Wnt signaling in sexual dimorphism

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Abstract:

The embryonic gonad of *Drosophila melanogaster* begins to display sexually dimorphic traits soon after its formation. Here we demonstrate the involvement of a *wnt* family ligand, *wnt-2* in the induction of these sex specific differences. We show that *wnt-2* contributes to the survival of a male specific population of somatic gonadal precursor cells (SGPs), the msSGPs that are located at the posterior of the male gonad. We also show that the Wnt-2 ligand synergizes with the JAK-STAT ligand Upd, which is produced by SGPs at the anterior of the gonad to activate the STAT pathway in male germ cells. We suggest that the use of two spatially separated signaling system to initiate the JAK-STAT stem cell maintenance pathway in germ cells provides a mechanism for increasing the pool of potential progenitors of the germline stem cells in the adult testes. Finally, we present evidence indicating that like the JAK-STAT pathway, *wnt-2* promotes germ cells in male embryos to re-enter the cell cycle.
Introduction:

The primitive embryonic gonad in *Drosophila melanogaster* is composed of two distinct cell types, the germ cells and the somatic gonadal precursor cells (SGPs) (Santos and Lehmann, 2004). These two cell types are formed at different locations in the embryo and are specified by distinct mechanisms. The germ cells arise as pole cells at the posterior end of the pre-cellular blastoderm embryo and their proper specification depends on maternal determinants that are assembled in the pole plasm during oogenesis. After cellularization of the blastoderm, the germ cells must make their way into the center of the embryo and then migrate towards the newly formed SGPs in parasegments (PS) 10-13. SGPs are derived from dorsolateral mesodermal tissue in these parasegments and are specified by the hierarchical action of zygotic patterning genes. The dorsolateral mesoderm of PS10-13 is formed under the control of *tinman*, and *zfh-1* (Moore et al., 1998) while the eventual specification of SGPs from these cells depends upon the bi-functional transcription factor *eyes absent* (*eya*) (Boyle et al., 1995; Moore et al., 1998). Although *eya* is required for SGP identity, it is differentially expressed in anterior and posterior SGPs. This difference depends upon the activity of the homeotic genes *abdominal-a* (*abd-A*) and *Abdominal-B* (*Abd-B*). The specification of anterior SGPs depends upon *abd-A*, while the specification of posterior SGPs depends upon both *abd-A* and *Abd-B* (De Falco et al., 2004; Boyle and Dinardo, 1997).

In addition to differences between anterior and posterior SGPs, the embryonic gonad is sexually dimorphic. One sex specific difference is in the activity of signaling pathways that mediate communication between the SGPs and the PGCs. Wawersik et al. (2005) found that the ligand for the JAK-STAT pathway, *unpaired* (*upd*) (Harrison et
al., 2003), is expressed in a small group of SGPs at that very anterior of the embryonic gonad in male but not female embryos. (The same sex-specific expression pattern is seen for the closely related upd-3 (Hombria et al., 2005). The upd ligand signals to the germ cells in male embryos upregulating the level and activity of the transcription factor STAT92E (Hou et al., 1996). By contrast, there is little, if any, STAT92E in the germ cells of female embryos. The activity of the JAK-STAT pathway in males and females is dependent upon the somatic sex determination pathway. The sex determination pathway can be bypassed in females by ectopic expression of upd (or upd-2 or upd-3) which, then activates STAT92E accumulation in their germ cells.

Another sex-specific difference is the presence of cell types in one sex but not the other. (De Falco et al., 2003, De Falco et al., 2008). One example of a cell type only found in males is the pigment precursor cells. These cells arise late in embryogenesis and are distributed around the outside of the embryonic gonad. Their specification depends upon the wingless ligand wnt-2, which is activated by the dsx gene. Another sex-specific cell type is the male specific SGPs (msSGPs), which are clustered at the posterior end of the coalesced gonad. msSGPs are specified by a mechanism that seems to be independent of tinman and zfh-1 and they express three different molecular markers namely Eya, the wnt-2 ligand and the transcription factor Sox100B. While Sox100B protein is detected just in the male gonads, wnt-2 expression is observed in gonads of both sexes around the time that the germ cells and SGPs first make contact. Subsequently, at the gonad coalescence stage, wnt-2 is greatly enriched in the male gonads in the msSGPs (De Falco et al., 2003). At this stage another SGP specific marker, Eyes absent (Eya), is also enriched in msSGPs.
Although msSGPs are only found in the coalesced gonads of male embryos, their initial specification is not sex specific. Thus, Sox100B/Abd-B positive cells are detected in PS13 of both male and female stage 13 embryos. However, survival of msSGPs is controlled by the doublesex (dsx) gene in a sex specific manner. In female embryos, the female Doublesex protein activates a conserved cell death pathway and the msSGPs are eliminated by stage 14-15. In contrast, msSGPs remain in the male embryos (De Falco et al, 2003).

In the studies reported here we have examined the role of wnt-2 in the development of the male gonad. We show that wnt-2 promotes survival of msSGPs in a sex specific manner. In addition, wnt-2 also plays an important role in the sex-specific development of the male germline. One of the instructive functions of wnt-2 is to potentiate the activation of the JAK-STAT pathway in male germ cells. As a consequence there are two signaling centers, a group of SGPs at the anterior of the gonad, which express the JAK-STAT ligand Upd, and the msSGPs at the posterior, which express Wnt-2, that mediate the induction of STAT expression in male germ cells. We speculate that the use of this dual but spatially separated signaling system to initiate the JAK-STAT stem cell maintenance pathway provides a mechanism for specifying not only the anterior but also the posterior germ cells in the male embryonic gonad as potential progenitors of the germline stem cells (GSCs) in the adult testes. This would maximize the number of germ cells that can be incorporated into the stem cell niche when it is established at the anterior of the embryonic gonad during stage 17 of embryogenesis (Le bras and Van Doren, 2006). In addition to a role in upregulating the JAK-STAT pathway, we find that wnt-2 induces male germ cells to re-enter the cell cycle
and begin dividing. Like the activation of the JAK-STAT pathway, the early proliferation of germ cells is a male-specific trait.

Results:

**Hyperactivation of the canonical wnt signaling pathway induces the formation of excess msSGPs**

Previous work has shown that *wnt-2* mutations cause sterility in males, but have no apparent effect on fertility in females. The testes of *wnt-2* males lack the pigment cells of the testes sheath, are much smaller than wild type and have an unusual oblong shape rather than the typical spiral (Kozopas *et al.*, 1998; Kozopas and Nusse, 2002; also see Linnemannstöns *et al*, 2014). However, since many of the characteristic somatic and germline cell types appear to be present, the precise mechanism(s) responsible for the sterility of *wnt-2* males has remained uncertain.

