the escort cells for differentiation and inx2 has been shown to regulate accumulation of DE-Cadherin (DE-Cad) [40]. As wg, a member of the Wnt signaling pathway, regulates inx2 in the developing foregut [41], we hypothesized that dWnt4 could regulate inx2 in the escort cells to mediate DE-Cad based adhesion for encapsulating Cbs. To test this, we first monitored the wild type expression of Inx2 using an inx2 lacZ reporter and found it to be expressed in the somatic niche, including escort cells (Fig 3A and 3A1) [41]. Next, we analyzed whether dWnt4 and inx2 act through the same pathway to promote CB differentiation by genetically removing a copy of dWnt4 and inx2 to create double heterozygous flies, and staining the ovaries for Vasa and 1B1. Undifferentiated cells accumulated in the trans-heterozygous germaria, as compared to heterozygous controls (10% in dWnt4 heterozygotes, n = 40; 14% in inx2 heterozygotes n = 57; 78% in transheterozygous n = 57) (Fig 3B and 3C) (Table 1). We found that LacZ levels, a reporter of inx2 transcription, were reduced in escort cells of dWnt4 mutant germaria compared to the control (Fig 3D and 3F). These results support the notion that inx2 acts in the same pathway as dWnt4 in the escort cells to promote CB differentiation.

inx2 mutants accumulate undifferentiated spectosome-containing cells [40]. These accumulated, undifferentiated cells show reduced DE-Cad accumulation where they abut the follicle cells [40]. To determine if loss of inx2 in the escort cells also leads to perturbed DE-Cad levels, we depleted inx2 in the escort cells using c587-GAL4 and found that DE-Cad levels were altered compared to control (Fig 3G–3H2). Because DE-Cad is part of the AJ complex, we also monitored for another critical AJ component protein, β-catenin. We found that compared to
control, β-catenin levels, like the DE-Cad levels, were perturbed in escort cells depleted for inx2 (Fig 3I–3J2). Selective RNAi depletion of DE-Cad and β-catenin in the escort cells using c587-GAL4 resulted in an accumulation of >3 undifferentiated cells as compared to the control (Fig 3K–3N and Table 1). This suggests that inx2 regulates AJ component proteins DE-Cad and β-catenin to promote CB differentiation.

Escort cell encapsulation of CBs is required for CB differentiation, but the escort cell intrinsic pathways that regulate this process have not been fully elucidated [9,39]. We hypothesized that Wnt signaling may promote CB differentiation by modulating AJ proteins via inx2 in the escort cell to promote encapsulation of the CB. To determine if dWnt4 regulates adhesion, we monitored the expression of the AJ components DE-Cad and β-catenin and found their expression was perturbed in dWnt4 mutants, as compared to controls (Fig 4A–4F). To determine if dWnt4 regulates escort cell encapsulation of CB, we visualized the cytoplasmic processes of the escort cell by labeling them with Fax (Failed axon-connections) tagged to GFP (FaxGFP) and antibodies against Coracle (Cora) [8,42]. Encapsulation was lost in dWnt4 mutants as compared to control germaria (Fig 4G–4J1). As a further test, we carried out transmission electron microscopy (TEM) and found that dWnt4 mutants failed to encapsulate CBs compared to WT.
germaria (Fig 4K and 4L). This was not due to a loss of escort cell fate specification or cell death, as monitored respectively by escort cell marker Zfh1 and cleaved Caspase 3 staining (S4A–S4D Fig) [42]. Somatic depletion of dWnt4 did result in a 13% reduction in the number of Zfh1 positive cells (13 ± 2 in dWnt4 mutants, n = 30, compared to 15 ± 2 in c587-GAL4, n = 30, P-value = 0.004). However, these escort cells all lost their ability to encapsulate the CBs (Fig 4G–4H1). Lastly, we found that depleting inx2 and AJ components, DE-Cad and β-
Transposons Modulate dWnt4 Signaling

