Submission date: 9-Oct-07

Jak/Stat signalling in niche support cells regulates *dpp* transcription to control germline stem cell maintenance in the *Drosophila* ovary

Lourdes López-Onieva[1], Ana Fernández-Miñán[1, 2] and Acaimo González-Reyes[1, *]

[1]Centro Andaluz de Biología del Desarrollo (CABD),
CSIC-Universidad Pablo de Olavide,
Carretera de Utrera km 1,
41013 Sevilla,
Spain

[2]Current address: European Molecular Biology Laboratory, Meyerhofstrasse 1, Heidelberg 69117, Germany.

[*]Correspondence to: AG-R (e-mail: agonrey@upo.es; phone: +34-954 348672; fax: +34-954 349376)

Running title: J/S regulates germline stem cells via *dpp*.
Key words: Jak/Stat; germline stem cells; niche signalling; BMP; *Drosophila* oogenesis.

SUMMARY

The existence of specialised regulatory microenvironments or niches that sustain stable stem cell populations is well documented in many tissues. However, the specific mechanisms by which niche support (or stromal) cells govern stem cell maintenance remain largely unknown. Here we demonstrate that removal of the Jak/Stat pathway in support cells of the *Drosophila* ovarian niche leads to germline stem cell loss by differentiation. Conversely, ectopic Jak/Stat activation in support cells induces stem cell tumours, implying the presence of a signal relay between the stromal compartment and the stem cell population. We further show that ectopic Jak/Stat signalling in support cells augments *dpp* mRNA levels and increases the range of Dpp signalling, a BMP2 orthologue known to act as a niche extrinsic factor required for female germline stem cell survival and division. Our results provide strong evidence for a model in which Jak/Stat signalling in somatic, support cells regulates *dpp* transcription to define niche size and to maintain the adjacent germline stem cells in an undifferentiated state.

INTRODUCTION

The generation, maintenance and repair of adult tissues and organs rely on populations of stem cells. In order to ensure an appropriate production of tissue cells during embryogenesis and adulthood, stem cells possess the ability to divide symmetrically or to undergo asymmetric divisions to self-renew and to produce differentiating progeny. The balance between stem cell proliferation and differentiation is brought about by regulatory microenvironments termed 'niches' in which a specialised cellular context provides signals and physical support to maintain stem cells. Thus, support (or stromal) cells of the niche play pivotal roles in organogenesis, in tissue homeostasis and repair, and in stem cell behaviour (Fuchs et al., 2004; Scadden, 2006; Spradling et al., 2001).

Germline Stem Cells (GSCs) are broadly conserved across animal species. Although the normal development of this type of stem cells is in some respects limited, as they normally give rise only to sexual gametes and accessory cells, they show a series of characteristics that make GSCs an important source of information useful for understanding stem cell behaviour (Wong et al., 2005). For instance, differentiating *Drosophila* germline cells have been shown to de-differentiate and to adopt a stem-cell fate under certain experimental conditions, thus opening the possibility to find new sources of progenitor cells for tissue repair (Brawley and Matunis, 2004; de Rooij and Russell, 2000; Kai and Spradling, 2004). Similarly, a number of niches hosting GSCs have been defined in several experimental systems such as mice, flies or worms. The *Drosophila* germline has emerged as one of the best

experimental systems in which to study the biology of stem cells and their niches. We have focused our investigations on the ovarian niche and on the behaviour of the GSCs contained within it. Ovarian GSCs are located in the anterior tip of the germarium, a structure composed of germline cells — including GSCs, and differentiating cystoblasts and cystocytes — and a few somatic cell types, namely Terminal Filament Cells (TFCs), Cap Cells (CpCs), Escort Stem Cells (ESCs) and Escort Cells (ECs). These somatic cells have been shown to provide physical support and signals to the GSC population (Decotto and Spradling, 2005; Xie and Spradling, 2000).

Communication between support cells and stem cells is crucial to control ovarian niche formation and to avoid depletion of stem cells. *decapentaplegic (dpp), glass bottom boat (gbb), fs(1)Yb, piwi* and *hedgehog* are known to be expressed in somatic support cells and to control GSC numbers (Cox et al., 1998; Cox et al., 2000; King and Lin, 1999; King et al., 2001; Song et al., 2004; Xie and Spradling, 1998). Although it is well established that the activity of the two BMP-like molecules Dpp and Gbb is required for GSC maintenance by directly repressing transcription of the differentiation-promoting gene *bag of marbles (bam)* and by modulating the activity of the putative regulator of translation Pelota (Chen and McKearin, 2003; Song et al., 2004; Szakmary et al., 2005; Xi et al., 2005; Xie and Spradling, 1998), the mechanisms that ensure appropriate BMP signalling in the GSC niche remain unknown. In this work, we identify a signalling pathway that modulates BMP signalling in the niche. The evolutionary conserved Janus kinase / Signal transducer and activator of transcription (Jak / Stat) signalling pathway has been identified as a key regulator of

the *Drosophila* germline niches (Decotto and Spradling, 2005; Kiger et al., 2001; Tulina and Matunis, 2001). Here we show that this pathway acts upstream of *dpp* transcription in ovarian support cells to ensure the maintenance of the adjacent GSC population.

MATERIALS AND METHODS

Staining procedures and microscopy

Immunohistochemistry was performed at room temperature using standard procedures (detailed protocols are available upon request). Primary antibodies were used at the following concentrations: mouse anti-Hts, 1/50 (Developmental Studies Hybridoma Bank (DSHB), University of Iowa); rabbit anti-Upd (Harrison et al., 1998), 1/250 ; rabbit anti β-Galactosidase (Cappel™), 1/10,000; rabbit anti-GFP (Molecular Probes™), 1/500; rabbit anti-PhosphoMad (a gift from Ginés Morata), 1/500. Secondary antibodies Cy2 and Cy3 (Jackson ImmunoResearch Laboratories, Inc.) were used at 1/200. DNA staining was performed using the DNA dyes TOPRO-3 (Molecular Probes™) at 1/1,000 or Hoechst (Sigma) at 1/1,000. Images were captured with a Leica TCS-SP2 confocal microscope and processed with Adobe Photoshop.

Fly Stocks

Flies were raised on standard *Drosophila* media at 25 ^{o}C unless indicated. *Stat92E*^{06346}, *dome*^{G0468}, *hop*^{27}, *hop*^{25}, *hop*^{2} and *upd*^{YM55} (also known as *os*$^{upd-4}$; although FlyBase refer to

the *upd* gene as *os* we have decided to keep the old name to avoid confusion as virtually all the published literature on this gene uses *upd*) have been described elsewhere (Binari and Perrimon, 1994; Brown et al., 2001; Hou et al., 1996; Perrimon and Mahowald, 1986; Wieschaus et al., 1984). To express UASt-*DsRed* (Bloomington Stock Center) or UASt-*dome*$^{\Delta CYT}$ (Brown et al., 2001) in somatic cells we used the *bab1*-Gal4 driver (Bolívar et al., 2006). In order to obtain adult females overexpressing *upd2* (Hombria et al., 2005) or *hop*^{Tum} (Harrison et al., 1995) under the control of *bab1*-Ga4, we crossed *w; tub*-Gal80^{ts}/CyO; *bab1*-Gal4/TM2 with *yw;* UASt-*upd2* or *yw*; UASt-*hop*^{Tum}, respectively. The offspring were grown at 18 ^{o}C and, upon eclosion, adult F1 flies were shifted to 31 ^{o}C for 4 days.