Studies by Boyle *et al.* (1997) indicate that the specification of the somatic component of the gonad, the SGPs, depends upon canonical *wnt* signaling. They found that ectopic expression of an activated form of the Armadillo protein, Arm-S10, in the mesoderm using a *twist-GAL4* driver or *wg* under a heat shock promoter) substantially enlarges the population of Eya expressing SGPs in the coalesced gonads of stage 15 embryos. Since most of these extra SGPs appear to be clustered at the posterior of the coalesced gonad where the Wnt-2 expressing msSGPs are located, it is reasonable to suppose that they correspond to msSGPs. If this is correct, it raises the possibility that Wnt-2 and canonical *wnt* signaling might be important in specifying and/or maintaining the msSGPs. On the other hand, since *wg* is known to play a role in SGP specification, it is also possible that *wnt-2* activity is not needed, or that *wnt-2* has some other
function. Consistent with the latter possibility, DeFalco et al (2008) have shown that wnt-2 activity is necessary and sufficient for the specification of pigment precursor cells in male gonads (De Falco et al., 2008).

To explore the function of wnt signaling in the male gonad further we first re-examined the effects of ectopic ArmS10 on SGPs, focusing specifically on the msSGPs in the coalesced gonad of stage 15 embryos. While all SGPs are thought to express Eya, msSGPs can be differentiated from other SGPs in that they also express Sox100B and Abd-B (De Falco et al., 2003; De Falco et al., 2004). To determine if the population of msSGPs is expanded by activation of the canonical wnt signaling pathway, we probed twist-GAL4/UAS-ArmS10 embryos with Vasa antibodies to identify the gonad, and with Sox100B and Abd-B antibodies to identify the somatic msSGPs. Wild type, stage 15 male embryos have a cluster of about 10 (n=10, mean=10; S.D. 1.08) Abd-B/Sox100B positive msSGPs at the posterior of the gonad, while there are no Abd-B/Sox100B positive msSGPs in stage 15 female embryos (n=10, mean =0.091). Fig. 1 shows that as would be expected from the results of Boyle et al, there is a substantial increase in the number of Abd-B/Sox100B positive msSGPs in the gonads of twist-GAL4/UAS-ArmS10 embryos (n=15. Mean=23, S.D. 4.65; p value< 0.00001). There was also no obvious sex specificity in the inductive effects of ectopic ArmS10.

**wnt-2 is required for the survival but not the specification of msSGP in male embryos**

The findings described above indicate that the canonical wnt signaling pathway promotes the formation of msSGPs. To assess the role of wnt-2, if any, in the
specification process we initially examined the effects of \textit{wnt}-2 mutations on the msSGP population in the coalesced gonads of stage 15 male embryos. In the first set of experiments (see Fig. 2), we probed embryos produced by \textit{wnt}-2/Cyo, \textit{en}:LacZ stocks for two different \textit{wnt}-2 alleles, \textit{wnt}-2$^0$ and \textit{wnt}-2$^+$, with Vasa antibodies to label the germ cells (imaged in blue) and with Abd-B and Sox100B antibodies to label the msSGPs in the embryonic gonad (see legend). To identify \textit{wnt}-2$^-$ males and their \textit{wnt}-2$^+$ male sibs we also probed the embryos with Sxl and b-galactosidase antibodies. Altogether we examined the coalesced gonads of 15 \textit{wnt}-2$^-$ and 15 \textit{wnt}-2$^+$ stage 15 male embryos.

The control (Beta-galactosidase positive and Sxl negative) \textit{wnt}-2/+ stage 15 male embryos had between 8 and 14 Abd-B positive Sox100B positive msSGPs, with an average of 10.4 msSGPs. This is comparable to the number of msSGPs found in stage 15 wild type male embryos. By contrast, as illustrated by several different examples in Fig. 2, there is a clear reduction in the number of Abd-B/Sox100B positive msSGPs in the \textit{wnt}-2 mutant males (b-galactosidase and Sxl negative). The number of msSGPs in the mutants ranged from 0 to 4 with an average of 2 Abd-B/Sox100B positive cells (n=10, mean=2; S.D. 1.63; p value< 0.00001).

In the second set of experiments we marked msSGPs in embryos from the two \textit{wnt}-2/Cyo \textit{en}:LacZ stocks using a combination of Sox100B and Eya antibodies. As before we localized the gonad in stage 15 embryos with Vasa antibody, and identified wild type and \textit{wnt}-2$^-$ male embryos by probing with Sxl and b-galactosidase antibodies. As illustrated in Fig. 3, while SGPs expressing Eya are observed in coalesced gonads of \textit{wnt}-2 mutant male embryos, Eya/Sox100B positive msSGPs couldn’t be detected in almost half of the \textit{wnt}-2 male embryos. In the remaining \textit{wnt}-2 embryos, 1-5 msSGPs
were observed (n=10, mean 1.8; S.D. 1.87; p value< 0.00001).

One interpretation of these results is that msSGP specification depends upon wnt-2. However, this does not seem to be the case. When we examined the msSGPs in younger male embryos (stages 12-13) we found that the number of Sox100B/Abd-B or Sox100B/Eya positive msSGPs in the wnt-2 mutant males (n=12, mean =8, S.D.=1.73; p value 0.012) was close to that seen in wild type (n=10, mean= 9.2, S.D. = 1.3) (see Supplemental Fig.1). The presence of normal numbers of msSGPs at this earlier stage would argue that wnt-2 is not essential for the initial specification of msSGPs. On the other hand, since the number of msSGPs is substantially reduced in older stage 15 wnt-2- male embryos, it would appear that wnt-2 activity is important for the survival of these cells when the male gonad coalesces.

**Ectopic wnt-2 can promote survival of msSGPs in female embryos**

The findings in the previous section argue that wnt-2 is required for the survival of msSGPs, but doesn't have an important role in their specification. To test this further, we ectopically expressed Wnt-2 in the mesoderm using a twist-GAL4 to drive expression of a UAS-wnt-2 transgene. We used Sxl antibody to distinguish between male and female embryos, Vasa antibody to mark the embryonic PGCs and Sox100B to identify the msSGPs. We found that there was a striking difference between the effects of ectopic ArmS10 and Wnt-2. While the former induced the formation of excess msSGPs, there was no obvious increase in the number of msSGPs with the latter (n=15; mean=11.6, S.D. 3.44; p value 0.051). In this context, it should be noted that the effects of wg misexpression using the same twist-GAL4 driver resembled that observed with ArmS10: n=10, mean = 26.4, S. D. 6.1; p value< 0.00001). These findings
together with the results described above would be consistent with the conclusion that
\(wnt-2\) does not function in the specification of the msSGPs.

We next asked whether ectopic Wnt-2 could promote the survival of msSGPs in
female embryos. As previously shown (De Falco et al., 2003), we found that Sox100B
positive msSGP cells are absent in the gonad of control stage 15 female embryos (Fig.
4, panels A & A'). In contrast, Sox100B positive msSGPs were observed in the
coalesced gonads of \(twist\)-GAL4 /UAS \(wnt-2\) female embryos (Fig.4, panel C & C').
While ectopic Wnt-2 clearly suppresses msSGP apoptosis in female embryos, it should
be noted that msSGPs are found in the coalesced gonads of only about 50-60% of the
\(twist\)-GAL4:UAS \(wnt-2\) female embryos and the number of Sox100B positive
cells/gonad ranges from 1-6 (n=11, mean = 2.8, S.D. 2.04, p value 0.000267) as
compared to around 10 in wild type males (compare panels B' and C'). Taken together
with the effects of \(wnt-2\) mutations in males (Fig. 2 & 3), this could mean that \(wnt-2\) is
important but not entirely sufficient to protect against apoptosis. Alternatively (or in
addition), since the msSGPs themselves appear to be a source of Wnt-2 in male
gonads, it is possible that the level of Wnt-2 accumulation in the gonad using the \(twist\)
driver is not equivalent to the autocrine signal produced by the male msSGPs
themselves. Potentially consistent with this latter possibility, we found that the extent of
rescue of msSGPs by ectopic Wnt-2 differs between different UAS \(wnt-2\) transgenic
lines (not shown).

