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 germline-soma communication. Here we demonstrate that gap junctions may also mediate direct, bi-directional signalling between the soma and germline. When gap junctions between the soma and germline 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 germline and the soma side, respectively. Overall, our results show that bi-directional gap junction-mediated signalling is essential to coordinate the soma and germline 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.

Introduction

In animals, the gonads are composed of two tissue types: the germline, 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 germline in order to maintain life-long gamete production. Communication between the soma and germline 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). While 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 somatic cyst cells that remain wrapped around the differentiating germline cyst until the end of spermatogenesis (Schulz et al., 2002). Soma-germline interactions are essential for the 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 (BMP) 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 independent of JAK-STAT signalling (Amoyel et al., 2013; Michel et al., 2012). Additionally, soma-germline communication mediated by the 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 program 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 germline-soma 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; Herve and Derangeon, 2013; Phelan, 2005). Genetic screens identified the gap junction gene innexin 4 or zero population growth (zpg) as being required for 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 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 reagents, 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 germline and 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 germline. 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 germline or by somatic knockdown of Inx2 produces severe defects in both the soma and the germline, resulting in sterility.

Results

zpg is required specifically in the germline for GSC maintenance and differentiation

To understand the role of zpg in regulating germline-soma 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 to wild-type (Figure 1A-B; Tazuke et al., 2002). Previous analysis of the germline did not distinguish between differentiating germ cells and GSCs (Tazuke et al., 2002), defined as Vasa-positive 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 wild-type sibling controls, 1.2±0.3(n=49) vs. 10.8±0.3(n=37) (Figure 1C, D, G) and in 62.1% of zpg testes, no GSCs were present at 1 day post-eclosion (DPE) (n=100), though 95% of zpg

testes contained germ cells (n=101). The reduction GSCs in zpg testes may 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 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 (Figure 1E-F). This suggested that, although GSCs in zpg 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% and 73% of testes, respectively (n= 32, n=41, Figure 1H, L). However, zpg mutant GSC clones were only detected in 3.2% testes at 4 dpci and never detected at 5 dpci (n = 32, n=59, Figure 1I, L). zpg mutant germline clones were always observed as single-cells several cell lengths away from the hub. In comparison, CySC maintenance was not affected by loss of zpg. When zpg clones were induced in CySCs (defined as Traffic jam (Tj)-positive cells less than 1 cell diameter from the hub) (Fairchild et al., 2015), they were found in 53% of the testes scored at 5 dpci in (n=15, Figure 1K, L) . This was similar to control wild-type CySC clones, which could be detected at 5 dpci in 69% of testes (n=13, Figure 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 GSCs are lost over time in zpg mutants (Tazuke et al., 2002). zpg is required germline differentiation and association with the soma

Since 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 germline 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 (Figure 2A). As described previously, GSCs and gonialblasts in zpg testes were observed to contain spectrosomes (Tazuke et al., 2002); Figure 2B). In some instances, dumbell-shaped fusomes were detected in zpg mutant testes between adjacent germ cells, indicating the presence of 2-cell stage spermatogonia. However, in the 2-cell stage spermatogonia seen in zpg mutant testes, unlike in wild-type testes, ectopic spectrosomes were also observed alongside early fusomes, consistent with a differentiation defect. (Figure 2B, inset). As a second readout 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 Figure 2C to D). However, Bam-positive germ cells in zpg 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 germline (Tazuke et al., 2002). We confirmed this result by labelling for the septate junction component Coracle, which is a useful marker for encapsulation expressed in the soma (Fairchild et al., 2015) (Figure 2E). This analysis showed that, in zpg testes, germ cell clusters lacked a detectable

belt of Coracle, indicating encapsulation defects (Figure 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 (Figure 3A): 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 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 testes was misregulated compared to wild-type. Wild-type testes contained an average of 37.1±1.5 Zfh-1-positive cells per testis (n=17, Figure 3B), whereas testes from zpg flies contained an average of 160.0±9.7 Zfh-1-positive cells per testis (n=27, Figure 3C), a 331% increase. Wild-type testes were observed to have an average of 98.4±1.8 Tj-positive cells per testis (n=23, Figure 3D), compared to an average of 160.6±8.2 in zpg testes (n=55, Figure 3E), a 63% increase. Finally, an average of 185.1±6.1 Eya-positive cells were detected in wild-type testes (n=19, Figure 3F), compared to 79.3±4.3 for zpg mutant testes (n=26, Figure 3G), a 67% reduction. These results suggest a large increase in the number of CySCs and early somatic cells in zpg testes, but a significant decrease in the late-stage somatic cells, relative to wild-type (Figure 3H). Furthermore, the stochastic variability in somatic cell numbers in zpg testes is consistent with the idea that loss of gap junction-mediated regulatory cues have large effects on the soma. Taken together, these results suggest that somatic differentiation is disrupted, and perhaps delayed or partially blocked in zpg mutant testes. zpg is required non-autonomously to regulate the proliferation of the early soma

The differentiation defect of somatic cells in zpg testes likely results from failures in gap junction-mediated germline-soma communication. However, the germline in zpg mutants is disrupted in two ways: first, there are fewer germ cells present; second, the residual germline is blocked in differentiation. We sought to determine which of these two germline defects might lead to the somatic defects we observed. While 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 analyzed in tudor mutant flies which lack a germline (Arkov et al., 2006; Boswell and Mahowald, 1985; Thomson and Lasko, 2004). In tudor mutant testes, the number of Tj-positive 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 (Figure 4A-B, E). This contrasts with the substantial and significant increase in the number of Tj-expressing cells observed in zpg mutant testes (Figure 3D-E). Our result are 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, may 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 testes does not by itself account for the somatic defects.

To test if the somatic defects observed in zpg 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-positive cells upon

activation of the BMP pathway, compared to controls, an average of 130.7±4.4(n=27) compared to 101.8±2.0(n=26) cells per testis respectively, a 28% increase (Figure 4C-E). This increase was similar to that observed in zpg testes and it is therefore possible that the somatic defects in zpg flies were related to disrupted germline differentiation.

To further explore the possibility that the zpg mutant phenotype resulted from the inability of the germline to differentiate, additional analysis was performed. We hypothesized that the increase in early somatic cells could possibly result from delays in somatic differentiation or from abnormal proliferation. Therefore, CySC and early daughter cell proliferation was assayed by labelling cells actively synthesizing new DNA with an 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 approximately 17 cells per testis (Figure 4H). This implies that constitutively-activating germline