Generation of Somatic and Germline Clones

Germline mutant clones were generated using the FLP/FRT technique. The following chromosomes were used: *y w* hs-*flp122*, FRT82B *Stat92E*^{06346}, FRT19A *dome*^{G0468} and FRT101 *hop*^{2}. 72-96 hour-old larvae were heat shocked for 1 hour at 37 ^{o}C; adult offspring were transferred to fresh food and kept at 25 ^{o}C until dissection at the appropriate time. To generate somatic mutant clones we used the following chromosomes: FRT101 *hop*^{2}and FRT101 *ubi*-GFP; *bab1*-Gal4 UASt-*flp.*

Reverse Transcriptase (RT)-PCR

mRNA was isolated from ~100 ovary pairs from *yw* virgin females or from 12-24 hour-old *yw* embryos and purified with the QuickPrep micro mRNA Purification Kit (Amershan Biosciences) according to the manufacter´s instructions. 1-2 µg of mRNA were used as a template for first strand cDNA synthesis together with 0.5 µg of oligo dT (Sigma Genosis) and the SuperscriptII RNase H Transcriptase (Invitrogen Lifetechnology) in a final volume of 20µl. 2 µl of the ovarian or embryonic cDNA libraries served as templates for the subsequent RT-PCR amplification. The following primers were used for cDNA detection:

ftz: sense, CGAGGAGACTTTGGCATCAGATTG; antisense, TGACTGTGACTGTGGCTGTAAGCG.

hh: sense, CAACAGGGACATCCTTTTTCCG; antisense, TGCCGTATTTGGACTGGTCG.

upd: sense, TTCTGGCTCCTCTGCTGCTTCT; antisense, TACCGCAGCCTAAACAGTAGC.

upd2: sense, AGCGCCAGCCAAGGACGAGTTATC; antisense, TTGGCTGGCGTGTGAAAGTTGAGA.

upd3: sense, ATGTCCCAGTTTGCCCTCTC; antisense, CTAGAGTTTCTTCTGGATCGCC.

Real Time PCR

~200 ovary pairs of the genotype *tub*-Gal80^{ts}/+; *bab1*-Gal4/+ or ~400 ovary pairs from *tub*-Gal80^{ts}/UASt-*upd2*; *bab1*-Gal4/+ females were used for *mRNA* isolation and cDNA synthesis following the above protocol (both types of females were grown at 18 ^{o}C and, upon eclosion, shifted to 31 ^{o}C for 4 days). 100 ng/reaction of the different cDNA libraries were used as a template for the subsequent Real Time PCR reactions. The relative quantification of *dpp* and *gbb* expression was carried out using the Comparative C_T Method (Separate Tubes; (Applied-Biosystems, 1997)) and TaqMan MGB probes, Fam dye-labeled. Primers and TaqMan Probes for the different cDNAs were obtained from the Assays-by-Design Service (Applied Biosystems) and were the following:

dpp: sense, GCCAACACAGTGCGAAGTTTTA; antisense, TGGTGCGGAAATCGATCGT; probe, CACACAAAGATAGTAAAATC.

gbb: sense, CGCTGTCCTCGGTGAACA; antisense, CGGTCACGTTGAGCTCCAA; probe, CCAGCCCACGTAGTCC.

RNA polymerase II: sense, ACTGAAATCATGATGTACGACAACGA; antisense, TGAGAGATCTCCTCGGCATTCT; probe, TCCTCGTACAGTTCTTCC.

RNA polymerase II was used as the endogenous control. Primer pairs were validated representing the Cycle Threshold (C_T) mean value of three replicates at increasing cDNA concentrations. The absolute value of the slope of log input amount

vs ΔC_T was < 0.1. Real Time-PCR was performed on an ABI-PRISM 7700 Sequence Detection System machine. Quantified mRNA levels were expressed as relative fold change normalized against *RNA polymerase II*. The comparative C_T method (Applied-Biosystems, 1997) was used to analyze the data by generating relative values of the amount of target cDNA. Relative quantification for any given gene, expressed as fold variation over control, was calculated from the determination of the difference between the C_T of the given gene (*dpp* or *gbb*) and that of the calibrator gene (*RNA polymerase II*). C_T values used were the result of three different replicas from three independent experiments.

RESULTS

Jak/Stat signalling in somatic niche cells in the germarium

Genetic studies in *Drosophila* have identified three cytokine-like ligands (Upd, Upd2 and Upd3), one transmembrane receptor (Dome), one kinase (Hop) and one transcription factor (Stat92E) as the positive transducers of Jak/Stat signalling (Agaisse et al., 2003; Binari and Perrimon, 1994; Brown et al., 2001; Chen et al., 2002; Gilbert et al., 2005; Harrison et al., 1998; Hombria et al., 2005). In order to assess the role of the Jak/Stat pathway in the ovarian niche we analysed the expression of several of its components in the germarium (Fig. 1). We made use of an antibody that recognises the Upd ligand (Harrison et al., 1998) and found that this protein strongly accumulates in TFCs and CpCs (Fig. 1B). Second, utilising a *Stat92E-lacZ* line (the bacterial *lacZ* gene inserted in the *Stat92E* gene) we observed β-Gal expression in all

of the somatic cell types present in the anterior half of the germarium: TFCs, CpCs, ESCs and ECs (Fig. 1C; Suppl. Fig. 1). In addition, the fact that the Jak/Stat pathway reporters 2xStat92E-GFP and 10xStat92E-GFP — consisting of tandem repeats of the Stat92E binding sequence upstream of GFP (Bach et al., 2007) — are expressed in CpCs indicates that the pathway is at least active in these somatic cells (data not shown). Next, we isolated ovarian mRNA from virgin females and performed RT-PCR analysis to confirm that the three known ligands of the pathway (*upd*, *upd2* and *upd3*) are expressed in wild-type ovaries (Fig. 1D).

It has been reported that overexpression of the Upd ligand in ESCs and ECs using the *c587*-Gal4 line leads to disorganised germaria and to rare ovarioles (3.5%) filled with GSC-like cells (Decotto and Spradling, 2005; Kai and Spradling, 2003). Considering the importance of CpCs for niche function (Song et al., 2007), we wished to study the effect of Upd or Upd2 ectopic expression using the *bab1*-Gal4 line, which induces strong expression of reporter genes in TFCs and CpCs and weaker levels in ESCs and ECs (Fig. 1E) (Bolívar et al., 2006). While the overexpression of Upd produced a mild increase in the number of GSC-like cells in experimental germaria (not shown), *bab1*-Gal4-driven expression of UASt-*upd2* gave a very consistent phenotype, as it caused hyperplastic stem cell growth in all of the ovarioles examined (n>100; Fig. 1F). In these germaria we never observed the gross organisational defects reported after *c587*-Gal4-driven Upd expression (Decotto and Spradling, 2005). Because it has been established that Gal4-mediated Upd2 overexpression results in ectopic activation of the pathway (Hombria et al., 2005), the above result demonstrates that strong Jak/Stat pathway overactivation in TFCs and CpCs, and at

lower levels in ESCs and ECs, is sufficient to increase greatly the number of GSCs present in the niche.

Jak/Stat signalling is required in the ovary for germline stem cell maintenance

It has been previously shown that the ovarian niche requires Jak/Stat signalling (Decotto and Spradling, 2005). Using viable, hypomorphic conditions of the pathway (*hop*^{25}/*hop*^{27} and *hop*^{25} *upd*^{YM55}/*hop*^{27}, analysed 2, 10 and 25 days After Eclosion (AE); Suppl. Table) we were able to confirm that the Jak/Stat pathway is required in the ovary for GSC maintenance and cyst production (Fig. 2A, B, E). In contrast to the wild-type controls, which show on average 2.57±0.5 GSCs per germarium 25 days AE (n=58), the average number of GSCs in germaria of the strongest mutant combination (*hop*^{25} *upd*^{YM55}/*hop*^{27}; n=33) dropped to just 0.9±0.8 25 days AE. Furthermore, ~25% of these mutant ovarioles were devoid of germline cells (not shown).

The morphology of the spectrosome has previously been used as a marker to assess GSC division (de Cuevas and Spradling, 1998). Early interphase spectrosomes display a characteristic 'exclamation mark' figure (Fig. 2C) in which the nascent cystoblast spectrosome on the basal side of the cytokinetic ring remains temporally linked to the apically-anchored GSC spectrosome material via the cytokinetic neck. This study also showed that GSC cytokinesis only occurs several hours later, after S-phase of the following cycle is completed in both the GSC daughter and its sister cystoblast (de Cuevas and Spradling, 1998). Surprisingly, during our analysis of Jak/Stat hypomorphic mutant germaria we found that a large proportion of GSCs undergoing cytokinesis exhibited a strikingly different spectrosome arrangement. In

these cases most of the GSC spectrosome loses its apical localisation and comes to lie next to the cytokinetic ring, adopting — together with the future cystoblast's spectrosome — a 'dumbbell-shape'. In addition, a small 'scar' of spectrosome material was frequently observed on the apical side of the GSC in contact with the CpCs, perhaps labelling the original, apical anchoring point of the interphase GSC spectrosome (Fig. 2D). GSC divisions harbouring this spectrosomal organisation are hereafter referred to as 'anchorless', the frequency of which depends on the severity of the mutant condition and on the age of the female (Suppl. Table). In fact, nearly 75% of GSCs of the strongest mutant combination analysed 25 days AE show 'anchorless' spectrosomes (Fig. 2D, F). A detailed study of control ovaries indicated that 'anchorless' figures are also found in wild-type niches. We observed that until 10 days AE a small percentage (11-13%) of control GSCs show 'anchorless' figures. However, there is a noticeable increase in the frequency of 'anchorless' figures 25 days AE (23.5%), as GSCs age (Fig. 2F).

