Development 129, 2529-2539 (2002)
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DEV5009

A germline-specific gap junction protein required for survival of differentiating early germ cells

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Accepted 1 March 2002

SUMMARY

Germ cells require intimate associations and signals from the surrounding somatic cells throughout gametogenesis. The *zero population growth* (*zpg*) locus of *Drosophila* encodes a germline-specific gap junction protein, Innexin 4, that is required for survival of differentiating early germ cells during gametogenesis in both sexes. Animals with a null mutation in *zpg* are viable but sterile and have tiny gonads. Adult *zpg*-null gonads contain small numbers of early germ cells, resembling stem cells or early spermatogonia or oogonia, but lack later stages of germ cell differentiation. In the male, Zpg protein localizes to the surface of spermatogonia, primarily on the sides adjacent to the somatic cyst cells. In the female, Zpg protein localizes to germ cell surfaces, both those adjacent to surrounding somatic cells and those adjacent to other germ cells. We propose that Zpg-containing gap junctional hemichannels in the germ cell plasma membrane may connect with hemichannels made of other innexin isoforms on adjacent somatic cells. Gap junctional intercellular communication via these channels may mediate passage of crucial small molecules or signals between germline and somatic support cells required for survival and differentiation of early germ cells in both sexes.

Key words: *Drosophila*, Gap junction, Innexin, Oogenesis, Spermatogenesis

INTRODUCTION

Communication between germ cells and intimately associated somatic support cells regulates germ cell fate choices, maintenance and differentiation. Close range signaling between germline and somatic cells via polypeptide ligand–transmembrane receptor pathways guides gamete differentiation in many animal species. In mammals, the ligands steel factor (Kitl) and glial cell line-derived neurotrophic factor (GDNF) produced in the somatic Sertoli cells regulate decisions between proliferation and differentiation in spermatogonia (Meng et al., 2000; Ohta et al., 2000). In *C. elegans*, the somatic distal tip cell signals via the Delta-like ligand *lag-2* to the Notch-like receptor *glp-1* on germ cells to maintain closely associated germ cells in mitotic proliferation, and to suppress the transition to meiosis and differentiation (Austin and Kimble, 1987; Berry et al., 1997; Kadyk and Kimble, 1998; Hall et al., 1999). In *Drosophila*, somatic cells at the tip of the gonad provide a niche that regulates germ line stem cell behavior via a TGFβ/SMAD signal transduction pathway in females (Xie and Spradling, 2000) and activation of the JAK-STAT pathway in males (Kiger et al., 2001; Tulina and Matunis, 2001).

In many cases, intimate interactions between germline and somatic support cells are required for normal germ cell behavior and differentiation, but the signaling pathways involved are not yet known. For example, in mammals, somatic cumulus cells regulate the cell cycle program of maturing oocytes (reviewed by Tsafiri, 1978). In *Drosophila*, interactions between germline and somatic cells are crucial for proper germ cell migration and gonad formation during embryogenesis (Moore et al., 1998; Boyle and Dinardo, 1995), and for germline sex determination (Cline and Meyer, 1996). In *Drosophila* males, both the early stages of spermatogonial differentiation (Kiger et al., 2000; Tran et al., 2000) and the transition from spermatogonia to spermatocytes (Matunis et al., 1997) require information from surrounding somatic cyst cells.

Other modes of intercellular signaling, in addition to ligand/receptor-based mechanisms, may also be important for

close range interactions between germline and somatic support cells. We show that a germline-specific gap junction protein encoded by the *zero population growth* (*zpg*) locus of *Drosophila* plays a crucial role in early germ cell differentiation and survival. The Zpg protein localizes to the surface of early germ cells and in some stages appears especially concentrated at the interface between germline and somatic support cells. Lack of *zpg* function leads to failure to differentiate and loss of spermatogonia in males and dividing germline cysts in females. Strikingly, germline stem cells were present in *zpg* males and newly eclosed *zpg* females, although female germ line stem cells were lost with age. Thus, stem cells and early germ cells initiating differentiation require *zpg* function.

MATERIALS AND METHODS

Drosophila strains and culture

Drosophila were raised on standard cornmeal and molasses medium at 25°C unless otherwise noted. The zpg^1 allele was induced on a *st e* background by Johannes Hackstein in a large scale screen for EMS induced male sterile mutations (Hackstein, 1991). zpg^2 was induced on a *red e* background in our laboratory, in a standard F_2 screen for EMS-induced mutations on third chromosome that failed to complement zpg^1. zpg^3 was recovered after mobilization of a $P(w^+)$ (Erdelyi et al., 1995). $zpg^{3\text{-}ex}$ was induced by mobilizing the $P(w^+)$ in zpg^3 and screening for lines that had lost the w^+ eye color but retained the *zpg* mutant phenotype and failed to complement zpg^1 and zpg^2. $zpg^{z\text{-}5352}$, $zpg^{z\text{-}2533}$, $zpg^{z\text{-}2552}$, $zpg^{z\text{-}0918}$, $zpg^{z\text{-}2679}$, $zpg^{z\text{-}3860}$ were isolated by B. Wakimoto and D. Lindsley as male sterile mutations from the collection of 22,000 viable EMS-treated lines generated in C. Zuker's laboratory (B. Wakimoto, personal communication) and identified as *zpg* alleles by failure to complement zpg^1 and zpg^2. *Df(3L)CH12* and *Df(3L)CH20* have been described previously (Hong and Hashimoto, 1995). All other mutations and chromosomal rearrangements are described in FlyBase (http://flybase.bio.indiana.edu/). Unless otherwise stated, the phenotypic analyses and the counts for viability were performed on the progeny of a cross between $zpg^{z\text{-}5352}$/*TM6B* females and *Df(3L)Zn47/TM3* or *Df(3L)CH12/TM3* males.

Mapping and molecular cloning of *zpg*

The zpg^1 allele was mapped to 20.9±1.9 m.u. proximal to the visible marker *ru* by meiotic recombination between *ru* and *h*, and to the 65A-65C1 interval by deficiency complementation tests. *zpg* was uncovered by *Df(3L)Zn47*, *Df(3L)CH12* and *Df(3L)CH20*. A 3 kb genomic region (proximal) to the P-*lacZ* insert in zpg^3 was cloned by plasmid rescue (Cooley et al., 1988) and used to screen a *Drosophila* genomic λEMBL3 library (Tamkun et al., 1992) and a λZAP *Drosophila* testis cDNA library (gift of T. Hazelrigg, Columbia University). P-element-mediated germline transformation was carried out using a 6.15 Kb *Bam*HI-*Hin*dIII fragment cut from a *Drosophila* genomic phage clone and subcloned into pCasper-4 (Rubin and Spradling, 1982). Two independent transformed lines were tested. In each case the tiny testis, small ovary and male and female fertility phenotypes of $zpg^{z\text{-}5352}$/*Df(3L)Zn47* animals were rescued by a single copy of the transgene insert.

The genomic region and the candidate cDNAs were sequenced on both strands by dideoxy chain termination (Sanger et al., 1977) using T3 and T7 primers and genomic region specific oligonucleotides (PAN facility, Stanford, CA). Unless otherwise stated, all molecular techniques were performed as described elsewhere (Sambrook et al., 1989). An ovary cDNA, GM13027, from the Berkeley Drosophila Genome Project (http://www.fruitfly.org/), matching the predicted transcript CG10125 (FlyBase), was obtained from ResGen. The amino acid sequence of the predicted protein was used to search nucleotide sequence databases translated in all reading frames (tBLASTn). Sequence alignments were generated using the ClustalW Multiple Sequence Alignment (Thompson et al., 1994) and Boxshade programs.