To confirm that Wnt-2 promotes the survival of msSGPs in the coalesced gonad
we used a \(nanos\)-GAL4 driver to express Wnt-2 only in the germ cells. As shown in Fig.
4 (panels D-F), this localized source of Wnt-2 can act as a survival factor for msSGPs in
female gonads and block apoptosis. Interestingly, expression of Wnt-2 in the germ cells is somewhat more effective in protecting msSGPs from cell death than is ubiquitous expression in the mesoderm by the twist driver and we find that around 75% of the coalesced gonads in nanos-GAL4: UAS-wnt-2 female embryos have Sox100B positive msSGPs (n=11, mean= 3.3, S.D. 2.34, p value= 0.000164). This finding also indicates that germ cells in the embryonic gonad are able to signal to their somatic partners.

**Does wnt-2 have functions in addition to sustaining msSGPs?**

An important question is whether wnt-2 has other roles in the development of the male embryonic gonad. It has recently been shown that SGPs at the anterior of the male embryonic gonad express Upd and that this ligand activates the JAK-STAT signaling pathway in male germ cells, leading to a substantial upregulation of STAT protein expression. By contrast, upd is not expressed by anterior SGPs in female gonads, and the JAK-STAT signaling pathway remains off in female germ cells. Since the Wnt/Beta-catenin pathway can up-regulate the expression of Stat3 in mouse embryonic stem cells (Hao et al, 2006)), we wondered whether the Wnt-2 produced by the msSGPs functions to synergize the activation of the JAK-STAT pathway in male germ cells.

To investigate this possibility we first examined the effects of wnt-2 mutations on STAT protein expression in the germ cells of male embryos. In wild type, high levels of STAT accumulate in the germ cells of male embryos (Fig. 5; panels A & A’) while the germ cells of female embryos have only low levels of STAT (Fig. 5; panels B & B’). As can be seen in Fig. 5 panels C & C’, STAT expression is appreciably reduced in the germ cells of wnt-2 mutant male embryos and the level of STAT protein is only
marginally greater than that seen in the germ cells of wild type females. This result indicates that Wnt-2 is required to promote the accumulation of high levels of STAT protein in male embryonic germ cells.

We next tested whether ectopic expression of Wnt-2 can induce STAT protein accumulation in female embryonic germ cells. For this purpose we used twist-GAL4 to drive wnt-2 expression in the mesoderm. Fig. 6 shows that wild type female embryos at stage 13-14 have barely detectable levels of STAT protein in their germ cells (see panels A-A”). In contrast, when wnt-2 is ectopically expressed in the mesoderm of female embryos, it can promote the accumulation of STAT protein in the female germ cells (see panels C-C”). STAT protein also seems to persist in female PGCs of stage 15 UAS-wnt-2/ nos-Gal4 embryos (panels D-D”). Note that the level of STAT protein observed in female germ cells in the presence of ectopic Wnt-2 is not equivalent to that seen in male germ cells (compare panels B” and D”). On the other hand, it is clearly elevated compared to that observed in the germ cells of wild type female embryonic PGCs (panel A”, also see Figure 8, panel B”). To confirm this finding, we tested whether ectopic expression of Wnt-2 using the germline specific nos-GAL4 driver induces STAT accumulation in female germ cells. We found that STAT accumulation could also be induced when Wnt-2 is expressed in female germ cells; however, as was observed with the twist driver, the level of STAT protein is less than that in the germ cells of wild type males (data not shown).

One likely reason why the level of STAT induced by ectopic Wnt-2 in female germ cells is less than in wild type male germ cells is that the anterior SGPs in male gonads express high levels of the JAK-STAT ligand Upd while this ligand is not
expressed by SGPs in female gonads. To better understand the respective functions of Wnt-2 and Upd in the germline and soma of the embryonic gonad, we compared the effects of ectopic Upd and Wnt-2 in female gonads. In contrast to Wnt-2, expression of Upd using the nanos-GAL4 driver did not promote the survival of msSGPs in female embryonic gonads (G.D.; not shown). On the other hand, ectopic Upd appeared to be more effective in inducing STAT protein accumulation in female germ cells than ectopic Wnt-2 (not shown). However, even in this case, the level of STAT accumulation induced in female germ cells by ectopic Upd was less than that observed in germ cells of wild type male embryonic gonads.

**How does wnt-2 upregulate STAT expression?**

Two different sorts of mechanisms could potentially explain how wnt-2 upregulates the expression of STAT protein in germ cells. In the first, Wnt-2 could exert its effect on the JAK-STAT pathway in germ cells indirectly by acting on other cells in the soma like the anterior SGPs. For example, production of the Upd ligands by the SGPs at the anterior of the male embryonic gonad might require input from the wnt-2 signaling pathway. In wnt-2 mutant males these SGPs would fail to express the Upd ligands and STAT expression would not be activated in the germ cells. In the second, Wnt-2 produced by msSGPs would signal directly to the germ cells to activate STAT expression. In this mechanism Upd expression by the SGPs at the anterior of the gonad would not in itself be sufficient to fully activate STAT expression in male germ cells in the absence of the synergistic activity of Wnt-2 produced by msSGPs at the posterior of the gonad.

Though we were unable to detect any obvious reduction in the level or distribution
of the Upd ligand in the embryonic gonads of \textit{wnt-2} mutant males (data not shown), \textit{wnt-2} could be required for expression of the Upd-3 or play some other role in the transmission of the Upd signal. For this reason, we focused on testing the second model. We first asked whether the \textit{wnt} signaling pathway needs to be activated in male germ cells in order to turn on high levels of STAT expression in these cells. To inhibit the reception of the \textit{wnt-2} signal specifically in the germ cells we overexpressed \textit{shaggy} (\textit{sgg}) which encodes a negative regulator of the canonical \textit{wnt} signaling pathway, Glycogen Synthase Kinase 3, in these cells using a \textit{nanos-GAL4} driver (Tolwinski et al., 2003). Overexpression of this kinase would be expected to prevent germ cells from responding fully to the Wnt-2 ligand because phosphorylation of the co-activator Armadillo (Arm) by Sgg promotes degradation of the Arm protein. Fig. 7 shows that STAT protein expression in germ cells of \textit{UAS-shaggy/nanos-GAL4} stage 15 male embryonic gonads is reduced (panel B’) compared to that in germ cells of similarly staged wild type male gonads (panel A’). Panels A” and B” show that the somatic msSGPs are not affected by downregulating the response to \textit{wnt} signaling specifically in male germ cells. Another protein that cell autonomously impedes the response to \textit{wnt} signaling when overexpressed is Axin (Willert \textit{et al.}, 1999). Axin interacts with both Sgg and Arm and is part of the Arm destruction complex (Willert and Jones, 2006). When \textit{nanos-GAL4} is used to drive Axin expression, the accumulation of STAT protein in male germ cells is also reduced as is seen for \textit{sgg} (Fig.7, panels C and C’).