The increased frequency of GSCs containing 'anchorless' spectrosomes in ageing wild-type niches and in Jak/Stat mutant niches raises the question of the significance for GSC niche function of the occurrence of 'anchorless' spectrosomes. Wild-type GSCs are known to be lost from the niche as flies age (Xie and Spradling, 1998; Xie and Spradling, 2000). Similarly, we have shown that the average number of Jak/Stat mutant GSCs per germaria is greatly reduced in comparison to the controls, a phenotype that worsens with time (Fig. 2E; Suppl. Table). Thus, there is a correlation between the rise in the frequency of 'anchorless' figures in wild-type niches and in Jak/Stat mutant niches and the occurrence of GSC loss. Hence, it is

possible that the high frequency of 'anchorless' spectrosome arrangement is generally related to stem cell loss. Both the experimental evidence provided below and the observation by Decotto and Spradling that the spectrosomes of the remaining GSCs present in mutant *Stat92E* germaria move away from cap cells, further support this possibility (Decotto and Spradling, 2005).

Jak/Stat signalling is required in the somatic cap cells.

Next we set out to dissect the requirement for the Jak/Stat pathway in the different compartments of the GSC niche. To this end, we removed the activity of the pathway in GSCs by making *hop*^{2}, *Stat92E*^{06346} or *dome*^{G0468} germline clones (n>70 for each of the genotypes). We analysed mosaic germaria 2, 10 and 25 days AE and observed that the removal of any of the above components of the pathway had no detectable effects on germ line development or on GSC maintenance (Fig. 3A). This observation is in agreement with recent results (Decotto and Spradling, 2005), confirming that the Jak/Stat pathway is not required in the germ line. Furthermore, it indicates that the reduction in the number of GSCs in Jak/Stat mutant ovaries is probably a consequence of the activity of the pathway in the somatic cells of the niche.

We took two experimental approaches to test the above hypothesis. First, we ectopically expressed a dominant negative form of the receptor Dome (Dome$^{\Delta CYT}$) (Brown et al., 2001) in the somatic cells of the niche and analysed its effect(s) on GSC behaviour. Experimental females grown at 25^{o}C for 25 days AE showed a small but significant decrease in the number of GSCs per germarium (control=2.61±0.62 GSCs/germarium, n=61; *bab1*-Gal4/UASt-*dome*$^{\Delta CYT}$=2.21±0.74 GSCs/germarium,

n=39; $p<0.05$). This reduction in the number of GSCs populating the ovarian niche was accompanied by an increase in the frequency of 'anchorless' figures 25 days AE, which rose from ~24% in controls to ~46.50% in experimental females. In fact, we observed germaria where all of the GSCs contained 'anchorless' spectrosomes, a phenotype never encountered in wild-type niches (Fig. 3B). Second, we generated CpCs mutant for a strong loss-of-function allele of the *hop* gene, hop^{2}. To this end, we utilised a *bab1*-Gal4 UAS-*flp* chromosome to manipulate genetically the support cells of the GSC niche (Bolívar et al., 2006). Wild-type CpCs adopt a rosette-like arrangement at the base of the terminal filament and come to lie in close contact with the underlying GSCs (reviewed in (González-Reyes, 2003; Spradling et al., 1997). The analysis of hop^{-} clones revealed that the activity of the Jak/Stat pathway in CpCs is essential to prevent GSC differentiation. Where wild-type GSCs abut both $hop^{-/-}$ and $hop^{+/-}$ CpCs they appear to be retained normally in the niche, as judged from possession of a normal-looking spectrosome 14 days AE (Fig. 3C). However, GSCs that made contact exclusively with $hop^{-/-}$ CpCs display characteristics of differentiating germline cells, as shown by the frequent appearance of 'anchorless' GSCs and by the development of cysts directly abutting mutant CpCs 14 days AE (55% of cases; n=17; Fig. 3D, E). It is interesting to note that the presence of *hop*-deficient CpCs did not affect the overall structure of the anterior germarium, as it is the case for $Stat92E^{-}$ ESCs (Decotto and Spradling, 2005). Finally, we assessed whether the removal of Jak/Stat signalling during gonadal development affects normal CpC specification. We analysed the pattern of expression of two CpC markers, the transcription factor Engrailed and nuclear Lamin-C, in *bab1*-Gal4-induced hop^{-} CpC clones (Forbes et al.,

1996; Xie and Spradling, 2000) (Fig. 4). We found that the loss of *hop* activity did not affect the expression of either of these markers, strongly suggesting that GSC differentiation induced by the loss of *hop* from CpCs is not due to a failure of normal CpC development.

Jak/Stat activity regulates *dpp* transcription and signalling.

The above results demonstrate that somatic Jak/Stat signalling has a specific effect on GSC maintenance and they strongly suggest that a signal is transmitted from the CpCs to the germline. To prove that this is the case, we expressed a constitutively active form of the Janus kinase, hop^{Tum} (Luo et al., 1995), in support cells using the *bab1*-Gal4 driver. As shown in Fig. 5A, overexpression of Hop^{Tum} in support cells blocks cyst differentiation and induces ectopic GSCs. Since this gain-of-function form of Jak activates the pathway in support cells in a cell-autonomous manner, the effect observed on the germline demonstrates the existence of a signal relayed from the support cells to the GSCs that is regulated by Jak/Stat. In an attempt to determine the nature of this signal, we examined whether the Jak/Stat pathway was regulating the transcription of the vertebrate Bone Morphogenetic Protein-2 (BMP2) orthologue *dpp*. This gene has been shown to encode an extrinsic signal required to prevent GSC differentiation in the germarial niche (Casanueva and Ferguson, 2004; Kai and Spradling, 2003; Song et al., 2004; Xie and Spradling, 1998; Xie and Spradling, 2000).

We analysed the level of *dpp* transcription after ectopic induction of the Jak/Stat pathway utilising the *bab1*-Gal4 driver. In our hands, the detection of *dpp* mRNA by *in situ* hybridisation on germaria did not yield consistent results. Thus, we

resorted to performing Real Time-PCR on control ovaries and on experimental ovaries that had overexpressed the Upd2 ligand for 4 days, using primers that recognise all the predicted spliced variants of *dpp* transcripts. We found that the ectopic activation of Jak / Stat in support cells led to a >3-fold increase in the levels of *dpp* mRNA, strongly suggesting that *dpp* transcription in support cells is controlled by the activity of the Jak / Stat pathway (Fig. 5B). In addition to *dpp*, another BMP-like ligand, Gbb, is also expressed in somatic cells of the germarium and is required for GSC maintenance (Song et al., 2004). However, in contrast to *dpp*, we found that the ectopic activation of Jak / Stat signalling did not significantly affect *gbb* transcription, suggesting that it is probably not a downstream target of the Jak / Stat pathway (Fig. 5B). Importantly, because both *dpp* and *gbb* are expressed in support cells (Song et al., 2004), the fact that *gbb* mRNA levels are not increased upon Upd2 overexpression strongly suggests that the effect of Jak / Stat ectopic activation on *dpp* expression is not due to an increase in support cell numbers but to transcriptional control. Finally, we wished to determine if the ectopic GSC-like cells produced after *bab1*-Gal4-driven activation of Jak / Stat were transducing the *dpp* signal. We utilised the presence of phosphorylated Mad (pMad) as a reporter of an active *dpp* pathway (Tanimoto et al., 2000). In wild-type germaria, pMad is found at high levels only in GSCs (Fig. 5C) (Kai and Spradling, 2003). In contrast, the ectopic spectrosome-containing cells in Upd2-induced tumorous germaria display strong pMad staining, even those located many cell diameters away from the CpCs (Fig. 5D). Taken together, our results strongly suggest that ectopic activation of the Jak / Stat pathway in support cells up-regulates *dpp* expression in these cells, which consequently enlarges the GSC niche as

witnessed by the expansion of Dpp signalling in the germ line. Moreover, our results suggest that, even though Dpp (or BMP) pathway activation is necessary and sufficient to prevent GSC differentiation, Jak/Stat signalling impinges on *dpp* in support cells to control the ovarian GSC niche.

