Without children is required for Stat-mediated zfh1 transcription and for germline stem cell differentiation

Iris Maimon, Malka Popliker and Lilach Gilboa*

ABSTRACT

Tissue homeostasis is maintained by balancing stem cell self-renewal and differentiation. How surrounding cells support this process has not been entirely resolved. Here we show that the chromatin and telomere-binding factor Without children (Woc) is required for maintaining the association of escort cells (ECs) with germ cells in adult ovaries. This tight association is essential for germline stem cell (GSC) differentiation into cysts. Woc is also required in larval ovaries for the association of intermingled cells (ICs) with primordial germ cells. Reduction in the levels of two other proteins, Stat92E and its target Zfh1, produce phenotypes similar to woc in both larval and adult ovaries, suggesting a molecular connection between these three proteins. Antibody staining and RT-qPCR demonstrate that Zfh1 levels are increased in somatic cells that contact germ cells, and that Woc is required for a Stat92E-mediated upregulation of zfh1 transcription. Our results further demonstrate that overexpression of Zfh1 in ECs can rescue GSC differentiation in woc-deficient ovaries. Thus, Zfh1 is a major Woc target in ECs. Stat signalling in niche cells has been previously shown to maintain GSCs non-autonomously. We now show that Stat92E also promotes GSC differentiation. Our results highlight the Woc-Stat-Zfh1 module as promoting somatic encapsulation of germ cells throughout their development. Each somatic cell type can then provide the germline with the support it requires at that particular stage. Stat is thus a permissive factor, which explains its apparently opposite roles in GSC maintenance and differentiation.

KEY WORDS: Stat92E, Zfh1, Woc, ZMYM, GSC, Niche, Drosophila

INTRODUCTION

Adult stem cells maintain tissue homeostasis by balancing self-renewal and differentiation. This balance depends on extensive communication between stem cells and their environment (niche). In many cases, the cues required for self-renewal differ from those directing differentiation. Whether the same signal might serve both is unclear.

Drosophila germline stem cells (GSCs) and their somatic niche cells are a convenient model for understanding the interactions between stem cells and their environment. The somatic niche for GSCs is composed of terminal filament (TF), cap cells and the anterior escort cells (ECs) (Fig. 1A), which produce the BMP2/4 homologue Decapentaplegic (Dpp) (Harris and Ashe, 2011; Lopez-Onieva et al., 2008; Wang et al., 2008; Xie and Spradling, 2000). Dpp signalling within GSCs results in phosphorylation of Mothers against Dpp (pMad), and in repression of the major differentiation gene bag of marbles (bam) (Chen and McKearin, 2003a,b; Xie and Spradling, 1998). Both GSCs and their first differentiating daughter cells (cystoblasts, CBs) contain a spherical organelle (fusome) that elongates and branches as differentiating CBs form germline cysts. Dividing cysts maintain tight contact with a group of somatic ECs, which are important for their differentiation (Fig. 1A) (Kirrily et al., 2011; Lim and Fuller, 2012; Schulz et al., 2002).

Many signalling pathways collectively control GSC biology (Fuller and Spradling, 2007; Gancz and Gilboa, 2013; Kirrily and Xie, 2007; Spradling et al., 2011). Among those, the Stat (signal transducer and activator of transcription) pathway functions in both males and females. In males, the activated Jak kinase (Hopscotch, Hop) and its target Stat (Stat92E) promote GSC and cyst stem cell (CySC) self-renewal cell-autonomously. In addition, Stat signalling within CySCs is required for GSC self-renewal (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). In females, Stat is also required non-autonomously for GSC maintenance (Decotto and Spradling, 2005; Lopez-Onieva et al., 2008; Wang et al., 2008). Stat target genes, such as Suppressor of cytokine signalling 36E (Socs36E), Chronologically inappropriate morphogenesis (chinmo) and zfh1, were found to function in male GSCs and CySCs (Flaherty et al., 2010; Issigonis et al., 2009; Leatherman and Dinardo, 2008; Singh et al., 2010). zfh1 encodes a transcriptional repressor with multiple zinc fingers and a homeodomain (Fortini et al., 1991). It is expressed in CySCs and their early daughter cells, and is required for their maintenance and for GSC self-renewal (Leatherman and Dinardo, 2008, 2010).