Point mutations of EMS-induced *zpg* alleles were identified by sequencing bulk PCR products amplified from genomic DNA from *zpg* homozygotes or *zpg/Df(3L)Zn47* flies using gene specific primers. Sequences were aligned and analyzed using Sequencher (Gene Codes) and MacVector (Oxford Molecular Group) DNA analysis software.

RNA blot analysis

RNA from whole adult flies and adult flies lacking germline (progeny of $oskar^{CE3}$/$oskar^{301}$ females) was isolated by homogenization in TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Poly(A)$^+$ RNA was selected in batch on oligo dT-cellulose beads (Pharmacia). The isolated RNAs (approximately 4-6 μg of poly(A)$^+$ RNA per sample) were then separated on a 1.2% agarose gel with formaldehyde, transferred onto Hybond nylon membrane (Amersham) in 10× SSPE, and fixed to the membrane by u.v. crosslinking (Stratagene Stratalinker model 2400). Probes were labeled using Rediprime II (Amersham Pharmacia Biotech) from gel-purified DNA fragments. Probes were: *zpg* [cDNA insert from GM13027 (ResGen)]; and *rp49* (PCR product using T7 and T3 universal primers from a pBluescript clone containing an *rp49* cDNA).

In situ hybridization

In situ hybridization to embryos and whole adult *Drosophila* testes was carried out as described previously (Tautz and Pfeifle, 1989) with modification for RNA probes (Klingler and Gergen, 1993). Single-stranded riboprobes were generated from the linearized GM13027 cDNA using the Genius System (Roche Molecular Biochemicals).

Anti-Zpg peptide antibody and other antibody reagents

Owing to multiple homologous regions among the eight innexin family members in the *Drosophila* genome (Flybase, 1999; Curtin et al., 1999), polyclonal antisera were raised in rabbits (Zymed) against a Zpg-specific oligopeptide representing amino acid residues 345-367 of the predicted Zpg protein. The resulting anti-Zpg antisera were used at 1:2500-1:5000 for immunofluorescence.

Mouse anti-α-Spectrin (1:5) and mouse anti-Fasciclin III (1:10) were obtained from the Developmental Studies Hybridoma Gene Bank (Iowa), and Rabbit anti-Vasa (1:5000) was provided by R. Lehmann. Rat anti-*Drosophila* E-cadherin (1:20) was provided by T. Uemura (Oda et al., 1993). As secondary antibodies, FITC/TRITC-conjugated anti-rabbit, anti-mouse or anti-rat IgG (Jackson ImmunoResearch Laboratories) were used at 1:200 after overnight pre-absorption with 0-24 hour fixed embryos.

Immunofluorescence

Adult ovaries were dissected in *Drosophila* Ringer's, fixed with 4% formaldehyde/PBS for 15 minutes at room temperature, rinsed three times in PBT (PBS with 0.1% Triton X-100), then blocked for 1 hour with 10% normal goat serum in PBT before incubation with primary antibodies. Larval and adult testes were dissected in testis buffer and processed as squashed preparations on glass slides as described elsewhere (Hime et al., 1996). Samples were incubated overnight at 4°C in primary antibody, washed extensively in PBT, blocked with PBTB (PBS with 0.1% Triton X-100 and 0.3% BSA) for 1 hour at room temperature, incubated with secondary antibody at 37°C for 2 hours, washed extensively in PBT, stained with 1 μg/ml DAPI for 5 minutes and mounted in VECTASTAIN for examination by epifluorecence on a Zeiss Axiophot microscope. Images were recorded by CCD camera (Princeton Instruments, Trenton, NJ; IPLab Software, Spectrum Software Signal Analytics) or a BioRad MRC-100 confocal imaging system connected to a Zeiss Axioskop

microscope (except Fig. 7E, which was obtained with Leica TCS NT imaging software for a Leica DM RBE confocal microscope). All images were processed with Adobe Photoshop (Mountain View, CA).

Electron microscopy

Ovaries were fixed using two different protocols. We used the standard procedure of fixing at room temperature for 30 minutes in 2% glutaraldehyde, buffered with 0.1 M PO_4 at pH 7.1, followed by postfixation in 1% OsO_4 at 4°C and staining with 0.5% uranyl acetate for 2 hours (also at 4°C); tissues were dehydrated with an alcohol series and embedded in Epon. In the second fixation, to highlight extracellular space and outline gap junctions, we added 1% lanthanum nitrate to both fixatives and buffer washes. The embedded ovaries were sectioned and stained with both uranyl acetate and lead citrate before viewing in a Philips TEM CM120.

RESULTS

zpg function is required for differentiation of early germ cells

Wild-type function of the *zero population growth* (*zpg*) locus of *Drosophila* is required for early steps in gamete differentiation in both sexes. Although animals carrying a null mutation in *zpg* were fully viable, they were sterile and had tiny gonads (Fig. 1B,D).

Testes from animals mutant for *zpg* contained only small numbers of early germ cells up to pre-spermatocyte stage. In wild-type adults, six to nine male germline stem cells lie in a rosette surrounding the cluster of somatic hub cells at the apical tip of the testis (Fig. 2A, arrowhead). Upon stem cell division, the daughter next to the hub maintains stem cell identity, while the other daughter becomes a gonialblast and initiates four rounds of synchronous mitotic division with incomplete cytokinesis to produce a cyst of 16 interconnected spermatogonial cells, which then differentiate into spermatocytes (Fig. 2A, arrows). Wild-type male germline stem cells and gonialblasts both have a spherical spectrin-rich subcellular structure, the spectrosome (Fig. 2C, arrowhead and small arrow, respectively). By contrast, interconnected spermatogonia and spermatocytes have a linear and branching spectrin rich fusome (Fig. 2C, large arrow). The tiny testes from newly eclosed (0-2 day old) *zpg* mutant males had only a small number of germ cells, based on immunostaining with germ cell-specific markers. The germ cells usually appeared as single or small clusters of cells near the apical tip (Fig. 2B, arrowhead, and arrow, respectively). Immunostaining revealed that these germ cells contained spherical spectrin rich structures, suggesting stem cell or gonialblast identity (Fig. 2D, arrowheads). Germ cells in clusters reminiscent of spermatogonia had round or slightly tapered spectrin-rich structures (Fig. 2D, lower arrow), rather than fusomes, which appeared larger than the spectrosomes in stem cells. This suggested that the *zpg* null mutant spermatogonia attempted, but were unable to complete, differentiation.