DISCUSSION

The creation of unique 'permissive zones' by support cells is a general principle of stem cell niches. Niches are dynamic systems where several signalling pathways are often integrated in order to coordinate different cell types and to respond to changing physiological conditions (Scadden, 2006). In this work, we have used the *Drosophila* ovarian germline niche to establish that Jak/Stat signalling in support cells regulates the production of the growth factor Dpp, an extrinsic signal transmitted from support cells and required for GSC division and perpetuation (Xie and Spradling, 1998; Xie and Spradling, 2000).

Two well-characterised extrinsic factors acting in the ovarian GSC niche are the BMP-like proteins Dpp and Gbb, known to block germline stem cell differentiation by repressing the transcription of the *bag of marbles* (*bam*) gene (Chen and McKearin, 2005; Song et al., 2004; Szakmary et al., 2005). In addition to its effect on *bam* expression, BMP signalling in the germline controls GSC maintenance through the activity of *pelota*, a putative regulator of translation that controls GSC self-renewal by repressing a Bam-independent differentiation pathway (Xi et al., 2005). The short-range signalling by Dpp and Gbb is restricted to GSCs and to, albeit at lower levels, cystoblasts (Kai and Spradling, 2003). In absence of Dpp or Gbb signalling, Bam is

expressed in GSCs and Pelo's repressor activity is probably reduced. As a result, GSCs differentiate and the niche is emptied (Song et al., 2004; Xi et al., 2005; Xie and Spradling, 1998). There are however clear differences between the roles of Dpp and Gbb in the female GSC niche. Dpp overexpression prevents stem cell differentiation and induces the formation of large tumours of GSC-like cells, partially by de-differentiating 'committed' cystocytes and partially by inducing GSC division (Kai and Spradling, 2004; Xie and Spradling, 1998). Thus, the reception of Dpp in the germ line is not only necessary to keep a stable population of GSCs, but also sufficient to specify stem cell fate. This conclusion points towards Dpp as the limiting factor that controls female GSC niche size and function. In contrast, Gbb is necessary but not sufficient to prevent female GSC differentiation (Song et al., 2004). In this work we have demonstrated that the Jak/Stat pathway is required in support cells to preserve GSCs, most probably by regulating *dpp* (but not *gbb*) transcription and by determining the extent of BMP pathway activation in the germ line. Therefore, considering the significance of Dpp in the proper functioning of the GSC niche, our results strongly suggest that the activity of the Jak/Stat pathway defines the GSC niche in the female ovary. Interestingly, Jak/Stat signalling in the *Drosophila* testis constitutes another extrinsic factor essential for GSCs to retain self-renewing potential, even though in this case the transduction of the pathway is required cell-autonomously in the germ line (Kiger et al., 2001; Tulina and Matunis, 2001). It would then appear that the same signalling pathway defines both male and female GSC niches. This conclusion correlates with previous results suggesting that male and female germline niches are governed by common signals (Decotto and Spradling,

2005; Gilboa and Lehmann, 2004).

The mechanism(s) by which Jak/Stat signalling modulates *dpp* transcription remain to be elucidated. Sequence analysis shows the existence of several consensus binding sites for the Stat transcription factor in the *dpp* gene (data not shown; (Bach et al., 2003), but their functionality has not been tested. Alternatively or in addition, the control of *dpp* mRNA levels by Jak/Stat may be indirect. Whatever the situation, it is unlikely that the increase in *dpp* mRNA after ectopic Jak/Stat signalling is an unspecific effect due to the global disruption of heterochromatic gene silencing that occurs in *Drosophila* larvae and adults upon Jak over-activation (Shi et al., 2006). First, *gbb* transcription is not affected in the same experimental conditions that cause an increase in *dpp* mRNA levels. Second, it has been suggested that *dpp* may function downstream of, or in parallel to, Jak/Stat signalling in *Drosophila* testes (Singh et al., 2006). Finally, the ectopic expression of Upd in eye discs results in a slight enhancement of *dpp* mRNA levels (Bach et al., 2003). Altogether these and our observations strongly suggest that Jak/Stat activation in support cells specifically regulates *dpp* transcription.

Our analysis of GSC spectrosomes has revealed a new organisation of the spectrosome that may constitute a useful tool to analyse niche function. 'Anchorless' figures are formed during post-mitotic (early interphase) stages and are observed in a low percentage of wild-type GSCs, suggesting that either this organisation of the spectrosome is very dynamic and lasts for a short period of time in GSCs undergoing cytokinesis, or that only a few of the GSCs present in an ovary develop it. In any case, because a significant increase in the frequency of these figures is associated with

stem cell loss when Jak / Stat signalling is impaired, the rise in the frequency of 'anchorless' spectrosomes may reflect the existence of defective niche signalling. We propose that Jak / Stat pathway activation in support cells prevents premature GSC loss by regulating the production of the relay signal Dpp. Thus, mutant niches may not achieve the right balance of survival factors, including Dpp, as to maintain a wild-type population of GSCs during the female's lifetime.

Given the importance of BMP signalling to avoid depletion of GSCs and to control their proliferation, the production of BMP ligands ought to be tightly regulated. In this context, cap cells and escort stem cells seem to act as a signalling centre where several signalling pathways might be integrated. In addition to *dpp* and *gbb*, other extrinsic factors with defined roles in the control of populations of GSCs and / or follicle stem cells such as *fs(1)Yb, piwi, wingless* and *hedgehog* are known to be expressed in cap cells (Cox et al., 1998; Cox et al., 2000; King and Lin, 1999; King et al., 2001; Song et al., 2004; Song and Xie, 2003; Xie and Spradling, 1998; Zhang and Kalderon, 2001). Our observations add the transduction of the Jak / Stat signal(s) to the complex network of signalling pathways that co-exist in the cap cells. Similarly, the Jak / Stat pathway is required in escort stem cells to maintain GSCs (Decotto and Spradling, 2005). Altogether, these evidence emphasise the contribution of support cells in direct contact with GSCs (cap cells and escort stem cells) in the determination of GSC niche size and function (Song et al., 2007). The signals that regulate Jak / Stat pathway activation in the niche are at present unknown, but clear candidates are any of the signalling molecules present in cap cells. In this regard, it is interesting to note that the expression of *piwi* and *hedgehog* in these cells is controlled by *fs(1)Yb* (King et

al., 2001). In addition, systemic signals such as the neural-derived insulin-like peptides, utilised in the ovary to sense nutritional input and to impinge on GSC niche activity to coordinate nutrient availability with egg production (LaFever and Drummond-Barbosa, 2005), may play a role. Deciphering the mechanism(s) that modulate Jak/Stat activity in ovarian support cells or determining the generality of Jak/Stat regulation of BMP signalling in other well-established niches are interesting questions that await further investigation.

ACKNOWLEDGMENTS

We thank J. C. Hombría, S. Noselli, T. Schupbach, A. Spradling, the Developmental Studies Hybridoma Bank (University of Iowa) and the Bloomington Stock Centre for fly stocks and reagents. F. Casares, M. D. Martín-Bermudo, J. Pearson and P. Rojas-Ríos provided useful comments on the manuscript. Research in our laboratory is funded by the Spanish Ministerio de Educación y Ciencia (BMC2003-01512 and BFU2006-10934) and by the Junta de Andalucía (CVI-280 and P06-CVI-01592). L. L-O and A. F-M were supported by FPI studentships from the Spanish Ministerio de Ciencia y Tecnología and by an I3P-CSIC contract (to A. F-M). The institutional support from the Junta de Andalucía to the CABD is acknowledged.