Upregulation of transposons leads to downregulation of dWnt4 signaling

As loss of dSETDB1 results in loss of dWnt4, we predicted that loss of dSETDB1 would also result in changes to AJ protein mediated encapsulation. We monitored DE-Cad and β-catenin in dSETDB1 mutants: both were downregulated in the dSETDB1 germaria compared to the control (S5A–S5F Fig). dSETDB1 mutants also showed downregulation of the Wnt reporter Fz3RFP and displayed a loss of escort cell encapsulation, as assessed with antibodies against FaxGFP and Cora (S5G–S5M Fig) [8,42]. Although we observed a reduction in the number of Zfh1 positive cells in dSETDB1 (7 ± 3 in dSETDB1mutants, n = 20 compared to 15 ± 2 in c587-GAL4, n = 30, P-value = 9.2x10E-14), all the escort cells present at the niche were clearly incapable of encapsulation (S5J–S5M2 Fig). Therefore, we conclude that dSETDB1 acts via dWnt4 to permit CB differentiation by regulating the levels of AJ proteins in escort cells needed to promote CB encapsulation.

Finally, we asked if dSETDB1 regulates dWnt4 through TEs via its role in the piRNA pathway [11]. We predicted that, if this was the case, mutations in the piRNA pathway that upregulate TEs should analogously display downregulation of dWnt4. This hypothesis is supported by the fact that mutations affecting the piRNA pathway in the somatic niche show similar differentiation defects [11,16,17]. We monitored the level of dWnt4 mRNA in escort knockdown of piwi (piwi KD), flamenco and aubergine mutants. The combination of these mutants allows us to test in which cell type the production of TEs results in dWnt4 downregulation, as they all affect the soma and germ line differently. Mutations in piwi KD and the flamenco piRNA cluster upregulate TEs only in the somatic cells while aubergine (aub) upregulate TEs only in the germ line [43–45]. flamenco mutants also have the advantage of altering piRNA production just through a mutation in the genomic region that encodes piRNAs and not through a block of a processing protein that could have more pleiotropic effects [44–46]. We compared dWnt4 mRNA levels in these three mutants to a housekeeping gene, RP49, and an escort cell marker, fax. We found using qRT-PCR that only mutations upregulating TEs in the soma but not in the germ line downregulated dWnt4 (Fig 5A) (S6A Fig). Additionally, we carried out in situ hybridization for dWnt4 and monitored the dWnt4 reporter and found that dWnt4 was downregulated in escort cells of dSETDB1 KD, piwi KD and flamenco mutants compared to wild type (S6B–S6J2 Fig). We also found that, like dSETDB1 mutants, piwi KD and flamenco downregulated β-catenin and DE-Cad (S7A–S7J Fig). Additionally, piwi KD showed downregulation of Fz3RFP in the escort cells (S7K and S7M Fig). Next, we monitored escort cell encapsulation using antibodies against Cora [42]. Similar to dSETDB1 and dWnt4 mutants, piwi KD and flamenco mutants showed an overall reduction in number of escort cells (6 ± 3 in piwi KD, n = 31, P-value = 2.6x10E-18 and 10 ± 3 in flamenco mutants, n = 40, P-value = 1.8x10E-09 compared to 15 ± 2 in c587-GAL4, n = 30), but the escort cells present were defective in encapsulation of the CB (Fig 5B–5D2). Although it was previously reported that dWnt4 mutants exhibited a reduction in piwi mRNA and protein levels, we found that piwi mRNA and protein levels were not altered in dWnt4 mutants (S8A–S8D Fig). This data suggests that that dWnt4 acts downstream of TEs and piRNA pathway to promote CB differentiation in the escort cells.

To test this more directly, we overexpressed dWnt4 in piRNA mutants to see if it would rescue the CB differentiation phenotype. We used c587-GAL4 to drive UAS dWnt4 in dSETDB1 mutants and stained them with Vasa and 1B1 [47]. Additionally, we depleted dSETDB1 and piwi only in the somatic cells, while simultaneously expressing dWnt4 there. We found that

catenin, in the escort cells led to a similar loss of encapsulation as seen in the dWnt4 mutants (S4E–S4H1 Fig). Thus, dWnt4 promotes encapsulation of CBs through regulation of inx2 and AJ components.
overexpression of $dWnt4$ in the soma of these mutant germaria resulted in the formation of fusomes, a hallmark of differentiation (Fig 6A–6G). However, the rescue was only partial, as the $dWnt4$ expressing $dSETDB1$ mutants were still sterile and exhibited loss of later...
differentiated stages. Thus, we conclude that \textit{dSETDB1} regulates \textit{dWnt4} via TEs to modulate CB differentiation.

