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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

A somatic permeability barrier around the germline is essential for *Drosophila* spermatogenesis

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ABSTRACT

Interactions between the soma and germline are essential for gametogenesis. In the *Drosophila* testis, differentiating germ cells are encapsulated by two somatic cells that surround the germline throughout spermatogenesis. *chickadee* (*chic*), the fly ortholog of Profilin, mediates soma-germline interactions. Knockdown of Chic in the soma results in sterility and severely disrupted spermatogenesis due to defective encapsulation. To study this defect further, we developed a permeability assay to analyze whether the germline is isolated from the surrounding environment by the soma. We find that germline encapsulation by the soma is, by itself, insufficient for the formation of a permeability barrier, but that such a barrier gradually develops during early spermatogenesis. Thus, germline stem cells, gonialblasts and early spermatogonia are not isolated from the outside environment. By late spermatocyte stages, however, a permeability barrier is formed by the soma. Furthermore, we find that, concomitant with formation of the permeability barrier, septate junction markers are expressed in the soma and localize to junctional sites connecting the two somatic cells that surround the germline. Importantly, knockdown of septate junction components also disrupts the permeability barrier. Finally, we show that germline differentiation is delayed when the permeability barrier is compromised. We propose that the permeability barrier around the germline serves an important regulatory function during spermatogenesis by shaping the signaling events that take place between the soma and the germline.

KEY WORDS: Blood-testis barrier, Septate junctions, Profilin, *Drosophila*

INTRODUCTION

Two tissue types populate animal gonads; the germline, which gives rise to the gametes; and the soma, which gives rise to all other tissues that support and maintain gamete formation. Gametogenesis requires ongoing cooperation between the soma and germline and disruption of somatic support cells can prevent the production of gametes and lead to sterility (Griswold, 1998; Korta and Hubbard, 2010; Zoller and Schulz, 2012). The soma provides support and instructive cues for the germline, including such roles as forming the stem cell niche that regulates germline stem cells (Kiger et al., 2001; Kimble and White, 1981; Meng et al., 2000; Xie and Spradling, 2000), providing signals that instruct the germline during gamete differentiation (Govindan et al., 2009; Kiger et al., 2000; Killian and Hubbard, 2005; Tran et al., 2000), supplying nutrients to the developing germline (Boussouar and Benahmed, 2004), and maintaining the tissue architecture required for gamete production (Tanentzapf et al., 2007; Vogl et al., 2008). Understanding soma-germline interactions is thus key to understanding gametogenesis.

Owing to the close cooperation of the soma and germline during gametogenesis, the germline is typically embedded in or surrounded by somatic tissue (França et al., 2012; Roosen-Runge, 1977). Somatic support cells often completely envelop the developing germline such that the soma can effectively isolate the germline from the rest of the organism. This isolation is secured by the presence of an occluding barrier formed between the somatic cells surrounding the germline. This soma-germline barrier is a feature that has been well-characterized in the testes (França et al., 2012) in both mammals (Setchell et al., 1969; Dym and Fawcett, 1970) and insects (Szöllösi and Marcaillou, 1977). The soma-germline barrier has been proposed to play diverse roles in the testis, but chief among them is its proposed function in regulating the germline signaling environment during differentiation (Cheng and Mruk, 2012; França et al., 2012; Lui et al., 2003b). Additionally, it is thought that the soma-germline barrier protects the germline from cytotoxic materials (Su et al., 2011) and from the immune system in vertebrates (Kaur et al., 2014).

The vertebrate soma-germline barrier, called the Sertoli cell barrier (SCB) or the blood-testes barrier (BTB), has been extensively studied (Cheng and Mruk, 2012; França et al., 2012; Lui et al., 2003b). The BTB is formed by an epithelial layer of somatic Sertoli cells and acts to separate the early phases of spermatogenesis, which occur in the basal compartment of the epithelium, from later phases, which occur in the apical compartment. The physical separation of the apical and basal compartments by the BTB is thought to be a direct consequence of an occluding function by a belt of tight junctions (Dym and Fawcett, 1970; Lui et al., 2003b). Disruption of the BTB in vertebrates leads to failures in germline differentiation and consequently to sterility (Gow et al., 1999; Mazaud-Guittot et al., 2010; Mok et al., 2012).