REFERENCES

Agaisse, H., Petersen, U., Boutros, M., Mathey-Prevot, B. and Perrimon, N. (2003). Signaling Role of Hemocytes in Drosophila JAK/STAT-Dependent Response to Septic Injury. *Developmental Cell* **5**, 441-450.

Applied-Biosystems. (1997). User Bulletin no. 2, ABI Prism 7700 Sequence Detection Systems.

Bach, E. A., Ekas, L. A., Ayala-Camargo, A., Flaherty, M. S., Lee, H., Perrimon, N. and Baeg, G. H. (2007). GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. *Gene Expr Patterns* **7**, 323-31.

Bach, E. A., Vincent, S., Zeidler, M. P. and Perrimon, N. (2003). A sensitized genetic screen to identify novel regulators and components of the Drosophila janus kinase/signal transducer and activator of transcription pathway. *Genetics* **165**, 1149-66.

Binari, R. and Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes Dev* **8**, 300-12.

Bolívar, J., Pearson, J., López-Onieva, L. and González-Reyes, A. (2006). Genetic dissection of a stem cell niche: the case of the *Drosophila* ovary. *Dev Dyn* **235**, 2969-79.

Brawley, C. and Matunis, E. (2004). Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. *Science* **304**, 1331-1334.

Brown, S., Hu, N. and Castelli-Gair Hombria, J. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. *Curr. Biol.* **11**, 1700-1705.

Casanueva, M. and Ferguson, E. (2004). Germline stem cell number in the Drosophila ovary is regulated by redundant mechanisms that control Dpp signalling. *Development* **131**, 1881-1890.

Chen, D. and McKearin, D. (2003). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Current Biology* **13**, 1786-1791.

Chen, D. and McKearin, D. (2005). Gene circuitry controlling a stem cell niche. *Curr Biol* **15**, 179-84.

Chen, H., Chen, X., Oh, S., Marinissen, M., Gutkind, J. and Hou, S. (2002). mom identifies a receptor for the Drosophila JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family. *Genes & Development* **16**, 388-398.

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

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

de Cuevas, M. and Spradling, A. (1998). Morphogenesis of the Drosophila fusome and its implications for oocyte specification. *Development* **125**, 2781-2799.

de Rooij, D. G. and Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *J Androl* **21**, 776-98.

Decotto, E. and Spradling, A. C. (2005). The Drosophila ovarian and testis stem cell

niches: similar somatic stem cells and signals. *Dev Cell* **9**, 501-10.
Forbes, A., Spradling, A., Ingham, P. and Lin, H. (1996). The role of segment polarity genes during early oogenesis in Drosophila. *Development* **122**, 3284-3294.
Fuchs, E., Tumbar, T. and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* **116**, 769-778.
Gilbert, M. M., Weaver, B. K., Gergen, J. P. and Reich, N. C. (2005). A novel functional activator of the Drosophila JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation. *Mech Dev* **122**, 939-48.
Gilboa, L. and Lehmann, R. (2004). How different is Venus from Mars? The genetics of germ-line stem cells in Drosophila females and males. *Development* **131**, 4895-905.
González-Reyes, A. (2003). Stem cells, niches and cadherins: a view from Drosophila. *J. Cell Sci.* **116**, 949-954.
Harrison, D., McCoon, P., Binari, R., Bilman, M. and Perrimon, N. (1998). Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes & Development* **12**, 3252-3263.
Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M. and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *Embo J* **14**, 2857-65.
Hombria, J. C.-G., Brown, S., Hader, S. and Zeidler, M. P. (2005). Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand. *Developmental Biology* **288**, 420-433.
Hou, X. S., Melnick, M. B. and Perrimon, N. (1996). Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs. *Cell* **84**, 411-9.
Kai, T. and Spradling, A. (2003). An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. *PNAS* **100**, 4633-4638.
Kai, T. and Spradling, A. (2004). Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. *Nature* **428**, 564-9.
Kiger, A., Jones, D., Schulz, C., Rogers, M. and Fuller, M. (2001). Stem cell self-renewal specified by JAK-STAT activation in response to a support clue. *Science* **294**, 2542-2545.
King, F. and Lin, H. (1999). Somatic signaling mediated by fs(1)Yb is essential for germline stem cell maintenance during Drosophila oogenesis. *Development* **126**, 1833-1844.
King, F., Szakmary, A., Cox, D. and Lin, H. (2001). Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the Drosophila ovary. *Mol. Cell* **7**, 497-508.
LaFever, L. and Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. *Science* **309**, 1071-3.
Luo, H., Hanratty, W. P. and Dearolf, C. R. (1995). An amino acid substitution in the Drosophila hopTum-l Jak kinase causes leukemia-like hematopoietic defects. *Embo J* **14**, 1412-20.
Perrimon, N. and Mahowald, A. P. (1986). l(1)hopscotch, A larval-pupal zygotic lethal with a specific maternal effect on segmentation in Drosophila. *Dev Biol* **118**, 28-41.
Scadden, D. T. (2006). The stem-cell niche as an entity of action. *Nature* **441**, 1075-9.

Shi, S., Calhoun, H. C., Xia, F., Li, J., Le, L. and Li, W. X. (2006). JAK signaling globally counteracts heterochromatic gene silencing. *Nat Genet* **38**, 1071-6.
Singh, S. R., Zhen, W., Zheng, Z., Wang, H., Oh, S. W., Liu, W., Zbar, B., Schmidt, L. S. and Hou, S. X. (2006). The Drosophila homolog of the human tumor suppressor gene BHD interacts with the JAK-STAT and Dpp signaling pathways in regulating male germline stem cell maintenance. *Oncogene* **25**, 5933-41.
Song, X., Call, G. B., Kirilly, D. and Xie, T. (2007). Notch signaling controls germline stem cell niche formation in the Drosophila ovary. *Development* **134**, 1071-80.
Song, X., Wong, M. D., Kawase, E., Xi, R., Ding, B. C., McCarthy, J. J. and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. *Development* **131**, 1353-64.
Song, X. and Xie, T. (2003). Wingless signaling regulates the maintenance of ovarian somatic stem cells in Drosophila. *Development* **130**, 3259-68.
Spradling, A., de Cuevas, M., Drummond-Barbosa, D., Keyes, L., Lilly, M., Pepling, M. and Xie, T. (1997). The Drosophila germarium: stem cells, germ line cysts, and oocytes. *Cold Spring Harbour Sym. Quant. Biol.* **LXII**, 25-34.
Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98-104.
Szakmary, A., Cox, D. N., Wang, Z. and Lin, H. (2005). Regulatory relationship among piwi, pumilio, and bag-of-marbles in Drosophila germline stem cell self-renewal and differentiation. *Curr Biol* **15**, 171-8.
Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of Dpp activity in Drosophila wing imaginal discs. *Mol. Cell* **5**, 59-71.
Tulina, N. and Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. *Science* **294**, 2546-2549.
Wieschaus, E., Nüsslein-Volhard, C. and Jürgens, G. (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster III. Zygotic loci on the X-chromosome and the fourth chromosome. *Roux's Arch. Dev. Biol.* **193**, 296-307.
Wong, M. D., Jin, Z. and Xie, T. (2005). Molecular Mechanisms of Germline Stem Cell Regulation. *Annu Rev Genet* **39**, 173-195.
Xi, R., Doan, C., Liu, D. and Xie, T. (2005). Pelota controls self-renewal of germline stem cells by repressing a Bam-independent differentiation pathway. *Development* **132**, 5365-74.
Xie, T. and Spradling, A. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell* **94**, 251-260.
Xie, T. and Spradling, A. (2000). A niche maintaining germ line stem cells in the Drosophila ovary. *Science* **290**, 328-330.
Zhang, Y. and Kalderon, D. (2001). Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. *Nature* **410**, 599-604.

