

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Bi-directional gap junction-mediated soma-germline communication is essential for spermatogenesis

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ABSTRACT

Soma-germline interactions play conserved essential roles in regulating cell proliferation, differentiation, patterning and homeostasis in the gonad. In the *Drosophila* testis, secreted signalling molecules of the JAK-STAT, Hedgehog, BMP and EGF pathways are used to mediate soma-germline communication. Here, we demonstrate that gap junctions may also mediate direct, bi-directional signalling between the soma and germ line. When gap junctions between the soma and germ line are disrupted, germline differentiation is blocked and germline stem cells are not maintained. In the soma, gap junctions are required to regulate proliferation and differentiation. Localization and RNAi-mediated knockdown studies reveal that gap junctions in the fly testis are heterotypic channels containing Zpg (*Inx4*) and *Inx2* on the germ line and the soma side, respectively. Overall, our results show that bi-directional gap junction-mediated signalling is essential to coordinate the soma and germ line to ensure proper spermatogenesis in *Drosophila*. Moreover, we show that stem cell maintenance and differentiation in the testis are directed by gap junction-derived cues.

KEY WORDS: Gap junctions, Spermatogenesis, Germline, Testis, *Drosophila*

INTRODUCTION

In animals, the gonads are composed of two tissue types: the germ line, which develops into gametes, and the soma, which forms all other tissues in the gonad required to support germ cell maintenance and development. Importantly, gametogenesis often uses specialized populations of stem cells that give rise to the soma and the germ line in order to maintain life-long gamete production. Communication between the soma and germ line is essential throughout gametogenesis; failure in soma-germline signalling can result in tumorigenesis or sterility (Hochstenbach and Hackstein, 2000). The *Drosophila* gonads provide a powerful genetically tractable model to study stem cell biology and soma-germline interactions in an *in vivo* context (White-Cooper, 2009). Studies in the *Drosophila* testis have illustrated that soma-germline signalling plays an instructive role in controlling stem cell behaviour during spermatogenesis (de Cuevas and Matunis, 2011; Yamashita et al., 2005). This body of work highlights remarkable conservation across metazoans of the mechanisms that mediate soma-germline communication (Hochstenbach and Hackstein, 2000; Yamashita et al., 2005).

In the *Drosophila* testis, a population of mitotically quiescent somatic cells, known as the hub, physically anchor germline stem cells (GSCs) and somatic cyst stem cells (CySCs). As a GSC

divides, one daughter cell remains in contact with the hub and CySCs, and maintains stem cell identity. The other daughter cell is displaced from the niche and begins to differentiate (de Cuevas and Matunis, 2011). Although CySCs do not divide asymmetrically, their divisions generate a daughter cell that is maintained as a CySC and a daughter cell that begins to differentiate (Cheng et al., 2011; Amoyel et al., 2014). As the displaced germ cell exits the niche, it is encapsulated by two CySCs that remain wrapped around the differentiating germline cyst until the end of spermatogenesis (Schulz et al., 2002). Soma-germline interactions are essential for germline differentiation and maturation (Gonczy and DiNardo, 1996; Tran et al., 2000).

The signalling pathways that mediate soma-germline communication within the niche have been characterized in detail. The hub secretes the JAK-STAT ligand Unpaired (Upd), Hedgehog (Hh) and bone morphogenetic proteins (BMPs) to regulate stem cell maintenance and repress differentiation in both GSCs and CySCs (de Cuevas and Matunis, 2011). Hub-derived Hh is required in CySCs for self-renewal independently of JAK-STAT signalling (Amoyel et al., 2013; Michel et al., 2012). Additionally, soma-germline communication mediated by the epidermal growth factor receptor (EGFR) pathway is required during and after encapsulation (Hudson et al., 2013; Sarkar et al., 2007; Schulz et al., 2002). Although much is currently known about the mechanisms that regulate stem cells in the niche, the mechanisms that drive differentiation of their daughter cells upon exit from the niche are not well understood. One factor known to promote germline differentiation is Bag of Marbles (Bam), a downstream repression target of BMP signalling (Gonczy et al., 1997; Kawase et al., 2004; Shivdasani and Ingham, 2003). Ectopic Bam expression promotes precocious germline differentiation and thus depletes GSCs (Kawase et al., 2004; Ohlstein and McKearin, 1997; Sheng et al., 2009; Shivdasani and Ingham, 2003). The EGFR pathway has also been implicated in preventing precocious differentiation of CySCs (Tran et al., 2000). Nonetheless, the complex programme of gametogenesis follows a defined set of stages, and much remains to be elucidated about the signalling mechanisms that mediate soma and germline differentiation during this process.

Previous studies in flies have suggested a role for gap junction components in regulating soma-germline communication (Gilboa et al., 2003; Tazuke et al., 2002). Gap junctions are composed of innexin proteins in invertebrates and connexins in vertebrates (Phelan, 2005). Innexins are four-pass transmembrane proteins which hexamerize to form a hemichannel. A hemichannel at the cell surface can dock with another hemichannel on an apposing cell to form a gap junction. Gap junctions can permit the passage of small molecules and ions, such as cAMP, IP3 or Ca²⁺, to mediate rapid cell-cell signalling between neighbouring cells (Bauer et al., 2005; Hervé and Derangeon, 2013; Phelan, 2005). Genetic screens identified the gap junction gene *innexin 4* (*inx4*), also known as *zero population growth* (*zpg*), as being required for

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male fertility (Tazuke et al., 2002). Further analysis in the ovary showed that GSCs require Zpg in order to differentiate and that forced differentiation induced by ectopic Bam expression resulted in cell death (Gilboa et al., 2003). However, this analysis did not resolve whether Zpg mediated communication between germ cells or between germ cells and the soma (Gilboa et al., 2003; Tazuke et al., 2002). This issue is complicated by the fact that innexins can either couple with the same type of innexin to form homotypic channels or with another type of innexin to form heterotypic channels (Bauer et al., 2005). Whether Zpg coupled homotypically with other Zpg molecules in neighbouring germ cells or heterotypically with other somatic innexins has not previously been established.

Here, we have performed a detailed analysis of the germline and somatic phenotypes of *zpg* mutants. Since the initial characterization of Zpg was carried out, many additional markers for soma and germline differentiation have been identified. Using these markers, we show that Zpg is required to regulate both soma and germline differentiation upon exit from the niche as well as for GSC maintenance. We demonstrate that gap junctions can be observed using electron microscopy between the germ line and the soma, and between germ cells. Furthermore, we show that Innexin2 (Inx2) is the somatic innexin that couples with Zpg to form gap

junctions between the soma and the germ line. Our evidence shows that heterotypic Zpg-Inx2 gap junctions mediate communication, allowing for coordination of soma and germline differentiation. Disruption of gap junctions either by removal of Zpg from the germ line or by somatic knockdown of Inx2 produces severe defects in both the soma and the germ line, resulting in sterility.

RESULTS

zpg is required specifically in the germ line for GSC maintenance and differentiation

To understand the role of *zpg* in regulating soma-germline communication, we expanded on previous analysis of the germline phenotypes of *zpg* mutant testes (Tazuke et al., 2002). As described, testes in *zpg* null flies are rudimentary and contain fewer germ cells compared with wild type (Fig. 1A,B; Tazuke et al., 2002). Previous analysis of the germ line did not distinguish between differentiating germ cells and GSCs (Tazuke et al., 2002), defined as Vasa⁺ germ cells that contact the hub (Chang et al., 2013; Davies et al., 2013). We found that *zpg*-deficient testes had far fewer GSCs than did wild-type sibling controls (1.2 ± 0.3 , $n=49$ versus 10.8 ± 0.3 , $n=37$; Fig. 1C,D,G), and in 62.1% of *zpg* mutant testes, no GSCs were present at 1 day post-eclosion (DPE) ($n=100$), although 95% of *zpg* mutant testes contained germ cells ($n=101$). The reduction in

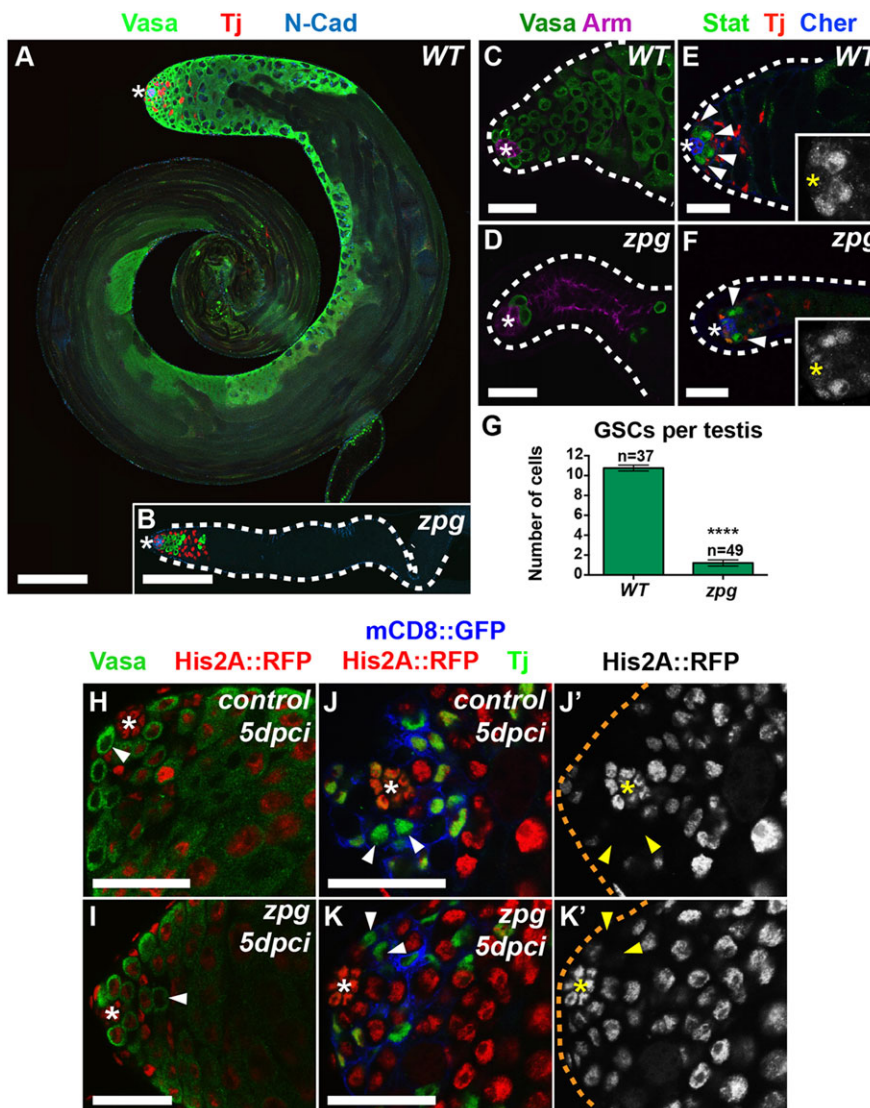


Fig. 1. *zpg* is required specifically in the germ line for GSC maintenance and differentiation.

(A,B) Compared with wild-type testes (A), *zpg* mutant testes (B) are rudimentary and contain few germ cells. (C) Wild-type testes contain GSCs arrayed around the hub. (D) *zpg* mutant testes contain few GSCs, if any. (E,F) In response to JAK/STAT pathway signals from the hub, Stat92E accumulates in GSCs (arrowheads) in both wild-type (E, inset) and *zpg* mutant testes (F, inset). (G) GSC quantification in wild-type and *zpg* mutant testes at 1 day post-eclosion. Error bars show mean \pm s.e.m., **** $P < 0.0001$; n , number of testes counted. (H,I) Negatively labelled germline clones were either wild-type controls or homozygous for a *zpg* null allele, *zpg*^{z-5352}. (H) Control clones (arrowhead) were maintained as GSCs at 5 days dpci. (I) *zpg*^{z-5352} clones were never successfully maintained as GSCs at 5 dpci, and rarely at 4 dpci. Where *zpg*^{z-5352} clones were detected, they existed as single-celled clones, displaced from the niche. (J-K') Negatively labelled somatic clones were either wild-type controls or homozygous for *zpg*^{z-5352}. Both control (J) and mutant clones (K) were detected in the CySC position at 5 dpci (arrowheads). Germline labelled for Vasa (green; A-D,H,I) CySCs and early soma with Tj (red in A,B,E,F; green in J,K), hub with N-Cad (CadN – FlyBase; blue; A,B), Arm (magenta; C,D), Cherio (blue; E,F), Stat92E detected by antibody (green; E,F), and clones labelled by loss of His2A::RFP (red), mCD8::GFP labels soma (blue; J,K). Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 100 μ m in A,B; 30 μ m in C-F,H-K.