Somatic support cells normally associated with early male germ cells were present in *zpg* mutant testes, although their morphological arrangement appeared abnormal. In wild type, two types of somatic cells, the hub and cyst cells, are in intimate contact with the germ cells. The area and number of the hub cells at the apical tip often appeared expanded in *zpg*-null males compared with wild type (Fig. 2E,F, arrows). Such

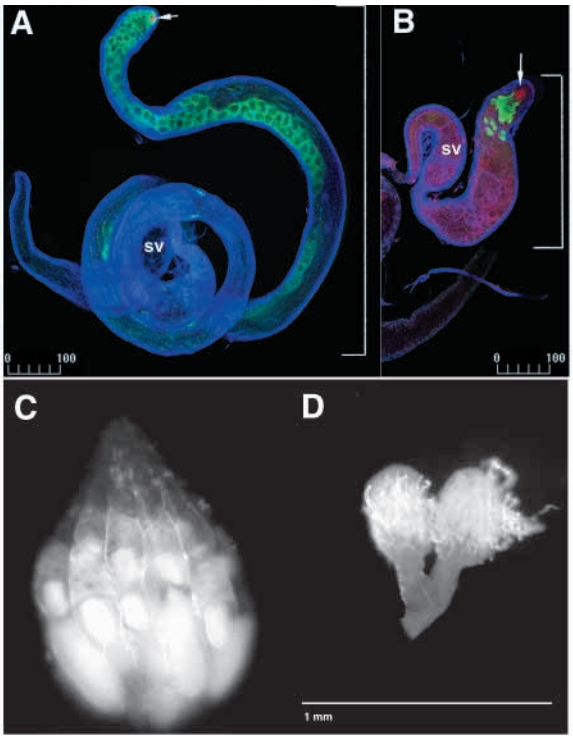

Fig. 1. *zpg* mutant animals have tiny testes and ovaries. (A,B) Whole testes from (A) wild type and (B) $zpg^{z-5352}/Df(3L)Zn47$ mutant male (2 days old after eclosion), all shown at the same magnification: early germ cells and spermatocytes stained with anti-Vasa (green); apical hub (arrow) stained with anti-*Drosophila* E-Cadherin (red); anti-α-Spectrin (blue). sv, seminal vesicle. Scale bar: 100 μm. (C) Whole wild-type ovary, and (D) a pair of ovaries from a $zpg^{z-5352}/Df(3L)CH12$ female (2 days old after eclosion), at same magnification.

abnormalities in the hub may be secondary to a defect in germ cells in *zpg* mutant testes, as similar abnormalities in hub morphology and cell number were described in testes lacking germ cells altogether (Gönczy and DiNardo, 1996). In wild type, a pair of somatic cyst progenitor cells enclose each germline stem cell. Their progeny, the somatic cyst cells, enclose the developing germ cells. Cyst progenitor and cyst cells were present in *zpg*-null testes, based on the appearance of GFP expressed in these cells under the control of a *ptc*-GAL4 driver (Fig. 2G,H). However, as only a few germ cells were present in the *zpg* mutant testes, many of the cyst cells did not appear to enclose germ cells, and so did not have the 'lacy' appearance characteristic of cyst cells in wild-type testes.

Wild-type function of *zpg* was also required for differentiation of early germ cells in females. The tiny ovaries from newly eclosed *zpg* mutant females lacked the strings of developing egg chambers characteristic of wild type (Fig. 3A,B). Instead, germaria from freshly eclosed females commonly contained only a few germ cells, which appeared as single cells at the apical tip of the germarium, located where female germline stem cells and cystoblasts reside (Fig. 3C,D, arrow). As in the male, female germ line stem cells and cystoblasts can be identified by spherical spectrin rich structures, spectrosomes, while the mitotically amplifying

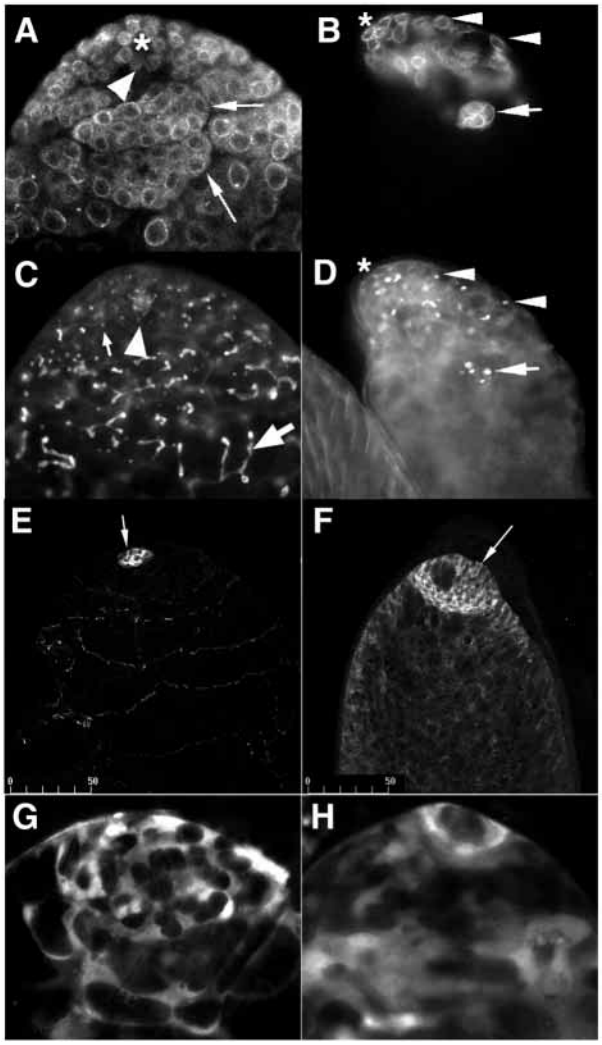

Fig. 2. Wild-type function of *zpg* is required for differentiation and survival of male germ cells. Apical tips of testes from (A,C,E,G) wild type and (B,D,F,H) $zpg^{z\text{-}5352}/Df(3L)Zn47$ males. (A,B) Germ cells at the testis apical tip labeled by staining with anti-Vasa. (A) Wild type. Rosette of single germline stem cells (arrowhead) surrounding the apical hub (*); cysts of spermatogonia or young spermatocytes (arrows). (B) $zpg^{z\text{-}5352}/Df(3L)Zn47$. Single germ cells (arrowheads) away from the hub (*); small cluster of germ cells resembling early spermatogonia (arrow). (C,D) Same samples as in A,B stained with anti-α-Spectrin to label spectrosomes in stem cells (arrowhead in C) and gonialblasts (small arrow in C, arrowhead in D), and the linear branched fusomes in wild-type spermatogonia (large arrows in C). (C) Wild type. (D) $zpg^{z\text{-}5352}/Df(3L)Zn47$. Germ cells resembling gonialblasts with spectrosomes (arrowheads); clusters of germ cells with abnormally large spectrosomes instead of fusomes (arrow). (E,F) Hub cells at the testes apical tip (arrows) labeled with anti-*Drosophila* E-Cadherin. (E) Wild type. (F) $zpg^{z\text{-}5352}/Df(3L)Zn47$. Note the expanded number of hub cells. Scale bar: 50 μm. (G,H) Somatic cyst progenitor and cyst cells marked by *ptc*-GAL4; UAS-GFP. (G) Wild type. Note 'lacy' network of cyst cells enclosing developing germ cell clusters. (H) $zpg^{z\text{-}5352}/Df(3L)Zn47$. Cyst cells are present but mostly either enclose single germ cells or no germ cells.

interconnected cysts contain branching fusomes (Fig. 3E). The germ cells remaining in *zpg* null mutant germaria had spherical spectrosomes rather than branched fusomes, suggesting stem cell or cystoblast identity (Fig. 3F, arrow). Occasionally, in freshly eclosed females, structures resembling egg chambers were observed (one per 2.9 ovaries, n=58 ovaries) further down the ovary, which contained abnormal number of germ cells that appeared to be degenerating (data not shown).

zpg encodes a germline-specific *Drosophila* gap junction protein

We cloned the *zpg* locus by plasmid rescue of sequences flanking the P-element insert in the zpg^3 allele (Fig. 4A), followed by isolation of genomic and testis cDNA clones from the region (see Materials and Methods). Sequence analysis of the flanking DNA revealed that the P-element in zpg^3 was inserted in the 5′ UTR, just upstream of the start of the open reading frame for CG10125. Sequence analysis of several EMS-induced *zpg* alleles revealed point mutations in the protein-coding region of CG10125, identifying it as *zpg* (Table 1; Fig. 4C). Three alleles with nonsense mutations that introduce premature stop codons and an allele with a missense mutation that changes a conserved proline residue to serine in a predicted extracellular loop of the protein had strong phenotypes. The *zpg*-coding region is contained within a large intron of a transcript on the opposite strand, identified through a testis cDNA, which encodes a predicted protein with a small region of homology to yeast RNase H (Fig. 4A). A 6.1 kb genomic fragment containing *zpg* but lacking the 3′ end of the protein-coding region of the RNase H-like gene fully rescued the male and female sterile and small gonad phenotypes when introduced into *zpg* mutant flies.