Although many effectors are known to control GSC biology, the list is by no means complete. In a screen designed to find new players in soma-germline communication (Gancz et al., 2011), we identified without children (woc) as a potential candidate. Woc was first isolated due to a sterility phenotype, and was shown to contain zinc fingers and an AT-hook domain, which suggest a function in transcription (Warren et al., 2001; Wismar et al., 2000). Indeed, Woc binds active chromatin domains and colocalise with initiating forms of RNA polymerase II (Rafia et al., 2005). Woc was further shown to recruit and regulate the binding of heterochromatin protein 1c (HP1c) to active sites of transcription (Font-Burgada et al., 2008). In addition, Woc binds telomeres and prevents telomere fusion, independently of other known telomere-capping proteins (Rafia et al., 2005).

Here, we report that Woc is a novel player in ovarian biology that, together with Stat and Zfh1, is required for GSC/CB differentiation. We further show that Zfh1 is haplo-insufficient for its function in ECs and that Woc is required for a Stat-mediated elevation in its transcription. The root of the seemingly opposing functions of Stat in GSC self-renewal and differentiation is a common role in promoting contacts between somatic cells and germ cells throughout development.

RESULTS

Woc is required for GSC and CB differentiation

To find novel regulators of GSC maintenance and differentiation, we expressed various RNAi lines in the somatic cells of the ovary,
To determine the stage at which germ cell differentiation was blocked, we stained wild-type and woc-RNAi ovarioles using an anti-SMAD3 antibody, which cross-reacts with pMAD and labels GSCs (Ahles and Drummond-Barbosa, 2010). The ovarioles were also stained using anti-GFP to detect bam-P-GFP, which recapitulates endogenous bam RNA expression in CBs and dividing cysts (Chen and McKearin, 2003b). The developmental state of all cells carrying a spherical fusome was scored. As expected for wild-type germlaria, 2-3 GSCs were exclusively labelled with pMAD (GSC in Fig. 1D,D’,F). On average, less than one pMAD+ cell was observed outside the niche (Fig. 1F), and an average of less than one cell was labelled neither by pMAD antibody nor by GFP (Fig. 1F). The latter may represent the pre-CB, a GSC daughter cell that has lost pMAD but has not yet upregulated bam expression (Gilboa et al., 2003; Ohlstein and McKearin, 1997; Rangan et al., 2011). An additional single cell, the cystoblast, was labelled by GFP (Fig. 1D,D’, outlined, IF). In most woc-RNAi germlaria, pMAD staining was strong in GSCs located in the niche (Fig. 1E,E’, arrowheads, 1F). A few pMAD-positive cells were located away from the niche (Fig. 1F). However, the majority of single cells that were not located at the niche were either pre-CBs or CBs (Fig. 1E’-F). In total, woc-RNAi ovarioles contained 16 single cells (n=60) compared with 4.7 in wild-type cells (n=61, Fig. 1F).

Significantly, in strongly affected woc-RNAi ovarioles, bam-positive cells did not form cysts, suggesting that Bam expression is insufficient to drive cyst development without somatic Woc input. To test this more rigorously, we forced germ cells to differentiate by overexpressing bam using a heat-shock promoter (Ohlstein and McKearin, 1997). Following heat shock, wild-type GSCs differentiated (Fig. 1G, arrow, n=73). However, whereas increased Bam levels were detected in woc-RNAi ovarioles (not shown), no rescue of the bam-phenotype was observed (Fig. 1H, arrowheads, n=123). In conclusion, the mixed nature of the single cells in woc-RNAi tumours suggests that somatic Woc is required for efficient GSC differentiation and for cyst formation following Bam expression.

**Woc is required in escort cells (ECs) for their association with germ cells**

To determine which cells express Woc, we stained wild-type germlaria using anti-Woc antibody (Raffa et al., 2005). Woc was expressed in all ovarian cells (Fig. 2A-C), raising the possibility that Woc may affect germ cells autonomously, as well as through the soma. To test which cells in the germlarium require Woc function to allow GSC/CB differentiation, we generated large somatic clones of woc-phenotype by germline clones or by reducing Woc in germ cells by either germline clones or by Bam expression.

Reducing Woc in germ cells by either germline clones or by expression of woc-RNAi with the germline driver nos-Gal4 did not result in differentiation defects (Fig. 2H-J). However, fewer germline clones were retrieved when compared with WT, suggesting Woc may be required cell-autonomously for germ cell viability and non-cell autonomously within ECs for GSC/CB differentiation.