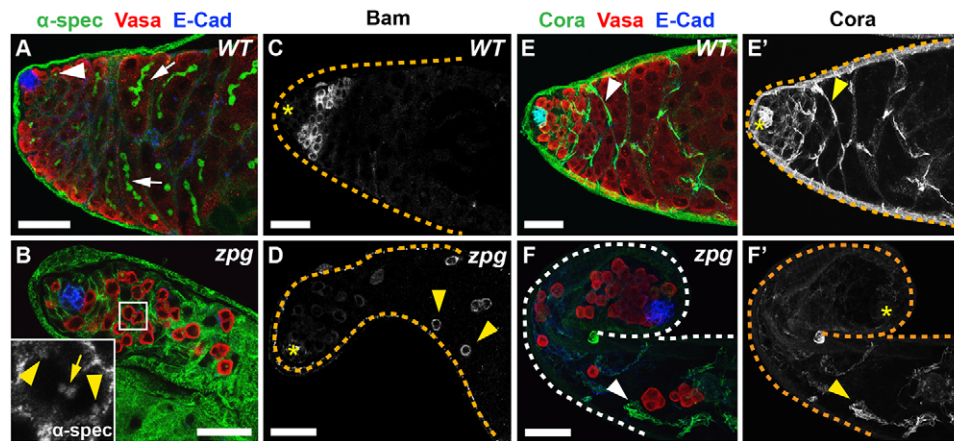


Fig. 2. *zpg* is required for germline differentiation and association with the soma. (A) In wild-type testes, small dot spectrosomes (arrowhead) enlarge and branch, forming fusomes (arrows). (B) In *zpg* mutant testes, germ cells contained spectrosomes and occasionally small dumbbell-shaped fusomes (inset, arrow). However, ectopic or fragmented spectrosomes were also detected, indicative of abnormal differentiation (inset, arrowheads). (C) Bag of Marbles (Bam) expression is repressed in GSCs by niche-derived BMP signalling. Bam expression is detected in germ cells beginning at the 2- to 4-cell stage in wild-type testes until the 16-cell stage. (D) In *zpg* mutant testes, Bam is detected in single-celled germ cells that are far from the hub (arrowheads). (E, E') In wild-type testes, germ cells are wrapped by somatic cells shortly after exiting the niche and remain encysted throughout spermatogenesis, detected by Cora staining (arrowhead). (F, F') In *zpg* mutant testes, germ cell clusters far from the niche are not encysted by the soma (arrowhead). Germine labelled for Vasa (red; A, B, E, F); spectrosomes/fusomes with α -spectrin (green; A, B); the hub with E-Cad (blue; A, B, E, F); and encystment by Cora (green; E, F). Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 30 μ m.

GSCs in *zpg* mutant testes might be due to an inability of GSCs to respond to stem cell maintenance signals secreted from the hub, such as Upd (Kiger et al., 2001; Tulina and Matunis, 2001). To test this possibility, wild-type and *zpg* mutant testes were stained for Stat92E, a downstream effector protein of JAK-STAT signalling, which accumulates in GSCs and promotes their maintenance (Leatherman and DiNardo, 2010). In both wild-type and *zpg* mutant testes, Stat92E protein was detected in GSCs (Fig. 1E, F). This suggested that although GSCs in *zpg* mutant testes can respond to JAK-STAT signalling, they are not maintained. This was tested directly by generating negatively labelled control and *zpg* clones and counting the number of GSC clones present. For the wild-type control, GSC clones were detected at 4 and 5 days post-clone induction (dpci) in 78% ($n=32$) and 73% ($n=41$) of testes, respectively (Fig. 1H, L). However, *zpg* mutant GSC clones were only detected in 3.2% ($n=32$) testes at 4 dpci and never detected at 5 dpci ($n=59$; Fig. 1I). *zpg* mutant germline clones were always observed as single cells several cell lengths away from the hub. By comparison, CySC maintenance was not affected by loss of *zpg*. When *zpg* clones were induced in CySCs [defined as Traffic jam (Tj)⁺ cells less than one cell diameter from the hub; Fairchild et al., 2015], they were found in 53% of the testes scored at 5 dpci ($n=15$; Fig. 1K, L). This was similar to control wild-type CySC clones, which could be detected at 5 dpci in 69% of testes ($n=13$; Fig. 1J, L). Overall, these findings argue that *zpg* is specifically required for GSC maintenance in the testis. These results are consistent with analysis of the *zpg* phenotype in the fly ovary, which showed that GSCs are lost over time in *zpg* mutants (Tazuke et al., 2002).

***zpg* is required for germline differentiation and association with the soma**

As work in the *Drosophila* ovary showed that germ cells in *zpg* mutants begin to differentiate but do not survive (Gilboa et al., 2003; Tazuke et al., 2002), we examined the differentiation of the germ line in *zpg* mutant testes. The maturation of the spectrosome, a round, cytoskeletal-rich organelle in the GSC, to become a branched

fusome within a spermatogonial cyst is a well-established indicator of germline differentiation (Fuller, 1993). In wild-type testes, the spectrosome could be detected in GSCs and gonialblasts, forming a large, branched fusome as the spermatogonia underwent further mitotic divisions (Fig. 2A). As described previously, GSCs and gonialblasts in *zpg* mutant testes were observed to contain spectrosomes (Tazuke et al., 2002; Fig. 2B). In some instances, dumbbell-shaped fusomes were detected in *zpg* mutant testes between adjacent germ cells, indicating the presence of 2-cell-stage spermatogonia. However, unlike in wild-type testes, in the 2-cell-stage spermatogonia seen in *zpg* mutant testes, ectopic spectrosomes were also observed alongside early fusomes, consistent with a differentiation defect (Fig. 2B, inset). As a second indicator of differentiation, we also examined expression of Bam. In wild-type testes, Bam is expressed in gonialblasts following displacement from the hub in 2- to 4-cell-stage spermatogonia, and is detected until the 16-cell stage (Gonczy et al., 1997). Analysis of Bam expression in *zpg* mutant testes revealed it to be expressed in germ cells which resided outside of the niche, similar to wild type (compare Fig. 2C with 2D). However, Bam⁺ germ cells in *zpg* mutant testes often appeared to be single-celled spermatogonia, rather than 2- to 16-cell-stage spermatogonia. Taken together, these results are consistent with earlier analyses (Gilboa et al., 2003; Tazuke et al., 2002), suggesting that germ cells initiate differentiation in *zpg* mutants but cannot complete the process.

Previous analysis of *zpg* mutant testes indicated defective association between the soma and the germ line (Tazuke et al., 2002). We confirmed this result by labelling for the septate junction component Coracle (Fig. 2E), which is a useful marker for encapsulation expressed in the soma (Fairchild et al., 2015). This analysis showed that in *zpg* mutant testes, germ cell clusters lacked a detectable belt of Coracle, indicating encapsulation defects (Fig. 2F).

***zpg* is required non-autonomously for differentiation of the soma**

Markers that label specific stages of soma development were identified following the initial characterization of the *zpg* mutant

phenotype (Tazuke et al., 2002). Three such stage-specific somatic cell markers were chosen (Fig. 3A): Zinc Finger Homeodomain 1 (*Zfh-1*) to label CySCs and their immediate daughters (Leatherman and DiNardo, 2008), *Tj* to label early-stage somatic cells (Li et al., 2003) and Eyes Absent (*Eya*) to label late-stage somatic cells (Fabrizio et al., 2003). Analysis with these markers revealed that the size of somatic cell populations in *zpg* mutant testes was misregulated compared with wild type. Wild-type testes contained an average of 37.1 ± 1.5 *Zfh-1*⁺ cells per testis ($n=17$; Fig. 3B), whereas testes from *zpg* flies contained an average of 160.0 ± 9.7 *Zfh-1*⁺ cells per testis ($n=27$; Fig. 3C), a 331% increase. Wild-type testes were observed to have an average of 98.4 ± 1.8 *Tj*⁺ cells per testis ($n=23$; Fig. 3D), compared with an average of 160.6 ± 8.2 in *zpg* mutant testes ($n=55$; Fig. 3E), a 63% increase. Finally, an average of 185.1 ± 6.1 *Eya*⁺ cells were detected in wild-type testes ($n=19$; Fig. 3F), compared with 79.3 ± 4.3 for *zpg* mutant testes ($n=26$; Fig. 3G), a 67% reduction. These results suggest a large increase in the number of CySCs and early somatic cells in *zpg* mutant testes, but a significant decrease in the late-stage somatic cells, relative to wild type (Fig. 3H). Furthermore, the stochastic variability in somatic cell numbers in *zpg* mutant testes is consistent with the idea that loss of gap junction-mediated regulatory cues have substantial effects on the soma. Taken together, these results suggest that somatic differentiation is disrupted, possibly delayed or partially blocked in *zpg* mutant testes, and that *zpg* is required non-autonomously to regulate the proliferation of the early soma.

The differentiation defect of somatic cells in *zpg* mutant testes likely results from failures in gap junction-mediated soma-germline communication. However, the germ line in *zpg* mutants is disrupted in two ways: first, there are fewer germ cells present; second, the residual germ line is blocked in differentiation. We sought to determine which of these two germline defects might lead to the somatic defects we observed. Although the somatic defects could be due to both fewer germ cells and disrupted germline differentiation, we tested these separately. To test if fewer germ cells are responsible for the somatic defects, soma differentiation was analysed in *tudor* mutant flies, which lack a germ line (Arkov et al., 2006; Boswell and Mahowald, 1985; Thomson and Lasko, 2004). In *tudor* mutant testes, the number of *Tj*⁺ early somatic cells was not significantly higher relative to a wild-type control, with an average of 112.0 ± 10.9 ($n=14$) cells per testes versus 103.3 ± 2.9 ($n=14$), respectively (Fig. 4A,B,E). This contrasts with the substantial and significant increase in the number of *Tj*-expressing cells observed in *zpg* mutant testes (Fig. 3D,E). Our results differ to previous work by Gonczy and DiNardo (1996), which suggested that agametic *oskar* mutant testes exhibited a large increase in early somatic cell number. This variance between *tudor* and *oskar*, which would be expected to have similar effects, might simply be because we performed this analysis in adult flies at <1 day post-eclosion versus 1–5 day post-eclosion for the previous study. Thus, the somatic phenotypes of *zpg* and *tudor* are at least partially distinct, arguing that the reduction in germ cells in *zpg* mutant testes does not by itself account for the somatic defects.

To test whether the somatic defects observed in *zpg* mutant testes were due to germline differentiation defects, constitutively active transgenes for the Type-I BMP receptors Thickveins (*Tkv*) and Saxophone (*Sax*) were expressed in early germ cells (Haerry et al., 1998). It has been previously shown that over-activation of the BMP pathway disrupts germline differentiation (Kawase et al., 2004; Shivdasani and Ingham, 2003). Quantification of early somatic cells revealed an increase of *Tj*⁺ cells upon activation of the BMP pathway, compared with controls, an average of 130.7 ± 4.4 ($n=27$) compared with 101.8 ± 2.0 ($n=26$) cells per testis, respectively; this

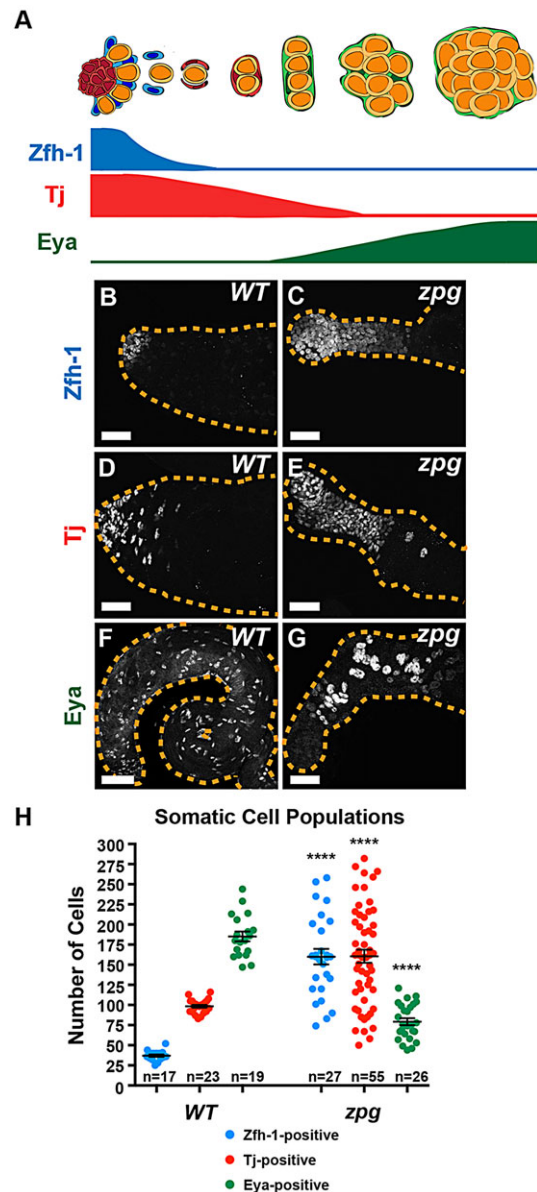


Fig. 3. *zpg* is required non-autonomously for differentiation of the soma.