Sequence analysis of a near full-length ovary cDNA revealed that *zpg* encodes Innexin 4, a member of the gap junction protein gene family in *Drosophila* (Curtin et al., 1999). The predicted Zpg protein has four probable transmembrane regions (Fig. 4B) and four signature conserved cysteines in the predicted extracellular loops, similar to other members of the *Drosophila* Innexin gene family. Similar conserved cysteines in mammalian gap junction proteins have been shown to be important for the docking of gap junction proteins across the two opposing cell membranes to form a functional intercellular channel (reviewed by White and Bruzzone, 1996).

The *zpg* locus encodes a 1.6 kb transcript detected in poly A+ mRNA from whole adult males and females but not from

Table 1. Molecular lesions in *zpg* alleles

zpg allele	Base pair change	Amino acid change	Phenotype*
zpg^1	A (150) to T	D (21) to V	Weak
zpg^2	C (919) to T	P (235) to S	Strong
zpg^3	P element insertion in 5′ UTR		Strong
$zpg^{3\text{-}ex}$	Excision of P insertion		Strong
$zpg^{z\text{-}5352}$	C (359) to T	R (91) to Stop	Strong
$zpg^{z\text{-}2533}$†	A (931) to T	K (239) to Stop	Strong
$zpg^{z\text{-}2552}$†	A (931) to T	K (239) to Stop	Strong
$zpg^{z\text{-}0918}$	G (926) to A	C (237) to Y	Strong
$zpg^{z\text{-}2679}$	G (366) to A	Splice acceptor site at residue 92	Weak
$zpg^{z\text{-}3860}$	G (1043) to A	W (276) to Stop	Strong

*Based on phenotype of $zpg^{allele}/Df(3L)Zn47$.

†*z-2533* and *z-2552* have identical molecular base pair changes in the *zpg*-coding region, but *z-2552* has slightly more germ cells in the gonad.

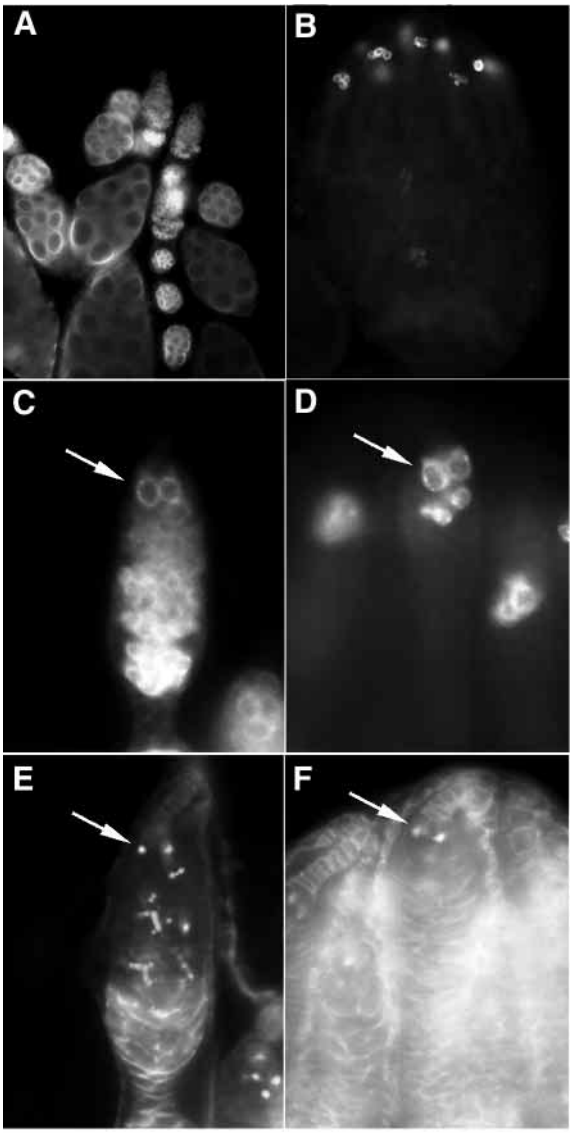

Fig. 3. Wild-type function of *zpg* is required for survival of differentiating female germ cells. (A,B) Ovarioles and (C-F) germaria from newly eclosed (A,C,E) wild type and (B,D,F) *zpg*$^{z\text{-}5352}$*/Df(3L)Zn47* females. (A-D) Germ cells labeled by immunofluorescence with anti-Vasa. (B,D) *zpg* mutant ovaries contained only a few early germ cells, usually located at the tip of the germarium. (C,D) Higher magnification views of individual germaria. (E,F) Same samples as in C,D stained with anti-α-Spectrin to show spectrosomes and fusomes and with anti-Fasciclin III to outline somatic cells. Germ cells (arrows in C-F) with a spherical spectrosome at the stem cell position in (C,E) wild type and (D,F) *zpg*$^{z\text{-}5352}$*/Df(3L)Zn47* germaria.

agametic animals (Fig. 5A). Consistent with the transcript being germline dependent, in situ hybridization to embryos revealed that *zpg* mRNA was concentrated in germ plasm and in the pole cells of wild-type embryos, from the syncitial blastoderm stage through gonad formation (Fig. 5C,D). Zpg protein was also detected in pole cells and primordial germ cells throughout embryogenesis (data not shown). In wild-type testes, *zpg* mRNA was detected in the spermatogonial region near the apical tip (Fig. 5B, arrow). The level of *zpg* mRNA decreased sharply to background at the transition from spermatogonia to spermatocytes.

Zpg protein is localized to the germ cell surface and enriched at points of contact between germ cells and somatic support cells

In testes, Zpg protein was expressed in spermatogonia and early spermatocytes, where it appeared to be concentrated at the interface between germ cells and somatic cyst cells. The anti-Zpg antibody (see Materials and Methods) detected discrete patches of protein on the surface of early spermatogonia during the mitotic amplification stage (Fig. 6). A similar pattern was seen in both larval and adult testes (data not shown). The appearance of Zpg protein on the surface of early spermatogonia correlated with the stage at which early germ cells were lost in *zpg* mutant males (Fig. 2B,D). In later spermatogonial and early spermatocyte cysts, anti-Zpg staining was distributed more evenly over the germ cell surface but was especially concentrated at the outer surface of the germ cell cluster, where the germ cells interface with the enveloping somatic cyst cells (Fig. 6). Staining with the anti-Zpg antibody became weaker and more diffuse during the subsequent primary spermatocyte stage. Pre-immune serum used at a similar dilution did not stain the cell surface of male germ cells, and pre-absorption of the anti-Zpg antiserum with the oligopeptide used as the immunogen blocked staining of the surface of spermatogonia (data not shown). In addition, no staining was detected in the adult or larval testes of *zpg*$^{z\text{-}5352}$*/Df* mutant animals, in which the mutant *zpg* transcript lacked the very C terminus against which the antiserum was raised. The spatiotemporal correlation between the appearance of Zpg protein on the surface of spermatogonia in wild-type testis and the defective differentiation and loss of spermatogonial cells in *zpg* mutant testes suggests that gap junctional communication between spermatogonia and somatic cyst cells may be required for normal differentiation and survival of spermatogonia.