To better understand the requirement for Woc in ECs, we examined their morphology using a GFP trap in the protein Failed axon connections (Fax-GFP), which labels their membranes (Buszczak et al., 2007). Normal ECs extend fine cytoplasmic processes that wrap and support dividing cysts (Fig. 2K) (Decotto...
Fig. 2. Woc is required in ECs to maintain cell protrusions and to allow GSC differentiation. (A–C) A wild-type germarium. Anti-Woc (green in A,B) stains somatic and germ cell nuclei (DAPI, blue in A, greyscale in C). (D–L) Anti-Hts is in magenta. (D–I) GFP marks wild-type cells. (D) Control ovary FRT82B with GFP-deficient somatic cells. GSCs differentiate into normal cysts (arrows). (E–G) When niche cells are WT (arrowheads) and somatic ECs are mutant (no GFP, arrows) for woc251 (E), wocO100 (F) or wocO29 (G), germ cells fail to differentiate and carry spherical fusomes. (H–I) Germ cells mutant for wocO29 (H, arrows), or woc251 (I, arrow) can develop into cysts. (J) woc-deficient germ cells (anti-Vasa, green) differentiate normally into cysts (arrows). (K,L) Somatic cell membranes are marked by Fax-GFP (anti-GFP, green). Arrows mark somatic cells. Several compressed z-sections are shown. In WT (K), somatic cell protrusions extend between cysts. (L) In woc-RNAi ovaries, ECs fail to send protrusions and GSCs fail to differentiate. Scale bars: 10 μm.

and Spradling, 2005; Kirilly et al., 2011; Morris and Spradling, 2011; Schulz et al., 2002). In contrast, whereas woc-deficient EC nuclei were observed in woc-RNAi ovaries, ECs failed to send cellular extensions into the germmarium, and to wrap germ cells (Fig. 2L, n=69). In addition, fewer ECs were present in woc-RNAi ovaries. Staining with the vital dye propidium iodide (PI) revealed that 25% (n=80) of all woc-RNAi germmaria contained a dying EC, as compared with only 3.7% (n=54) of wild-type germmaria. Combined, these data suggest that Woc is required in ECs for viability, for proper soma-germmline contact and for GSC/CB differentiation.

Woc is already required in the forming ovary for soma-germmline association

Intercellular contact is a major driving force of cell behaviour not only in adult organ function, but also during organ formation. We therefore asked whether Woc affects the formation of the GSC unit. In wild-type late larval third-instar (LL3) ovaries, primordial germ cells (PGCs) occupied the medial part of the ovary, and the somatic intermingled cells (ICs) were interspersed between them (Fig. 3A) (Gilboa and Lehmann, 2006; Li et al., 2003). PGCs in woc-RNAi ovaries were still medially localised. However, the majority of ICs remained outside of the germ cell region (Fig. 3B, outlined, 73% of ovaries, n=49). Similar phenotypes were observed in ovaries containing large mutant clones of wocB111 (D) and wocO29 (E) mutant ICs (GFP-negative) organise outside the germ cell region (outlined) and very few cells intermingling with PGCs. (F) Overexpression of Woc results in increased IC numbers (compare F with A). Scale bars: 10 μm.

To determine whether Woc may have additional effects on IC biology, we overexpressed it using a line carrying a UAS insertion into the woc locus. Woc overexpression resulted in a significant increase in IC numbers (compare Fig. 3A with 3F, supplementary material Table S1). Collectively, these data suggest that Woc is essential for proper contact of somatic cells with germ cells, and can affect IC survival and specification or proliferation.

As Woc is already required for soma-germmline interactions at larval stages, we wondered whether the woc adult phenotypes might result from the earlier, larval, defects. We therefore used the Gal80ts system to remove Woc function in adult ovaries only. Defective EC extensions, coupled to a lack of germ cell differentiation, were also observed under these experimental settings (supplementary material Fig. S2), demonstrating that Woc is required both in larval and adult somatic cells for correct ovarian morphology and function.

Similar ovarian phenotypes of woc, stat and zfh1

It has been previously shown that Stat activation in adult germmaria increases EC numbers (Decotto and Spradling, 2005; Lopez-Onieva et al., 2008). Considering the similarity in gene expression between adult ECs and larval ICs, and the possible origin of ECs from ICs, we tested whether Stat activation may also increase IC numbers. Indeed, similar to Woc overexpression, somatic overexpression of
either the ligand Upd or the constitutively active Jak kinase hop\textsuperscript{tum-1} resulted in extensive IC over-proliferation (100% of ovaries, \(n=30\) each, compare Fig. 3A with Fig. 4A,B). Repression of the Stat pathway also resulted in woc-like phenotypes; large clones of ICs that are mutant for stat92E\textsuperscript{197} or stat92E\textsuperscript{5-9} failed to intermingle with PGCs and mostly remained at the periphery of the germ cell region (Fig. 4C,D, respectively, 100% of ovaries, \(n=52\)). This further extends the similarity between woc and stat phenotypes.