BMP signalling leads to an increase in Tj-positive cells by delaying their differentiation rather than through changes in proliferation. In comparison, in zpg mutant testes the number of proliferating somatic cells increased by 113% (Figure 4H; 19.0±1.3 in the control to 40.6±4.6; n=18 and 14 respectively). Furthermore, while proliferating somatic cells were only detected proximal to the hub in wild-type testes (Figure 4F, arrowheads), Zfh-1-/EdU-positive cells could be detected many cell lengths away from the hub in zpg testes (Figure 4G, arrowheads). These results show that the increase in the number of Tj-positive cells in zpg mutants is at least partially due to a specific defect in proliferative regulation of the early soma. While we cannot rule out that disrupted germline differentiation and a reduction in germ cells both contribute to the effects we observe, the phenotype of zpg mutants is distinct from that obtained by either removing the germline or blocking germline differentiation (Figure 4I). This suggests that the somatic misregulation observed in zpg mutants likely represents a specific defect in gap junction-mediated germline-soma 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 germline. Since Zpg is expressed only in the germline (Tazuke et al., 2002), it must interact with other innexin proteins on the somatic side of germline-soma 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 (Figure 5A; Figure 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 (Figure 5A; Figure 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 co-localize 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 analyzed in the testis (Figure 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 germline-soma boundary (Figure 5B, S2G, 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 (Figure S2F-F', see insets). As previously published, Zpg also localized to the germline-soma boundary, visualized with a Zpg-specific antibody (Figure 5C-D) and a GFP-tagged Zpg rescue construct (Figure 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 (Figure S1). Labelling Inx2 in testes expressing the Zpg::GFP transgene showed that Zpg co-localized with Inx2 (Figure 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 (Figure 5C). As in the ovary, Inx3 also co-localized with Zpg at germline-soma boundaries, though its knockdown did not give rise to detectable defects (Figure 5A, F; Figure 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 (Figure 6A), gap junctions were observed between GSCs and adjacent CySCs (Figure 6B-B'). Gap junctions could also be observed between 1-cell stage gonialblasts and cyst cells immediately outside of the niche (Figure 6C-C'). In differentiating spermatogonia, gap junctions were visible between germline cysts and cyst cells (Figure 6 D-D'). Interestingly, germline-germline gap junctions were observed in spermatogonia (Figure 6E-E'), although these were infrequent and small in comparison to germline-soma gap junctions (compare Figure 6E' to B'-D'). Together, these results indicate that gap junctions are formed early during spermatogenesis, these junctions persist during early stages of germline differentiation, and 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 germline. If Inx2 was indeed the main somatic innexin and it coupled to Zpg on the surface of germ cells, then Inx2 knockdown could affect Zpg's distribution. 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 (Figure 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 to background staining (Figure 7C). Upon knockdown of Inx2 in the soma, Zpg redistributed from the soma-germline to the germline-germline interface (Figure 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 to background cytoplasmic staining (Figure 7C). In comparison, a disruption in the subcellular localization of Zpg was not observed upon knockdown of Inx3 (Figure 7D-F). This shows that Inx2 is required in the soma to maintain Zpg's distribution at the soma-germline interface, consistent with a coupling of somatic Inx2 with germline Zpg. Moreover, since Zpg is the only fly innexin known to be expressed in the germline (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 was 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 (Figure 8A-B). Because the inx2 locus is located on the X chromosome, clonal analysis of inx2 mutants in the testis proved 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 in the ovary (Figure S2H-I, Figure 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 in the testes, we stained zpg and inx2RNAi testes for E-Cad. We did not observe differences between zpg, inx2RNAi, and control testes (Figure 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 as a readout. Again, we did not detect changes in Armadillo levels in zpg over-expression clones, nor did cell-cell adhesion appear altered (Figure 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, Figure 8C-E). Second, somatic knockdown of Inx2 partially blocked germline differentiation (Figure 8F-N). Although most germline cysts developed a fusome (Figure 8F-G) almost half did 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), Figure 8L-N). However, Bam expression was similar to that observed in wild-type (Figure 8H-I). Furthermore, encapsulation, as judged by staining for the somatic marker Cora, was largely normal (Figure 8J-K). This is consistent with the Inx2 knockdown phenotype being less severe than the zpg phenotype in some aspects (compare Figure 8J-K to Figure 2E-F). Nonetheless, testes at the severe end of the phenotypic spectrum greatly resembled zpg mutant testes (Figure 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 (Figure S3C-H). Knockdown of Inx3 alone in the soma did not disrupt spermatogenesis, despite reducing Inx3 protein levels, determined by antibody staining (Figure S3C-D). Knockdown of Inx2 disrupted Inx3's localization, knocked down Inx2 protein levels, and disrupted spermatogenesis (Figure S3E). Simultaneous knockdown of Inx2 and Inx3 phenocopied the Inx2 knockdown phenotype (Figure S3F-I). These results suggested that Inx3 is dispensible in the early soma for spermatogenesis, and Inx3 may require Inx2 for its localization. Inx2 is required to regulate the proliferation and differentiation of the early soma

Since the zpg mutant germline phenotypes resembled those observed upon Inx2 somatic knockdown, we analyzed 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 (Figure 9I). The number of Zfh-1 positive cells compared to controls was 48% higher (67.5±2.9 per testes, n=30 versus 45.5±1.4, n=23 respectively, Figure 9A-B, I). The number of Tj-positive cells compared to control was slightly lower, though not statistically-significant (99.8±3.0 per testes, n=12 versus 92.6±2.7, n=43, Figure 9C-D, I). Finally, the number of late somatic Eya-positive cells was decreased in Inx2 knockdown testes compared to controls by 19% (149.8±6.2 per testes, n=13 versus 184.8±7.5, n=9 respectively, Figure 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 to 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-positive cells were detected (n=23, Figure 9G, J). In Inx2 knockdown testes, an average of 18.5±1.3 Zfh-1-/EdU-positive cells were observed (n=30, Figure 9H, J). Taken by itself, this result appeared to suggest that early somatic proliferation was not misregulated in Inx2 somatic knockdown testes. However, since the Zfh-1 population is in general larger in Inx2 knockdown testes compared to 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-positive cells per testis was quantified in addition to Zfh-1-/EdU- positive cells which were less than 1 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 with EdU. In Inx2 somatic knockdown and zpg mutant testes, only 13% and 7% of CySCs were labelled, respectively (Figure 9K). This result suggested that CySC proliferation is disrupted upon somatic Inx2 knockdown, similar to zpg mutant testes.

It was further noted that in Inx2 knockdown testes, cells proliferated further away from the hub, similar to what was observed in zpg testes (Figure 4G, 9L). The average distance from the hub of Zfh-1-/EdU-positive cells was determined. While Zfh-1-/EdU-positive cells were detected within 4.9µm±0.2 (n=310) of the hub in control testes, upon knockdown of Inx2, the average distance of Zfh-1-/EdU-positive cells from the hub grew by 43% to 7.0µm±0.3 (n=338). This was comparable to zpg testes, where proliferation events occurred on average 97% further from the hub, or 11.0µm±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 germline 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 germline

The work presented here demonstrates that gap junctions between the soma and germline are essential for fly spermatogenesis. Previous work showing an essential role for Zpg in the 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 the 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 germline 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 germline in the fly testis is a heterotypic channel coupling Inx2 in the soma and Zpg in the germline. Importantly, disrupting gap junctions in the soma by knocking down Inx2 phenocopies the zpg mutant phenotype in both the germline 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; Speder 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 germline 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 germline

Following the stem cell stage, strong expression and co-localization 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 germline 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 germline, isolating the germline

from the outside environment (Fairchild et al., 2015). This transition corresponds with a switch occurs whereby soma-germline communication shifts from predominantly exocrine to juxtacrine signalling. Thus, as the germline becomes increasingly isolated, it becomes more dependent on differentiation signals that arrive via gap junctions from the soma. Once the germline becomes isolated, gap junctions may also play an important nutritive role and permit the movement of essential small metabolites between the germline and soma. Similarly, the soma requires gap junction-mediated signals to allow it to accommodate the increasingly expanded, differentiated, and specialized germline.