\section*{Discussion}

Our results indicate that \textit{dSETDB1} controls the expression of \textit{dWnt4} to facilitate differentiation. We find that \textit{dWnt4} is expressed in the escort cells and acts on these same cells to promote CB differentiation. We have identified \textit{inx2} and proteins of the AJ complex, DE-Cad and β-catennin, as downstream targets of \textit{dWnt4}. These proteins regulate the encapsulation of the CB by the escort cells via altering the adhesion of the soma to the germ line to control differentiation (Fig 6H). We find that mutations in the piRNA pathway downstream of \textit{dSETDB1} also downregulate \textit{dWnt4} expression, suggesting that TEs modulate the expression of this signaling pathway through an as yet unidentified mechanism (Fig 6H). Additionally, the overexpression of \textit{dWnt4} in the soma of these mutant germaria only partially rescued differentiation, suggesting that there are yet unknown factors that function in parallel to \textit{dWnt4} downstream of the piRNA pathway to regulate CB differentiation (Fig 6H).

Our findings are consistent with recently published work that shows \textit{dWnt4} acts in an autocrine manner from the escort cells to promote germ cell differentiation. Wang et al find that Wnt4 along with Wnt2 signaling acts through the canonical pathway to promote proliferation.
and formation of escort cell processes [48]. They identify reactive oxygen species (ROS) as a main target of Wnt signaling, which maintains reduced redox state to promote CB differentiation. We find that TEs modulate \(dWnt4\) levels in the escort cells to regulate \(inx2\) and AJ proteins, which are then required for CB encapsulation. Thus, we both independently identified separate pathways downstream of \(dWnt4\) in the escort cells, which promote GSC differentiation. Intriguingly, it has been shown that low redox levels are required to maintain DE-Cad levels [49,50]. We speculate that downstream of \(dWnt4\), both ROS and \(inx2\) could be acting in parallel to regulate AJ proteins to promote differentiation via escort cell encapsulation.

Wang et al find a reduction in number of escort cells in germaria with abrogated Wnt signaling [48]. Analogously, we find a 13% reduction in \(dWnt4\) germaria. However, we find that these escort cells present at the niche do not extend processes that promote differentiation. Wang et al’s data shows that overexpression of Wnt signaling leads to an increase in number of escort cells but this increase does not lead to germ cell differentiation defects. This suggests that the mere presence of escort cells is not sufficient to promote differentiation. Thus, in addition to its required role in maintaining escort cell proliferation, our data suggests that \(dWnt4\) signaling also serves a critical function in driving encapsulation of the stem cell daughter.

Our findings and those of Wang et al [48], contradict published work showing that \(piwi\) lies downstream of \(dWnt4\) [51]. It was reported that \(dWnt4\) mutants lose Piwi expression in escort cells and upregulate TEs. However, we find no change in Piwi expression, TE levels or double strand breaks in \(dWnt4\) mutants. We suggest two reasons for this discrepancy. First, Hamada-Kawaguchi et al., used semi-quantitative PCR to measure TE levels, as compared to our use of qRT-PCR [51]. Second, the altered morphology of escort cells in \(dWnt4\) mutants could make them difficult to find, perhaps explaining Hamada-Kawaguchi et al.’s perceived loss of Piwi staining in the escort cells of \(dWnt4\) mutants. Thus, our work has discovered \(dWnt4\) signaling acts downstream of the piRNA pathway to control CB differentiation.

Cohen et al [22], have reported that \(dWnt4\) is essential for the ensheathment of the entire ovariole in which germline stem cells reside. We find that upregulation of TEs results in \(dWnt4\) downregulation. However, \(dSETDB1\), \(piwi\) and \(flamenco\) mutants have not been reported to exhibit disrupted ovariole ensheathment as seen in \(dWnt4\) mutants. This suggests that \(dWnt4\) acts to promote ovariole ensheathment independently of TEs at earlier developmental times.