As woc- and stat-mutant or -overexpression larval phenotypes overlap, we asked whether these genes share phenotypes in the adult. Stat signalling is required for GSC maintenance in both males and females (Decotto and Spradling, 2005; Kiger et al., 2001; Lopez-Onieva et al., 2008; Tulina and Matunis, 2001; Wang et al., 2008). Because our data suggest that woc is required in ECs for differentiation of GSCs and CBs, we tested whether, in addition to GSC maintenance, Stat signalling might affect GSC differentiation. Such a function could have been missed previously due to masking by the earlier function of Stat in GSC maintenance, and because observing EC-mediated control of GSC differentiation requires mutating the majority of ECs within a germarium (using the Minute technique).

Germaria carrying large populations of stat-mutant ECs displayed aberrant GSC differentiation. In contrast to the WT, cells carrying spherical fusomes were observed far from the niche (Fig. 4E, arrowheads, all ovarioles, \(n=124\)). In line with the mutant analysis, somatic removal of stat by three different RNAi lines resulted in an excess of single cells (Fig. 4F, \(n=115\), supplementary material Table S2), suggesting that Stat signalling in ECs is required for GSC differentiation.

To further establish a second role for Stat in GSC differentiation, we analysed the outcome of reducing Zfh1 expression in ECs. Zfh1 is a transcriptional target of Stat, which maintains CySCs in the Drosophila testis (Leatherman and Dinardo, 2008). Similar to woc and stat phenotypes, removal of Zfh1 by RNAi from ovarian somatic cells (supplementary material Fig. S1) resulted in dissociation of ICs and germ cells in larval ovaries (Fig. 4G, 95% of ovaries, \(n=21\)), and a failure of GSC differentiation in adults. Approximately 70% of the ovaries tested (\(n=35\)) were filled with single cells carrying spherical fusomes (Fig. 4H).

We next analysed EC protrusions in stat-RNAi and zfh1-RNAi ovaries. EC extensions were labelled by anti-Coracle, whereas their nuclei were marked by anti-Tj. Extensions in wild-type germaria were easy to note (Fig. 4I). However, in stat-RNAi ovaries, EC extensions in region 1 of the germarium were either missing (Fig. 4J) or reduced (Fig. 4K). Extensions of ECs closest to the niche were sometimes observed (Fig. 4K, arrowhead), suggesting that these ECs may be less affected. zfh1-RNAi ovaries exhibited a similar lack of EC extensions (Fig. 4L). Our combined analyses show that Woc, Stat and Zfh1, the target gene of Stat, are all required for GSC differentiation and for soma-germline association.

**Woc is required for proper Zfh1 expression**

Considering the remarkable phenotypic similarity between zfh1, woc and stat, we addressed the relationship between Woc and the Stat pathway. Epistasis analysis showed that Woc did not regulate Stat levels, neither did Stat affect Woc expression (supplementary material Fig. S3). Furthermore, activation of Stat by expression of Upd or Hop\textsuperscript{tum-1} did not rescue the woc phenotype, suggesting that Woc should act in parallel or downstream of Stat activation (supplementary material Fig. S3). We then tested whether Woc might induce zfh1 expression in concert or in parallel to Stat. In early larval third-instar ovaries, Zfh1 protein was expressed in all somatic cell nuclei. Staining was stronger in nuclei that were in contact with germ cells (Fig. 5A, compare arrowheads with arrows, all ovaries, \(n=20\)). These cells likely become ICs during the late third instar. Interestingly, ICs not only express higher levels of Zfh1, but also show higher levels of Stat labelling than do non-IC cells from larval ovaries (Fig. 5C,C'). Significantly, increased Zfh1 levels in somatic cells that contact germ cells were not prominent in woc-RNAi ovaries (Fig. 5B, compare arrowheads with 5A, all ovaries, \(n=23\)), suggesting that Woc normally regulates this elevation.

To determine whether Woc controls Zfh1 levels in adult ECs, ovaries were co-stained with anti-Tj antibody to detect ECs, and with anti-Zfh1 antibody. Tj and Zfh1 staining colocalised (Fig. 5D, arrowheads), indicating that Zfh1 is expressed in ECs, but not in germ cells. In woc-RNAi ovaries, Tj levels remained normal (compare Fig. 5D,E, arrowheads), suggesting that protein expression in woc-mutant ECs was not generally reduced. In contrast, Zfh1 levels were significantly reduced (Fig. 5F, arrowheads), suggesting that Zfh1 expression is driven by Stat and Woc in ECs.
Woc is required for a Stat-mediated elevation of zfh1 expression

Removing both Stat and Woc from ECs did not result in a significant reduction in Zfh1 levels compared with removing woc alone (Fig. 5G, P = 0.09), suggesting that Woc and Stat act in concert to increase Zfh1 levels. To determine how Woc might control Zfh1 (Fig. 5F-G), similar results were obtained in woc-mutant cell clones [Fig. 5F, compare wild-type cell (arrow) with mutant cell (arrowhead)]. Quantification of Zfh1 in protein levels revealed an average decrease of 27%-38% in Zfh1 protein levels following woc reduction (Fig. 5G).