The effects of excess Sgg (and Axin) on STAT accumulation in male germ cells would be consistent with the second mechanism—namely that Wnt-2 produced by somatic gonadal cells synergizes with the Upd ligand to induce a high level of STAT
expression in male germ cells. To provide further evidence that activation of the canonical Wnt pathway in germ cells can promote STAT accumulation we generated germline clones of the negative regulator sgg and analyzed STAT expression in the germ cells of their female progeny (Fig. 8). We found that unlike wild type females (Panels B and B′), STAT protein accumulated in the germ cells of sgg<sup>M-Z+</sup> females (compare Fig.8 panels C and C′). Thus, the effects of inappropriately activating the canonical <i>wnt</i> signaling pathway in female germ cells are also consistent with the idea that the Wnt-2 ligand functions to synergize the activation of the STAT pathway in male germ cells by Upd. This suggestion is supported by the finding that ectopic expression of the gain-of-function ArmS10 protein in the germline using a <i>nanos-GAL4</i> driver also induced STAT accumulation in female germ cells (data not shown). Though these results are most easily explained by a <i>wnt-2</i> dependent activation of the canonical <i>wnt</i> pathway in male germ cells, they don’t exclude the possibility that <i>wnt-2</i> activates the production of some other <i>wnt</i>-like ligand that actually signals to the male germ cells.

**Do msSGPs participate in the signaling process?**

Since the msSGPs are a major source of Wnt-2 in the male embryonic gonad, we wondered whether one of the functions of this male-specific group of somatic cells is to send the Wnt-2 signal to the male germ cells. To investigate this possibility we needed to protect the msSGPs in female gonads from apoptosis by a mechanism that did not depend upon ectopic expression of Wnt-2. DeFalco <i>et al.</i> (2003) have shown that the baculovirus anti-apoptosis protein p35 can partially protect the msSGPs in female embryos from cell death and that unlike wild type females, the p35 expressing msSGPs are able to persist once the female gonad has coalesced. We used the <i>twist-GAL4</i>
driver to express p35 and after confirming that it promotes the survival of msSGPs in female embryonic gonads we examined the expression of STAT in the germ cells of these embryos (see Supplemental Fig. 2). As shown in Supplemental Fig., STAT expression is upregulated in female embryonic gonads when msSGPs are protected from cell death by ectopic p35 (compare panel B’ with panels C’ and D’). This finding indicates that one function of the male specific msSGPs is to signal germ cells to upregulate STAT accumulation.

**Wnt signaling promotes cell division in the germline**

When the pole cells are formed in the pre-cellular blastoderm embryo, they exit the cell cycle and don’t divide during their migration from the exterior of the embryo to the somatic gonad. After the coalescence of the embryonic gonad, the germ cells in males re-enter the cell cycle and begin dividing, slowing expanding the germline population in the gonad. By contrast, the germ cells in the female gonad do not start dividing until much later in development. Wawersik *et al.* (2005) have shown that the difference between males and females depends in part upon the activation of the JAK-STAT pathway in the male embryonic gonad. If *wnt-2* signaling functions to potentiate STAT activation in male germ cells, then it should also influence the sex-specific choice of whether to re-enter the cell cycle or not. To test this possibility we used antibodies against phosphohistone H3 (pH3) to identify germ cells that are undergoing cell division in the embryonic gonads of wild type and *wnt-2* mutant males and females. We observed at least one pH3 positive germ cell in about 40% of the wild type male embryos, while pH3 positive germ cells were never detected in wild type female embryos (Table 1). Wawersik *et al* (2005) also found that pH3 positive germ cells are never
detected in female embryonic gonads, while pH3 positive cells are readily detected in male embryonic gonads. As was seen for mutations in stat and upd (Wawersik et al. 2005), the frequency of male embryos with pH3 positive germ cells is reduced in wnt-2 males (see Table 1); however, the effects of wnt-2 are not as strong as those observed for JAK-STAT pathway mutations. To demonstrate that re-entering the cell cycle depends upon a response by the male germ cells to the Wnt-2 signal, we ectopically expressed the pathway inhibitor Axin in the germ cells using a nanos-GAL4 driver. As indicated in Table 1, excess Axin in the male germ cells attenuated cell cycle entry, and the frequency of gonads with pH3 positive germ cells also dropped to about 15%.

To confirm these findings we asked whether it was possible to induce female germ cells to begin dividing by inappropriately activating Wnt-2 signaling. We first expressed Wnt-2 in female germ cells using the nanos-GAL4 driver. As shown in Table 1, ectopic Wnt-2 can induce cell division in female germ cells, and a small (5%) but significant number of the nanos-GAL4:UAS-wnt-2 female embryos had pH3 positive germ cells. To show that activation of the canonical Wnt pathway in germ cells can induce cell division in females, we examined the gonads of the female progeny of sgg* germline clone mothers. As was observed for ectopic wnt-2, loss of maternal sgg activity induces the female germ cells to undergo premature cell division and pH3 positive germ cells are observed (see Fig. 9 and Table 1).

wnt-2 activity is required in for proper germline function in the adult testes

While adult wnt-2 males are sterile, the nature of the defect(s) has not yet been established. Since our findings indicate that communication between the soma and germline is sufficiently perturbed in wnt-2 mutants to alter the behavior of the germ cells
in the embryonic gonad, we wondered whether there was evidence for similar abnormalities in adult testes. To investigate this possibility, we stained wild type and \textit{wnt-2} mutant testes with the Vasa antibody and the DNA dye Hoechst. We observed several potentially related phenotypic abnormalities in the stem cell niche region of \textit{wnt-2} testes. In wild type testes, the highest levels of Vasa protein are found in the stem cells that are associated with the hub. As shown in Sup Fig. 3, the levels of Vasa protein in the germ cells near the hub were reduced in the mutant testes compared to wild type. Moreover, instead of being tightly associated with the hub as in wild type, the Vasa positive cells were often displaced from the hub. This lack of tight association with the hub does not appear to be due to a defect in hub formation as the morphology and arrangement of the hub cells in the mutant testes is the same as in wild type (data not shown).

To assess if there were any other defects, we also probed the testes with antibodies against a component of the fusome i.e. Spectrin. The fusome is a germline specific organelle. In germline stem cells and their daughter gonialblasts, the fusome is a small spherical structure. When the gonialblasts divides, the fusome maintains connections between the two daughters cells and begins to enlarges form a more branched-like structure. In wild type gonads the cluster of GSCs and newly formed gonialblasts at the tip of the testes have small spherical fusomes, while branched and more heavily stained fusome structures characteristic of differentiating cysts are only evident in germ cells that are displaced from the tip of testes. Unlike wild type, cells with branched and more heavily stained fusome structures characteristic of differentiating cysts can be seen close to the tip of the \textit{wnt-2} mutant testes (not shown). These cells
also show reduced levels of Vasa protein compared to wild type. In wild type there are typically around 11 cells at the apical tip of the testes that have the small spherical fusomes characteristic of GCSs and gonialblasts. In contrast, we found that the average number of cells with spherical fusomes in \textit{wnt}-2 mutant testes (n= 30) was only about 7.