(A) Schematic summarizing stage-specific expression of somatic cell differentiation markers *Zfh-1*, *Tj* and *Eya* in the testis. (B) In wild-type testes, *Zfh-1* is highly expressed in CySCs and weakly in their immediate daughters. (C) In *zpg* mutant testes, the apical tip is filled by *Zfh-1*⁺ cells. (D) In wild-type testes, *Tj* is expressed in the hub, CySCs and early somatic cells. (E) In *zpg* mutant testes, *Tj*⁺ cells fill the tip of the testis, similar to expression of *Zfh-1* in *zpg* mutant testes. (F) In wild-type testes, *Eya* is detected in the soma beginning near the 4-cell cyst stage until the end of spermatogenesis. (G) In *zpg* mutant testes, fewer *Eya*⁺ cells were observed than in the wild type. Dashed lines outline the testis. (H) Quantification of *Zfh-1*⁺, *Tj*⁺ and *Eya*⁺ somatic cells in wild-type and *zpg* mutant testes. *n*, number of testes counted. Error bars show mean ± s.e.m.; *****P* < 0.0001. Scale bars: 30 μm in B–E,G; 100 μm in F.

equates to a 28% increase (Fig. 4C–E). This increase was similar to that observed in *zpg* mutant testes and it is therefore possible that the somatic defects in *zpg* mutant flies were related to disrupted germline differentiation.

To explore further the possibility that the *zpg* mutant phenotype resulted from the inability of the germ line to differentiate, additional analysis was performed. We hypothesized that the increase in early somatic cells could possibly result from delays in somatic

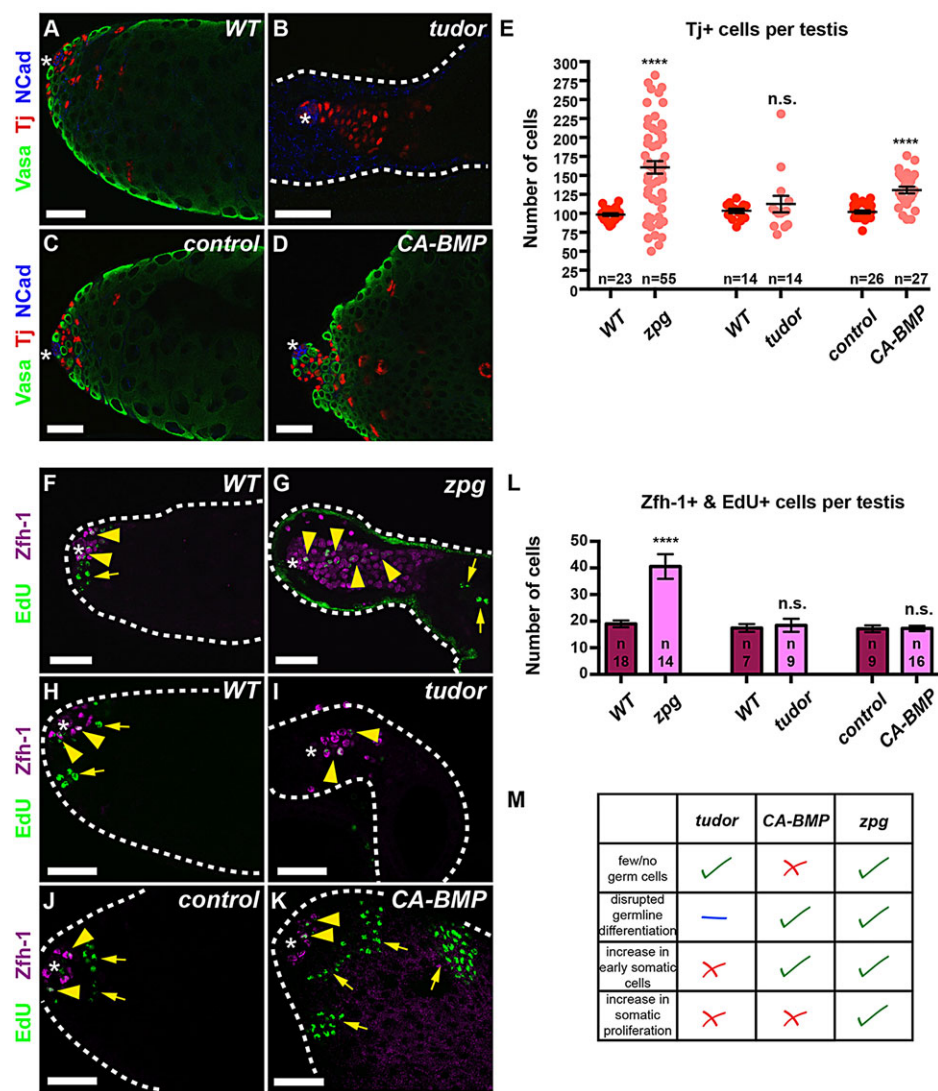


Fig. 4. *zpg* is required non-autonomously to regulate the proliferation of the early soma. (A-E) Analysis of early somatic cell numbers in agametic testes and testes expressing constitutively active BMP receptors in the early germ line using *nanos-GAL4-VP16*. Germline labelled by Vasa (green), early soma by Tj (red) and the hub by N-Cad (blue). (A,B) Tj⁺ cells in *w¹¹¹⁸* (WT) controls (A) and in *tudor* mutant testes (B), which do not form a germ line. (C,D) Tj⁺ cells in control (*w¹¹¹⁸; UAS-*tkv^{Act}*, *sax^{Act}/+**) testes (C) and in testes expressing CA-BMP receptors in the early germ line (*nanos-GAL4-VP16>UAS-*tkv^{Act}*, *sax^{Act}/+**; D). (E) Quantification of Tj⁺ cells in *zpg*, *tudor* and *nos>CA-BMP* testes versus their respective controls (in order: *zpg/+*, *w¹¹¹⁸*, *w¹¹¹⁸; UAS-*tkv^{Act}*, *sax^{Act}/+**). Loss of *Zpg* or constitutive-activation of BMP signalling led to a significant increase in Tj⁺ cells. (F-K) Quantification of S-phase somatic cells labelled with Zfh-1 and EdU in wild-type, *zpg*, *tudor*, control and CA-BMP testes. (F) In wild-type testes, Zfh-1⁺/EdU⁺ cells were detected adjacent to the hub (arrowheads). (G) In *zpg* mutant testes, Zfh-1⁺/EdU⁺ cells were detected throughout the apical tip (arrowheads). Germ cells in S phase were observed in both backgrounds (arrows). (H,I) Zfh-1⁺/EdU⁺ cells (arrowheads) were detected adjacent to the hub in wild-type and *tudor* testes, as were germ cells in wild-type testes (arrows). (J,K) In control and CA-BMP testes, Zfh-1⁺/EdU⁺ cells were detected near the hub (arrowheads), in addition to germ cells in S phase (arrows). (L) Quantification of Zfh-1⁺/EdU⁺ cells in *zpg*, *tudor* and *nos>CA-BMP* testes versus their respective controls (in order: *zpg/+*, *w¹¹¹⁸*, *w¹¹¹⁸; UAS-*tkv^{Act}*, *sax^{Act}/+**). A significant increase in proliferating somatic cells was only seen in *zpg* mutant testes. (M) Table summarizing germline and somatic phenotypes in the different genotypes studied. *n*, number of testes. Error bars show mean±s.e.m.; *****P*<0.0001. Asterisks indicate the hub, detected by N-Cad or Cheerio staining. Dashed lines outline the testis. Scale bars: 30 μm.

differentiation or from abnormal proliferation. Therefore, CySC and early daughter cell proliferation was assayed by labelling cells actively synthesizing new DNA with an 5-ethynyl-2'-deoxyuridine (EdU) pulse and co-staining for Zfh-1 (Leatherman and DiNardo, 2008). Interestingly, in agametic *tudor* testes or when BMP signalling is constitutively activated, the number of proliferating somatic cells was similar to controls at ~17 cells per testis (Fig. 4H). This implies that constitutively activating germline BMP signalling leads to an increase in Tj⁺ cells by delaying their differentiation rather than through changes in proliferation. By comparison, in *zpg* mutant testes the number of proliferating somatic cells increased by 113% (Fig. 4H; 19.0±1.3 in the control to 40.6±4.6; *n*=18 and 14, respectively). Furthermore, whereas proliferating somatic cells were only detected proximal to the hub in wild-type testes (Fig. 4F, arrowheads), Zfh-1⁺/EdU⁺ cells could be detected many cell lengths away from the hub in *zpg* mutant testes (Fig. 4G, arrowheads). These results show that the increase in the number of Tj⁺ cells in *zpg* mutants is at least partially due to a specific defect in proliferative regulation of the early soma. Although we cannot rule out the possibility that disrupted germline differentiation and a reduction in germ cells both contributed to the effects we observed, the phenotype of *zpg* mutants is distinct from that obtained by either

removing the germ line or blocking germline differentiation (Fig. 4I). This suggests that the somatic misregulation observed in *zpg* mutants likely represents a specific defect in gap junction-mediated soma-germline communication.

Analysis of innexins in the testis

Our results showing specific somatic defects in *zpg* mutant testes point to a possible role for innexin-mediated signalling between the soma and germ line. Because *Zpg* is expressed only in the germ line (Tazuke et al., 2002), it must interact with other innexin proteins on the somatic side of soma-germline contact sites to form gap junctions. To identify which of the eight fly innexins might be implicated in *Zpg*-dependent, gap junction-mediated communication during spermatogenesis, we carried out a small RNAi-based fertility screen using *tj*-GAL4 to drive RNAi expression specifically in the soma (Fig. 5A; supplementary material Fig. S2A–D). Somatic knockdown of only one innexin, *Inx2*, resulted in a phenotype. Somatic knockdown of *Inx2* resulted in sterility and subsequent histological analysis revealed small, rudimentary testes (Fig. 5A; supplementary material Fig. S2E).

Previous work in the fly ovary demonstrated that both Innexin2 (*Inx2*) and Innexin3 (*Inx3*) are present in the somatic follicle cells in developing egg chambers. Furthermore, *Inx2* was found to colocalize with *Zpg* at soma-germline boundaries (Bohrmann and Zimmermann, 2008). Phenotypic analysis of a hypomorphic *inx2* mutation in the ovary suggested a role for *Inx2* in the soma (Mukai et al., 2011). Therefore, expression of *Inx2* was analysed in the testis (Fig. 5B). Previously published *Inx2* antibodies did not yield good results in immunohistochemical analysis so a new *Inx2* antibody was generated (see Materials and Methods). Staining of wild-type testes using this new antibody revealed that *Inx2* localized to the soma-germline boundary (Fig. 5B; supplementary material Fig. S2G; Fig. S3). Our *Zpg* data suggested that *Inx2* should be expressed at the earliest stages of spermatogenesis. An *inx2* enhancer trap line expressing GFP revealed that *Inx2* expression could be detected weakly in the hub and CySCs, and, more strongly, in differentiating somatic cells (supplementary material Fig. S2F,F', insets; Quiñones-Coello et al., 2007). As previously published, *Zpg* also localized to the soma-germline boundary, visualized with a *Zpg*-specific antibody (Fig. 5C–D) and a GFP-tagged *Zpg* rescue construct (Fig. 5E; *Zpg::GFP*; see Materials and Methods). This GFP-tagged *Zpg* rescue construct was able to fully rescue spermatogenesis when introduced into a *zpg* null background, restoring fertility to wild-type levels (supplementary material Fig. S1). Labelling *Inx2* in testes expressing the *Zpg::GFP* transgene showed that *Zpg* colocalized with *Inx2* (Fig. 5B). Intriguingly, although weak expression of *Zpg* was detected in GSCs at the hub interface, *Inx2* was not detected at this stage by antibody staining (Fig. 5C). As in the ovary, *Inx3* also colocalized with *Zpg* at soma-germline boundaries, although its knockdown did not give rise to detectable defects (Fig. 5A,F; supplementary material Fig. S2E). Overall, these results argue that *Inx2* is required in the soma for spermatogenesis and its expression largely overlaps with that of *Zpg*.