We therefore queried whether this Woc/Stat-induced mild elevation of Zfh1 expression following exposure to the Stat ligand Upd (Baeg et al., 2005). In control cells, Upd elicited a normal response associated with Stat signalling, as demonstrated by a ~6-fold elevation of Socs36E, a known target of the pathway (supplementary material Fig. S4) (Baeg et al., 2005; Karsten et al., 2002). zfh1 expression was elevated by ~25% upon addition of Upd (Fig. 5I). This modest elevation was statistically significant and resembled in magnitude the increase in either Zfh1 mRNA or protein levels observed in woc or stat mutant ECs (Fig. 5G,H).

When cells were exposed to RNAi directed against woc in a medium that did not contain Upd, zfh1 levels did not change compared with control, lacZ-RNAi cells (Fig. 5I). This confirms that Woc does not change Zfh1 levels in the resting state, independently of Stat activation. Significantly, when Upd was added to woc-RNAi cells, zfh1 expression remained at its uninduced level (Fig. 5I). Combined, these data strongly support the conclusion that the Stat-mediated increase in zfh1 transcription requires Woc activity.

Zfh1 is haplo-insufficient and a major Woc target in ECs

Our results thus far suggest that Stat-mediated upregulation of Zfh1 requires Woc, and that this upregulation determines soma-germline association and GSC/CB differentiation. However, removal of Woc or Stat results in only a mild reduction in Zfh1 expression (Fig. 5). We therefore queried whether this Woc/Stat-induced mild elevation in Zfh1 levels is functionally important. To test this, we examined germaria of heterozygous flies, in which one of three null zfh1 alleles was observed, when compared with the WT (Fig. 6A-D, supplementary material Table S2). The increase in single cells was correlated with reduced levels of Zfh1 protein (Fig. 6E-H′, supplementary material Table S3). Thus, Zfh1 function significantly reduced (compare Fig. 5D′ with 5E′, G). Similar results were obtained in woc-mutant cells carrying Stat activity could be induced upon ligand addition, and which had previously been shown to activate gene transcription in ECs. (A) Wild-type somatic cells in proximity to germ cells exhibit stronger Zfh1 labelling (compare arrowheads with arrows). (B) In woc-RNAi ovaries, Zfh1 levels in somatic nuclei abutting PGCs are not as high as in WT (compare arrowheads in A,B). (C,C′) Wild-type larval ovaries stained with anti-Stat (green in C, greyscale in C′). Higher Stat levels are present in ICs (PGCs are outlined in C). (D,E) Anti-Tj (green) and anti-Zfh1 (D′, E′, greyscale) co-stain EC nuclei. Zfh1 staining is reduced in woc-RNAi ECs (arrowheads E′, compare with D′). woc-RNAi sheath cells outside the outlined germinarium still express high Zfh1 levels. Tj levels are unaffected (compare D with E). (F) GFP (green) marks wild-type cells. In woc mutant cells (arrowhead), Zfh1 (magenta in F, greyscale in F′) staining is reduced compared with a neighbouring wild-type cell (arrow). The nuclei of the marked cells are at the same confocal plane and can therefore be compared. (G) Quantification of Zfh1 protein expression in woc- and stat-deficient cells. P-values of Student’s t-test and s.e.m. bars are indicated. (H) Real-time qPCR of zfh1 transcripts comparing bam mutant ovaries with bam mutants that were also woc deficient. Two different recombinant lines produced similar results in two independent experiments (shown combined). (I) Real-time qPCR of zfh1 transcripts comparing control cells (lacZ dsRNA) to woc dsRNA, exposed to control or Upd-containing media. Student’s t-test P-values of five independent experiments are shown. Scale bars: 10 µm (bar in A applies to A,B; bar in D applies to D-F).
in ECs is haplo-insufficient, and correct GSC differentiation requires Woc to ensure high levels of this protein.