TEs occupy a large part of our genome and controlling them is a critical task to protect genomic integrity [52]. It has been shown that the gypsy class of TEs, which are upregulated in somatic cells upon loss of the piRNA pathway, can infect the germ line to be passed on to the next generation [53–55]. We propose that TE-mediated modulation of \(dWnt4\) signaling in the somatic cells of the gonad acts to prevent the TE damaged eggs from being propagated by controlling somatic encapsulation of the germ line. Somatic encapsulation can act as a differentiation cue by either protecting the germ line from receiving self-renewal cues or by passing a differentiation cue through the gap junctions present between the soma and germ line [40,56,57]. Wnt signaling is a conserved signaling pathway whose dysregulation has been implicated in several human cancers [58]. Furthermore, some cancers have been associated with increased levels of TEs [52]. Our work suggests a chromatin-transposon-Wnt signaling axis could act as a driver of both differentiation and cancers by perturbing Wnt expression.

**Material and Methods**

**Microarrays**

Microarrays were performed at the NYU genomics core as described in [59] and analyzed at the CFG Core Facility, University at Albany SUNY using Genespring GX v12.6.
Transmission electron microscopy was performed at the NYU EM core as described in [60].

Fly stocks

The following fly stocks were used in the study: dWnt4EMS23 bw1/CyO, dWnt4C1/CyO, w1118; def (2L) Bsc291/CyO, eggx1473/CyO, Df(2R)Dll-Map SM6a, hs-bam, bamGFP, c587-GAL4, nos-GAL4::VP16, y1 w+/C3; Mi{MIC}Wnt4MI03717/SM6a, ArmadilloRNAi (Bloomington 31304), ShgRNAi (Bloomington 32904), Fz2RNAi (27568), PiwiRNAi (Bloomington 33724), flamencoKG/Fm7, faxGFP, W(67c23) P[w+mC] = lacW][Int2][G0035]/Fm7c (Bloomington), aubergineHS2/CyO (Bloomington); aubergineH1/CyO, Sco/CyO;MKRS/TM6 (Lehmann Lab); dSETDB1HA (Bontron Lab); Sco/CyO;Fz3RFP (Bach Lab); w- P[w+UASdwnt4];P[w+UASdwnt4];P[w+UASdwnt4] (Newfeld Lab; Pandur Lab); Wnt4RNAi (V104671), Innexin2RNAi (V102194) (VDRC); eggRNAi (TriP) and dSETDB1HA,dWnt4C1/CyO (This study).

Collection and fixation of tissues

Collection and fixation of ovaries was carried out as previously described (de Cuevas et al., 1996).

Immunostaining

Immunostaining of the ovaries was carried out with the following Primary antibodies: Rb Vasa (1:5000, this study), Ch Vasa (1:500, Lehmann Lab and this study), Ch GFP (1:1000, abcam AB13970), Rb pMAD (1:200, abcam AB52903), Mo BamC (1:200, DSHB), Rat HA (1:100, santa cruz sc53516, Mo β-catenin (Armadillo) (1:15, DSHB), Rat DE-CAD (DCAD2) (1:5, DSHB), Mo H4K20Me3 (1:500, abcam AB78517), Mo LacZ (1:50, Promega), Mo 1B1 (1:20, DSHB), Rb PH3 (1:200, Cell Signaling 9705), Rb Piwi (1:500, Lehmann Lab), Rb zfh1 (1:1000, Lehmann Lab), Rb Orb (1:500, Lehmann Lab), Mo Coracle (1:200, DSHB), Mo Lamin C (1:20, DSHB), Rb H2Av (1:500, Rockland 600-401-914), and Rb Caspase3 (1:300, Cell Signaling 96615).

Alexa 488 (Molecular Probes), Cy3 and Cy5 (Jackson Labs) conjugated secondary antibodies were used at a concentration of 1:500.

Fluorescence in situ hybridization

FISH of the ovaries was carried out with a mixture of 48 probes against dWnt4, which were obtained from Stellaris FISH probes (Custom assay with quasar 570 dye, SMF-1063-5). The ovaries were dissected in PBS, fixed in 4% formaldehyde in PBS for 30 minutes and washed 3 times with PBT (0.3% TritonX 100). Next, they were treated with 3ug/ml Proteinase K in PBS and incubated on ice for an hour. The tissue was blocked in 2mg/ml glycine in PBT twice for 2 minutes each, rinsed twice with PBT. The ovaries were fixed again for 30 minutes in 4% formaldehyde in PBS at room temperature. The tissue was then washed with PBT 5 times for 2 minutes and washed with fresh pre-hybridization mix (10% deionized formamide in 2x SSC) for 10 minutes at 37°C. 60ul per sample of hybridization mix (10% deionized formaldehyde, 1mg/ml of t-RNA and sperm ssDNA, 50 ng/ul probe, 10% Dextran sulphate, 2mg/ml BSA, 2x SSC and 2ul of RNase In) was added and the sample was incubated overnight at 37°C. The sample was washed in pre-hybridization mix (warmed at 37°C) for 15 minutes, and thrice with 1x PBS for 45 minutes each and mounted.