**Discussion**

The primitive embryonic gonad in \textit{D. melanogaster} consists of the primordial germ cells and somatic gonadal precursor cells. Recent studies have shown that both the somatic and germline components of the embryonic gonad display sex specific differences (Casper and Van Doren, 2006). One difference is the presence of msSGPs at the posterior end of the male embryonic gonad. The msSGPs are derived from parasegment 13 and unlike other SGPs they are Sox100B and Abd-B positive and express high levels of the Wnt-2 ligand (De Falco \textit{et al.}, 2003). msSGPs are initially formed in both sexes, but subsequently undergo apoptosis in females during gonad coalescence and disappear by stage 15 (De Falco \textit{et al.}, 2003). Ectopic expression experiments by Boyle \textit{et al.} (1997) more than a decade ago showed that the population of SGPs at the posterior of the gonad could be substantially increased by inappropriately activating the canonical \textit{wnt} pathway. We have confirmed their findings and shown that these extra SGPs correspond to msSGPs. However, while the canonical \textit{wnt} pathway induces the formation of msSGPs, this doesn't seem to be the role of \textit{wnt}-2. Two observations argue against such a function. First, we found that ectopic expression of \textit{wnt}-2 using either mesodermal or germline drivers does not induce the formation of
excess msSGP (or other SGPs). Second, there does not appear to be any defect in the specification of msSGPs in \textit{wnt-2} mutant embryos.

Instead, our results suggest that \textit{wnt-2} is one of the factors responsible for the survival of msSGPs in the male embryonic gonad is Wnt-2. Thus, though the msSGPs appear to be properly specified in the absence of \textit{wnt-2} activity, these cells do not persist as they should in males and by the time the gonad has fully coalesced in stage 15 male embryos the number of msSGPs in \textit{wnt-2}\textsuperscript{-} gonads is greatly reduced compared to the wild type control. Conversely, ectopic expression of Wnt-2 in females can protect against apoptosis and msSGPs can be found in female stage 15 gonads. Since the msSGPs express Wnt-2, it would appear that this Wnt ligand normally functions as an autocrine survival factor. However, it can also promote survival of the msSGPs in females when expressed throughout much of the mesoderm or even when it is expressed in the germ cells. For reasons that we do not fully understand, the number of msSGPs that survive in female embryos when Wnt-2 is ectopically expressed is less than that in wild type males. One likely problem is that the level of the ectopic Wnt-2 signal produced by either the mesodermal \textit{twist} driver or the germline \textit{nanos} driver is not equivalent to the autocrine signal produced by the male msSGPs themselves. Two observations are consistent with this idea. First, the number of surviving msSGPs differed with different \textit{twist} drivers. Second, we found that the number of surviving msSGPs induced by ectopic Wnt-2 was roughly equivalent to that observed when the anti-apoptosis factor p35 was expressed in females using the \textit{twist} driver (not shown). On the other hand, it is possible that other autonomous or non-autonomous factors besides Wnt-2 also contribute to the survival of the msSGPs in males. This suggestion is
supported by the finding that a few surviving msSGPs are often observed \textit{wnt-2} mutant males. One factor that could substitute for \textit{wnt-2} would be \textit{wg}. An alternative, and more likely explanation is that other signals, probably from anterior SGPs, could promote survival (De Falco \textit{et al}., 2008).

\textit{wnt-2} signaling from the msSGPs upregulates the JAK-STAT pathway in germ cells and helps induce cell division

Previous studies have shown that the JAK-STAT pathway is activated in male, but not female germ cells (Wawersik \textit{et al}., 2005; Hombria \textit{et al}., 2005). Activation of this pathway in the primordial germ cells depends upon SGPs at the anterior of the male embryonic gonad, which unlike their female counterparts, express two of the fly JAK-STAT ligands, Upd and Upd-3. Our results indicate that the initial upregulation of the JAK-STAT pathway in male germ cells also depends upon a second signaling molecule, Wnt-2, that is expressed by the msSGPs at the posterior of the gonad (see diagram in Fig. 1). Several lines of evidence support this conclusion. First, upregulation of the JAK-STAT pathway in male germ cells requires \textit{wnt-2}, and in \textit{wnt-2} mutant males the level of STAT accumulation is only marginally higher than in the germ cells of wild type females. Second, it is possible to induce STAT accumulation in female germ cells by ectopically expressing Wnt-2 in either the mesoderm or in germ cells. Not surprisingly, the level of STAT induced in female germ cells by Wnt-2 expression is much less than that induced by ectopic Upd; however, Upd itself is also not sufficient to generate the same level of STAT accumulation in female germ cells as in wild type males. Third, inhibition of the canonical \textit{wnt} pathway in male germ cells by overexpression of Sgg (or Axin) downregulates STAT expression. Fourth, activation of the \textit{wnt} pathway in female germ
cells by mechanisms that bypass the Wnt-2 signal, \((sgg\) germline clones\) upregulates STAT accumulation.

Although our results indicate that Wnt-2 promotes the activation of the JAK-STAT pathway in male germ cells by the Upd ligands, the mechanism for this synergistic activity of the Wnt signaling pathway is unknown. One plausible idea is that the nuclear Arm-\(dTCF\) complexes formed after reception of the Wnt-2 signal activate the expression of some factor(s) that is needed to turn-on STAT transcription. Alternatively, it is possible that \(dTCF\) interacts directly with regulatory elements in the STAT promoter. In the absence of the Wnt-2 signal \(dTCF\) would negatively regulate the STAT gene, opposing activation by Upd, while it would positively regulate the STAT gene when associated with nuclear localized Arm. In either case, our results indicate that the induction of high levels of STAT accumulation in embryonic male germ cells depends upon input from both Upd and Wnt-2 signaling.

One of the early functions of the JAK-STAT pathway in male gonads is to induce the germ cells to re-enter the cell cycle and begin dividing again. This is a sexually dimorphic trait, as the germ cells in female embryos do not divide until much later in development. We found that \(wnt-2\) also contributes to this sex-specific difference in the embryonic gonad. When \(wnt-2\) signaling is inappropriately activated in females, it induces female germ cells to start dividing prematurely. Conversely, loss of \(wnt-2\) signaling interferes with the induction of germ cell division in males. Moreover, as is the case for the potentiation of STAT accumulation, the induction of germ cell division depends upon the activation of the canonical Wnt pathway in the germ cells. While the effects of gain and loss of \(wnt-2\) signaling on cell cycle re-entry are not as strong as
those reported for components of the JAK-STAT pathway, they closely parallel those seen when the activity of JAK-STAT pathway is manipulated in males and females. At this point it seems likely that the induction of cell division by \textit{wnt-2} is mediated, at least in part, by its role in potentiating STAT accumulation in male germ cells. The JAK-STAT pathway has already been implicated in cell cycle re-entry and factors that facilitate the activation of this pathway in male germ cells would indirectly promote cell division. On the other hand, it is possible that \textit{wnt-2} signaling simultaneously promotes cell division by a mechanism that is independent of its role in the JAK-STAT pathway. Potentially supporting this later possibility is the finding that mutations in the STAT pathway do not appear to completely eliminate germ cell division in male embryos.