Ultrastructural analysis of gap junctions in the testis

Previous work has demonstrated the presence of gap junctions between GSCs and niche cells in the fly ovary (Tazuke et al., 2002). To determine when gap junctions form in the testis, we performed ultrastructural studies using electron microscopy. At the apical tip of the testis (Fig. 6A), gap junctions were observed between GSCs and adjacent CySCs (Fig. 6B,B'). Gap junctions could also be observed

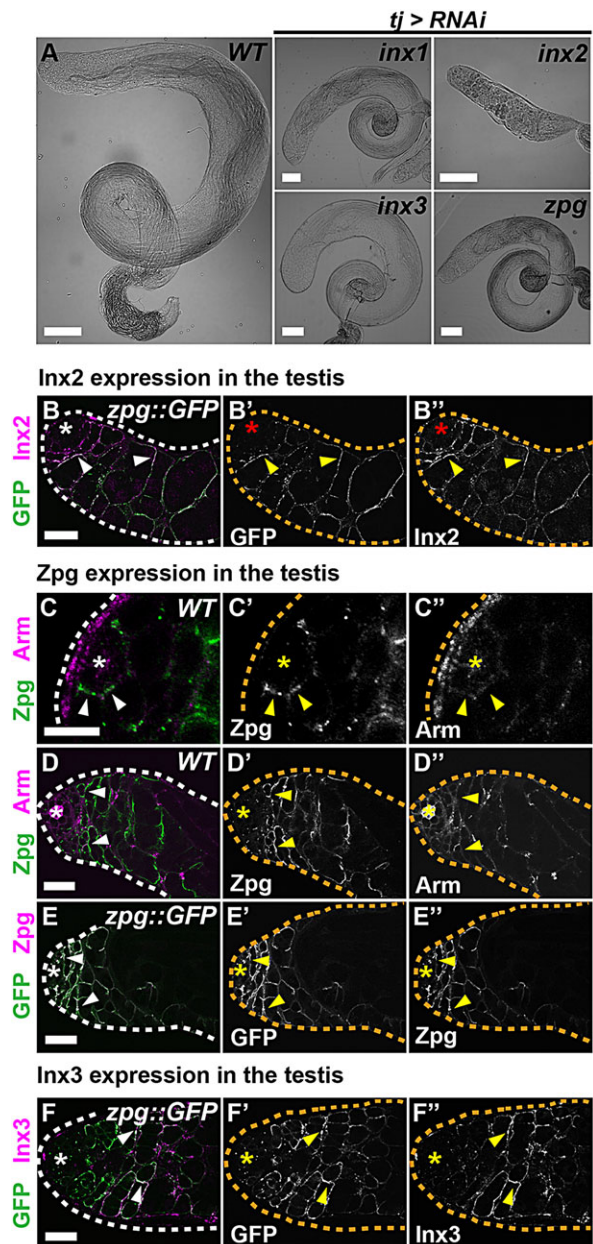


Fig. 5. Analysis of innexins in the testis. (A) Representative differential interference contrast images of wild-type testis and testes in which Innexins 1–4 were somatically knocked down using RNAi driven with *tj*-GAL4. Only Innexin 2 (*Inx2*) knockdown produced a mutant phenotype. (B) *Inx2* (magenta) prominently colocalized (arrowheads) with *Zpg* (green; visualized using a GFP-tagged rescue construct) beginning at the 4-cell cyst stage. (C–C'') Weak *Zpg* (green) expression was detected at the hub-GSC interface (arrowheads, hub marked with Arm in magenta). (D–D'') Strong *Zpg* expression (arrowheads; green) was observed in 4- to 16-cell-stage cysts (hub marked with Arm in magenta). (E–E'') The GFP-tagged *Zpg* rescue construct expression (arrowheads) mimicked endogenous *Zpg* protein expression, detected with *Zpg*-specific antibody (magenta). (F–F'') *Inx3* (magenta) also colocalized (arrowheads) with *Zpg* (green; GFP-tagged rescue construct) during early spermatogenesis. Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 100 µm in A; 10 µm in C; 30 µm in all other panels.

between 1-cell-stage gonialblasts and cyst cells immediately outside of the niche (Fig. 6C,C'). In differentiating spermatogonia, gap junctions were visible between germline cysts and cyst cells (Fig. 6D,D'). Interestingly, germline-germline gap junctions were observed in spermatogonia (Fig. 6E,E'), although these were

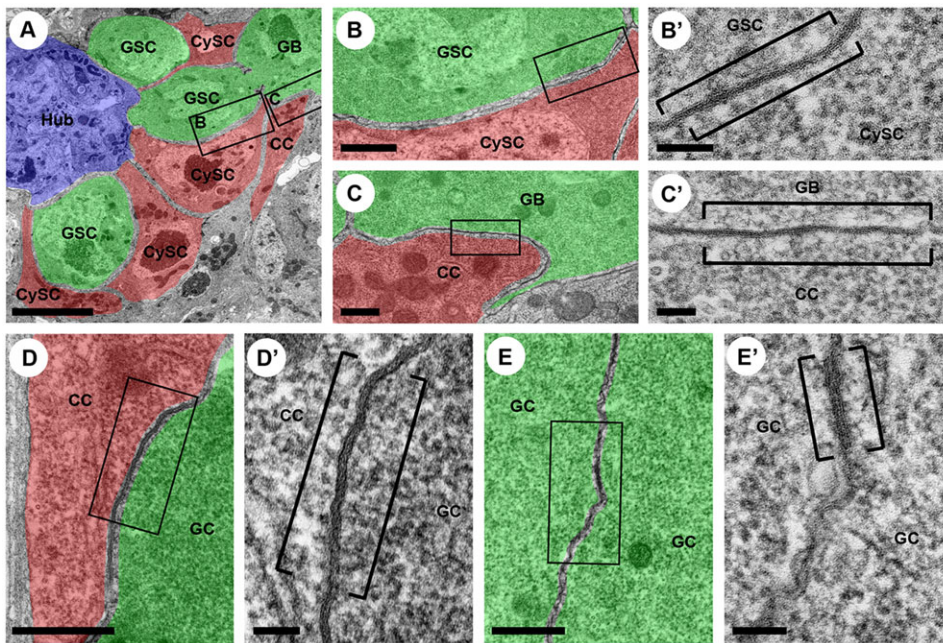


Fig. 6. Ultrastructural analysis of gap junctions in the testis. (A-E) Electron micrographs of wild-type testes. (A) Overview of the hub cell niche. (B, C) Gap junctions were detected early during spermatogenesis between both GSCs and CySCs (B, B') and between cyst cells (CCs) and 1-cell-stage gonialblasts (GBs; C, C'). (D, D') Outside the niche, germ cells (GCs) exhibited large gap junctions with associated CCs. (E, E') Gap junctions were also observed between neighbouring germ cells (E', brackets), although these were less common and smaller than those between germ cells and cyst cells (compare E' with B'-D'). The hub is highlighted in blue, germ line in green and soma in red. Square brackets indicate gap junctions. Scale bars: 5 µm in A; 1 µm in B, C; 500 nm in D, E; 100 nm in B'-E'.

infrequent and small in comparison to soma-germline gap junctions (compare Fig. 6E' with 6B'-D'). Together, these results indicate that gap junctions are formed early during spermatogenesis, that these junctions persist during early stages of germline differentiation and that gap junctions occur within a germline cyst.

Inx2 is required for the subcellular localization of Zpg

The expected mode of innexin function would predict coupling between innexins in the soma and the germ line. If *Inx2* was indeed the main somatic innexin and it coupled to Zpg on the surface of germ cells, then *Inx2* knockdown could affect distribution of Zpg. This prediction was verified directly by knocking down *Inx2* in the

soma and staining for Zpg. Quantification of the relative enrichment of Zpg at soma-germline and germline-germline interfaces demonstrated that in wild-type testes (Fig. 7A) there is 3.5 ± 0.2 -fold enrichment of Zpg at soma-germline interfaces and a 1.5 ± 0.1 -fold enrichment of Zpg at germline-germline interfaces, compared with background staining (Fig. 7C). Upon knockdown of *Inx2* in the soma, Zpg redistributed from the soma-germline to the germline-germline interface (Fig. 7B), with an approximately 1.2 ± 0.1 -fold enrichment of Zpg at soma-germline interfaces and a 3.7 ± 0.1 -fold enrichment of Zpg at germline-germline interfaces, compared with background cytoplasmic staining (Fig. 7C). By comparison, a disruption in the subcellular localization of Zpg was not observed

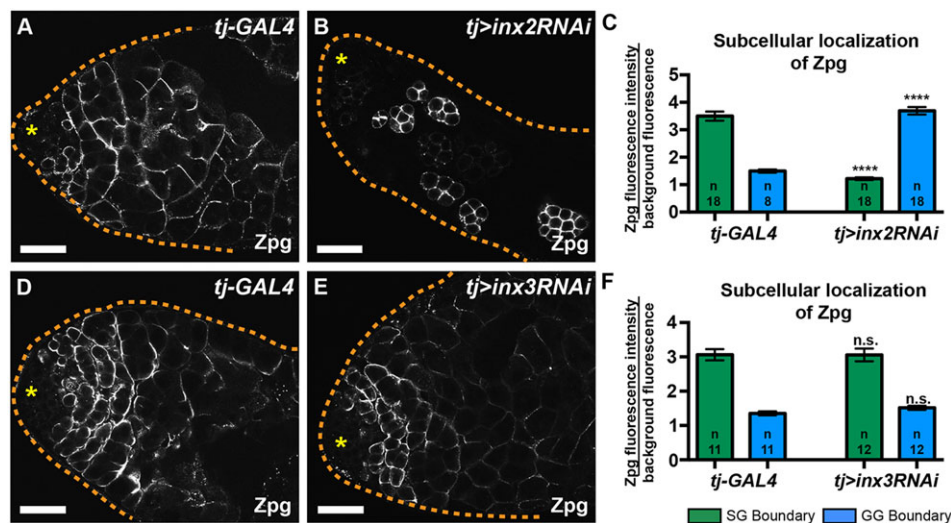


Fig. 7. *Inx2* is required in the soma for the subcellular localization of Zpg. (A, B) Control (*tj-GAL4*) and *tj>inx2RNAi* testes labelled with anti-Zpg antibody. (A) Zpg localized predominantly to soma-germline boundaries. (B) Following *Inx2* somatic knockdown, Zpg was enriched at germline-germline boundaries. (C) Quantification of Zpg enrichment based on normalized fluorescence intensity. Zpg was recruited to soma-germline boundaries at approximately three times greater levels than to germline-germline boundaries in control testes; this recruitment was disrupted following *Inx2* knockdown and Zpg became enriched at germline-germline boundaries compared with controls. (D-F) Analysis of Zpg recruitment upon somatic *Inx3* knockdown. *Inx3* knockdown did not affect the subcellular localization of Zpg compared with controls. *n*, number of testes quantified. Error bars show mean \pm s.e.m.; *****P* < 0.0001. Significance indicates differences between either soma-germline boundaries between samples, or germline-germline boundaries between samples, respectively. Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 30 µm.