Fluorescence imaging

The tissues were visualized under 10X, 20X, 40X and 63x objective lenses. The images were acquired using a Zeiss LSM-510/710 confocal microscope under 40X and 63x objective.
Quantification analysis

In order to calculate intensities for LacZ, DE-Cadherin, β-catenin, Piwi and Fz3RFP, both, control and mutant germaria were taken under the same confocal settings and Z stack planes were obtained. For DE-Cadherin and β-catenin quantification, specific planes showing both the CB and Zfh1 positive escort cell were chosen and the area of Zfh1 positive surrounding the CB was outlined. For Fz3-RFP and Piwi quantification, the Zfh1 positive cell was outlined. The intensity of this region was analyzed using ImageJ. The ratio between mean and area was calculated. Average of all these ratios, per image was calculated and a comparison was made between control and mutants. P-value was determined by two-tailed equal variance t test by comparing the intensities of mutants vs. wild type strains. A minimum of 5 germaria were chosen for the each quantification.

Materials and reagents

Fly food was created using the procedures from the Ruth Lehmann lab at NYU (summer/winter mix), and used to fill narrow vials to approximately 12mL.

Real-time PCR

Expression levels of dWnt4 in dWnt4, dSETDB1, bam, piwi, flamenco, and aubergine mutants were analyzed using real-time, quantitative PCR.

RNA isolation

RNA was isolated using the Trizol reagent protocol. The ovaries were first homogenized in Trizol and kept for 5 minutes at room temperature. Choloform was added and the sample was centrifuged at 12000g for 15 minutes at 4°C. The aqueous phase was aspirated out in a new tube. 100% isopropanol was added and the sample was incubated for 10 minutes at room temperature, followed by centrifugation at 12000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol at 7500g for 5 minutes at 4°C. The wash was discarded and the pellet was allowed to air dry for 10 minutes and later re-suspended in RNase free water. The Turbo DNase (Life Technologies, Catalog Number: AM1907) protocol was used to remove any DNA contamination.

Reverse transcription was performed with Super Script III (Life Technologies, Catalog Number: 1808051) The cDNA samples were diluted to 50ng/μl. dWnt4 and inx2 specific primer was used (IDT). All real-time PCR reactions were carried out using the ABI 7700 sequence detection system (Applied Biosystems) and the amplifications were done using the iTaq Universal Probes Supermix (Biorad, Catalog Number: 172–5130). The thermal cycling conditions consisted of 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15s, and 60°C for 60s. The experiments were carried out in technical triplicate and three biological replicates for each data point.

To calculate fold change in dWnt4 and piwi mRNA levels compared to fax mRNA levels, ratio and then average of the same was calculated between the two for each of the three biological replicate. Error bars were plotted using Standard deviation of the ratios. P-value was determined by one-tailed equal variance t test by comparing ratios of mutants vs. wild type strains.

To calculate fold change in dWnt4 and piwi mRNA levels to RP49 mRNA levels, average of the 2^ΔCt for three biological replicates was calculated. Error bars were plotted using Standard deviation of the ratios. P-value was determined by one-tailed equal variance t test by comparing ratios of mutants vs. wild type strains.
Primers used for qRT-PCR

The following primers were used to perform qRT-PCR.

F-Wnt4 (5'-CAACTGTGGCA CCGTGCTGC-3'), R-Wnt4 (5'-GTCTCAGCCGCTGGCAG-3'), F-Piwi (5'-CTTTGGACAA ACTCGAAATCAG-3'), R-Piwi (5'-GCAGCGGCTGATTGTCG-3'), F-ZAM (5'-CTTTGGACAA GACTCCGAAATCAGC-3'), R-ZAM (5'-GCAGCGGCTGATTGTCG-3'), F-Idefix (5'-AACAAATCGTGGCAGGAAG-3'), R-Idefix (5'-GCTTCTTTGGTTGGTCTGGA-3'), F-Fax (5'-CGTTCATCGAGCTGAATGGG-3'), R-Fax (5'-CCCGAGTCCAGGTACTTCT-3'),

F-RP49 (5'-ATGACCATCCGCCCAGCATAC-3') and R-RP49 (5'-CTGCATGAGCAGGACTCCGAGCC-3').