To further test the importance of maintaining the correct levels of Zfh1 by Woc, we tested whether increased Zfh1 expression from a
UAS promoter would rescue the woc-RNAi phenotype. As a control, we also over-expressed a mutant form of Zfh1 (Zfh1*) that cannot
function as a repressor (Postigo and Dean, 1999). Overexpression of either of these proteins alone did not result in overt ovarian phenotypes, and germline cysts were produced normally (Fig. 7A,B). Significantly, expression of the WT Zfh1 in woc-RNAi ovaries resulted in a very strong phenotypic suppression; ~70% of ovarioles (n=72) contained a
normal complement of cysts (Fig. 7C, compare with Fig. 1C). Anti-
Coracle staining of somatic EC extensions revealed a similar restoration of this feature in the rescued ovarioles. Whereas
extensions were lost in woc-RNAi ovaries (compare Fig. 7D
with 7E), prominent extensions could readily be observed between
cysts in the rescued germaria (Fig. 7F). In line with normal cyst
development, egg chambers were observed in all rescued ovarioles, as
opposed to an almost complete lack of egg chambers in woc-RNAi
ovaries (Fig. 7G,H). By contrast, overexpression of Zfh1* could not
rescue woc-RNAi ovaries, which still contained many spherical
fusomes (Fig. 7I). This suggests that the repressor function is required
for Zfh1 activity in ECs. The Woc phenotype by Zfh1
suggests that this transcriptional repressor is a major effector of
Stat-mediated response in ECs and a major Woc target.

DISCUSSION
The balance between stem cell self-renewal and stem cell
differentiation must be strictly maintained to allow organ
homeostasis. We identify the chromatin-binding factor Woc as a
novel player in GSC differentiation. We further show that efficient
GSC differentiation requires high Zfh1 levels in somatic support cells.
Woc achieves this by assisting a Stat-mediated increase in zfh1
transcription, demonstrating that precise control of gene transcription is
required for correct stem cell differentiation. Stat signalling has been
recognised as a self-renewal signal in both male and female Drosophila
gonads. Our data demonstrate that Stat is also required for GSC
differentiation, and is therefore a cue that controls both maintenance and
differentiation (Fig. 7J).

Stat signalling within ECs is required for GSC differentiation
Stat signalling has long been recognised as a stem cell self-renewal
cue in both males and females (Brawley and Matunis, 2004; Decotto
and Spradling, 2005; Issigonis et al., 2009; Kiger et al., 2001;
Leatherman and Dinardo, 2008; Lopez-Onieva et al., 2008; Tulina
and Matunis, 2001; Wang et al., 2008). Here, we uncover a novel Stat
activity by showing that it is required for proper differentiation of the
GSC progeny. Stat is expressed in two distinct cell populations in the
germinarium: cap cells and ECs (Decotto and Spradling, 2005; Wang
et al., 2008). GSCs contact cap cells and the anterior-most ECs.
Clonal analysis defined Stat within cap cells as being required
for GSC maintenance by enhancing Dpp expression, which is
indispensable for GSC maintenance (Lopez-Onieva et al., 2008;
Wang et al., 2008). Some contribution to GSC maintenance may also
be provided by ECs that are located at the anterior and contact GSCs,
as they also produce Dpp (Decotto and Spradling, 2005; Lopez-
Onieva et al., 2008; Rojas-Rios et al., 2012; Wang et al., 2008).

In contrast to GSCs, their differentiating daughter cells contact
only ECs. Our data show that removal of Stat from ECs by either
RNAi or mutations results in a surplus of undifferentiated germ cells
(Fig. 4E,F; supplementary material Table S2). Thus, the function of
Stat in distinct cell populations – cap cells or ECs – determines GSC
self-renewal or differentiation, respectively. One possibility is that
the gene expression profile in cap cells and ECs following Stat
activation is different, and that these different targets direct GSC
maintenance or cyst differentiation. Otherwise, Stat response genes
within Cap and ECs may be similar, but the combination with other
cell type-specific signalling pathways will produce differential
responses in germ cells. One emerging common feature of Stat
signalling in all tested ovarian somatic cells is its requirement for
soma-germline adherence.

Soma-germline association and germ cell differentiation
Previous studies suggested that the primary role of Stat is to support
adhesion of stem cells to the niche, thereby promoting exposure of
stem cells to self-renewal cues that are produced by the niche
(Issigonis et al., 2009; Leatherman and Dinardo, 2010). Our work
extends this principle by showing that, in addition to stem-cell niche
adhesion, Stat, Zfh1 and Woc maintain the association of somatic
Physical association between ECs and germ cells is crucial for GSC differentiation (Jin et al., 2013; Kirilly et al., 2011; Schulz et al., 2002; Shields et al., 2014). Thus, loss of extensive physical contact with ECs per se could account for the downregulation phenotypes of \textit{stat}, \textit{woc} and \textit{zfh1}. It is interesting to note that the germ cell differentiation phenotype can be observed only when the entire population of ECs within the germarium is mutated, either by RNAi or by generating very large clones. The fact that few wild-type ECs could rescue GSC differentiation within an entire germarium suggests that EC extensions are motile and may contact GSC daughters that are not necessarily close to them (Morris and Spradling, 2011).