In ovaries, Zpg protein was present on the surface of developing germ cells (Fig. 7A), at least up to stage 10 of oogenesis (data not shown). In developing egg chambers, anti-Zpg antibody staining was particularly striking at the germ cell/somatic follicle cell interface, where under conditions of lighter staining, Zpg protein appeared to be concentrated on the germ cell surface in a discrete patch under each follicle cell (Fig. 7A-C, arrows). The distribution of Zpg protein appeared more continuous at the nurse cell/nurse cell interface (Fig. 7A-C, arrowheads). In the germarium, Zpg protein was detected on the surface of all germ cells, including stem cells (Fig. 7E,F). Zpg protein appeared to be concentrated in discrete patches on the surface of dividing cysts (Fig. 7E,F arrowheads), where germ cells are in contact with cytoplasmic extensions from the somatically derived inner germarium sheath cells (C. S., S. I. T. and M. T. F., unpublished) (Margolis and Spradling 1995). Pre-absorption with the Zpg C-terminal oligopeptide used as the immunogen eliminated the staining of the surface of female germ cells in both the germarium and egg chambers (Fig. 7D,G).

In female germline stem cells, Zpg protein also appeared to localize to a small plaque adjacent to the spectrosome at the interface between female germline stem cells and somatic apical cap cells (Fig. 7F,I,J,L,M arrows), under conditions where less overall anti-Zpg staining was detected. In an

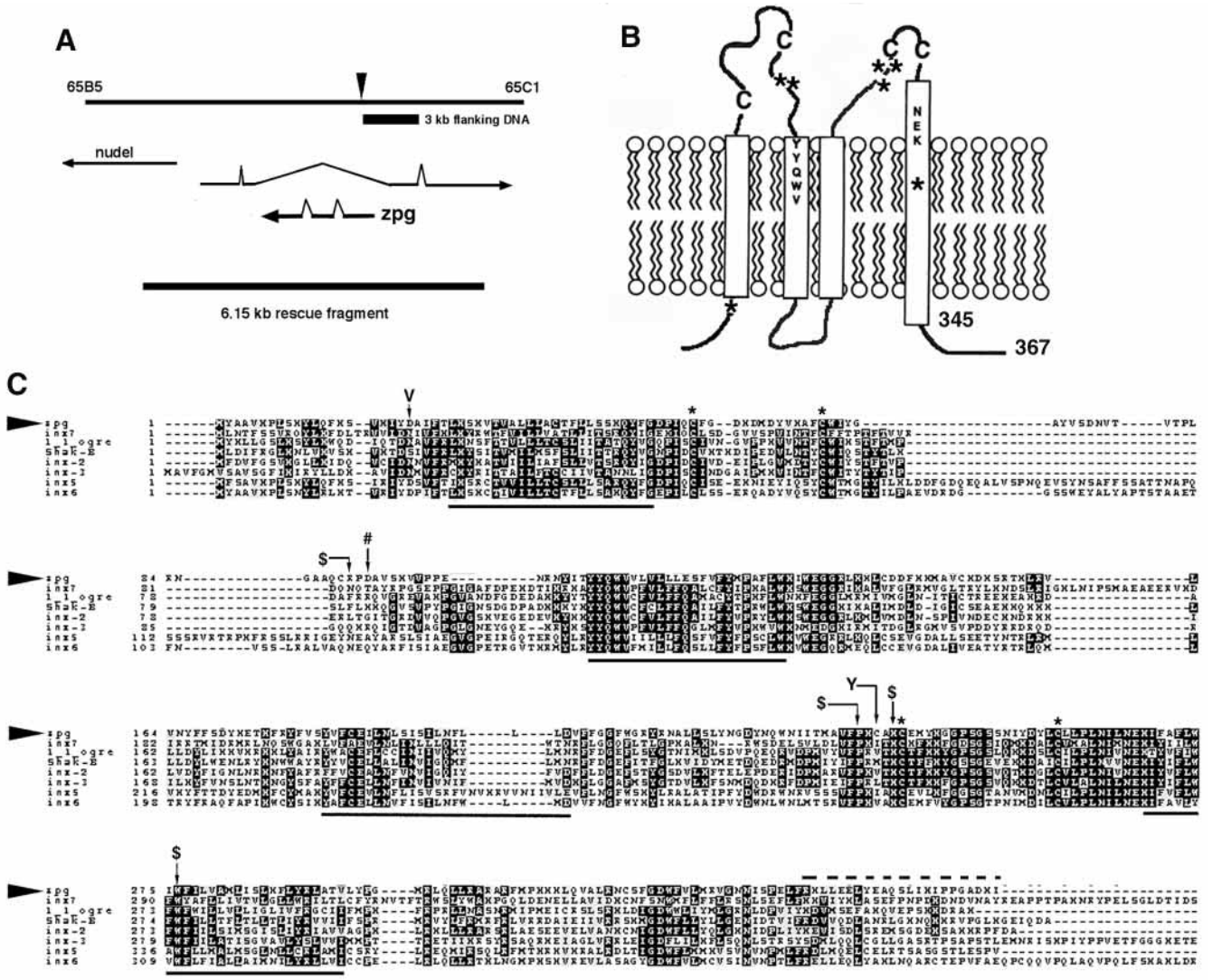

Fig. 4. *zpg* encodes a *Drosophila* gap junction protein – Innexin-4. (A) *zpg* genomic region in 65B5-65C1 (top) with position of P element insert in zpg^{3}. Predicted transcripts (horizontal arrows); 6.15 kb genomic fragment that rescued *zpg* (bold bottom line). (B) Predicted topology of the Zpg protein, showing four transmembrane domains and conserved extracellular cysteine residues. Asterisks indicate sites of mutations in EMS-induced *zpg* alleles. (C) Multiple alignment and comparison (ClustalW) of eight innexin family members in *Drosophila*. Predicted transmembrane domains (unbroken bars); conserved predicted extracellular cysteine residues (asterisks); the oligopeptide used to generate the anti-sera is based on residues 345-367 (broken line). Amino acid changes in *zpg* alleles indicated above the protein sequence. ($) Stop codon; (#) base pair change at the splice acceptor site in $zpg^{z\text{-}2679}$ allele leads to an altered predicted amino acid sequence starting at amino acid residue D(93).

experiment where wild-type ovaries were stained with anti-α-Spectrin and anti-Zpg antibodies, this dot was detected in 258 of the 289 stem cells scored from 10 different ovaries. The small plaque of anti-Zpg staining next to the spectrosome at the tip of the germarium was not detected in germ cells from $zpg^{z\text{-}5352}$/*DfZn47* third adult ovaries (data not shown), confirming the specificity of the antibody.