Supporting Information

S1 Fig. dWnt4 affects differentiation at the CB stage. (A) GO term analysis of dSETDB1 mutants compared to bam mutants showing Wnt signaling to be primarily downregulated. (B-C) dWnt4 mutants stained with 1B1 (red), Vasa (green) and DAPI (blue) showing pleiotropic differentiation defect and loss of later stages. (D-E) dWnt4 heterozygote and dWnt4 mutant stained for 1B1 (red) and Orb (green) (white arrow) showing an oocyte specification defect. (F-G) Zoomed images (63X) of dWnt4 heterozygote and dWnt4 mutant stained for pMad (red) (white asterisk), BamC (green) and Vasa (blue) showing an accumulation of undifferentiated CBs. (H-I) dWnt4 heterozygote and dWnt4 mutant stained for LaminC (red) and Vasa (green) showing no increase in the somatic niche size (white arrow). (J-K) dWnt4 heterozygote and dWnt4 mutant stained for PH3 (red), Vasa (green) and DAPI (blue) showing similar cell division rate. (L-M) dSETDB1 heterozygote and dWnt4, dSETDB1 trans-heterozygote stained with 1B1 (red), Vasa (green) and DAPI (blue) showing an accumulation of >3 undifferentiated single cells (white line).

(TIF)

S2 Fig. dWnt4 acts downstream of dSETDB1. (A-B) c587-GAL4 and escort cell knock down (KD) of dSETDB1 stained with H4K20me3 (red) and Vasa (green) showing loss of heterochromatin expression in the soma (white arrow). (C-D) dWnt4 heterozygote and dWnt4 mutant stained with H4K20me3 (red) and Vasa (green) showing similar heterochromatin expression in the soma (white arrow). (E-F) dWnt4 KD do not show an upregulation of ZAM and Idefix levels compared to dSETDB1 KD. (G-H) dSETDB1 heterozygote and dSETDB1 mutant stained with H2Av (red), and Vasa (green) showing upregulated H2Av expression in the somatic cells (white line). (I-J) dWnt4 heterozygote and dWnt4 mutant stained with H2Av (red), 1B1 (blue) and Vasa (green) showing similar H2Av expression during meiosis (white line). (K-L) dWnt4 heterozygote and dWnt4 mutant stained for dSETDB1 that is tagged with HA (red) and Vasa (green) showing similar dSETDB1 expression. The accumulation of dSETDB1 in dWnt4 mutant is due to accumulation of undifferentiated CBs.

(TIF)

S3 Fig. dWnt4 is not required in the germ line or in the terminal filament and cap cells for differentiation. (A-B) nos-GAL4 and dWnt4 RNAi where dWnt4 has been specifically knocked down in the germ line, stained with 1B1 (red), Vasa (green) and DAPI (blue) showing 2–3 undifferentiated cells. (C-D) hh-GAL4 and dWnt4 RNAi where dWnt4 has been specifically knocked down in the terminal filament and cap cells, stained with 1B1 (red), Vasa (green) and DAPI (blue) showing 2–3 undifferentiated cells. (E) Quantification of the percentage of nos-dWnt4 and hh-dWnt4 RNAi germaria showing lack of differentiation defects.