FIGURE LEGENDS

Figure 1. Components of the Jak/Stat pathway are expressed in the GSC niche. (A) Schematic diagram of a germarium. Terminal Filament Cells (TFCs), Cap Cells (CpCs), Escort Stem Cells (ESCs) and Escort Cells (ECs) are of somatic origin. Germline Stem Cells (GSCs) are in close contact with CpCs and contain apical spectrosomes that evolve into 'exclamation mark' figures in post-mitotic GSCs. Cystoblasts (CB) also contain spectrosomes, but these do not keep an apical localisation. CBs undergo four incomplete rounds of division giving rise to cysts of two, four, eight, and sixteen cells interconnected by branched fusomes. (B) Wild type germarium double stained to visualize spectrosomes and fusomes (anti-Hts; red) and the Upd ligand (anti-Upd; green). The accumulation of Upd protein is clearly visible in TFCs and CpCs. In addition, a weaker, speckled distribution is present in more posterior cells, possibly corresponding to ESCs and ECs, cell types where the Jak / Stat pathway is active (Decotto and Spradling, 2005). (C) *Stat92E*^{06346} germarium double stained to visualize Hts (red) and β-Gal (green). *Stat92E* directs *lacZ* expression in TFCs, CpCs, ESCs and ECs (see also (Decotto and Spradling, 2005). (D) Detection of *upd*, *upd2* and *upd3* mRNAs by RT-PCR in ovaries from virgin females. The expression of the embryonic gene *ftz* was used as a control for the specificity of the ovarian cDNA library (lanes 1 and 2). The expression of *hedheghog* (*hh*; lane 3) was utilised as a positive control. Lanes 4, 5 and 6 show that *upd*, *upd2* and *upd3* are expressed in the ovary. (E) Germarium from a *bab1*-Gal4 / UASt-*DsRed* female stained with anti-Hts (red). *bab1*-Gal4 directs *DsRed* expression (shown in green) in TFCs, CpCs, ESCs and ECs. (F) Germarium from a *tub*-Gal80^{ts} / UASt-*upd2*; *bab1*-Gal4 / +

female stained with anti-Hts to show the tumour of spectrosome-containing cells produced after *bab1*-Gal4-driven overexpression of Upd2 in adult germaria.

Scale bar =10 μm. Anterior is up in all figures unless otherwise stated. Open arrowheads: TFCs; arrowheads: CpCs; arrows: ESCs and ECs; asterisks: GSCs.

Figure 2. Jak/Stat signalling is required in the ovary for GSC maintenance

(A, B) Wild type (A) and hop^{27}/hop^{25} upd^{YM55} (B) germaria stained with anti-Hts to visualise the clear reduction in the number of GSCs and developing cysts in the mutant condition. (C, D) *nanos*-Gal4 / UASt-*Src:GFP* (C) and hop^{27}/hop^{25}; *nanos*-Gal4 / UASt-*Src:GFP* (D) germaria dissected 10 days after eclosion. They have been double stained with anti-Hts (red) and anti-GFP (green) to visualize spectrosomes and to outline the germline cells, respectively. The spectrosome in (C) displays the typical 'exclamation mark' shape (de Cuevas and Spradling, 1998); the spectrosome in (D) has lost its apical anchoring while still maintaining its connection with the cystoblast spectrosome and was thus classified as an 'anchorless' GSC spectrosome. The small 'scar' of spectrosomal material left on the apical side, adjacent to the cap cells, suggests that the GSC spectrosome has severed its apical connection prior to accumulating basally. (E) Graph representing the mean number of GSCs (± s.d.) per germarium in hop^{27} / FM7 (control), hop^{27}/hop^{25} and hop^{27}/hop^{25} upd^{YM55} germaria. Ovaries were dissected 2, 10 and 25 days after eclosion. Black triangles indicate a statistically significant difference between the given experimental condition and its control

(Student's *t* test: $p<0.01$). (F) Bar graph showing the percentage of 'anchorless' GSC figures in hop^{27} / FM7 (control), hop^{27}/hop^{25} and hop^{27}/hop^{25} upd^{YM55} germaria dissected 2, 10 and 25 days after eclosion. (E,F) The number of germaria analyzed for each experiment (n) is shown in (E).

Scale bar =10 μm. Asterisks: GSCs; CB: cystoblast. The white, dotted lines delineate GSC-CB pairs.

Fig 3. Somatic Jak/Stat signalling is essential to prevent GSC differentiation.

(A) *yw hs-flp122*; FRT82 $Stat92E^{06346}$ / FRT82B *ubi*-GFP germarium dissected 25 days after eclosion. It has been double stained with anti-Hts (red) and anti-GFP (green) to document that *Stat92E* loss-of-function in the germline does not affect stem cell maintenance. (B) *w*; UASt-$dome^{\Delta CYT}$ / +; *bab1*-Gal4 / + germarium grown at 25 °C and dissected 25 days after eclosion. The staining with anti-Hts shows that the two GSCs present in the niche possess an 'anchorless' spectrosome. (C, D, E) FRT101 hop^{2} / FRT101 *ubi*-GFP; *bab1*-Gal4 UASt-*flp* germaria stained with anti-Hts (red), anti-GFP (green) and the DNA dye Hoechst (white) to label hop^{2} mutant CpCs. (C, C') GSC in contact with both mutant and wild type CpCs showing a normal spectrosome. (D, D') GSCs in contact with mutant CpCs displaying 'anchorless' spectrosomes. (E, E') Mutant CpCs in direct contact with a differentiating 8-cell cyst.

Scale bar =10 μm. Asterisks: GSCs; yellow and red dotted lines: CpCs; white dotted

line: germline cyst.

Figure 4. Loss of *hop* activity does not affect the acquisition of Terminal Filament Cell and Cap Cell fates. Germaria harbouring *hop*^{c111} somatic clones stained with Topro-3 (blue), anti-GFP (green) and anti-Engrailed (A-A'') or anti-Lamin-C (B-B'') in red. The expression of Engrailed or Lamin-C proteins is not altered in mutant cells when compared to wild-type neighbours. Yellow, empty arrowheads: wild-type TFCs; yellow arrowheads: wild-type CpCs; White, empty arrowheads: mutant TFCs; white arrowheads: mutant CpCs. The genotype of these germaria is *hop*^{c111} FRT-101 / GFP FRT-101; *bab1*-Gal4 UASt-*flp* / +.

Figure 5. Jak/Stat regulates *dpp* signalling in the GSC niche

(A) Germarium from a *tub*-Gal80^{ts} / UASt-*hop*^{Tum}; *bab1*-Gal4 / + female stained with anti-Hts showing the extra-GSC-like cells and blocked cyst differentiation produced by *bab1*-Gal4-driven overexpression of *hop*^{Tum} in adult germaria. (B) Detection by Real Time-PCR of the relative levels of *dpp* and *gbb* mRNAs in ovaries from *tub*-Gal80^{ts} / UASt-*upd2*; *bab1*-Gal4 / + females compared to *tub*-Gal80^{ts} / +; *bab1*-Gal4 / + controls. On average, *dpp* mRNA levels were increased ~3.25 fold in experimental ovaries, whereas the amount of *gbb* mRNA did not vary substantially in the same experimental condition (the black triangle denotes that the mean difference was statistically significant in the case of *dpp* mRNA but not in the case of *gbb* mRNA; Student's *t* test: $p<0.01$; the mean values are averages of 3 different replicas from 3 independent experiments). (C, D) Wild type (C) and *tub*-Gal80^{ts} / UASt-*upd2*; *bab1*-

Gal4 / + (D) germaria stained with anti-Hts (red), anti-pMad (a reporter of Dpp-pathway activation, green) and the DNA dye TOPRO-3 (blue). (C, C´, C´´) Wild type germarium showing pMad protein restricted to the GSCs. (D, D', D'', D''') A *tub*-Gal80^{ts} / UASt-*upd2*; *bab1*-Gal4 / + germarium showing expanded pMad staining within the ectopic GSC tumour.

Scale bar =10 μm. Asterisks: GSCs; the white, dotted lines demarcate the areas magnified in C', C'', D', D'' and D'''.