The testis of *Drosophila melanogaster* is an important model for studying soma-germline interactions (Jemc, 2011; Zoller and Schulz, 2012). The fly testes are coiled tubular structures that contain a stem cell niche in their apical region, known as the hub (Hardy et al., 1979). The hub is composed of 8-15 somatic cells and functions to adhere to and regulate both germline stem cells (GSCs) and somatic cyst stem cells (CySCs). GSCs undergo asymmetric divisions to create a gonialblast, which then undergoes incomplete divisions to form syncytial spermatogonia. After four rounds of transit-amplifying divisions, the germline forms 16 postmitotic spermatocytes that grow, undergo meiosis, and form 64 spermatids (Davies and Fuller, 2008; Fuller, 1993). CySCs also undergo asymmetric divisions producing cyst cells, two of which surround the gonialblast and encapsulate it such that the differentiating germline develops within the lumen formed between the two somatic cyst cells (Hardy et al., 1979; Cheng et al., 2011; Jemc,

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2011; Zoller and Schulz, 2012). During encapsulation, germ cells activate EGFR and MAPK signaling in the soma, which act on the actin cytoskeletal regulators Rac and Rho to coordinate the somatic envelopment of the germline. Disruption of EGF signaling inhibits encapsulation and leads to defects in germline differentiation resulting in spermatogonia-like tumors (Kiger et al., 2000; Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000).

In this study, we sought to identify genes that act in the soma to regulate soma-germline interactions during spermatogenesis, specifically during encapsulation. Our work identified *chickadee* (*chic*), the sole *Drosophila* ortholog of the actin-binding protein Profilin, as being essential for this process. To further understand how encapsulation regulates germline development, we developed a novel assay to assess the formation and maintenance of a somatic permeability barrier around the germline. Using this assay, we demonstrate that encapsulation and the formation of a permeability barrier are separate processes, both of which require Chic. Moreover, we show that the formation of the permeability barrier correlates with the expression of septate junction components, which are functionally analogous to vertebrate tight junctions (Banerjee et al., 2006). In addition, knockdown of septate junction proteins disrupts the formation of the permeability barrier. Finally, we provide evidence that the permeability barrier is required to restrict the range of niche-derived BMP signaling. Together, our work identifies a somatic permeability barrier in the fly testis that plays a role in regulating germline differentiation and suggests that this barrier might modulate access of the germline to signaling molecules from the outside environment.

RESULTS

Chic is required in the soma for fertility

We sought to identify genes required in the soma for germline development. We carried out an RNAi-based fertility screen with the soma-specific driver *tj*-Gal4 (Li et al., 2003) to target gene knockdown in early somatic cyst cells of the fly testis (M.J.F. and G.T., unpublished). This screen identified *chic*, the fly homolog of the actin-polymerizing protein Profilin (Cooley et al., 1992; Jockusch et al., 2007; Verheyen and Cooley, 1994), as being required in the soma for spermatogenesis. We used three RNAi lines that target independent regions of the *chic* mRNA to confirm that sterility was specifically due to Chic knockdown (Fig. 1G). Expression of any of these *chic* RNAi lines in the soma led to sterility, with or without the co-expression of Dicer2, which has been shown to enhance RNAi knockdown phenotypes (Dietzl et al., 2007) (Fig. 1E). Morphological analysis of Chic knockdown testes revealed a phenotypic range, which we categorized as strong, moderate or weak (Fig. 1A-F). Strong phenotypes were defined as a lack of testis structures or the presence of testes lacking detectable somatic cells (Fig. 1B). Moderate phenotypes were defined as rudimentary testes that lacked differentiated somatic or germ cells (Fig. 1C). Weak phenotypes were defined as testes that contained large germline tumors (Fig. 1D).