Our observations that gap junctions regulate germline differentiation and soma proliferation are in line with studies from both vertebrate and invertebrate models. In C. 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; Sridharan et al., 2007b). 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 germline-soma gap junctions is the expression of different gap junction proteins, resulting in heterotypic gap junctions (Gunther 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; Herve and Derangeon, 2013), which have been implicated in regulating meiosis in the germline (Conti et al., 2012; Von Stetina and Orr-Weaver, 2011). Our attempts to study cAMP and Ca2+ in the testis have proven inconclusive (CMS and GT, 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 (Kruger and Bohrmann, 2015). Overall, multiple signals are likely exchanged between the soma and germline 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 provide a supporting role, allowing the passage of cues from the soma that facilitate long-term GSC maintenance. After stem cell division germline undergoes rapid differentiation. The germline becomes increasingly isolated from the outside environment, and a permeability barrier is formed by the soma. As outside signals from the niche are lost, the germline 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 germline, signals pass from the germline through the gap junctions into the soma. Taken together, our work 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 germline 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 germline during spermatogenesis.

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Competing Interests

The authors declare no competing 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. W.V. performed the electron microscopy and analysis. C.M.S., W.V., and G.T. interpreted data and prepared the manuscript.

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Materials & Methods

Image Analysis

All analyses were performed on males <1DPE, unless otherwise stated. GSCs were defined as Vasa-positive or Tj-negative cells contacting the hub. CySCs were defined as Zfh-1-positive or Tj-positive cells <10µm from the hub. Somatic cells in S-phase were labeled by vivisecting testes in Testis Buffer (TB) (White-Cooper, 2004) and culturing for 30 minutes 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-positive nuclei edge. Electron Microscopy

Testes were dissected in PBS and fixed for 2 hours 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 minutes with 0.1 M sodium cacodylate (pH 7.3) and post-fixed for 1 hour in 1% osmium tetroxide in 0.1M Na cacodylate on ice. Samples were washed three times for 10 minutes with ddH2O, stained for 1 hour ‘en bloc’ with 1% aqueous uranyl acetate, washed three times for 10 minutes with ddH2O, then dehydrated through an ascending concentration ethanol series, ending with three changes of 100% ethanol for 10 minutes each. Dehydration was followed by two changes for 15 minutes each of 100% propylene oxide. 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 hrs 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.

Zpg Subcellular Localization Measurements

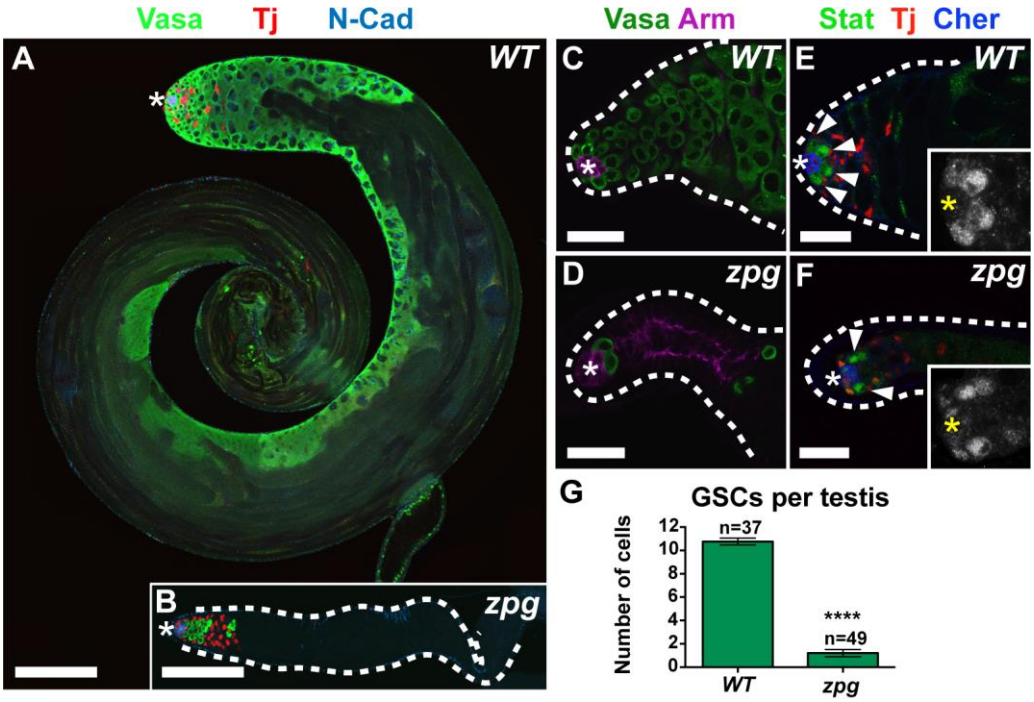
Fluorescence-intensity measurements were performed in ImageJ (NIH, Bethesda, Maryland) on confocal images of germline cysts, using the line tool. Germline-soma boundaries were defined as borders where Zpg co-localized with Armadillo. Background intensity was measured in the cytoplasm of germline cysts. In tj>inx2RNAi testes, the germline-soma boundary was defined as a cell boundary without an adjacent germ cell (based on Zpg staining). A minimum of 7 measurements were made from germline-soma and germline-germline boundaries, and cytoplasm from multiple cysts within each testis and averaged. Antibody Generation

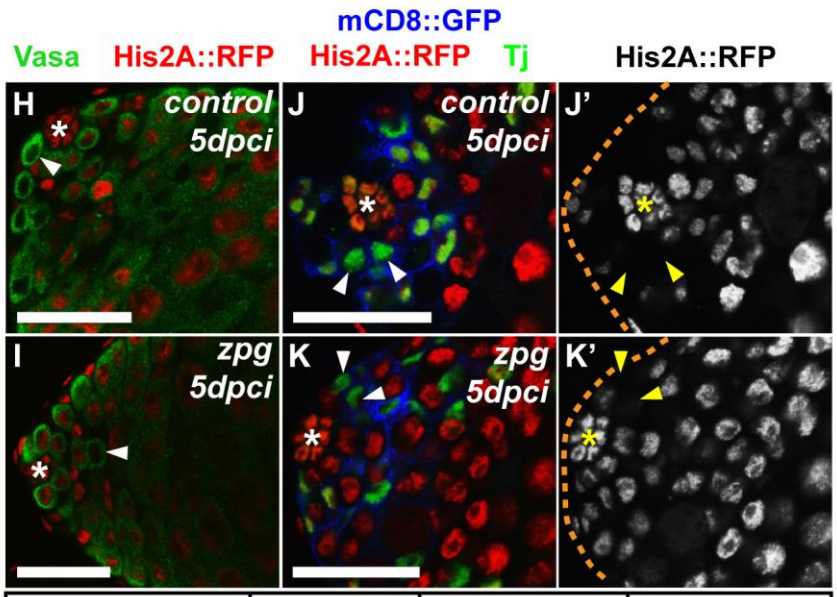
Polyclonal antibodies were raised in rabbits (GenScript) to peptides corresponding to the C-terminal amino acids (345-367, RKLLEELYEAQSLIKIPPGADKI) of Zpg. A peptide corresponding to amino acids 348-367 of Inx2, (DLSREMSGDEHSAHKRPFDA), was injected into guinea pigs (GenScript). Polyclonal antisera were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