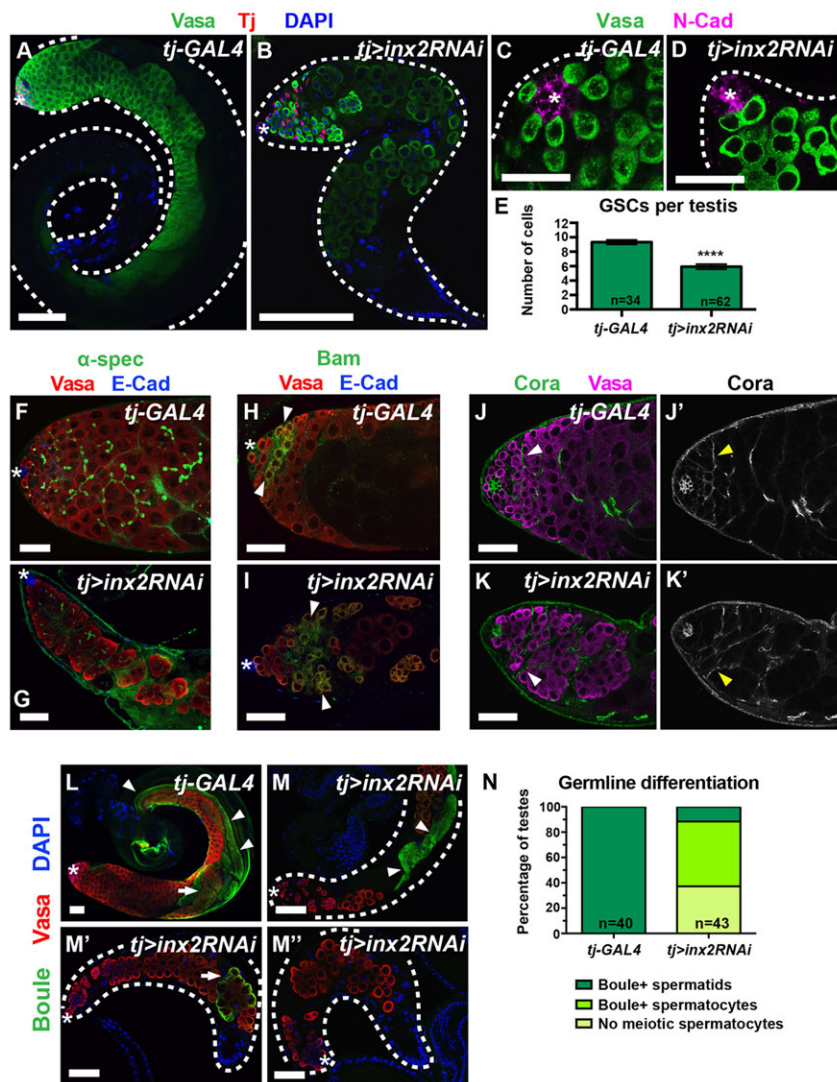


Fig. 8. Inx2 is required in the soma for GSC maintenance and germline differentiation.

(A,B) Control (*tj-GAL4*) and *tj>inx2RNAi* testes labelled for Vasa (green), Tj (red) and DAPI (blue). Somatic Inx2 knockdown resulted in small rudimentary testes. (C,D) Close-up of the apical tip of control and *tj>inx2RNAi* testes (hub highlighted with N-Cad in magenta, Vasa in green). (E) GSC number was significantly reduced upon Inx2 knockdown in the soma compared with controls. (F,G) Labelling with the spectrosome/fusome marker α -spectrin (green) revealed mostly normal fusomes in *tj>inx2RNAi* and control testes (Vasa in red; E-Cad in blue). (H,I) Bam expression (green) was similar in control and *tj>inx2RNAi* testes (arrowheads; Vasa in red; E-Cad in blue). (J-K') Encystment, marked by labelling for Cora (green; Vasa in magenta) appeared largely normal in *tj>inx2RNAi* testes. Arrowheads indicated encystment, detected by Cora staining. (L) In wild-type testes, meiotic spermatid stages were marked by expression of Boule protein (green; Vasa in red; DAPI in blue). (M-M'') Examples of the phenotypic range observed in *tj>inx2RNAi* testes arranged from the least (M) to most (M'') severe. (L-M'') Arrowheads indicate Boule⁺ spermatids and arrows indicate Boule⁺ spermatocytes. (N) Quantification of the effect of Inx2 knockdown on germline differentiation, showing the percentage of testes observed in each phenotypic class in control and *tj>inx2RNAi* testes. Nearly one-third of *tj>inx2RNAi* testes exhibited no Boule expression. *n*, number of testes. Error bars show mean \pm s.e.m.; *****P* < 0.0001. Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 100 μ m in A-B, L-M; 30 μ m in all other panels.

upon knockdown of Inx3 (Fig. 7D-F). This shows that Inx2 is required in the soma to maintain the distribution of Zpg at the soma-germline interface, consistent with a coupling of somatic Inx2 with germline Zpg. Moreover, as Zpg is the only fly innexin known to be expressed in the germ line (Bohrmann and Zimmermann, 2008; Stebbings et al., 2002), these results suggest a possible homotypic coupling of Zpg at germline-germline boundaries that competes with the heterotypic coupling of Inx2-Zpg at soma-germline boundaries.

Inx2 function in the soma is required for GSC maintenance and germline differentiation

Our data thus far suggested that germline signals travel through Zpg-Inx2 gap junctions to regulate somatic differentiation and proliferation and vice versa. If this were the case, somatic knockdown of Inx2 should resemble the *zpg* mutant phenotype; indeed the small, rudimentary testes we observed following somatic Inx2 knockdown closely mirrored the *zpg* phenotype (Fig. 8A,B). Because the *inx2* locus is located on the X chromosome, clonal analysis of *inx2* mutants in the testis proved to be exceptionally difficult. To draw further comparisons between Zpg and somatic Inx2 knockdown, we extended our Inx2 analysis using the RNAi line that gave the strongest and most penetrant phenotypes. RNAi-mediated Inx2 knockdown in the soma using this line strongly

reduced Inx2 protein levels in both the testis and the ovary (supplementary material Fig. S2H,I; Fig. S3A,B).

Previous work has suggested that innexins may affect cadherin-mediated cell-cell adhesion (Bauer et al., 2006; Giuliani et al., 2013; Lehmann et al., 2006). To determine if disruption of gap junctions altered levels of E-Cad (Shg – FlyBase) in the testes, we stained *zpg* and *inx2RNAi* testes for E-Cad. We did not observe differences between *zpg*, *inx2RNAi* and control testes (supplementary material Fig. S4A-D).

Furthermore, we used clonal over-expression of a wild-type *zpg* transgene in the ovary follicular epithelium to investigate whether Zpg could modify cell-cell adhesion, using Armadillo (β -catenin) expression as a marker. Again, we did not detect changes in *zpg* over-expression clones, nor did cell-cell adhesion appear to be altered (supplementary material Fig. S4E,F).

In addition to having similar morphology, Inx2 knockdown testes resembled *zpg* mutants in several other regards. First, Inx2 knockdown reduced GSC numbers, consistent with the GSC maintenance defects observed in *zpg* mutants (an average of 5.9 ± 0.3 per knockdown testis, *n* = 62 versus 9.6 ± 0.3 per control testis, *n* = 16; Fig. 8C-E). Second, somatic knockdown of Inx2 partially blocked germline differentiation (Fig. 8F-N). Although most germline cysts developed a fusome (Fig. 8F,G) almost half did

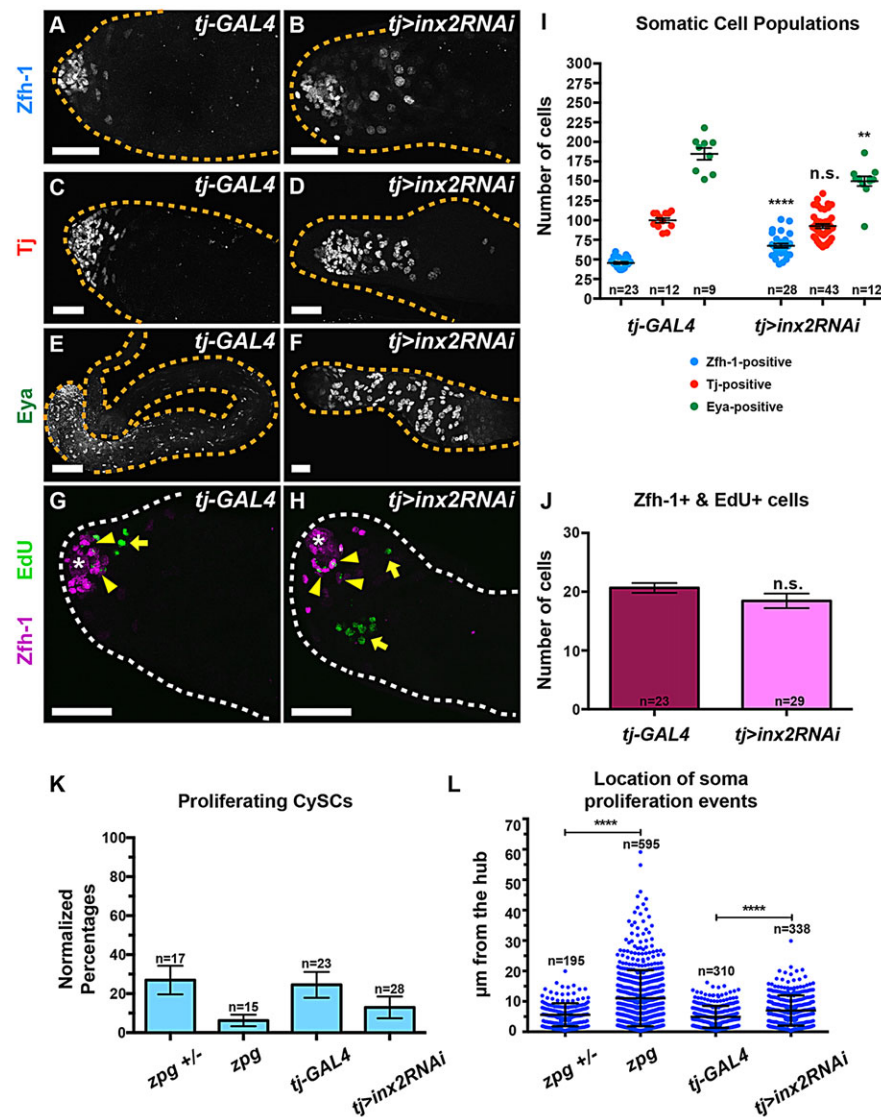


Fig. 9. Inx2 is required to regulate the proliferation and differentiation of the early soma.

(A–F) Expression of the stage-specific somatic cell markers Zfh-1 (A,B), Tj (C,D) and Eya (E,F) in control (*tj-GAL4*) and *tj>inx2RNAi* testes. (G,H) S-phase somatic cells are labelled for Zfh-1 (magenta) and EdU (green) in control and *tj>inx2RNAi* testes. Arrowheads indicate proliferating CySCs, arrows indicate germ line. (I) Quantification of Zfh-1⁺, Tj⁺ and Eya⁺ cells in control and *tj>inx2RNAi* testes. (J) Quantification of Zfh-1⁺/EdU⁺ cells. (K) Percentage of proliferative CySCs in wild-type (*zpg*^{+/−}), *zpg*, control and *tj>inx2RNAi* testes normalized to total number of Zfh-1⁺ cells per testis. (L) Quantification of distance of somatic proliferation events from the hub in wild-type, *zpg*, control and *tj>inx2RNAi* testes. *n*, number of testes quantified in I–K; single Zfh-1⁺/EdU⁺ cells in L. Error bars show mean±s.e.m.; ***P*<0.01; *****P*<0.0001. Asterisks indicate the hub. Dashed lines outline the testis. Scale bars: 30 μm in A–D,F–H; 100 μm in E.

not reach meiotic stages and late-spermatid stage cysts were observed in only 10% of testes (identified by Boule protein staining; Cheng et al., 1998; Fig. 8L–N). However, Bam expression was similar to that observed in wild type (Fig. 8H,I). Furthermore, encapsulation, as judged by staining for the somatic marker Cora, was largely normal (Fig. 8J,K). This is consistent with the Inx2 knockdown phenotype being less severe than the *zpg* phenotype in some aspects (compare Fig. 8J,K with Fig. 2E,F). Nonetheless, testes at the severe end of the phenotypic spectrum greatly resembled *zpg* mutant testes (Fig. 8M). The weaker phenotype may reflect the limitations of RNAi or a partial redundancy with Inx3, which is also expressed in the soma. To test for this latter option, a double RNAi-knockdown experiment targeting both Inx2 and Inx3 was performed (supplementary material Fig. S3C–H). Knockdown of Inx3 alone in the soma did not disrupt spermatogenesis, despite reducing Inx3 protein levels, determined by antibody staining (supplementary material Fig. S3C–D). Knockdown of Inx2 disrupted localization of Inx3, reduced protein expression levels of Inx2 and disrupted spermatogenesis (supplementary material Fig. S3E). Simultaneous knockdown of Inx2 and Inx3 phenocopied the Inx2 knockdown phenotype (supplementary material Fig. S3F–I). These results suggested that

Inx3 is dispensable in the early soma for spermatogenesis, that Inx3 may require Inx2 for its localization and that Inx2 is required to regulate the proliferation and differentiation of the early soma.