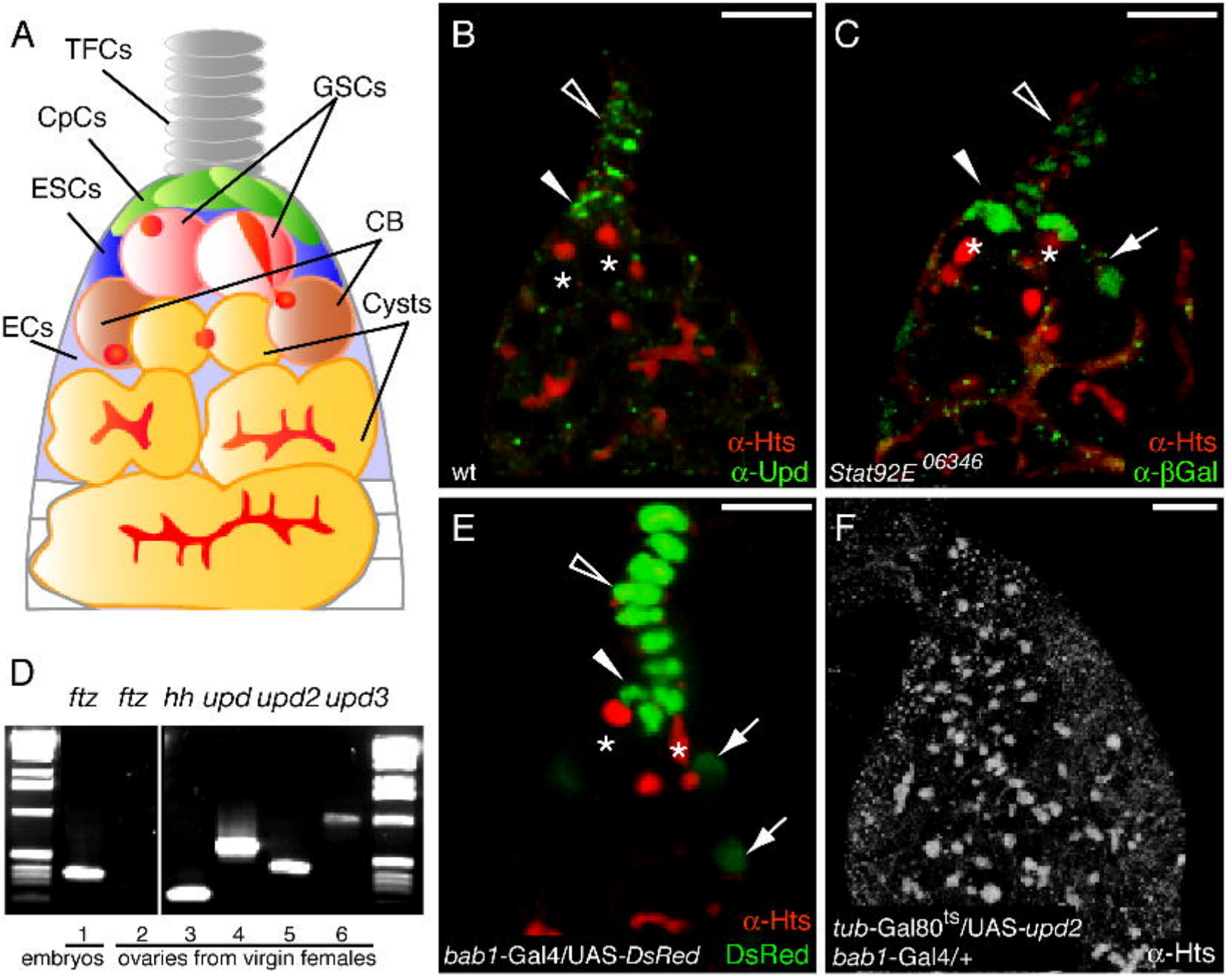

Fig. 1 López-Onieva et al

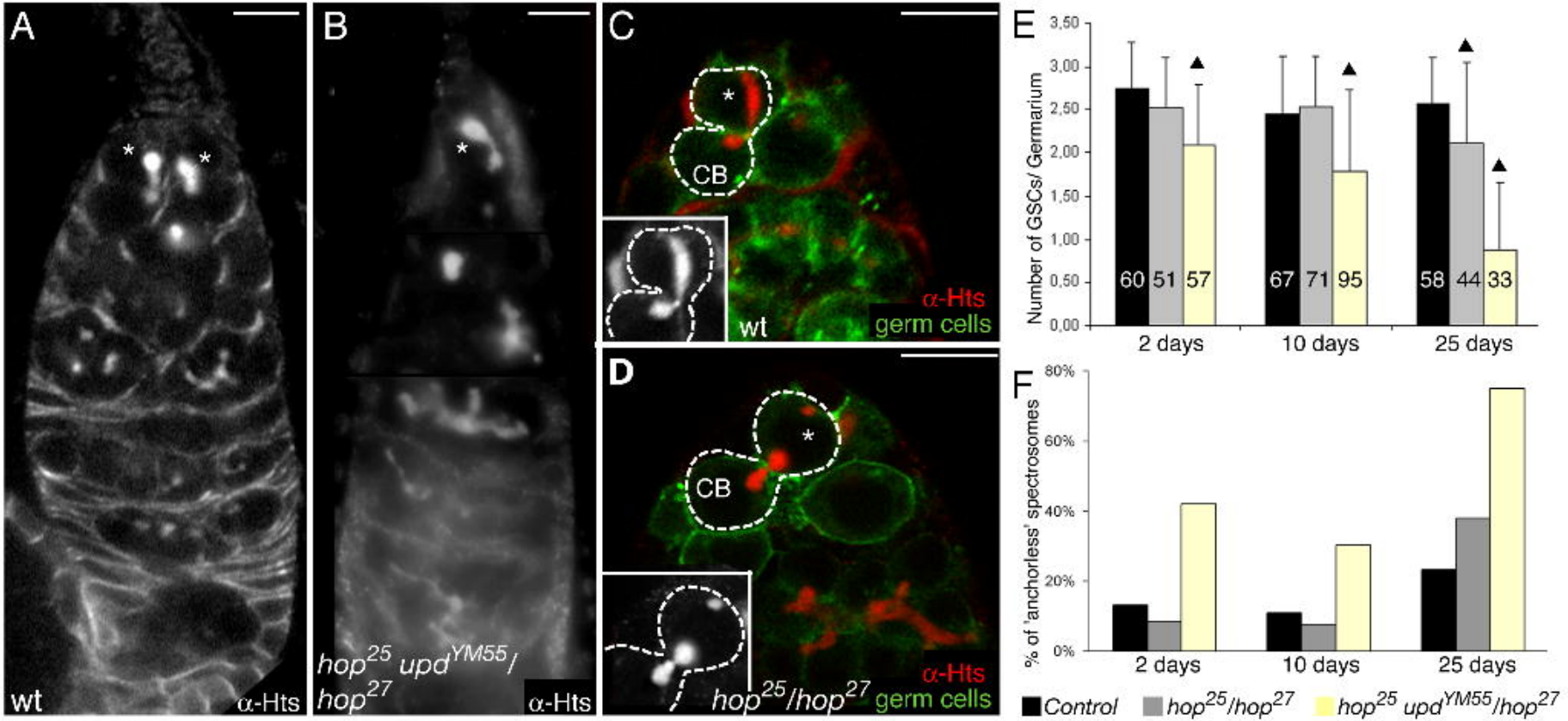

Fig. 2 López-Onieva et al

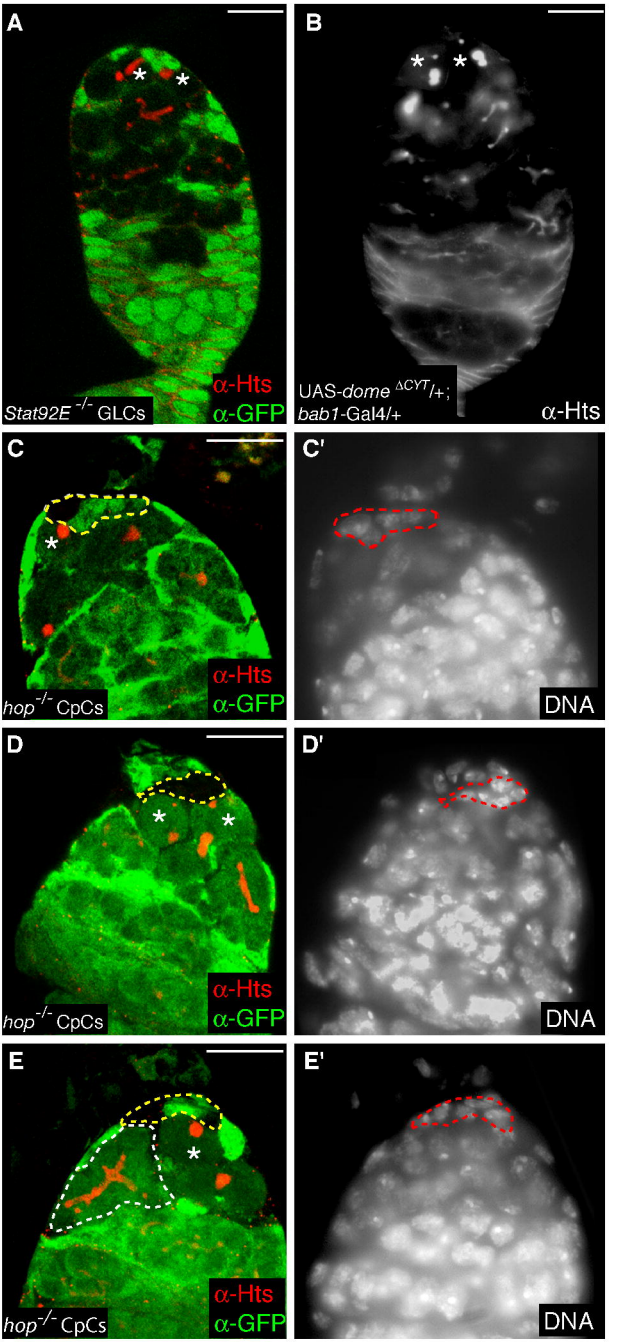