Statistics

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

Figures





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genotype	clones	4dpci	5dpci
FRT2A	GSC	78% n=32	73% n=41
zpg^{z-5352},*FRT2A*		3.2% n=32	0% n=59
FRT2A	CySC		69% n=13
zpg^{z-5352},*FRT2A*			53% n=15

Figure 1 - zpg is required specifically in the germline for GSC maintenance and differentiation Compared to 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 testes at 1 day post-eclosion. (H-I) Negatively-labelled germline clones were either wild-type controls or homozygous for a zpg null allele, zpgz-5352. (H) Control clones (arrowhead) were maintained as GSCs at 5 days post-clone induction (dpci). (I) zpgz-5352 clones were never maintained as GSCs at 5dpci, and rarely at 4dpci. When zpgz-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 zpgz-5352. Both control (J) and mutant clones (K) were detected in the CySC position at 5dpci (arrowheads). (L) Table summarizing GSC and CySC clone maintenance. Germline labelled with 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 (blue, A-B), Arm (magenta, C-D), E-Cad (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). n is number of testes counted in G-K. Asterisks indicate hub. Scale bars are 100µm in A-B; 30µm in C-F, H-I, K-L.

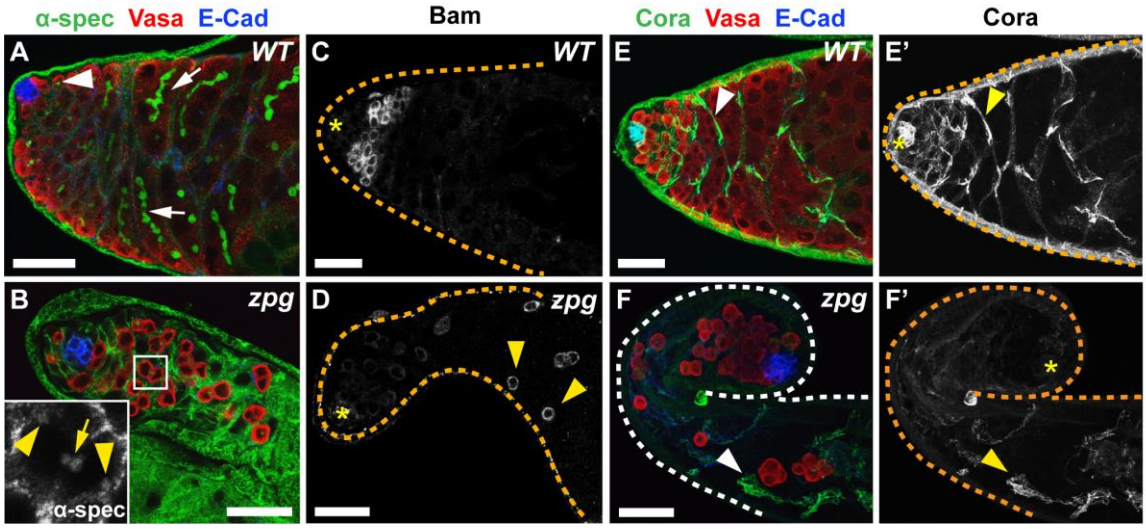

Figure 2 - zpg is required for germline differentiation and association with the soma (A) In wild-type testes, small dot spectrosomes (arrowhead) enlarge and branch, forming fusomes (arrows). (B) In zpg testes, germ cells contained spectrosomes and occasionally small dumbbell-shaped fusomes (inset, arrow). 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 2-4 cell stage in wild-type testes until the 16-cell stage. (D) In zpg testes, Bam is detected in single-celled germ cells that are far from the hub (arrowheads). (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) In zpg testes, germ cell clusters far from the niche are not encysted by the soma (arrowhead). Germline labelled with 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. Scale bars are 30μm in A-F.

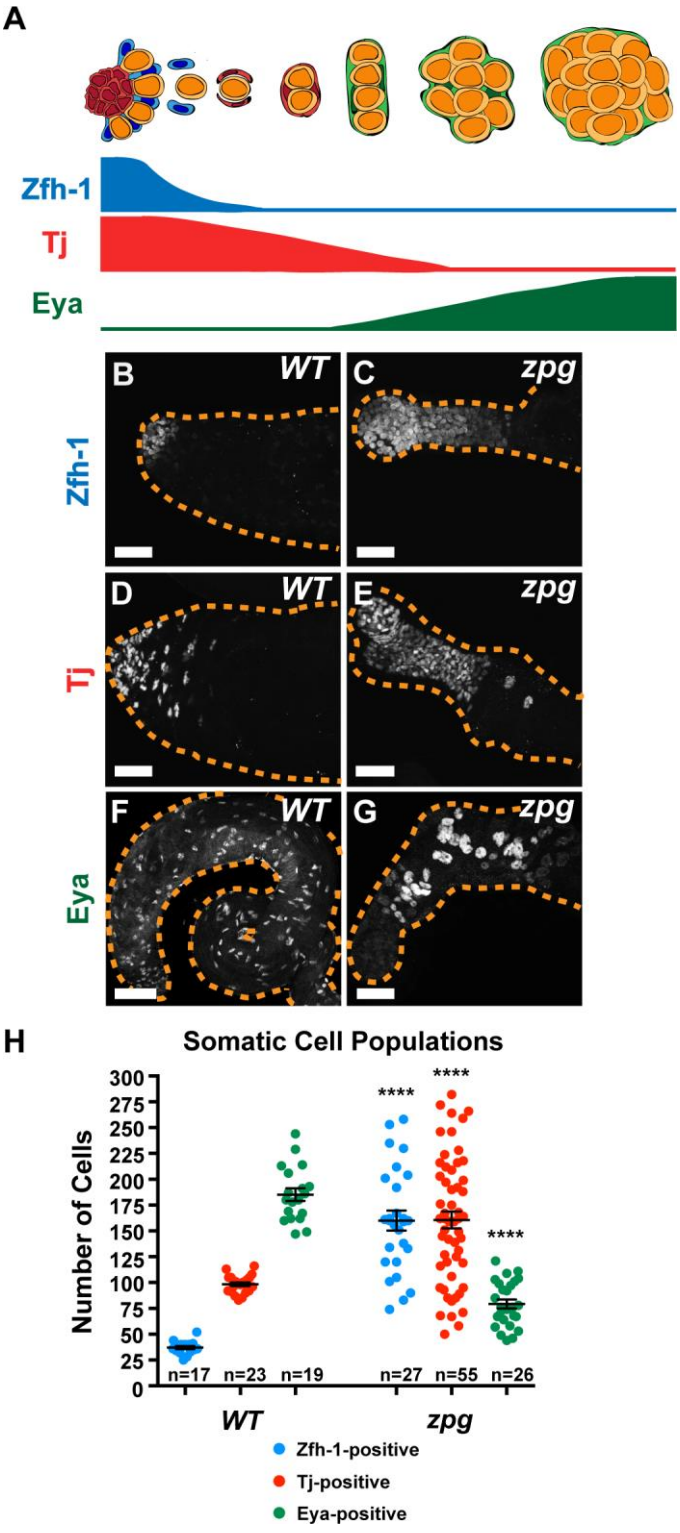

Figure 3 - zpg is required non-autonomously for differentiation of the soma (A) Schematic summarizing stage-specific expression of somatic cell differentiation markers Zfh-1, Tj, and Eya in the testis. (B) In wild-type testes, Zfh-1 is highly expressed in CySCs and weakly in their immediate daughters. (C) In zpg testes, the apical tip is filled by Zfh-1-positive cells. (D) In wild-type testes, Tj is

expressed in the hub, CySCs, and early somatic cells. (E) In zpg mutant testes, Tj-positive cells fill the tip of the testis, similar to Zfh-1. (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-positive cells were observed. (H) Quantification of Zfh-1-, Tj-, and Eya-positive somatic cells in wild-type and zpg testes. n is number of testes counted. Scale bars are 30µm in B-E, G; 100µm in F.