The position of the spot of Zpg detected just apical to the spectrosome in female germline stem cells by immunofluorescence suggested the possibility that there are gap junctions between the female germline stem cells and the overlying somatic cap cells. The presence of gap junctions in early female germ cells was confirmed by ultrastructural studies. In two separate sets of serial sections through the spectrosome region of female germline stem cells, gap junctions with the characteristic 2×10^{-9} m (20 Å) intermembrane spacing were clearly evident between female germline stem cells and adjacent apical cap cells (Fig. 8A-C, arrow). We do not know whether these gap junctional structures between female germline stem cells and apical cap cells correspond to the spots of Zpg detected adjacent to the spectrosome by immunofluorescence, although their relative positions were the same. In addition, we observed that the intercellular space between germline stem cells and apical cap cells directly abutting the spectrosome was large (>200 Å; $>2\times10^{-8}$ m) and filled with lanthanum when stained with this substance (Fig. 8B, arrowheads). The components of this distinctive space are not known, although the space was characteristic of the five germaria studied by electron microscopy. Adherens junction were also seen between

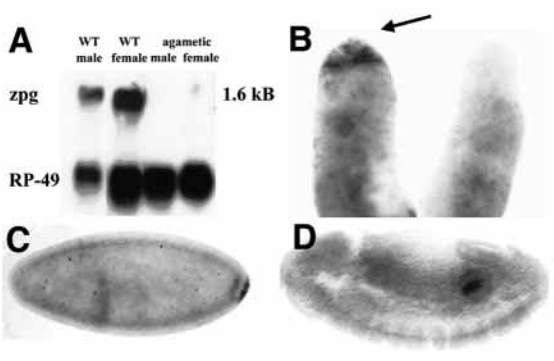

Fig. 5. Germline specific expression of *zpg*. (A) Northern blot showing 1.6 kb *zpg* transcript detected in adult males and females containing germ cells but not in agametic adults. (B) Expression of *zpg* mRNA in wild-type adult testes assayed by in situ hybridization: left testis probed with *zpg* antisense RNA. Right testis probed with sense strand control. *zpg* transcript is in spermatogonia at the testis apical tip (arrow). (C,D) Expression of *zpg* mRNA in wild-type embryos assayed by in situ hybridization with *zpg* antisense probe. Anterior is towards the left. (C) Blastoderm stage embryo showing *zpg* mRNA in pole cells, (D) *zpg* mRNA in primordial germ cells in coalescing gonads.

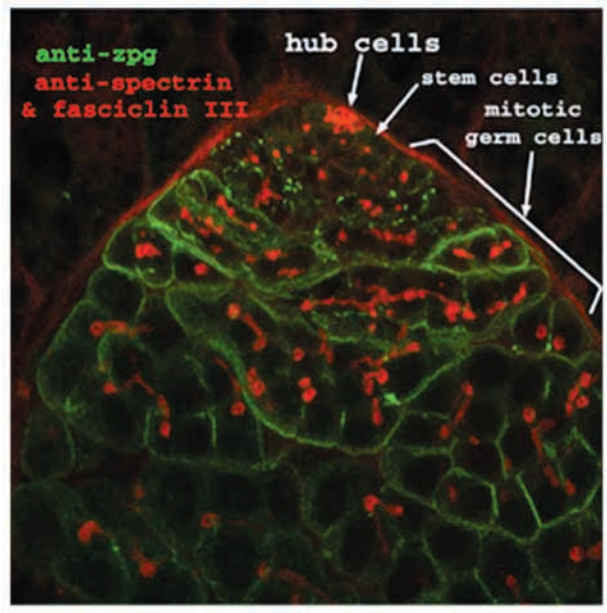

Fig. 6. Zpg protein (green) concentrated at the interface between germ cells and surrounding somatic cells in spermatogonia and early spermatocytes. Confocal image of apical tip of wild-type third instar larval testis. Apical hub cells stained by immunofluorescence with anti-Fasciclin-III (red), plus spectrosomes and fusomes in germ cells stained by anti-α-Spectrin (red).

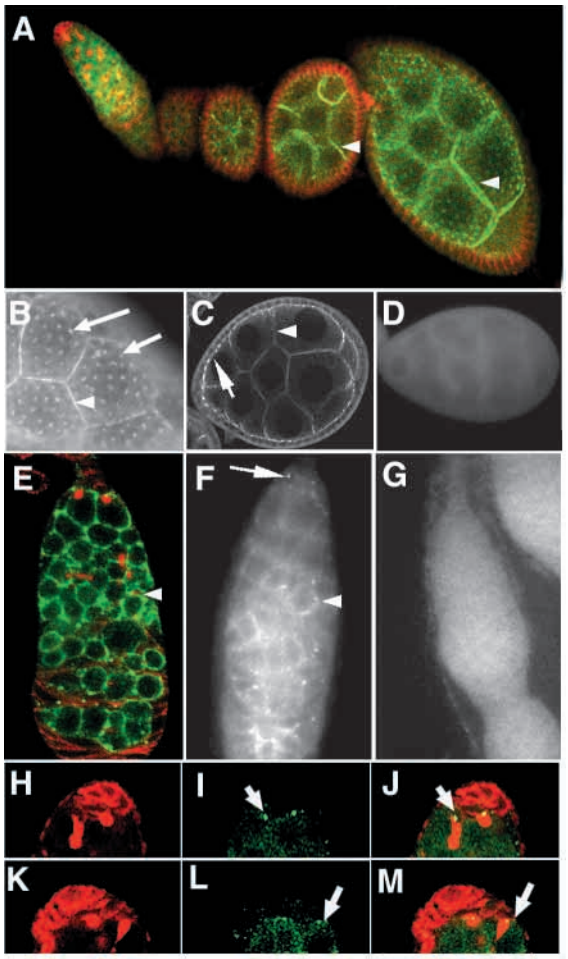

Fig. 7. Zpg protein is localized on the surface of germ cells in the ovary. (A) Wild-type ovariole through stage 8-9, stained with anti-Zpg (green) and anti-Fasciclin-III (red) plus anti-α-Spectrin (red). (B-D) Wild-type egg chambers, (B) stage 6 and (C,D) stage 7, stained with (B,C) anti-Zpg or (D) anti-Zpg sera pre-adsorbed with the C-terminal Zpg peptide immunogen. Plaques of Zpg staining (arrows) at the nurse cell-follicle cell interface; diffuse Zpg staining (arrowheads) at the nurse cell-nurse cell interface. (E,F) Wild-type germaria stained with anti-Zpg antisera. In both cases, anti-Zpg antibody stained the surface of early germ cells in the germarium. Zpg staining (arrowheads) in the dividing cysts. (E) Anti-Zpg (green) and anti-α-Spectrin (red). (F,I,J,L,M) Under conditions with lower levels of overall staining, Zpg protein was detected in a discrete dot (arrow) adjacent and just apical to the spectrosome in female germline stem cells. (G) Wild-type germaria stained with anti-Zpg sera pre-adsorbed with the C-terminal Zpg peptide immunogen. (H-M) High magnification views of the apical tip of two different wild-type germaria co-stained with anti-Zpg (green) (I,J,L,M) and anti-Fasciclin III plus anti-α-Spectrin (H,J,K,M) (red). (J,M) Merged images of H,I and K,L respectively. Dots of Zpg staining at the germline stem cell-cap cell interface (arrows).

germline stem cells and apical cap cells (Fig. 8C). Gap junctions were also observed at the ultrastructural level between adjacent germline stem cells, between cystoblasts, between cysts, between cystoblasts and inner sheath cells, and between adjacent nurse cells (data not shown). A cluster of multiple gap junction structures was visible by electron microscopy between follicle cells and underlying nurse cells in developing egg chambers (data not shown), consistent with the patch of Zpg staining at the base of each follicle cell observed by immunofluorescence light microscopy. As already stated, we do not know whether these gap junctional structures contain Zpg protein.