\textit{Does bi-polar signaling promote the formation of a more symmetric niche environment in the male embryonic gonad?}

From studies on the adult testes it is clear that the Upd-JAK-STAT pathway, together with Dpp signaling, is required to maintain GSC identity. When the Upd-JAK-STAT signaling pathway is compromised, the GCSs fail to self-renew and instead undergo differentiation. Conversely, when Upd-JAK-STAT signaling is inappropriately upregulated, GSCs proliferate without differentiating. The fate of the germ cells in the testes depends upon their proximity to the somatic cells, the terminal hub cells, which express the Upd ligand. The germ cells in close proximity to the hub retain GSC identity, while cells that are displaced from the hub as the GSCs divide are reprogrammed and undergo differentiation (Keiger \textit{et al.}, 2001; Tulina and Matunis, 2001). While it was initially thought that STAT regulates self-renewal of germline stem cells (GSCs) in a cell autonomous manner, more recent analysis has suggested that it likely controls GSC
adhesion to hub cells by maintaining the somatic cyst stem cell (CySCs) population possibly via BMP signaling (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). Given the apparent connection between \textit{wnt-2} signaling and the upregulation of the JAK-STAT signaling in the embryonic gonad, it is interesting that a somewhat similar phenotype is observed in the testes of \textit{wnt-2} mutant males. We find that the tight association of germline stem cells with hub is not properly maintained in the \textit{wnt-2} mutant, and the number of germline stem cells surrounding the hub is reduced.

In this context, it is interesting to note that when the male embryonic gonad coalesces in stage 14-15 embryos there is a similar spatial arrangement of primordial germ cells and the source of Upd signaling. Germ cells at the anterior are in close proximity to the Upd source and thus would be in an environment conducive to inducing association with anterior SGPs and assuming GSC identity. In contrast, cells in the middle and at the posterior are displaced from the Upd source and would be in a much less favorable environment for establishing appropriate contacts. However, this deficiency would be counteracted because the male embryonic gonad has a second somatic signaling source, the msSGPs, at the posterior which produces a ligand, Wnt-2, that functions in the initial upregulation of the JAK-STAT pathway. The presence of two distinct signaling centers at the opposite ends of the embryonic male gonad that synergistically activate the JAK-STAT pathway would tend to favor an initial equivalence in the potential of germ cells located at different positions along the anterior-posterior axis of the gonad. This would be important because it would effectively increase the pool of that can be drawn upon when the stem cell niche is assembled at the anterior of the male embryonic gonad during stage 17. If GSC fate is determined during this stage
as has been suggested by the work of Le Bas and Van Doren (2006), a gonad populated exclusively by germ cells with the capacity to adhere to the somatic cells in the niche and thus develop as GSCs would ensure that the niche is fully occupied when it is formed. In this case, the number of GSCs that are ultimately specified by incorporation into the niche would be limited by the capacity of the niche in the stage 17 gonad and not by the developmental potential of the embryonic germ cells. Consistent with this model, Asaoka and Lin (2004) found that both anterior and posterior germ cells in the male embryonic gonad could give rise to GSCs in the adult testes. As would be expected from the fact that there is a small excess of germ cells in the embryonic gonad, the frequency of GSC identity is not identical for the two populations. While all anterior cells formed GSCs, this was true for only about half of the posterior cells. This difference could be explained by the proximity of the anterior germ cells to the nascent stem cell niche (Le Bras and Van doren, 2006). Interestingly, the female embryonic gonad is much more asymmetric: germ cells in the anterior become GSC, while cells in the posterior differentiate directly into cystoblasts and do not become GSCs (Zhu and Xie, 2003; Aasoka and Lin, 2004). This asymmetry would suggest that the female embryonic gonad has a signaling center at the anterior that promotes GSC identity, while it does not have a signaling center at the posterior equivalent to that provided by the msSGPs in stage 14-15 male gonad.

Materials and Methods:
**Immunohistochemistry:** The embryo stainings were performed essentially as described in Deshpande *et al.* (1995). Embryos were fixed for 20 min in 3.7% formaldehyde/PBS/heptane and devitellinized in heptane:methanol for staining with the primary antibodies: Conjugated Secondary antibodies were obtained from Molecular Probes. Embryos were mounted in AquaPolymount (Polysciences, Inc.). Images were collected using a Zeiss LSM 510 confocal microscope and Adobe Photoshop was used to generate the final figures. The microscope settings including the gain and the laser intensity were maintained constant for the duration of a complete experiment after the initial standardization to minimize the variability. In each imaging experiment both experimental and control embryos were imaged under the same conditions.

To render older i.e. stage 17, embryos accessible to immunostaining, sonication was used (for a detailed description see ref. (Le bras and Van doren 2006). Embryos were rehydrated by washing several times in PBS containing 0.1% Triton (PBST), sonicated in 500 µl of PBST with a 3 second constant pulse using a Branson Sonifier 250 (set at 100% duty cycle and output setting 1), washed several times with PBST. The embryos were subsequently stained with the primary antibodies as described above.

Following antibodies were used. Vasa (rabbit; 1:1000; From Paul Lasko), Anti- Vasa (rat, 1:1000; from Paul Lasko), Sox100B (Rabbit, 1:2000; from Steve Russell), STAT (Rabbit, 1:1000; from Steven Hou), Eyes Absent (mouse, 1:15, from the Developmental Hybridoma Bank), Upd (1:500, from Doug Harrison), Sxl (mouse, 1:10, from the Developmental Hybridoma Bank), β-galactosidase (Rabbit, 1:2000, purchased from Kappel; mouse, 1:10 from the Developmental Hybridoma Bank).

**Clonal Analysis:** Females carrying germ line clones for *sgg* were produced as
previously described (Chou and Perrimon, 1992). Clones were induced in females homozygous for FRT insertion on X chromosome (FRT 101) and heterozygous for the dominant female sterile mutation, ovoD1, and zw3(M11). Females of the genotype: zw3, FRT 101/FM7 were mated to males that are: ovoD FRT 101/Y; Flp/Flp. Progeny was heat-shocked at 2\textsuperscript{nd} and 3\textsuperscript{rd} instar larval stages, for one hour each. The virgin females recovered after the heat shock, were crossed to OregonR males. The male progeny will not be rescued whereas females are zygotically rescued.

**Fly stocks:** Two different wnt-2 null alleles (31) wnt-2\textsuperscript{O} and wnt-2\textsuperscript{L}, were used for loss-of-function studies. They gave similar results. The following UAS and GAL4 stocks were used for the misexpression studies: UAS-wnt-2 (three different transgene inserts were used), UAS-upd (two different transgene inserts were used), UASArm-S10 (two different inserts were used), twist-GAL4, nanos-GAL4, UAS-\textbeta galactosidase. In most experiments, males carrying two copies of UAS-wnt-2 or UAS-upd transgenes were mated with virgin females carrying two copies of the GAL4 transgene. The resulting progeny embryos were fixed and stained for subsequent analysis (Brand and Perrimon, 1993).