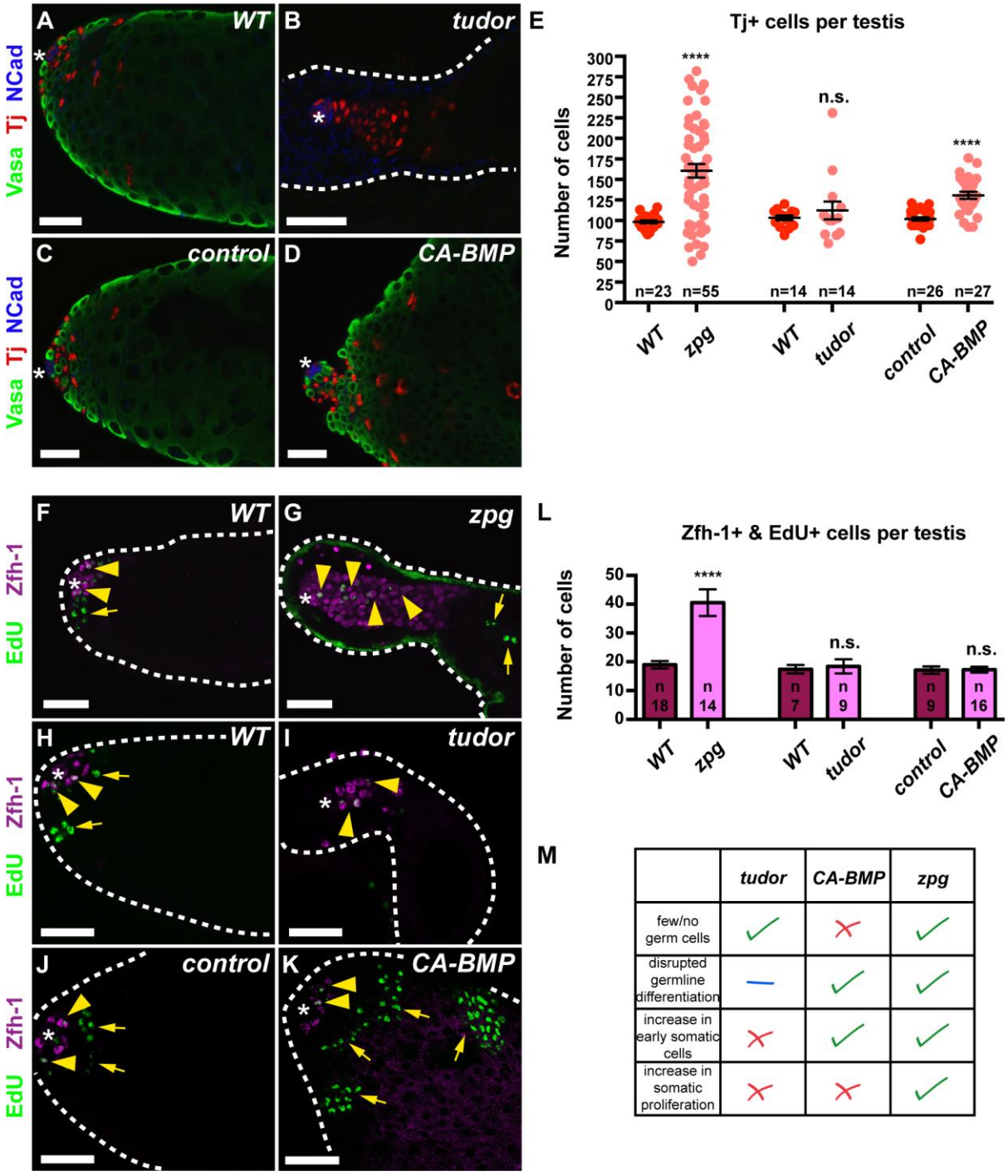

Figure 4 - zpg is required non-autonomously to regulate the proliferation of the early soma (A-E) Analysis of early somatic cell numbers in agametic testes and testes expressing constitutively-active BMP receptors in the early germline using nanos-GAL4-VP16. Germline labelled by Vasa (green), early soma by Tj (red), and the hub by N-Cad (blue). (A-B) Tj-positive cells in w1118 controls (A) and in tudor mutant testes (B) which do not form a germline. (C-D) Tj-positive cells in control (w1118; UAS-tkvAct, saxAct/+) testes (C) and in testes expressing CA-BMP receptors in the early germline (nanos-GAL4-VP16/>UAS-tkvAct, saxAct/+), (D). (E) Quantification of Tj-positive cells in zpg, tudor,

nos>CA-BMP testes versus their respective controls (in order: zpg/+, w1118, w1118; UAS-tkvAct, saxAct/+). Loss of Zpg or constitutive-activation of BMP signalling led to increased Tj-positive cells. (F-K) Quantification of S-phase somatic cells labeled with Zfh-1 and EdU in wild-type, zpg, tudor, control, and CA-BMP testes. (F) In wild-type testes, Zfh-1-/EdU-positive 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-positive cells (arrowheads) were detected adjacent to the hub in wild-type and tudor testes, as were germ cells in wild-type testes (arrows). (J) In control and CA-BMP testes, Zfh-1-/EdU-positive cells were detected near the hub (arrowheads), in addition to germ cells in S-phase (arrows). (L) Quantification of Zfh-1-/EdU-positive cells in zpg, tudor, and nos>CA-BMP testes versus their respective controls (in order: zpg/+, w1118, w1118; UAS-tkvAct, saxAct/+). An 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 is the number of testes. Asterisks indicate the hub, detected by N-Cad staining or Cheerio. Scale bars are 30µm throughout.