Recently, \textit{piwi} mutants have been shown to regulate both IC and EC association with germ cells (Jin et al., 2013). \textit{Piwi} has been shown to interact with Tj, with both sharing the IC dissociation phenotype (Li et al., 2003; Saito et al., 2009). We observed no change in Tj labelling in \textit{woc}-RNAi ovaries, suggesting that several pathways may regulate the association of ECs with GSC daughter cells.

**Requirement for Woc in Stat-mediated Zfh1 expression**

RT-qPCR and protein labelling of ECs in adult germaria show that Woc is required for an elevation in Zfh1 levels. Significantly, in larval ovaries the cells in contact with germ cells display high Stat and Zfh1 levels, whereas cells at the anterior of the ovary, which do not contact PGCs, contain lower Stat and Zfh1 levels. Reduction of Woc does not affect Zfh1 levels in anterior cells, but does reduce Zfh1 levels in cells that contact germ cells. This mirrors the tissue-culture experiments and suggests that the Woc-Stat-Zfh1 connection might be conserved in more than one cell type.

In \textit{stat}-mutant male cyst cells, Zfh1 protein is reduced by only about 25-35% (Leatherman and Dinardo, 2008). We show a similar reduction in both \textit{stats2E} and \textit{woc} mutant EC clones in females. Despite this mild effect on Zfh1 levels, cyst differentiation defects in \textit{woc}-RNAi ovaries are rescued by Zfh1 overexpression. This suggests that Zfh1 is a major target of Woc in ECs, and that correct levels of Zfh1 in these cells are of particular importance. Indeed, our data show a haplo-insufficiency of Zfh1 function in the germarium (Fig. 6, supplementary material Table S2). Interestingly, heterozygosity of Zfh1, a human homologue of the fly Zfh1, causes the Mowat-Wilson mental retardation syndrome in humans (Zweier et al., 2002). Thus, haplo-insufficiency of this protein in specific cells may be a feature of this transcriptional repressor. Further studies will be needed to determine whether Woc is required for increased expression of other haplo-insufficient genes.

Whereas promoting EC extensions through Zfh1 seems a major route of Woc function in ECs, the possibility of additional Woc/Stat-targets in ECs, which help differentiate GSCs, has not been ruled out. Supporting the hypothesis of additional target genes is the observed overproliferation of ICs in \textit{woc}-RNAi ovaries, which is induced by overactivation of Stat or overexpression of Woc (Figs 3, 4), but not by Zfh1 (not shown). To resolve this matter, identifying additional targets of Stat and a better understanding of how Zfh1 affects ECs must be achieved. In addition, Woc may have other main targets in other cell types. Supporting this notion is the fact that whereas Zfh1 could rescue \textit{woc}-mutant germaria, egg chamber development was still aberrant, suggesting a different target of Woc in follicle cells.

Woc is most closely related to the mammalian MYM-type (ZMYM: zinc finger, myeloproliferative and mental retardation motif) family of zinc-finger transcription factors. Aberrations in ZMYM2 (Znf198) and ZMYM3 (Znf261) proteins are associated with a myeloproliferative syndrome and with mental retardation, respectively (Smedley et al., 1999). Our findings that Woc controls ICs with PGCs in larval ovaries, and of ECs with GSC daughters following their departure from the niche, Stat signalling in larval ovaries and in the germarium appears to be required primarily for soma-germline association. At each stage of germ cell development, somatic cells that adhere to germ cells would provide these with different instructions. The permissive nature of the ovarian function of Stat can explain the seemingly opposing roles of Stat in GSC maintenance and differentiation.
Zfh1 expression downstream of Stat activation, and the similar haplo-insufficiency of Zfh1 in both systems, open new avenues for research into ZMYM2 and ZMYM3 function in mammalian development and human disease.

MATERIALS AND METHODS

Fly stocks

Stocks that were used in this study are listed in the supplementary material Table S4. Germline clones were generated using hs-flp.; FRT82B, nls-GFP. Somatic clones were generated using c587-Gal4, UAS-flp.; FRT82B, nls-GFP. The Minute technique (Newsome et al., 2000) was used to generate large somatic clones, mutant clones or wild-type (FRT82B) clones with c587-Gal4, UAS-flp.; FRT82B, nls-GFP, RpS3.