The phenotypic analysis of freshly eclosed *zpg*-null mutant ovaries suggests that Zpg function is not required for the initial

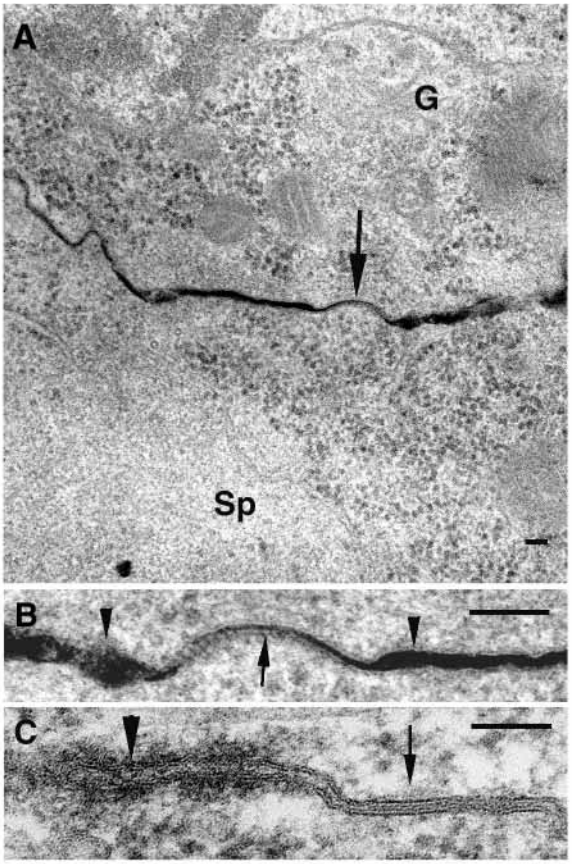

Fig. 8. Gap junctions between germline stem cell and cap cell viewed by electron microscopy. Scale bar=100 nm. (A) Low-magnification infiltrated with lanthanum, showing the spectrosome (Sp) of the stem cell (lower cell) and the adjacent cap cell (upper cell) with a prominent Golgi apparatus (G). Gap junction (arrow). (B) Higher magnification of A. Gap junction with characteristic 2×10^{-9} m (20 Å) spacing (arrow). Wide lanthanum-filled intercellular space (arrowheads in B and C) (>200 Å; 2×10^{-8} m) adjacent to gap junction. (C) A second germarium, fixed without lanthanum, showing a gap junction (arrow) between a stem cell (below) and cap cell (above).

placement of germ cells in the stem cell niche during ovary morphogenesis, although we can not rule out the possibility that minute undetectable amount of maternally derived Zpg product perdured during the larval stages. Analysis of older *zpg*-null mutant females revealed that the number of germaria with early germ cells in the stem cell niche at the apical tip decreased with age (Fig. 9). In three separate experiments, 70-93% of germaria from newly eclosed *zpg*/*Df* females had at least one and usually two or more early germ cells in the stem cell position at the apical tip (Fig. 9B). By contrast, in 3-week-old *zpg*-null mutant females of the same genotype, only 17-24% of germaria had even one Vasa-positive cell at the apical tip (Fig. 9C), when compared with 100% of wild-type control germaria. In some ovaries, ovarioles that lacked germ cells at the tip of the germarium had one or a few differentiating egg chambers further down the ovariole (Fig. 9C, lower right), as if germline stem cells were not maintained but instead initiated differentiation in the absence of *zpg* function in older females. The differentiating egg chambers in aged *zpg* females commonly appeared abnormal.

DISCUSSION

The *zpg* locus of *Drosophila* encodes a germline specific gap junction protein required for early steps of gamete differentiation and survival in both sexes. Gap junctions are intercellular channels assembled from connexin (vertebrate) or innexin (invertebrate) subunits, six of which oligomerize to form a cylindrical hemichannel in the plasma membrane (Bruzzone et al., 1996; White and Bruzzone, 1996; Phelan et al., 1998; Curtin et al., 1999). Hemichannels on two adjacent cell surfaces dock end-to-end to form gap junctions, which are commonly voltage gated and permit passage of ions and small molecules, such as nucleotides between the coupled cells. Vertebrates and invertebrates both have several gap junction protein isoforms, which can combine to form gap junctions with different permeability properties and regulation (Bruzzone et al., 1996; White and Bruzzone, 1996; Phelan et al., 1998; Curtin et al., 1999). The *zpg* protein has been shown to form functional, voltage-gated, heterotypic gap junctions in the paired *Xenopus* oocytes system, with one oocyte expressing *zpg* and the partner oocyte expressing a different *Drosophila* gap junction protein, Inx2 (J. Davies, personal communication). Strikingly, functional channels did not form when both oocytes expressed the Zpg protein, suggesting that Zpg forms heterotypic but not homotypic gap junctions (J. Davies, personal communication).

In both sexes, the Zpg protein was detected on the surface of germ cells where they interface with adjacent somatic cells. Gap junctions have been observed at the ultrastructural level between germ cells and associated somatic cells in both sexes in insects including *Drosophila* (Szöllösi and Marcaillou, 1980; Huebner, 1981; Adler and Woodruff, 2000). We propose that hemichannels made of Zpg on the surface of germ cells dock with hemichannels made of other innexin isoforms on the surface of somatic cells to form functional gap junctions. Of the eight innexins in the *Drosophila* genome (Curtin et al., 1999; Phelan and Starich, 2001), *ogre*, *inx2*, and *inx3* have been found to be expressed in follicle cells (Stebbings et al., 2002). Although the expression pattern of other innexins in testes has not been reported, we found that *inx2* message was expressed at the apical tip of the testis and follicle cells of egg chambers (S. I. T. and M. T. F., unpublished). Furthermore, ESTs matching *inx2*, *inx5* and *ogre* transcripts are found in adult testis cDNA library (Berkeley Drosophila Genome Project, http://www.fruitfly.org/), suggesting that, in both sexes, other innexins are expressed in the *Drosophila* gonad, in addition to *zpg*. Heterotypic gap junctions between germline and soma, which are required for gametogenesis, are reminiscent of connexin-derived gap junctions in the mammalian gonad. The mammalian connexin *Cx37* (*Gja4* – Mouse Genome Informatics), which is expressed on the mouse oocyte surface, is thought to form a heterotypic channel with a gap junction hemichannel containing *Cx43* (*Gja1* – Mouse), which is expressed on the surrounding somatic cumulus cells (Sutovsky et al., 1993; Juneja et al., 1999). Mice with targeted disruption of *Cx37* have defects in follicular growth with premature granulosa cell luteinization, resulting in infertility (Simon et al., 1997). Zpg protein was also detected on the surfaces between adjacent germ cells, where it may form a hemichannel together with other innexin isoforms possibly expressed in germ cells in small amounts to give rise to functional gap