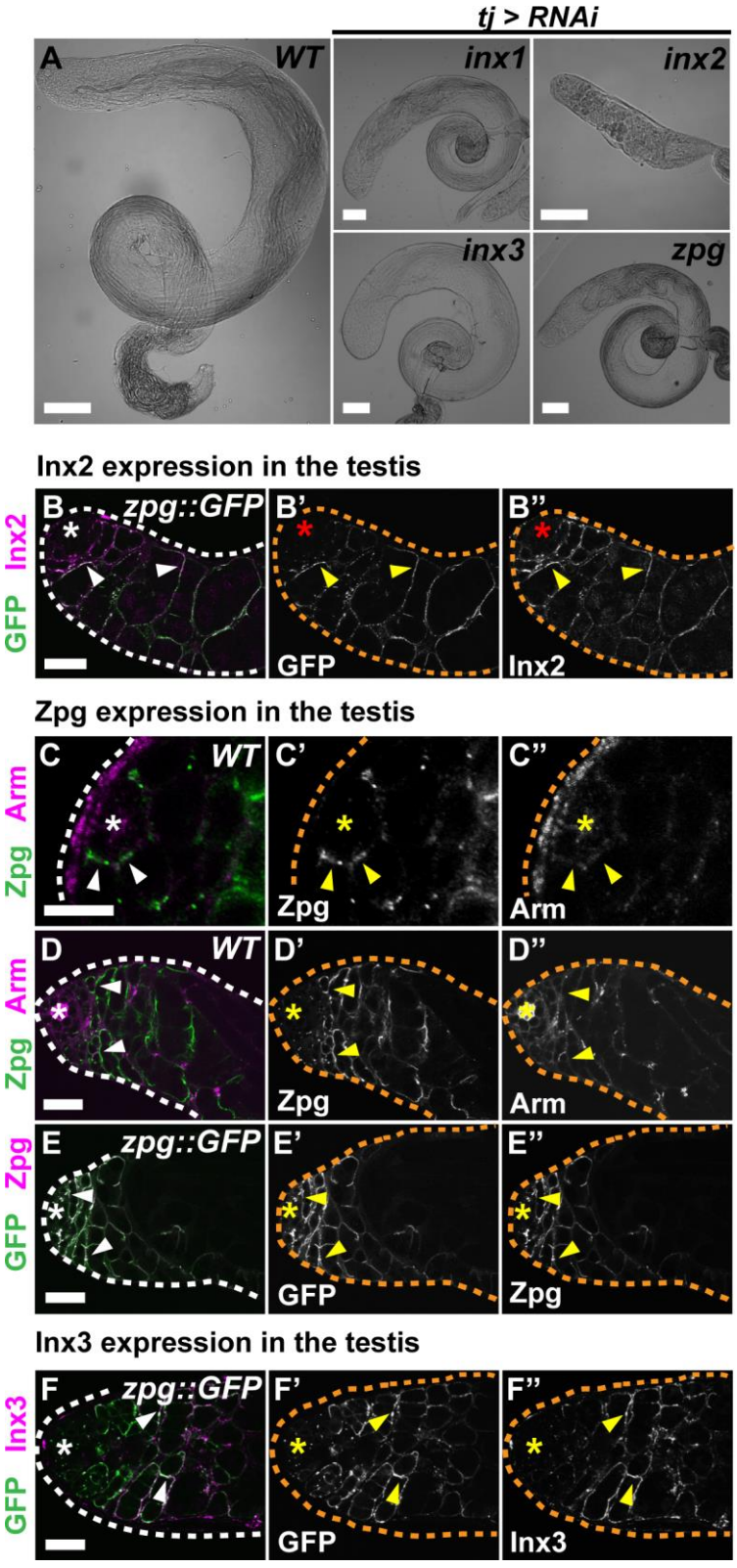

Figure 5 – Analysis of innexins in the testis (A) Representative DIC images of wild-type testis and testes where Innexins 1-4 were somatically knocked down using RNAi driven with tj-GAL4. Only

Innexin 2 (Inx2) knockdown produced a phenotype. (B) Inx2 (magenta) prominently co-localized (arrowheads) with Zpg (green; visualized using a GFP-tagged rescue construct) beginning at the 4-cell cyst stage. (C) Weak Zpg (green) expression was detected at the hub-GSC interface (arrowheads, hub marked with Arm in magenta). (D) Strong Zpg expression (arrowheads; green) was observed in 4-16 cell-stage cysts (hub marked with Arm in magenta). (E) The GFP-tagged Zpg rescue construct expression (arrowheads) mimicked endogenous Zpg protein expression, detected with Zpg-specific antibody (magenta). (F) Inx3 (magenta) also co-localized (arrowheads) with Zpg (green; GFP-tagged rescue construct) during early spermatogenesis. Asterisks indicate hub. Scale bars are 100µm in A; 10µm in C; 30µm in all other panels.