Antibody staining

Antibodies were used in the following concentrations: mouse monoclonal anti-Hts (1B1; 1:20) and anti-Concorde (1:200, catalogue no. C615.16) were from the Developmental Studies Hybridoma Bank (DSHB); rabbit anti-Vasa (1:5000) and anti-Zfh1 (1:5000) were a gift from Dr Ruth Lehmann (HHMI, New York University, USA); rabbit anti-Woc (1:2000) was a gift from Dr Maurizio Gatti (Università di Roma, Italy); rabbit anti-Stat92E (1:1000), which recognises the whole pool of Stat protein in the cell, was a gift from Dr Erika Bach (NYU School of Medicine, USA); guinea pig anti-Tj (1:7000) was from Dr Dorothea Godt (University of Toronto, Canada); rabbit anti-actin (1:5000) and anti-Zfh1 (1:5000) were a gift from Dr Ruth Lehmann (HHMI, New York University, USA); rabbit anti-Woc (1:2000) was a gift from Dr Dorothea Godt (University of Toronto, Canada); rabbit anti-pSMAD3 (1:100, catalogue no. A01122) was from Invitrogen. Secondary antibodies were from Jackson ImmunoResearch or from Invitrogen and used according to instructions. Young adult ovaries and late third-instar larval gonads were obtained as previously described (Maimon and Gilboa, 2011). Fixation and immunostaining were performed as described before (Gancz et al., 2011). Confocal imaging was performed with Zeiss LSM 710 on a Zeiss Observer Z1. Cell counts were carried out with the DeadEasy plug-in in ImageJ.

Quantification of Zfh1 staining intensity

Control and experimental animals were dissected and stained on the same day. Images were acquired on the same day, with the same acquisition parameters. For each gerarium, consecutive 1 μm z-sections were taken. The brightest section for each EC was measured with the measure tool in ImageJ software. A minimum of 18 cells from two to four independent experiments are shown.

Cell culture, transfection and RNAi

S2-NP cells were a gift from Dr Norbert Perrimon (Harvard Medical School, USA) and were maintained at 25°C in Drosophila Schneider’s medium (Biological Industries, Israel) containing 10% foetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco). dsRNA synthesis was carried out according to a Drosophila RNAi Screening Center (DRSC) protocol using Readymix (ABgene), MEGAscript T7 kit (Ambion) and RNAAeasy (Qiagen). Two different amplicons chosen from the DRSC database were tested for each gene to ensure a transcript-specific reduction; results from one amplicon are shown. Amplicon IDs DRSC15928 and DRSC36479 were used for woc, DRSC16870 and DRSC37655 for stat and DRSC24562 for lacZ.

Using a standard protocol, 6 μg of dsRNA against either woc or lacZ were applied to 0.4×10^6 cells in 12-well plates 72 h prior to a 2 h incubation with Upd-containing or control medium. To obtain Upd-containing medium, 0.6×10^6 cells were transfected with 54 ng act5-udp (a gift from Dr Norbert Perrimon) or act5-gal4 and pUASy (gifts from Dr Talila Volk, Weizmann Institute, Israel) as control, using Escort IV (Sigma) in a 1:1 ratio according to the manufacturer’s protocol. Medium containing Upd or control medium were collected 72 h later and added to RNAi-treated cells.

Reverse transcription quantitative PCR (RT-qPCR)

Approximately 40 ovaries were collected from very young females (a few hours to 2 days old) and RNA was purified either using Tri-Reagent (MRC) followed by a DNase treatment or with the RNaseq kit (Qiagen) for harvested cells. Reverse transcription was performed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR (qPCR) used SYBR Green (Invitrogen) with the following primers (forward and reverse): CCACCAGGAAGTCTCT and GCAGTCGAAGTCTGGTA for Socs36E; GAGCCACGTGACGTTCAGT and GTACATCTTCCCCAGGTCGAT for stat92E; CAAAGCTTCCGTTCTTCAA and GAACATGCAGCAGGT for woc; GCCGCAGGTTCTGAGT and CATTGACCCGATGCTGTGAGT for zfh1; CTCAAGTGGTTATATGTGGCA and AGACGACACACGCTGTA for bgcn (all from Sigma-Aldrich). Per reaction, 40 ng cDNA were used for qPCR, performed in triplicates in Applied Biosystems StepOne, analysed by DDCT and normalised to RpS17.

Statistical analyses

Experiments were repeated at least three independent times. For statistical analyses, two-tailed Student’s t-tests were performed. P-values are reported and s.d. or s.e.m. bars, as indicated, are shown.

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Competing interests

The authors declare no competing financial interests.

Author contributions

I.M. designed and performed most experiments, carried out data analysis and handled the manuscript. M.P. conducted RT-PCR experiments. L.G. designed the experiments and handled the manuscript.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.109611/-/DC1

References