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References:


**Figure legends:**

**Figure 1:** Upregulation of canonical wnt signaling leads to excess number of msSGPs.

Embryos were probed with Vasa antibodies (imaged in blue) to mark the germ cells and with Abd-B (imaged in green) and Sox100B (imaged in red) antibodies to mark the msSGPs. All the panels show coalesced gonads from stage 15 embryos. Panels A, A' and A'": Control male embryo. Panel B, B' and B'": twist-GAL4/UAS-ArmS10 embryo. Approximately 10 Sox100B positive cells (n =10) i.e. msSGPs are visible in the control embryo (Panel A'). By contrast, ArmS10/twist-Gal4 embryo shows an increased number (~23, n =15) of Sox-100B positive cells (Panel B'). Panels A"" and B"" (anti-Abd-B) show same embryos as in Panels A and B respectively.
Diagrams at the bottom of the figure show schematic representation of the coalesced male and female gonad. In the males, SGPs at the anterior of the gonad express the JAK-STAT ligand Upd and are indicated in the diagram in yellow. These anterior SGPs as well as all of the other SGPs in the male (and female) gonad are Eya positive. At the posterior, there is a special group of Sox100B (and Eya) positive msSGPs. These SGPs express Wnt-2 and in stage 15 embryonic gonad, are detected just in male but not female gonads. Germ cells in both males and females are Vasa positive (Blue).

**Figure 2:** *Wnt-2 is required for msSGP survival in male embryos.*

Embryos from *wnt-2/Cyo engrailed-LacZ* stock were probed with Sxl (not shown), β-galactosidase (not shown) Vasa (imaged in blue), Sox100B (imaged in red) and Abd-B (imaged in green) antibodies simultaneously. All the panels show coalesced gonads from stage 15 embryos. Panels A, A’ and A’”: Control male embryo carrying the balancer. Panels B, C and D: three different *wnt-2* male embryos. The *wnt-2* male embryo (Panel B-D) and the control embryo carrying the balancer (Panel A) show similar number of germ cells (blue). Roughly 10 msSGPs i.e. Sox100B positive cells are visible in the control embryo (Panel A’, n=15). By contrast, *wnt-2* embryos show reduced number (between 0-4) of Sox-100B positive cells (Panels B’, C’ and D’; n=10). Panels A’’, B’’, C’’ and D’’ (anti-Abd-B specific signal) show same embryos as in Panels A-D respectively.

**Figure 3:** *wnt-2 is required for survival of Eya/Sox100B positive msSGPs in male embryos.*
Embryos from a wild type stock and from \textit{wnt-2/Cyo engrailed-LacZ} stocks were stained with \(\beta\)-galactosidase (not shown), Sxl (not shown), Eya (imaged in green), Vasa (imaged in red), and Sox100B (imaged in blue) antibodies simultaneously. All the panels show coalesced gonads from stage 15 male embryos as indicated by the absence of Sxl specific staining. Panel A: Control male embryo carrying the balancer. Panels B: \textit{wnt-2} male embryo. Both the \textit{wnt-2} male embryo (Panel B) and the control embryo carrying the balancer (Panel A) show similar number of germ cells (red). Roughly equal number of SGPs are also seen in both the embryos (compare panels A' and B'); however, the Sox100B positive cells i.e. msSGPs are present in the control embryo (Panel A'') but not in the \textit{wnt-2} embryo (Panel B''). Like the example shown here, about 65\% (n=20) of the \textit{wnt-2} embryos had no Sox100B positive msSGPs. About 25\% of the \textit{wnt-2} male embryos had 1-2 msSGPs and about 10\% had 3-5. In the control males the number of Sox100B positive msSGPs varied from 8-14 (n=10).

\textbf{Figure 4:} \textit{A few msSGPs survive in female embryonic gonads upon ectopic expression of wnt-2 in the vicinity.}

Ectopic expression of \textit{wnt-2} was achieved by crossing \textit{twist-GAL4} females (Panel C) to \textit{UAS-wnt-2} males. Stage 15 embryos of the denoted genotype were stained with Sxl (not shown), Vasa (imaged in red) and Sox100B (imaged in blue) antibodies. Embryos in panels A, B, and C show composite images of the two fluorophores whereas Panels A’, B’, and C’ show just the Sox100B specific antibody staining (blue). Panels A & A’: Control female embryonic gonad from a stage 15 embryo. Note the absence of Sox100B specific signal (blue). Panel B: Control male embryonic gonad from a stage
15 embryo. Panel B': Same embryo as in panel B showing msSGPs that stained with Sox100B antibodies. Panels C: Stage 15 gonads of the UAS-wnt-2 / twist-GAL4 female embryos. This female embryo has a few Sox100B positive cells located at the posterior of the gonad seen in C'.

Ectopic expression of wnt-2 was achieved by crossing nos-GAL4 transgenes (Panel F) to UAS-wnt-2 males. Panels D-F: Stage 15 embryos of the genotype denoted below were stained with Sxl (imaged in blue), Vasa (imaged in red) and Sox100B (imaged in green) antibodies. Embryos in panels D, E and F show composite images of the three fluorophores whereas Panels D', E' and F' show just the Sox100B specific antibody staining (green). Panel D & D': Control wild type female embryonic gonad from a stage 15 embryo. Note the absence of Sox100B specific signal (imaged in green) in panel D'. Panel E: Control male embryonic gonad from a stage 15 embryo. Panel E': Same embryos as in panel E showing msSGPs that stained with anti-Sox100B antibodies. Panels F and F': Stage 15 gonad of a UAS-wnt-2 /nos-GAL4 female embryo. This female embryo has several Sox100B positive cells at the posterior of the gonad. For each of these experiments at least 15 embryos of the appropriate genotype was scored. The experiments shown here were conducted using UAS-wnt-2 transgene inserted on the X chromosome.

Figure 5: wnt-2 activity is necessary for the accumulation of high levels of STAT in male germ cells.
Panels A-C': Embryos from the *wnt-2*/ Cyo-engrailed Lac-Z stock or from wild type were stained with STAT (imaged in green) and VASA (imaged in red) antibodies. Male and female embryos were distinguished using Sxl specific staining (imaged in blue) whereas homozygous *wnt-2* embryos were identified by the absence of Beta-galactosidase specific staining (not shown). Panel A: Male embryonic gonad from a stage 15 embryo. Note the absence of Sxl specific signal. Panel A': Same embryo as in panel A. Wild type male germ cells show enrichment of STAT protein. Panel B: Female embryonic gonad from a stage 15 embryo. Panel B': Same embryo as in panel B. Background level of anti-STAT specific staining in the control female germ cells is seen. Panels C: Stage 15 gonad of the *wnt-2* male embryo. Panels F: Same embryos as in panels C'. The *wnt-2* embryo displays reduced levels of anti-STAT specific signal compared to the control (panel C'). The level of the STAT protein in the germ cells is comparable to that seen in the female germ cells shown in Panel B'. Levels of STAT protein were consistently different between *wnt-2* (n=15) male embryos as opposed to the control (n=10).