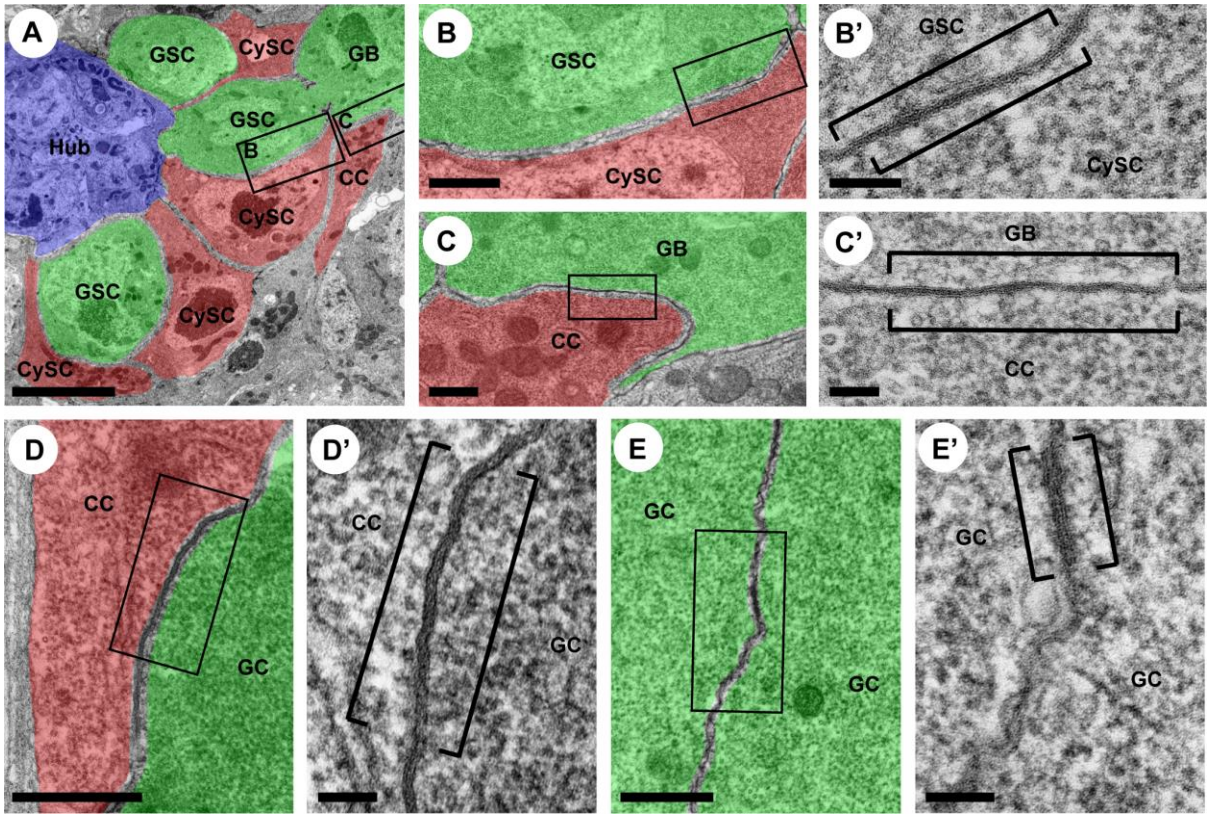

Figure 6 – Ultrastructural analysis of gap junctions in the testis (A-E) Electron micrographs of wild-type testes. (A) Overview of the hub cell niche. (B) Gap junctions were detected early during spermatogenesis between both GSCs and CySCs (see inset B') and (C) between cyst cells (CC) and one-cell stage gonialblasts (GB, see inset C'). (D) Outside the niche, germ cells (GC) exhibited large gap junctions with associated CCs (see inset D'). (E) Gap junctions were also observed between neighbouring germ cells (see inset E', brackets), though these were less common and smaller than those between germ cells and cyst cells (compare E' to B'-D'). The hub is highlighted in blue, germline in green, and soma in red. Square brackets indicate gap junctions. Scale bare are 5µm in A; 1µm in B-C; 500nm in D-E' 100nm in B'-E'.

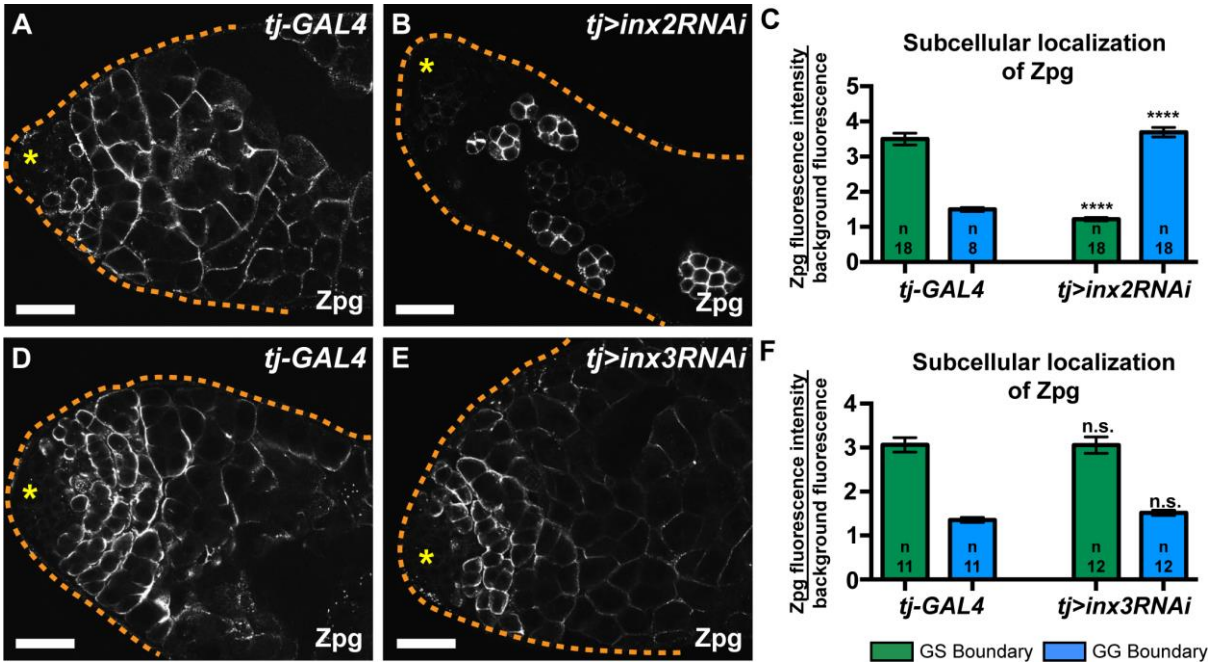

Figure 7 - Inx2 is required in the soma for the subcellular localization of Zpg (A-B) Control and tj>inx2RNAi testes labeled with Zpg antibody. (A) Zpg localized predominantly to germline-soma boundaries. (B) Following Inx2 somatic knockdown, Zpg was enriched at germline-germline boundaries. (C) Quantification of Zpg enrichment based on normalized fluorescence intensity. Zpg was recruited to germline-soma 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. (D-F) Analysis of Zpg recruitment upon somatic Inx3 knockdown. Inx3 knockdown did not effect on the subcellular localization of Zpg, compared to controls. n is number of testes quantified. Scale bars are 30µm throughout.