Fig. 3 López-Onieva et al.

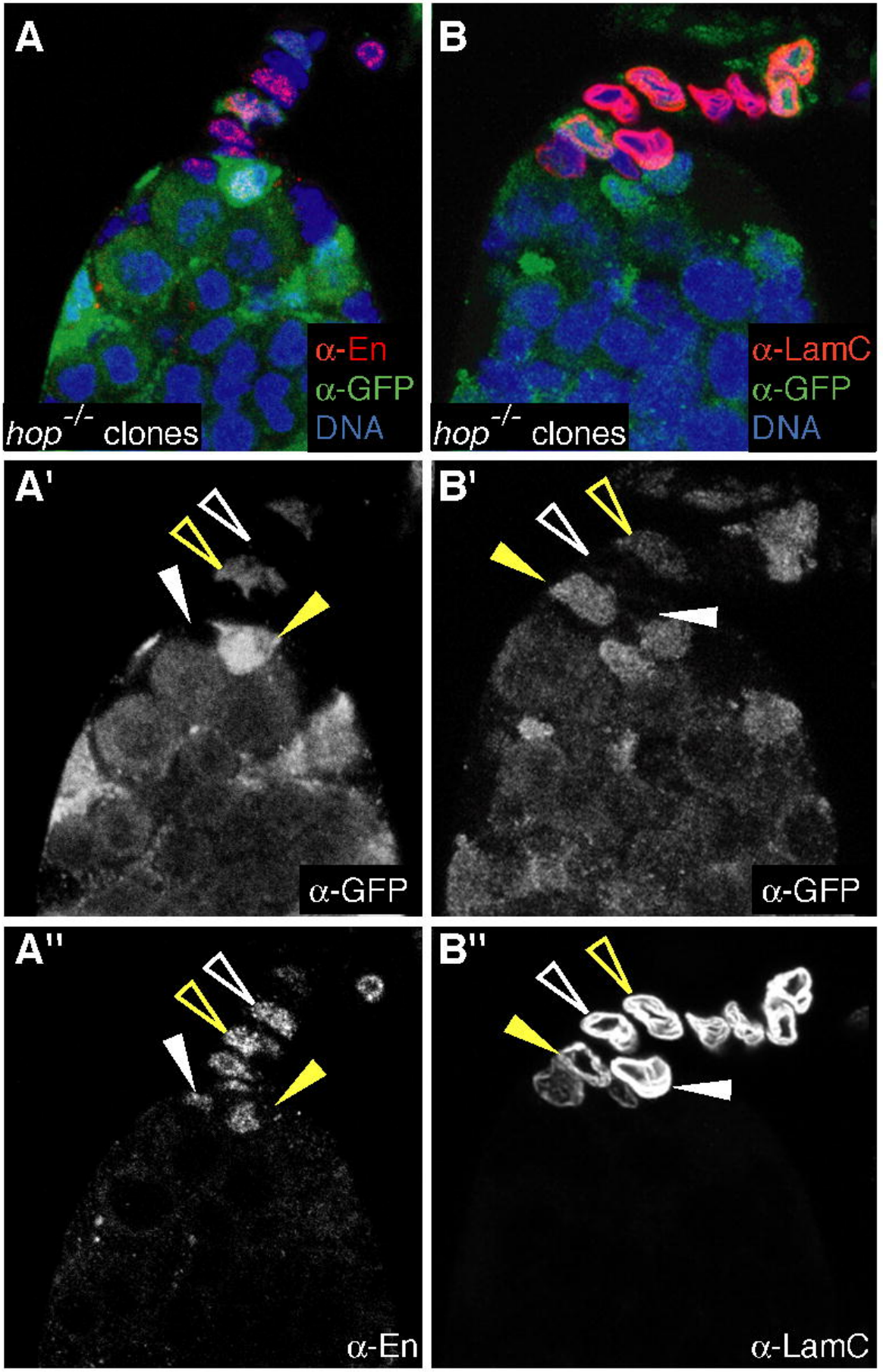

Fig. 4 López-Onieva et al

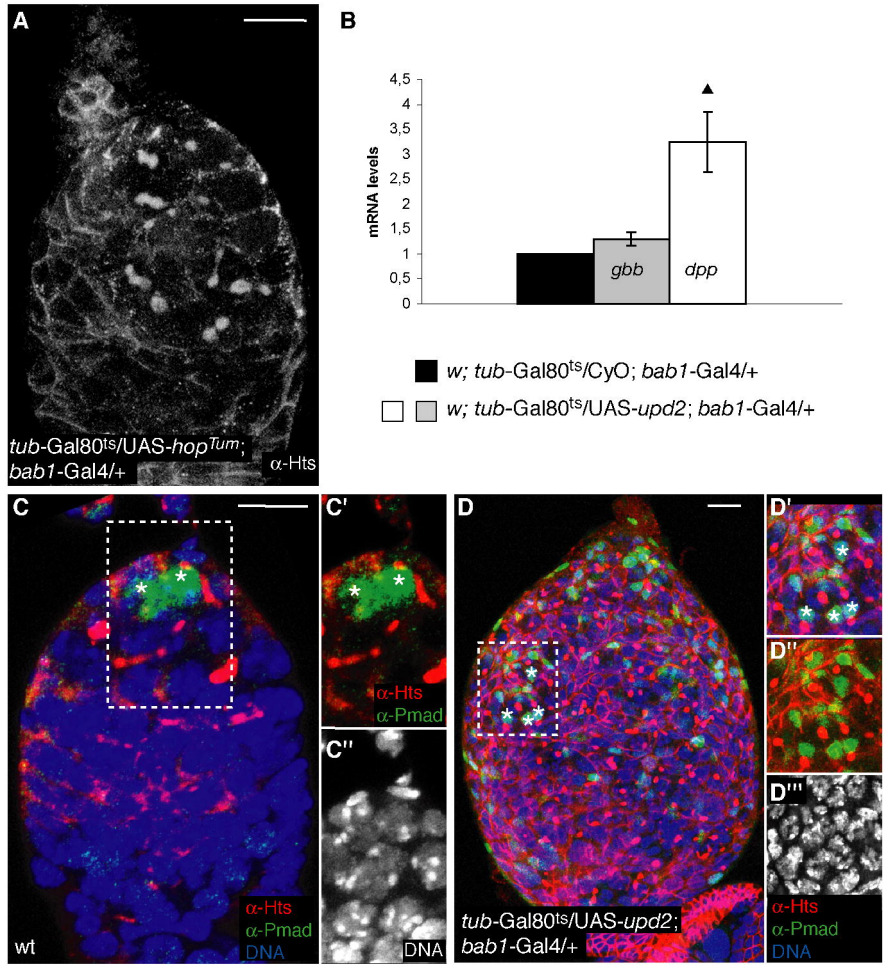

Fig. 5 López-Onieva et al