**Figure 6:** STAT expression is upregulated in the female germline by ectopic expression of *wnt-2* in the mesoderm

Embryos were labeled with Sxl (blue), Vasa (red) and STAT (green) antibodies. Panels A, B, C, and D show composite images whereas Panels A', B', C' and D' show magnified view of the PGCs from the same embryos respectively. Panels A'', B'', C'' and D'' show just the STAT antibody staining. Panels A-A'': wild type stage 13 female embryo. Background level of STAT specific signal is observed in wild type female germ cells at this stage. Panels B-B'': Coalesced gonad from a wild type stage 15 male embryo. Very
high level of STAT antibody staining is seen in the germ cells of male embryo. Panels C-C'': *UAS-wnt-2/twist-GAL4* female embryo. Unlike wild type females at this stage (panels A-A''), which have only background levels of staining STAT protein can be readily detected in the germ cells of *UAS-wnt-2/twist-GAL4* female embryos (compare panels A'' and C''). Panel D-D'': Coalesced gonad from a stage 15 *UAS-wnt-2/twist-GAL4* embryo. As with the female embryo in Panels C elevated levels of STAT protein are seen in this *UAS-wnt-2/twist-GAL4* female embryo. Note that while the levels of STAT antibody staining seen in the *UAS-wnt-2/twist-GAL4* female embryos is higher than that observed in wild type females, it is not equivalent to that seen in wild type males. Upregulation of STAT protein due to over expression of *wnt-2* in the female embryos was variable. Roughly 50% of the embryos (n=15) displayed elevated levels of STAT protein compared to the female PGCs from the wild type control.

**Figure 7:** *Germ cell specific over-expression of either sgg or axin leads to reduction of STAT levels in male germ cells.*

*UAS-sgg* or *UAS-Axin* males were mated with *nos-GAL4* females to achieve germ cell specific overexpression of *sgg* or *axin*. Embryos were stained with STAT (imaged in red), VASA (Green) and EYA antibodies (imaged in Blue) Male and female embryos were distinguished using Sxl specific staining (not shown). All panels show male embryos of defined genotypes. Panels A-A'': Control wild type male embryo (n=5). Panels B-B'': *UAS sgg / nos-GAL4*. Panels C- C'': *UAS-axin / nos-GAL4*. Overexpression of either *sgg* (n=7) or *axin* (n=6) results in reduction in STAT levels whereas it does not seem to influence the total number
of SGPs (Eya positive cells).

**Figure 8:** *Female germ cells compromised for sgg function show elevated STAT expression.*

Wild type embryos derived from females carrying sgg germline clones were probed with Sxl (blue), Vasa (red) and STAT (green) antibodies. Embryos in panels A-C show composite images while A’-C’ show only the STAT antibody staining. Panels A & A’: Gonad from a wild type stage 15 male embryo. Note the absence of Sxl protein (blue) and the high level of STAT (green) in the germ cells. Panels B & B’: Gonad from a wild type female (n=6). Note the presence of Sxl and the background level of STAT protein. Panels C & C’: Stage 15 female embryo derived from sgg germline clone carrying female (n=8). As in the case of wild type female embryo, high levels of Sxl are present in this embryo (C). However, unlike wild type, the sgg'' female germ cells have elevated levels of STAT (C’).

**Figure 9:** *Female germ cells compromised for sgg function undergo premature mitosis.*

Both wild type and embryos derived from females carrying sgg germline clones were probed with Sxl (not shown), Vasa (blue) and Phospho-histone H-3 (red) antibodies. Embryos in panels A-C show female embryos of the denoted genotype. Wild type stage 14 embryo in panel A is devoid of pH3 specific staining (magnified view of just the PGCs in panel A’) whereas sgg'' germ cells shown in panels B (stage 14) and C (stage 14) display pH3. Panels B’ and C’ show magnified view of just the PGCs from the same embryos with a clear presence of pH3 specific signal. For quantitation see Table 1.
Embryos between stages 13 to 15 were used for analysis.

**Supplementary Figure 1.** *wnt-2 is not required for the initial specification of msSGPs.*

Embryos from *wnt-2/Cyo engrailed-LacZ* stocks were probed with Sxl (imaged in green, not shown), Vasa (imaged in blue), Beta-galactosidase and Sox100B (both imaged in red) antibodies simultaneously. Both the panels show gonads from stage 13 embryos. Panels A: Control Beta-galactosidase positive *wnt-2/Cyo engrailed-LacZ* male embryo. Panels B: *wnt-2* male embryo. The *wnt-2* male embryo (Panel B; n=12) and the control embryo (Panel A; n=10) on an average displayed 10 Sox100B positive cells i.e. msSGPs.

**Supplementary Figure 2.** Mesodermal ectopic expression of the apoptosis inhibitor, p35, can activate STAT expression in the female germ cells.

Wild type embryos or embryos produced by twist-GAL4 females mated to males carrying a UAS p35 transgene were stained with Sxl (imaged in blue), Vasa (imaged in red) and STAT (imaged in green) antibodies. Embryos in panels A-D show composite images for all three antibodies, Panels A’-D’ show only the STAT specific antibody staining (green). Panels A & A’: Male embryonic gonad from a stage 15 embryo. Note the absence of Sxl specific signal and the high levels of STAT in the male germ cells. Panels B & B’: Female embryonic gonad from a wild type stage 15 embryo. Note the Sxl antibody staining and the very low level of STAT. Panels C & D and C’ & D’: Stage 15 gonads of *UAS-p35/twist-GAL4* female embryos. Note the presence of Sxl protein and the elevated levels of STAT protein compared to the wild type female control (compare B’ with C’ & D’).
Supplementary Figure 3. A subset of wnt-2 stem cells display altered adhesion with the hub and reduced level of Vasa.

WT (OregonR) and wnt-2 testis were probed with DNA dye (blue), and Vasa (red), antibodies. Panels A-C show composite images while panels A’-C’ show just Vasa specific signal. Panel A: WT testis; Panels B, and C testis from wnt-2 males. Vasa specific signal, which marks germ cells, appears to be reduced in the experimental panels as compared to wild type control. The typical rosette like arrangement observed in wild type testis is missing in wnt-2 male testes (compare panels A’ and C’).
Table 1: Influence of the wnt signaling pathway on sex specific germ cell division.

Embryos of the given genotype between stages 13 -15 were stained with Vasa, Sxl and pH3 antibodies and individual germ cells were carefully examined for the presence of pH3 specific signal within the germ cells. Total number of embryos that displayed one or more pH3 positive germ cells were counted. Wild type female and male embryos served as negative and positive controls respectively.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage (and number) of pH3+ embryos</th>
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<tbody>
<tr>
<td>WT male</td>
<td>40% (8/20)</td>
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<tr>
<td>WT female</td>
<td>0% (0/20)</td>
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<tr>
<td>wnt-2 male</td>
<td>20% (5/25; p value=0.0337)</td>
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<td>UAS axin X nos GAL4 male</td>
<td>13% (2/15; p value= 0.0443)</td>
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<td>sgg glc female</td>
<td>13% (4/30; p value= 0.0460)</td>
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Figure 1
Figure 2
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Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3