(TIF)
S4 Fig. Loss of AJ proteins leads to loss of CB encapsulation. (A–B) dWnt4 heterozygote and dWnt4 mutant stained for 1B1 (red) and Zfh1 (green) showing presence of escort cells (white arrows). (C–D) dWnt4 heterozygote and dWnt4 mutant stained for Caspase3 (red), Vasa (green) and 1B1 (blue) showing similar cell death. (E–H) c587-GAL4, and escort cell knock down (KD) of Inx2, DE-Cadherin and β-catenin stained for Coracle (red), Vasa (green) and DAPI (blue) showing loss of encapsulation in Inx2 KD, DE-Cadherin KD and β-catenin KD. (TIF)

S5 Fig. dSETDB1 mutants show loss of CB encapsulation. (A–B) c587-GAL4 and escort cell knock down (KD) of dSETDB1 stained for DE-Cadherin (red), and Zfh1 (green) (white arrow) showing perturbed DE-Cadherin expression in dSETDB1 KD. (C) Quantification (n = 5) of DE-Cadherin levels in c587-GAL4 and dSETDB1 KD showing a significant difference in dSETDB1 KD. (D–E) c587-GAL4 and dSETDB1 KD stained for β-catenin (red), and Zfh1 (green) (white arrow) showing perturbed β-catenin expression in dSETDB1 KD. (F) Quantification (n = 5) of β-catenin levels in c587-GAL4 and dSETDB1 KD showing a significant difference in dSETDB1 KD. (G–I) Control and dSETDB1 KD stained for GFP (green), Vasa (blue), and 1B1 (red) showing loss of encapsulation in dSETDB1 mutants. (J–K) Control and dSETDB1 KD stained for Coracle (red), Zfh1 (green) (white arrow), and Vasa (blue) showing loss of encapsulation in dSETDB1 KD. (TIF)

S6 Fig. piRNA pathway mutants show downregulation of dWnt4 in the escort cells. (A) qRT-PCR analysis showing a significant downregulation of dWnt4 mRNA levels compared to RP49 mRNA levels in escort cell specific knockdowns (KD) of dWnt4 KD, dSETDB1 KD, piwi KD and flamenco were compared to bam mutants. No significant change in dWnt4 mRNA levels was observed compared to RP49 mRNA levels was seen between aubergine mutants compared to its the heterozygous control. (B–F) Fluorescent in situ hybridization (FISH) for dWnt4 mRNA in wild type, dWnt4 KD, dSETDB1 KD, piwi KD and flamenco showing downregulation of dWnt4 in the soma compared to wild type. (G–G2) Germarium of a minos GFP (dWnt4 reporter) stained for Zfh1 (red), GFP (green) and 1B1 (blue) showing the expression of GFP primarily in the escort cells (white arrow). (H–I) Germarium of dSETDB1 KD and piwi KD carrying dWnt4 reporter stained for Zfh1 (red), GFP (green) and 1B1 (blue) showing a downregulation of dWnt4 in the escort cells. (TIF)

S7 Fig. piRNA pathway mutants show downregulation of β-catenin, DE-Cadherin and Fz3RFP levels. (A–C) Wild type, escort cell knock down of piwi (piwi KD) and flamenco mutants respectively, stained for DE-Cadherin (red), and Zfh1 (green) (white arrow) showing perturbed DE-Cadherin expression in piwi KD and flamenco mutants. (D–E) Quantification (n = 5) of DE-Cadherin levels in wild type, piwi KD and flamenco mutants showing a significant decrease in mutants. (F–H) Wild type, piwi KD and flamenco mutants stained for β-catenin (red), and Zfh1 (green) (white arrow) showing perturbed β-catenin expression in piwi KD and flamenco mutants. (I–J) Quantification (n = 5) of β-catenin levels in wild type, piwi KD and flamenco mutants showing a significant decrease in mutants. (K–L) Germarium carrying a fz3-RFP transgene stained with RFP (red), Zfh1 (green) (white arrow) and Vasa (blue) showing expression of Fz3RFP in the escort cells. (M) Quantification of RFP in the escort cells showing a significant downregulation
in piwi KD.

**S8 Fig. dWnt4 acts downstream of the piRNA pathway.**  (A) qRT-PCR analysis showing no significant change in piwi mRNA levels between c587-GAL4 and germaria where dWnt4 has been specifically depleted in the escort cells. (B–C1) dWnt4 heterozygote and dWnt4 mutant fly stained for Piwi (red) (white arrows), GFP (green) and 1B1 (blue) showing similar Piwi expression in the escort cells. (D) Quantification (n = 6) of Piwi in escort cells showing a no difference in dWnt4 heterozygotes and dWnt4 mutants.

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**Author Contributions**
Conceived and designed the experiments: PR MU. Performed the experiments: MU YMC LT SWD SS PF CH MAN. Analyzed the data: PR MU PF SC. Wrote the paper: PR MU.

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