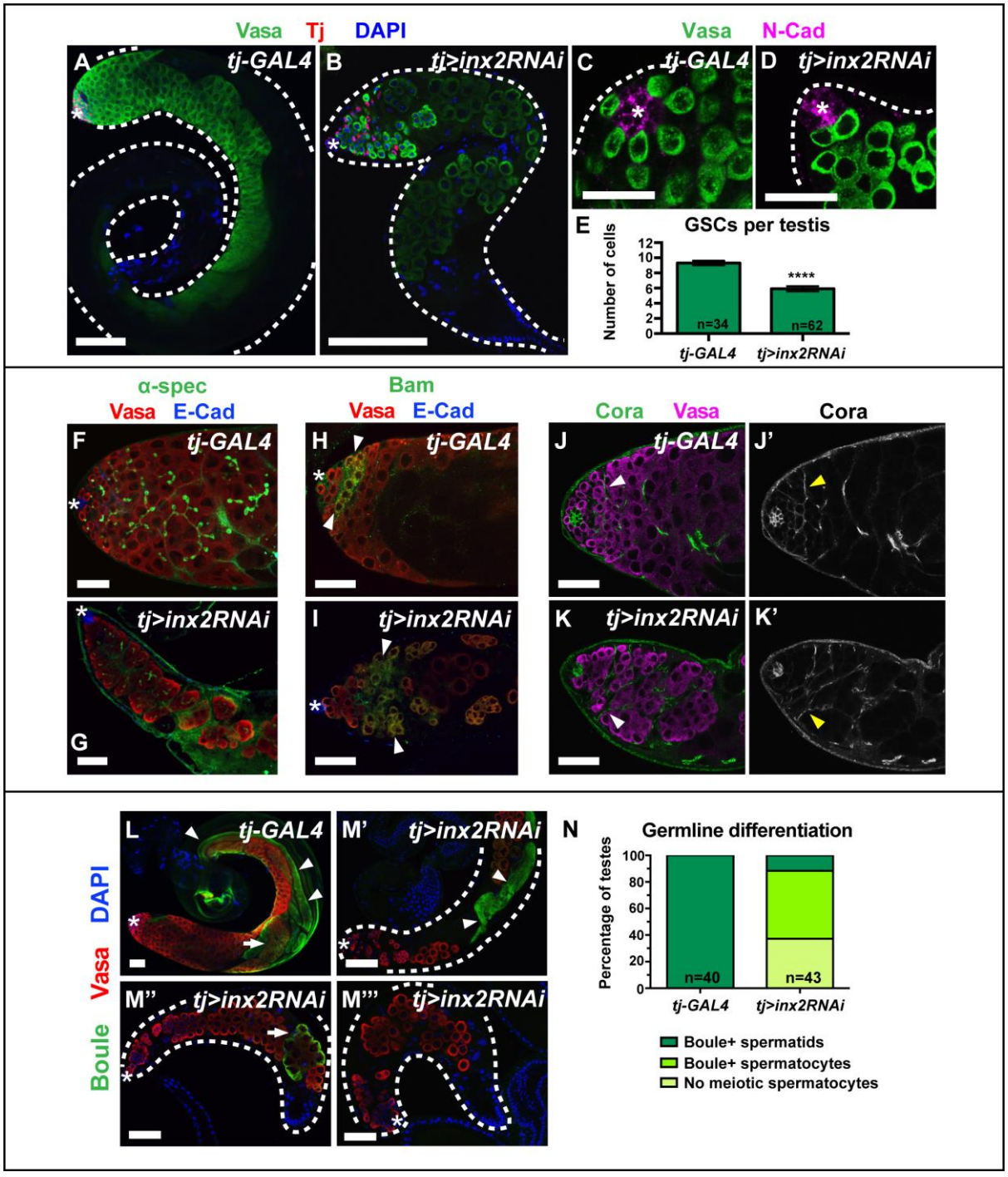

Figure 8 - Inx2 is required in the soma for GSC maintenance and germline differentiation (A-B) Control and tj>inx2RNAi testes labeled with Vasa (green), Tj (red) and DAPI (blue). Somatic Inx2 knockdown resulted in small rudimentary testes. (C-D) Close-up of the apical tip of control and tj>inx2RNAi testes (hub highlighted with N-Cad in magenta; Vasa in green). (E) GSC number was reduced upon Inx2 knockdown in the soma compared to controls. (F-G) Labeling 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 labeling for Cora (green; Vasa in magenta) appeared largely normal in tj>inx2RNAi testes. (L) In wild-type testes, meiotic spermatid stages were marked by expression of Boule protein (green; Vasa in red; DAPI in blue). (M) Examples of the phenotypic range observed in tj>inx2RNAi testes arranged from the least- to most-severe. (N) Percentage of testes observed in each phenotypic class. Nearly one-third of testes exhibited no Boule expression. n is number of testes. Scale bars are 100µm in panels A-B, L-M; 30µm in all other panels.

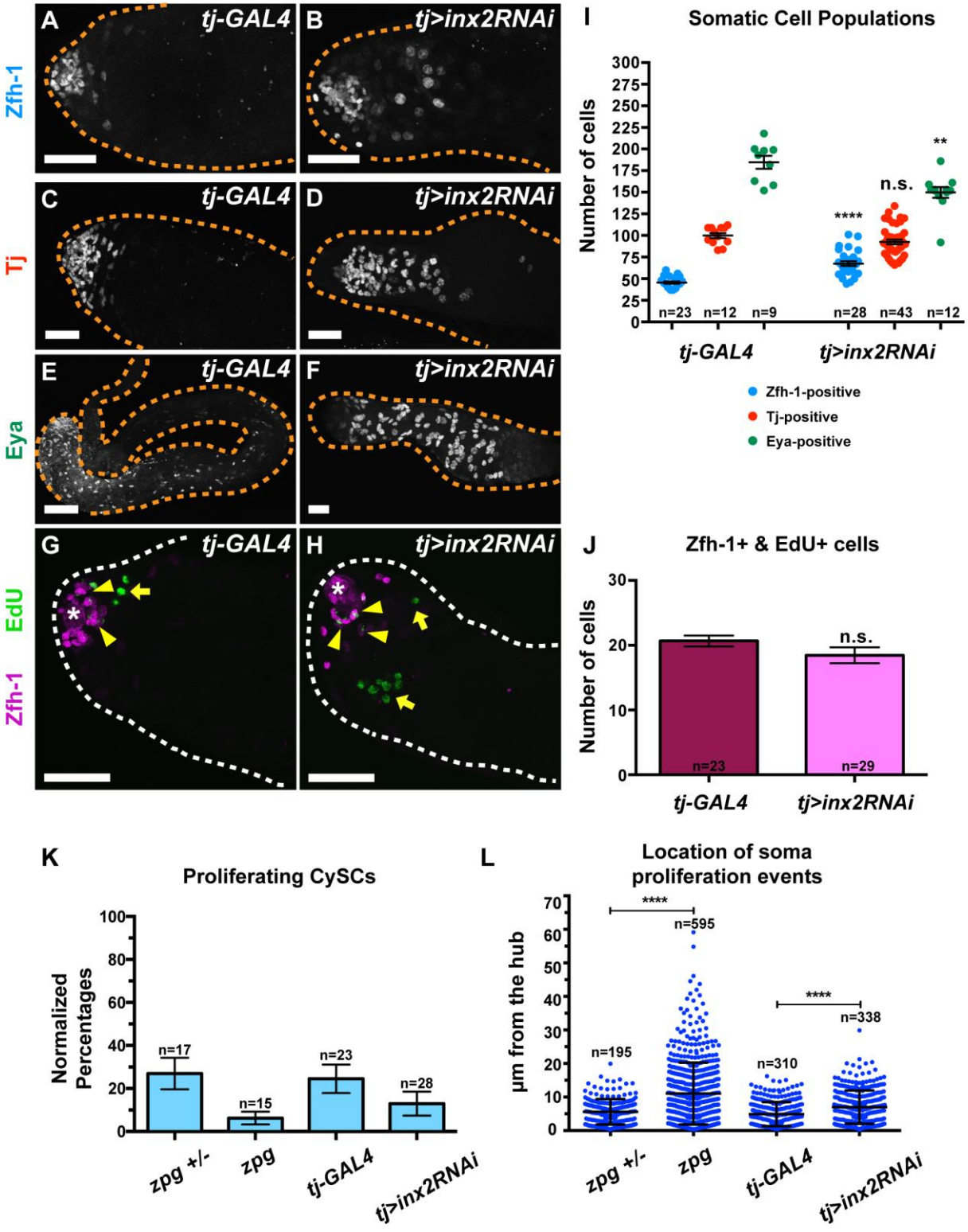

Figure 9 - Inx2 is required to regulate the proliferation and differentiation of the early soma (A-F) Expression of stage-specific somatic cell markers in control and tj>inx2RNAi testes. (A-B) Zfh-1-positive cells in control and tj>inx2RNAi testes, respectively. (C-D) Tj-positive cells in control and tj>inx2RNAi testes, respectively. (E-F) Eya-positive cells in control and tj>inx2RNAi testes,

respectively. (G-H) S-phase somatic cells are labelled with Zfh-1 (magenta) and EdU (green) in control and tj>inx2RNAi testes. Arrowheads indicate proliferating CySCs, arrows indicate germline. (I) Quantification of Zfh-1-, Tj-, and Eya-positive cells in control and tj>inx2RNAi testes. (J) Quantification of Zfh-1/EdU-positive cells. (K) Percentage of proliferative CySCs in wild-type, zpg, control, and tj>inx2RNAi testes normalized to total number of Zfh-1-positive cells per testis. (L) Quantification of distance of somatic proliferation events in zpg/+, zpg, control, and tj>inx2RNAi testes. n is number of testes quantified in I-K; single Zfh-1-/EdU-positive cells in L. Asterisks indicate the hub. Scale bars are 30µm in A-D, F-H; 100µm in E.

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