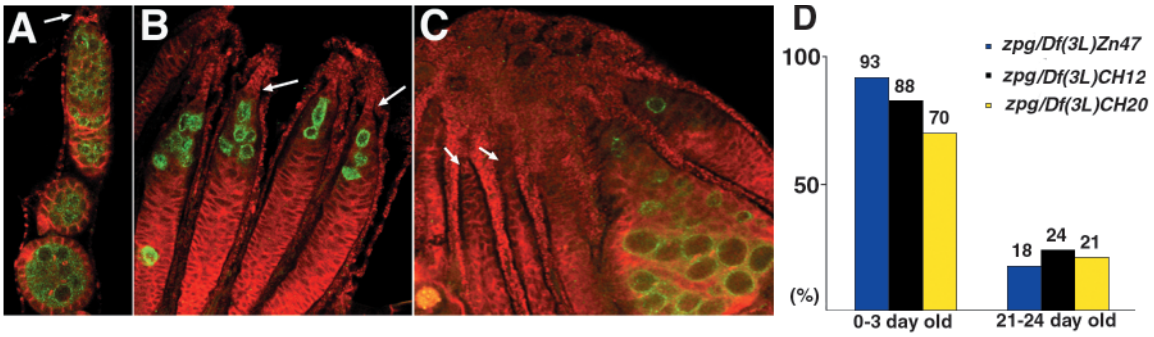

Fig. 9. Loss of female germline cells in stem cell niche with age in *zpg* mutants. (A) Wild-type tip of an ovariole. (B,C) Whole ovaries from newly eclosed (B) and 3-week-old (C) zpg^{z-5352}*/Df(3L)Zn47* females labeled with anti-Vasa (green) and anti-Fasciclin-III (red). A-C are at same magnification. Terminal filament (arrow). (D) Percent of germaria containing germ cells at the tip for 0- to 3-day-old versus 21- to 24-day-old *zpg/Df* females. Blue bars: zpg^{z-5352}*/Df(3L)Zn47* (*n*=324 germaria from 0- to 3-day-old females, *n*=415 germaria from 21- to 24-day-old females). Black bars: zpg^{z-5352}*/Df(3L)CH12* (*n*=388 from 0- to 3-day-old females, *n*=430 from 21- to 24-day-old females). Yellow bars: zpg^{z-5352}*/Df(3L)CH20* (*n*=526 from 0- to 3-day-old females, *n*=389 from 21- to 24-day-old females).

junctions between adjacent germ cells. Alternatively, the Zpg protein at the interface between adjacent germ cells may not form functional channels.

The requirement for *zpg* function appears to be different in germ cells occupying the stem cell niche than in dividing cyst cells or spermatogonia, as stem cells were initially present in newly eclosed *zpg*-null animals. The striking loss of early germ cells at the onset of gamete differentiation in *zpg*-null animals raises the possibility that gap junctions may mediate passage of small molecule nutrients or signals from the surrounding somatic cells that are required for germ cell differentiation or survival. Gap junctional intercellular communication could be required for early stages of gamete differentiation, with germ cells undergoing cell death if unable to follow the normal differentiation program properly. The observation that spectrin-rich structures remained spherical and never reached the branched fusome stage, even in clustered germ cells resembling mitotic spermatogonia or cyst cells, suggests that the earliest stages of gamete differentiation are defective in *zpg*-null gonads. The spectrin-rich structures in the clustered *zpg*-null spermatogonia were larger than the usual spherical spectrosomes and often had abnormal morphology, suggesting that the differentiation program may have initiated but failed to complete. Although *zpg* germ cells did not accumulate, no striking increase in Acridine Orange staining was detected in *zpg* gonads (data not shown), suggesting that *zpg* germ cells maybe rapidly lost after the onset of differentiation. Furthermore, the small number of germ cells present in a *zpg* mutant gonad was not due to failure in mitosis, as germline stem cells appeared to divide at the same frequency in newly eclosed *zpg* null mutant females as in wild type (L. G. and R. L., unpublished).

Interactions between early germ cells and somatic cells are known to play an essential role in early germ cell differentiation in both sexes. In males, for example, normal differentiation of spermatogonia from male germline stem cells requires a functional EGFR signaling pathway in the surrounding somatic cells (Kiger et al., 2000; Tran et al., 2000). Later, after mitotic amplification of spermatogonial cells, activation in somatic cyst cells of a receptor in the TGFβ signaling pathway is essential for germ cells to transition from the mitotic amplification program to spermatocyte growth, meiosis and spermiogenesis (Matunis et al., 1997). In neither case have the crucial signals from somatic support cells to the germ cells they enclose been identified. Our data on the mutant phenotype and the molecular identity of *zpg* gene product raise the possibility that crucial small molecule nutrients or signals regulating *Drosophila* germ cell differentiation and survival may be transmitted via gap junctions. Intriguingly, in mammals, gap junction permeability is regulated by EGFR pathway signaling via phosphorylation of the cytoplasmic tails of connexins by MAPK (Warn-Cramer et al., 1998). Activation of the EGFR in somatic cyst cells could signal to germ cells by changing the permeability of gap junctions for small molecule second messengers between germline and soma.

Gap junctions in the *Drosophila* gonad may also mediate transfer of small molecule nutrients between germline and soma. Mammalian follicle cells have been shown to take up and phosphorylate labeled nucleotides from the culture medium, then release them to the oocyte (Heller and Schultz, 1980), possibly via gap junctional intercellular channels. In developing egg chambers, Zpg protein was especially concentrated at the interface between each follicle cell and the underlying germ cell, consistent with the observation of gap junctions between germ cells and follicle cells of other insects by electron microscopy. Because Zpg function is required during the earlier steps of oogenesis, we could not determine the precise function of Zpg-derived gap junctions in egg chambers. However, electrical coupling and permeability to Lucifer Yellow dye, both characteristics of gap junctions, have been observed between germ cells and follicle cells in *Drosophila* and other insects (Woodruff, 1979; Huebner, 1981; Adler and Woodruff, 2000). Thus, it is possible that insect follicle cells also function to contribute to the growth of the oocyte by the uptake, metabolic conversion and intercellular transfer of small molecules via gap junctions.

Gap junctional communication between female germline stem cells and somatic apical cap cells may play a role in long term stem cell maintenance at the tip of the ovariole. Under specific staining conditions, *zpg* protein in female germline stem cells localized to a distinct dot adjacent to the spectrosome at the side where the germline stem cells abut the

somatic apical cap cells. The terminal filament and cap cells at the apical tip of the germarium regulate germline stem cell behavior (Lin and Spradling, 1993), in part through a signaling pathway involving the TGFβ homolog, *decapentaplegic* (*dpp*) (Xie and Spradling, 1998; Xie and Spradling, 2000). The loss of female germline stem cells with age in *zpg* mutants raises the possibility that gap junctional communication dependent on *zpg* might also be required to mediate signaling from apical cap cells for stem cell maintenance. Alternatively, gap junctions containing *zpg* may help maintain female germline stem cells in their niche by contributing to mechanical adhesion between stem cells and the apical cap cells (Watt, 2001), perhaps in conjunction with the adherens junctions observed adjacent to gap junctions between germline stem cells and adjoining cap cells (Fig. 8C) (A. P. M., unpublished).

We thank B. Wakimoto and D. Lindsley for kindly providing the Zuker male sterile lines, T. Uemura for rat anti-*Drosophila*-E-cadherin antibody, T. Hazelrigg for the testis cDNA library, the EM core facility of the University of Chicago Cancer Research Center, John Perrino for technical assistance with EM, and members of the Fuller laboratory for critical review and editing of the manuscript. This work was supported by the NIH-Reproductive Scientist Development Program (S. I. T.), Burroughs-Wellcome Fund (S. I. T.), an EMBO postdoctoral fellowship (C. S.), the Human Frontier Science Program at New York University Medical Center (L. G.), a Ministère de la Recherche et du Travail predoctoral fellowship (A. G.), NIH grant HD17608 (A. P. M.), the Howard Hughes Medical Institute (R. L.) and NIH grant P01-DK53074 (sub project #4) (M. T. F.).

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