Because the *zpg* mutant germline phenotypes resembled those observed upon Inx2 somatic knockdown, we analysed somatic differentiation and proliferation in Inx2 knockdown testes. Similar to *zpg*, somatic Inx2 knockdown gave rise to an increase in early somatic cells and a decrease in late somatic cells (Fig. 9I). The number of Zfh-1⁺ cells compared with controls was 48% higher (67.5±2.9 per testes, *n*=30 versus 45.5±1.4, *n*=23, respectively; Fig. 9A,B,I). The number of Tj⁺ cells compared with control was slightly lower, although not statistically significant (99.8±3.0 per testes, *n*=12 versus 92.6±2.7, *n*=43; Fig. 9C,D,I). Finally, the number of late somatic Eya⁺ cells was decreased in Inx2 knockdown testes compared with controls by 19% (149.8±6.2 per testes, *n*=13 versus 184.8±7.5, *n*=9, respectively; Fig. 9E,F,I). Thus, similar to *zpg*, somatic knockdown of Inx2 shows an increase, albeit smaller, in early somatic cells and a significant decrease in late somatic cells compared with wild type. These results show that Inx2 acts to promote differentiation of the soma. To determine whether somatic Inx2 knockdown resulted in aberrant proliferation of the early soma, as observed in *zpg* mutants, the number of proliferating early somatic

cells was quantified by co-labelling for EdU and Zfh-1. In control testes, an average of 20.7 ± 0.8 Zfh-1⁺/EdU⁺ cells were detected ($n=23$; Fig. 9G,J). In Inx2 knockdown testes, an average of 18.5 ± 1.3 Zfh-1⁺/EdU⁺ cells were observed ($n=30$; Fig. 9H,J). Taken by itself, this result appeared to suggest that early somatic proliferation was not misregulated in Inx2 somatic knockdown testes. However, because the Zfh-1 population is in general larger in Inx2 knockdown testes compared with controls, the actual proportion of proliferating early somatic cells following Inx2 knockdown was in fact much lower than in controls. In addition, it was suspected that non-CySCs might be proliferating upon somatic knockdown of Inx2. To correct for this and ensure that only CySC proliferation was being assayed, the total number of Zfh-1⁺ cells per testis was quantified in addition to Zfh-1⁺/EdU⁺ cells that were less than one cell-length from the hub. We have previously used this method to gain insight into patterns of CySC proliferation (Fairchild et al., 2015). This analysis revealed that in control testes, 25–27% of CySCs were labelled for EdU. In Inx2 somatic knockdown and *zpg* mutant testes, only 13% and 7% of CySCs were labelled, respectively (Fig. 9K). This result suggested that CySC proliferation is disrupted upon somatic Inx2 knockdown, similar to observations in *zpg* mutant testes.

It was further noted that in Inx2 knockdown testes, cells proliferated further away from the hub, similar to observations in *zpg* mutant testes (Fig. 4G, Fig. 9L). The average distance from the hub of Zfh-1⁺/EdU⁺ cells was determined. Zfh-1⁺/EdU⁺ cells were detected within $4.9 \mu\text{m} \pm 0.2$ ($n=310$) of the hub in control testes, whereas upon knockdown of Inx2, the average distance of Zfh-1⁺/EdU⁺ cells from the hub grew by 43% to $7.0 \mu\text{m} \pm 0.3$ ($n=338$). This was comparable to *zpg* mutant testes, where proliferation events occurred on average 97% further from the hub, or $11.0 \mu\text{m} \pm 0.4$ ($n=595$), relative to wild-type controls.

Taken together, these results show substantial overlap between Inx2 somatic knockdown and *zpg* mutant phenotypes. These results suggest that gap junction signalling between the germ line and soma is required to regulate the soma by controlling CySC proliferation rates, by limiting proliferation to near the hub cell niche, and by promoting differentiation of the soma.

DISCUSSION

Gap junctions link the soma and germ line

The work presented here demonstrates that gap junctions between the soma and germ line are essential for fly spermatogenesis. Previous work showing an essential role for Zpg in fly gonads raised the possibility that signals either from the soma or from other germ cells travel through gap junctions to regulate germline survival and differentiation (Tazuke et al., 2002). Subsequent work in fly ovaries showed that Zpg was also required for GSC maintenance (Gilboa et al., 2003). Our analysis supports and extends these conclusions by finding a cell-autonomous requirement for Zpg in GSC maintenance in the fly testis and demonstrates a role for Inx2 in the soma. Furthermore, we find that gap junction-mediated signals from the germ line also play unique and essential roles in the soma during spermatogenesis, independent of general germline defects. In particular, gap junctions are required to control the proliferation of CySCs and promote the differentiation of their daughters. Our work illustrates that the main type of gap junction between the soma and the germ line in the fly testis is a heterotypic channel coupling Inx2 in the soma and Zpg in the germ line. Importantly, disrupting gap junctions in the soma by knocking down Inx2 phenocopies the *zpg* mutant phenotype in both the germ line and soma. Therefore, gap junction-mediated soma-germline regulation in the testis is bi-directional.

Gap junctions contribute to stem cell regulation in the testis

Recent work has highlighted the importance of gap junctions in stem cell regulation in a number of systems (Foss et al., 2009; Spéder and Brand, 2014; Starich et al., 2014; Wong et al., 2008). In line with results from other stem cell models, our data illustrates a specific role for gap junctions in both GSCs and CySCs. The role of gap junctions in stem cell regulation in the testes was illustrated by the requirement for Zpg in the germ line and Inx2 in the soma for GSC maintenance. Moreover, loss of Zpg or somatic knockdown of Inx2 also affected CySC proliferation. Furthermore, ultrastructural analysis revealed the presence of gap junctions between GSCs and CySCs. These results, as a whole, suggest a requirement for gap junction-mediated soma-germline communication in both stem cell populations and at the earliest stages of sperm differentiation.

Gap junctions facilitate signalling between the soma and germ line

Following the stem cell stage, strong expression and colocalization of Zpg and Inx2 was consistently detected starting at the 4-cell cyst stage. Expression of Zpg and Inx2 began to diminish after the early spermatocyte stages and was not detected past meiotic stages. The timing at which Inx2 and Zpg expression were most prominent corresponds to a period during which niche signals such as BMP are lost (Kawase et al., 2004; Shivdasani and Ingham, 2003). Loss of these signals causes the germ line to undergo rapid differentiation and specialization (de Cuevas and Matunis, 2011; Fuller, 1993). Recently, we have shown that as somatic cells move away from the niche and begin differentiating, the soma forms a permeability barrier around the germ line, isolating the germ line from the outside environment (Fairchild et al., 2015). This transition corresponds with a switch whereby soma-germline communication shifts from predominantly exocrine to juxtacrine signalling. Thus, as the germ line becomes increasingly isolated, it becomes more dependent on differentiation signals that arrive via gap junctions from the soma. Once the germ line becomes isolated, gap junctions may also play an important nutritive role and permit the movement of essential small metabolites between the germ line and soma. Similarly, the soma requires gap junction-mediated signals to allow it to accommodate the increasingly expanded, differentiated and specialized germ line.

Our observations that gap junctions regulate germline differentiation and soma proliferation are in line with studies from both vertebrate and invertebrate models. In *Caenorhabditis elegans*, it was recently shown that gap junction-mediated signals are required to maintain GSCs in the niche and for germline differentiation (Starich et al., 2014). Similarly, work in vertebrates has shown that loss of gap junction-mediated signalling in the soma increased proliferation in post-mitotic Sertoli cells (Gilleron et al., 2009; Sridharan et al., 2007a,b). It is therefore likely that an early role for gap junctions in coordinating soma-germline differentiation is an evolutionarily conserved mechanism. One recurring feature of soma-germline gap junctions is the expression of different gap junction proteins, resulting in heterotypic gap junctions (Günther et al., 2013; Starich et al., 2014), exemplified by the Inx2-Zpg gap junctions we observe in flies. A key problem in understanding the role of gap junctions in mediating soma-germline communication is identifying the transported signalling cargos. Some possible signals are cAMP, Ca²⁺ and cGMP (Bauer et al., 2005; Hervé and Derangeon, 2013), which have been implicated in regulating meiosis in the germ line (Conti et al., 2012; Von Stetina and Orr-Weaver, 2011). Our attempts to study cAMP and Ca²⁺ in the testis have proven inconclusive (C.M.S. and G.T., unpublished data).

However, recent work in *Drosophila* ovaries has suggested that somatic gap junctions may play roles in regulating pH, membrane potential and ion transport (Krüger and Bohrmann, 2015). Overall, multiple signals are probably exchanged between the soma and germ line through gap junctions and elucidating their respective functions is a complex task that should be further studied.

Based on the results presented here and on previous studies, we propose the following model: GSCs receive multiple cues that control their behaviour, with gap junctions mostly providing a supporting role, allowing the passage of cues from the soma that facilitate long-term GSC maintenance. After stem cell division, the germ line undergoes rapid differentiation, becoming increasingly isolated from the outside environment, and a permeability barrier is formed by the soma. As outside signals from the niche are lost, the germ line relies more heavily on gap junctions to allow the passage of small molecules and metabolites from the soma to promote differentiation and provide nourishment. To ensure coordinated growth and differentiation of the soma and germ line, signals pass from the germ line through the gap junctions into the soma. The work presented here defines gap junction-mediated juxtacrine signalling as an additional signalling mechanism in the fly testis. Furthermore, our study provides a clear illustration of the bi-directional regulatory action of soma-germline gap junctions. As we demonstrate, disrupting innexins in the soma or germ line leads to a specific regulatory effect in the other tissue. Therefore, bi-directional gap junction-mediated signalling plays a vital role in ensuring proper coordination of the soma and germ line during spermatogenesis.

MATERIALS AND METHODS

Fly stocks and genetics

For details on fly stocks used and mutant genotype generation please see Supplementary Materials and Methods.

Antibody generation

Polyclonal antibodies were raised in rabbits (GenScript) to peptides corresponding to the C-terminal amino acids (345–367, RKLLEELYEA-QLIKIPPADKI) of Zpg. A peptide corresponding to amino acids 348–367 of Inx2 (DLSREMSGDEHSAHKRPFDA) was injected into guinea pigs (GenScript). Polyclonal antisera were affinity-purified for Zpg and Inx2, and determined to be epitope-specific by ELISA.

Immunostaining

Flies <1DPE were dissected in phosphate buffered saline (PBS) pH 7.4, fixed in 4% paraformaldehyde in PBS, rinsed in 0.2% Tween20, and washed and incubated in 0.3% Triton X-100 in PBS with 0.5% BSA. For a list of primary antibodies used please see Supplementary Materials and Methods. Bam stainings were supplemented with additional Bam over three days, followed by a one-day wash, before addition of secondary antibodies. For Stat stainings, samples were incubated with primary antibodies overnight at room temperature (M. Amoyel and E. Bach, personal communication). Alexa Fluor 488-, Cy3- and Cy5-conjugated secondary antibodies were used at 1:500 (Molecular Probes).

Imaging and analysis

All analyses were performed on males <1DPE, unless otherwise stated. GSCs were defined as Vasa⁺ or Tj⁺ cells contacting the hub. CySCs were defined as Zfh-1⁺ or Tj⁺ cells <10 µm from the hub. Somatic cells in S phase were labelled by vivisectioning testes in Testis Buffer (TB) (White-Cooper, 2004) and culturing for 30 min with EdU in TB prior to fixation and staining using a Click-iT Kit (Life Technologies). Distance of proliferation events from the hub was measured as the linear distance from the hub edge to the nearest Zfh-1⁺/EdU⁺ nuclei edge. Images were acquired on an Olympus FV1000 inverted confocal microscope using an UplanSApo 20x0.75, an UplanFL N 40x, 1.30NA oil objective, and an UplanSApo 60x, 1.35 NA oil objective. Image analysis was performed in Olympus Fluoview (Ver.1.7c)

unless otherwise stated. Images were processed using Adobe Photoshop CS4 (version 11.0.2).

Zpg subcellular localization measurements

Fluorescence-intensity measurements were performed in ImageJ (NIH) on confocal images of germline cysts, using the line tool. Soma-germline boundaries were defined as borders where Zpg colocalized with Armadillo. Background intensity was measured in the cytoplasm of germline cysts. In *tj>inx2RNAi* testes, the soma-germline boundary was defined as a cell boundary without an adjacent germ cell (based on Zpg staining). A minimum of seven measurements were made from soma-germline and germline-germline boundaries, and cytoplasm from multiple cysts within each testis and averaged.

Electron microscopy

Testes were dissected in PBS and fixed for 2 h in fixative [1.5% paraformaldehyde (Sigma-Aldrich), 0.1 M sodium cacodylate (Electron Microscopy Sciences), 1.5% glutaraldehyde (Electron Microscopy Sciences), pH 7] at room temperature. Samples were washed three times for 10 min with 0.1 M sodium cacodylate (pH 7.3) and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M Na cacodylate on ice. Samples were washed three times for 10 min with ddH₂O, stained for 1 h 'en bloc' with 1% aqueous uranyl acetate, washed three times for 10 min with ddH₂O, then dehydrated through an ascending concentration ethanol series, ending with three changes of 100% ethanol for 10 min each. Dehydration was followed by two changes of 100% propylene oxide for 15 min each. Samples were placed in a 1:1 mixture of propylene oxide:EMBED 812 Resin (Electron Microscopy Sciences) overnight. Testes were embedded in 100% EMBED 812 Resin and polymerized for 48 h at 60°C. Thin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems), placed on 200 mesh copper grids (Electron Microscopy Sciences) and stained with uranyl acetate and lead citrate. Sections were imaged on a Tecnai G2 Spirit electron microscope (FEI North America NanoPort) operated at 120 kV.

Statistics

The mean and standard error of the mean (s.e.m.) are shown. Prism (GraphPad) was used to test significance using unpaired *t*-tests with Welch's correction. *P*-values indicated are ***P*<0.01, *****P*<0.0001.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

G.T. conceived of the initial project and supervised the project. G.T. and C.M.S. designed the experiments. C.M.S. performed all experiments. A.M. designed the Zpg and Inx2 antibodies and performed all the molecular biology work. A.W.V. performed the electron microscopy and analysis. C.M.S., A.W.V. and G.T. interpreted data and prepared the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.123448/-/DC1>

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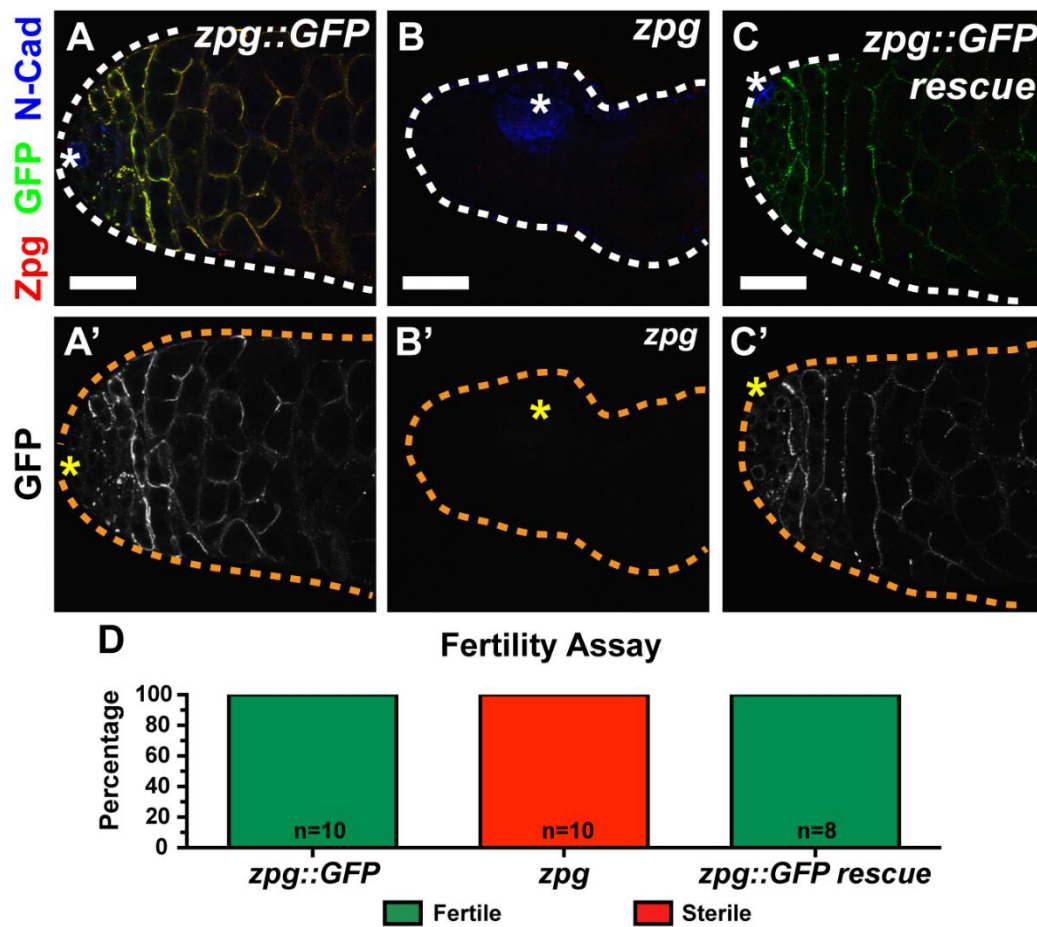
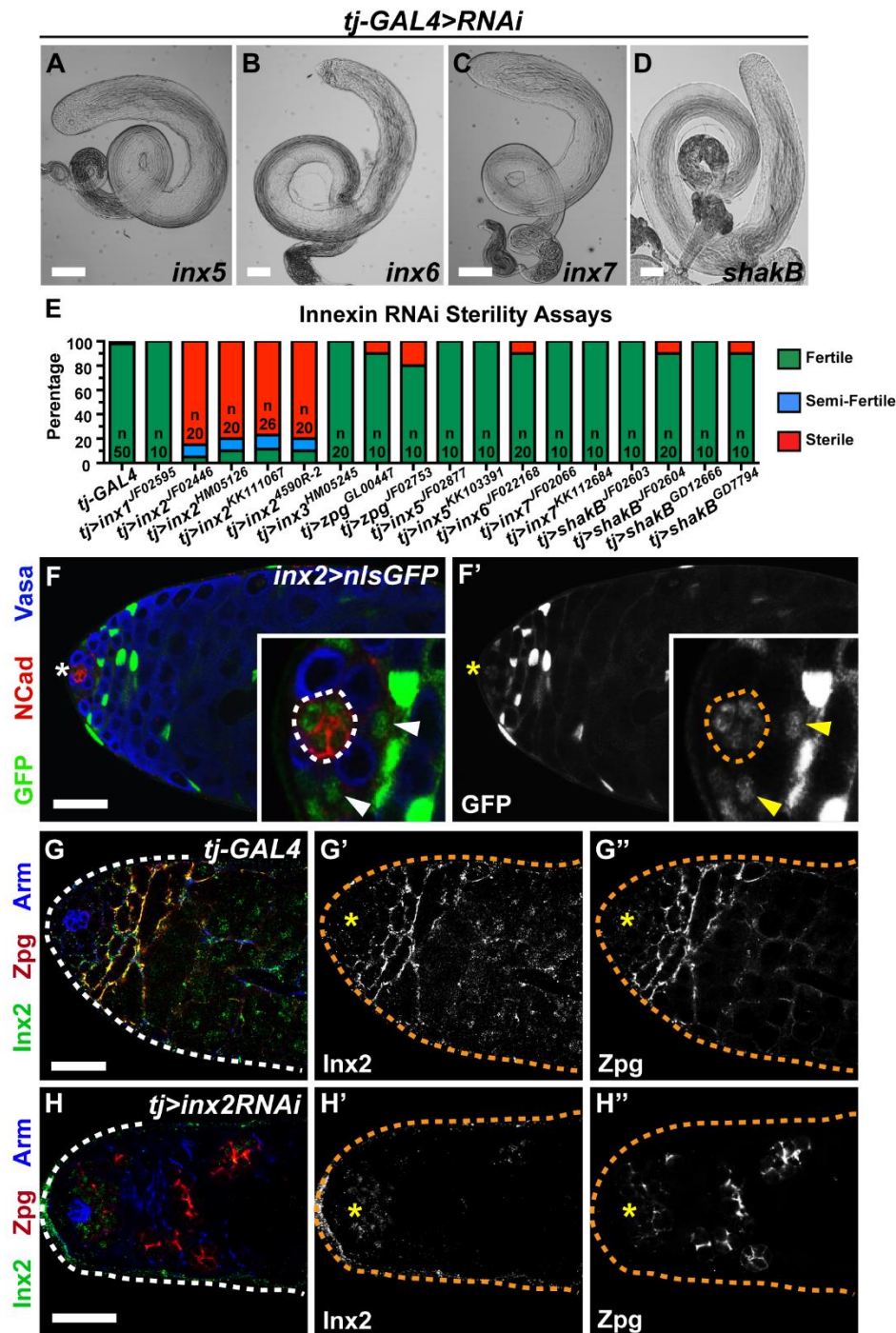


Figure S1 – A GFP-tagged, wild-type *zpg* transgene rescues fertility in *zpg* mutant testes.

(A-C) Expression of a GFP-tagged *zpg* transgene under the putative endogenous promoter of *zpg* co-localized with endogenous Zpg (A). The *zpg::GFP* transgene (C) was able to rescue the *zpg* mutant (B) phenotype, observed by immunohistochemistry. (D) Fertility assays demonstrate that the *zpg::GFP* transgene restored fertility in *zpg* mutant males to wild-type levels. Testes were stained with antibodies to detect Zpg (red), GFP (green), and N-Cad (blue). Asterisk indicates the hub. Dashed lines outline the testis. Scale bars are 30µm in all panels.



Summary of fertility assays carried out with various RNAi lines targeting every fly innexin driven by *tj*-GAL4 in the soma (n=number of independent fertility assays). *Inx2* was the only innexin that was consistently required for fertility. (F-F') Expression of nls-GFP under the control of the *inx2* promoter. Reporter expression can be detected weakly in hub cells and CySCs, before increasing in early somatic cells. Arrowheads indicate CySCs. (G) control and (H) *tj>inx2RNAi* testes that were stained with antibodies to detect *Inx2* (green) and *Zpg* (red) proteins show that RNAi mediated knockdown substantially reduced *Inx2* expression (Armadillo in blue was used to highlight the soma and the hub). Asterisk indicates the hub. Dashed lines outline the hub in F'F', and the testis in G-H. Scale bars A-D are 100µm and 30µm in all other panels.

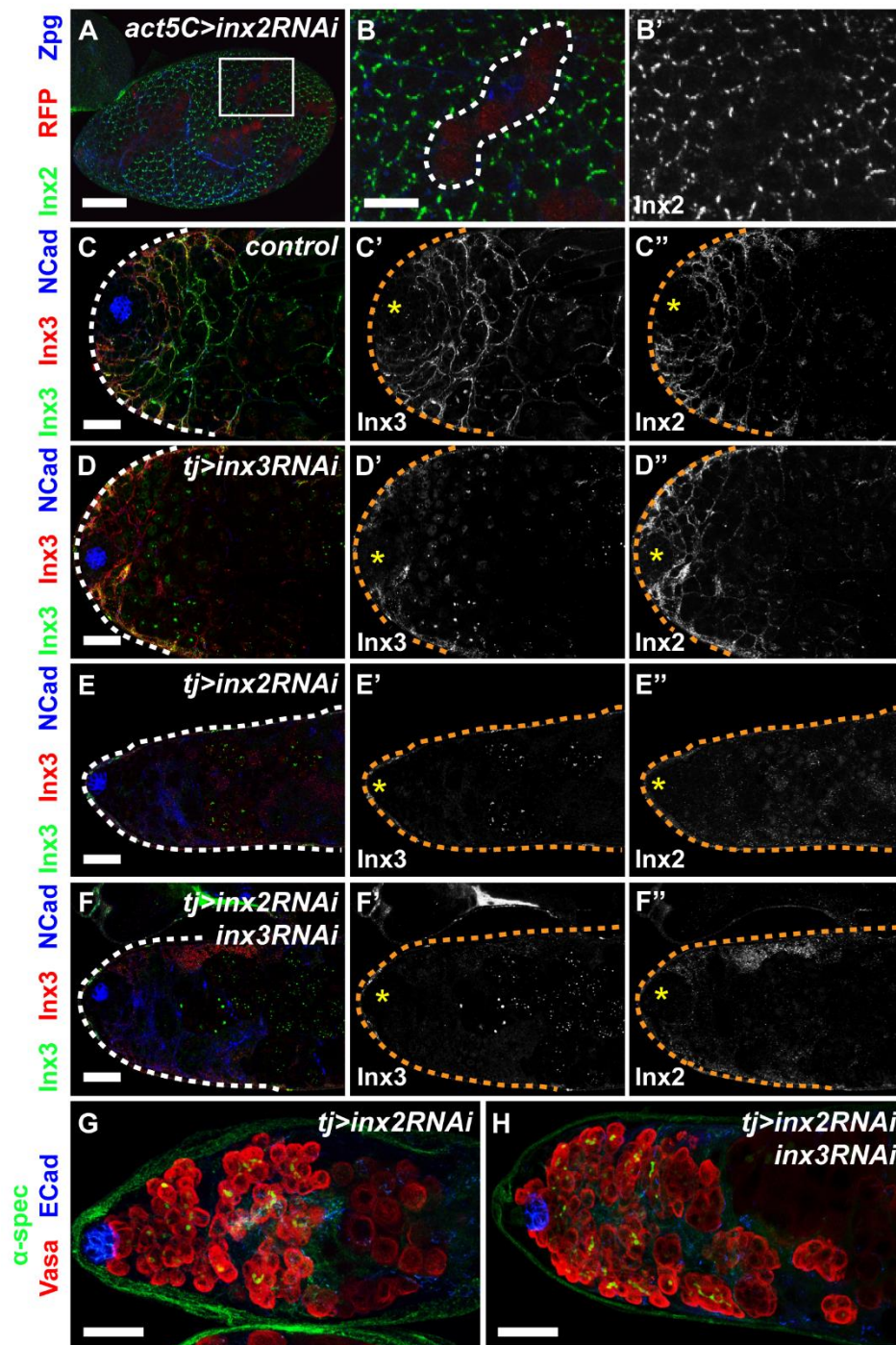


Figure S3 – Inx3 is not required in the soma for spermatogenesis, but Inx2 is required in the soma for the localization of Inx3. (A-B) Clonal overexpression of *inx2RNAi* in developing egg chambers. At 2 days post-clone induction, Inx2 protein levels were greatly reduced in clones, compared to neighbouring cells based on Inx2 antibody staining. (C-F)

Control, *inx3RNAi*, *inx2RNAi*, and *inx2RNAi+inx3RNAi* testes stained for Inx3 (green), Inx2 (red), and N-Cad (blue). (C) Inx3 and Inx2 colocalized in control testes. (D) Upon knockdown of Inx3, Inx3 staining was reduced, but Inx2 remained similar to controls. (E) Somatic knockdown of Inx2 disrupted Inx3 localization, in addition to reducing Inx2 protein levels. (F) Knockdown of both Inx2 and Inx3 in the soma greatly decreased levels of both Inx2 and Inx3. (H) *tj>inx2RNAi* and *tj>inx2RNAi, inx3RNAi* testes show that double-knockdown of Inx2 and Inx3 in the soma results in a phenotype similar to that obtained from knockdown of Inx2 alone (Vasa in red; E-Cad in blue; α -spectrin in green). Asterisk indicates the hub. Dashed lines outline the clone in B, the testis in C-F. Scale bars are 30 μ m in all panels.

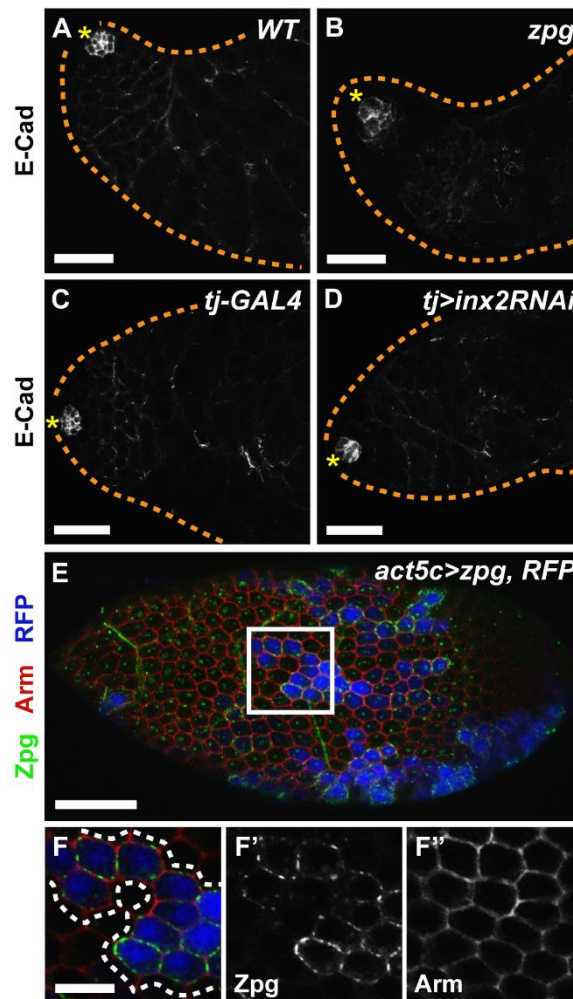


Figure S4 – Cadherin expression is not misregulated upon changes in *zpg* or *inx2*

expression. (A-B) Wild-type and *zpg* testes stained for E-Cad. In both wild-type (A) and *zpg* testes (B), E-Cad was enriched in the hub and could be detected in differentiating germline cysts. (C-D) In control testes and upon somatic knockdown of *Inx2*, E-Cad staining showed a similar staining pattern, with enrichment in the hub, and lower levels of expression outside of the niche. (E) Overview of a stage 9 developing egg chamber. A heat-shock-inducible *act5c-GAL4* drives expression of RFP (blue) and a *UAS^t-zpg^{cDNA}* transgene (green). Armadillo (red) labels cell-cell junctions. (F) Inset of clone from E. Expression of Zpg (F') does not visibly alter levels of Armadillo within clones (F''). Asterisk indicates the hub. Dashed lines outline the testis in A-D, the clone in F. Scale bars are 30µm in A-E, 10µm in F.

Fly Stocks

The following lines were used: the somatic drivers *tj-Gal4* and *c587-Gal4*; the germline driver *nos-Gal4-VP16*; *zpg^{z-2533},st/TM3,Sb*; *zpg^{z-5352}/TM6B*; *hs-flp/FM7;FRT2a/TM3,Ser*; *His2a::RFP/TM3,Ser*; *UAS-mCD8::GFP*; *w¹¹¹⁸*; *tud¹/CyO*, *tud^{B42}/CyO*, and *tud^{B45}/CyO*, (Arkov et al., 2006); *UAS-*tkv^{Act}**, *UAS-*sax^{Act}**/CyO (Haerry et al., 1998); *hs-flp*; +; *Dr/TM3,Sb*; *w*; +; *act5c-GAL4-FRT-stop-FRT-RFP/TM3, Sb*; *UAS-anti-*inx1* RNAi JF02595*; *UAS-anti-*inx2* RNAi JF02446*; *UAS-anti-*inx2* RNAi HM05126*; *UAS-anti-*inx2* RNAi KK111067*; *UAS-anti-*inx2* RNAi 4590R-2*; *UAS-anti-*inx3* RNAi HM05245*; *UAS-anti-*zpg* RNAi GL00447*; *UAS-anti-*zpg* RNAi JF02753*; *UAS-anti-*inx5* RNAi JF02877*; *UAS-anti-*inx6* RNAi JF02168*; *UAS-anti-*inx7* RNAi JF02066*; *UAS-anti-*inx7* RNAi KK112684*; *UAS-anti-*shakB* RNAi JF02603*; *UAS-anti-*shakB* RNAi JF02604*; *UAS-anti-*shakB* RNAi GD12666*; and *UAS-anti-*shakB* RNAi GD7794*. The *innexin2* enhancer trap line, *inx2>nlsGFP* P01999, was obtained from the Cooley lab (Quiñones-Coello et al., 2007).

Genetics

Crosses were set up at room temperature, flipped each day, and raised at 25°C on standard media. To generate *zpg* flies, *zpg^{z-2533}/TM3,Sb* flies were crossed to *zpg^{z-5352}/TM6B* flies; heterozygous siblings were used as a control. *tudor* flies were generated using *tud^{B42}/CyO* or *tud^{B45}/CyO* crossed to *tud¹/CyO*, which were then crossed to *w¹¹¹⁸* males to generate germ cell-less flies. *CA-BMP* flies were generated by crossing *nos-Gal4::VP16* flies to *UAS-*tkv^{Act}**, *UAS-*sax^{Act}**/CyO flies. RNAi knockdowns, were performed using *UAS-Dcr2*; *tj-Gal4/CyO* crossed to the corresponding UAS-anti-innexin-RNAi line. For *inx2RNAi* phenotypic analysis, *UAS-JF02446* was used as it provided the strongest knockdown based on testis morphology and sterility assays. Controls were *UAS-Dcr2*; *tj-Gal4/CyO* males. Clones were *hs-flp*, *c587-GAL44>UAS-mCD8::GFP*; +; *His2a::RFP*, *FRT2a/TM3,Ser* crossed to *w*; +; *FRT2a* for control clones and to *w*; +; *zpg^{z-5352},FRT2a/TM6B* for mutant clones. Progeny were raised at 25°C, clones were induced in males less than 1 day post-eclosion (DPE), using two 60-minute heat shocks at 37°, 90-minutes apart, and aged for 4-5 days at room temperature. Clones in

the ovary follicular epithelium were *hs-flp; +; act5c-GAL4-FRT-stop-FRT-GAL4-RFP/TM3, Sb* crossed to *UAS-JF02446* for the *Inx2* knockdown experiment and *w; UAS-zpg^{CDNA}* for the *Zpg* over-expression experiment. Clones were induced in females <1DPE as above and aged for three days prior to dissection.

Fertility Assays

Fertility assays were performed using single males, 5DPE, crossed to three *w¹¹¹⁸* virgin females, aged 15 days, and scored as sterile if no larvae/pupae were present, and semi-sterile if <50 larvae/pupae were present.

Immunostaining

Primary antibodies used were rabbit-anti-Vasa (R. Lehmann, P. Lasko, 1:5000), guinea-pig-anti-Traffic jam (D. Godt, 1:3500), rabbit-anti-Zfh-1 (R. Lehmann, 1:1000), guinea pig-anti-Zfh-1 (J. Skeath, 1:500), rabbit-anti-Stat92E (E. Bach, 1:500), rabbit-anti-*Inx3* (J. Davies, 1:1000), rabbit-anti-Boule (S. Wasserman 1:1000), mouse-anti-Cheerio/Filamin (L. Cooley, 1:1000), rabbit-anti-*Zpg* (1:20000), guinea pig-anti-*Inx2* (1:1000), rat-anti-DN-Cadherin (Developmental Studies Hybridoma Bank (DSHB), 1:50), rat-anti-DE-Cadherin (DSHB, 1:50), mouse-anti-spectrin (DSHB, 3A9, 1:5), mouse-anti-Bam (DSHB and D. McKearin, Bam, 1:50), mouse-anti-Coracle (DSHB, C566.9 and C615.16 1:500), mouse-anti-Armadillo (DSHB, N2 7A1, 1:1000), mouse-anti-Eya (DSHB, eya10H6, 1:500), mouse-anti-GFP (Invitrogen, A11120, 1:1000), and rat-anti-dsRed (Chromotek, 5f8, 1:1000).

Molecular Biology

A 6.15kb rescue fragment, encompassing the *zpg* genomic locus and an additional 1.5kb upstream and downstream of the locus (Tazuke *et al.*, 2002) was cloned into a pattB using *Bam*HI. A short linker sequence (LAAA) was inserted after the last amino acid of *Zpg* (Arai *et al.*, 2001) followed by a GFP cassette. The construct sequence was verified prior to injection (BestGene) into *Drosophila w¹¹¹⁸* flies. Transformants were generated using *attP40* integration sites on the second chromosome.

For *UASt-zpg^{cDNA}*, a vector containing the cDNA of *zpg* was obtained from the Berkley *Drosophila* Genome Project, care of the *Drosophila* Genomics Resource Centre (cDNA clone RE18536). The *zpg* cDNA was removed from the vector and cloned into a *UASt-pattB* vector. The construct sequence was verified prior to injection (BestGene) into *Drosophila w¹¹¹⁸* flies. Transformants were generated using *attP40* integration sites on the second chromosome.