Subsequent analysis focused on the *chic* RNAi line 9553R-3, which predominantly yielded weaker phenotypes (Fig. 1F). This was because the other two *chic* RNAi lines tested gave rise to a substantial number of flies with rudimentary or even absent testes, which were less informative. Importantly, expressing the *chic* RNAi line 9553R-3 in the soma resulted in a reduction of Chic protein levels, such that it was no longer detectable in early somatic cells, where it is enriched in wild-type controls (Fig. 1H,I). In summary, we find that somatic RNAi knockdown of Chic leads to depletion of the protein and results in severe defects during spermatogenesis.

Chic is required in the soma to regulate proliferation of both the soma and the germline

To analyze the phenotypes resulting from Chic knockdown in the soma in greater detail, somatic cell numbers were quantified using markers for early somatic differentiation, including the transcription factors Traffic jam (Tj) and Zfh1 (Fig. 2A,B). We found that control testes maintained consistent numbers of Tj-positive early somatic cells [88.2±2.07 and 81.59±1.59 at 1 and 7 days post-eclosion (DPE), respectively], whereas Chic knockdown testes had significantly more Tj-positive cells (108.0±3.34 and 129.2±7.83 at 1 and 7 DPE, a 22% and 58% increase over controls, respectively) (Fig. 2C). To further characterize this expansion in the Tj-positive cell population, we analyzed the size of the somatic population expressing Zfh1, a marker for CySCs and early daughter cells (Leatherman and DiNardo, 2008). This analysis revealed that this population was nearly the same size in control and Chic knockdown testes at both 1 and 7 DPE (Fig. 2D). Our results differ somewhat from those in a recent analysis of Chic by Shields et al. (2014), who found that Chic knockdown resulted in fewer Tj-positive cells. This difference is likely to be due to our use of a different UAS driver and RNAi line to obtain a weaker knockdown of Chic. Our data therefore suggest that the expansion in the Tj-positive population was due to an increase in somatic proliferation or a delay in somatic differentiation.

To determine whether the expansion in the Tj-positive population was due to increased proliferation or delayed differentiation, the population of proliferating somatic cells was quantified by labeling S-phase cells with a 30-min pulse of EdU and staining for early somatic cells with Zfh1 (Fig. 2E,F). This analysis showed that the absolute and relative number of cells that co-stained with both markers was not significantly different between control and Chic knockdown testes at 1 or 7 DPE (Fig. 2G,H). Moreover, because only CySCs proliferate in wild-type testes (Cheng et al., 2011), we examined whether cyst cells rather than CySCs were abnormally proliferating upon Chic knockdown. To this end, we determined the relative position at which somatic proliferation occurred in control and Chic knockdown testes by staining testes for Zfh1 and with EdU and measuring the distance of proliferative events from the hub. We found that cell proliferation occurred a similar distance from the hub in control and Chic knockdown testes (Fig. 2I). These results suggest that there are no major differences in somatic cell proliferation rates or location upon somatic Chic knockdown. Overall, our data support a model whereby expansion in the Tj-positive population is due to delayed somatic differentiation.

In comparison, knockdown of Chic in the soma caused a germline proliferation defect, resulting in large spermatogonia-like tumors (Fig. 2J,K). In control testes, mitotically active spermatogonia are only present near the apical tip of the testis, being identifiable by their size, shape and characteristic patterns of Vasa and DAPI staining (Fig. 2J). In Chic knockdown testes, large numbers of spermatogonia-like germ cells filled the basal regions of the testis (Fig. 2K) and were confirmed to be mitotically active using the marker phospho-Histone H3 (pH3) (Fig. 2L,M). In a recent study, Shields et al. (2014) obtained results consistent with those that we describe here, supporting our findings that somatic Chic knockdown leads to defects in somatic differentiation and germline proliferation.

Chic knockdown in the soma leads to defects in encapsulation

As germline tumors have previously been associated with failures in encapsulation (Kiger et al., 2000; Sarkar et al., 2007; Shields et al., 2014; Schulz et al., 2002; Tran et al., 2000) we investigated whether

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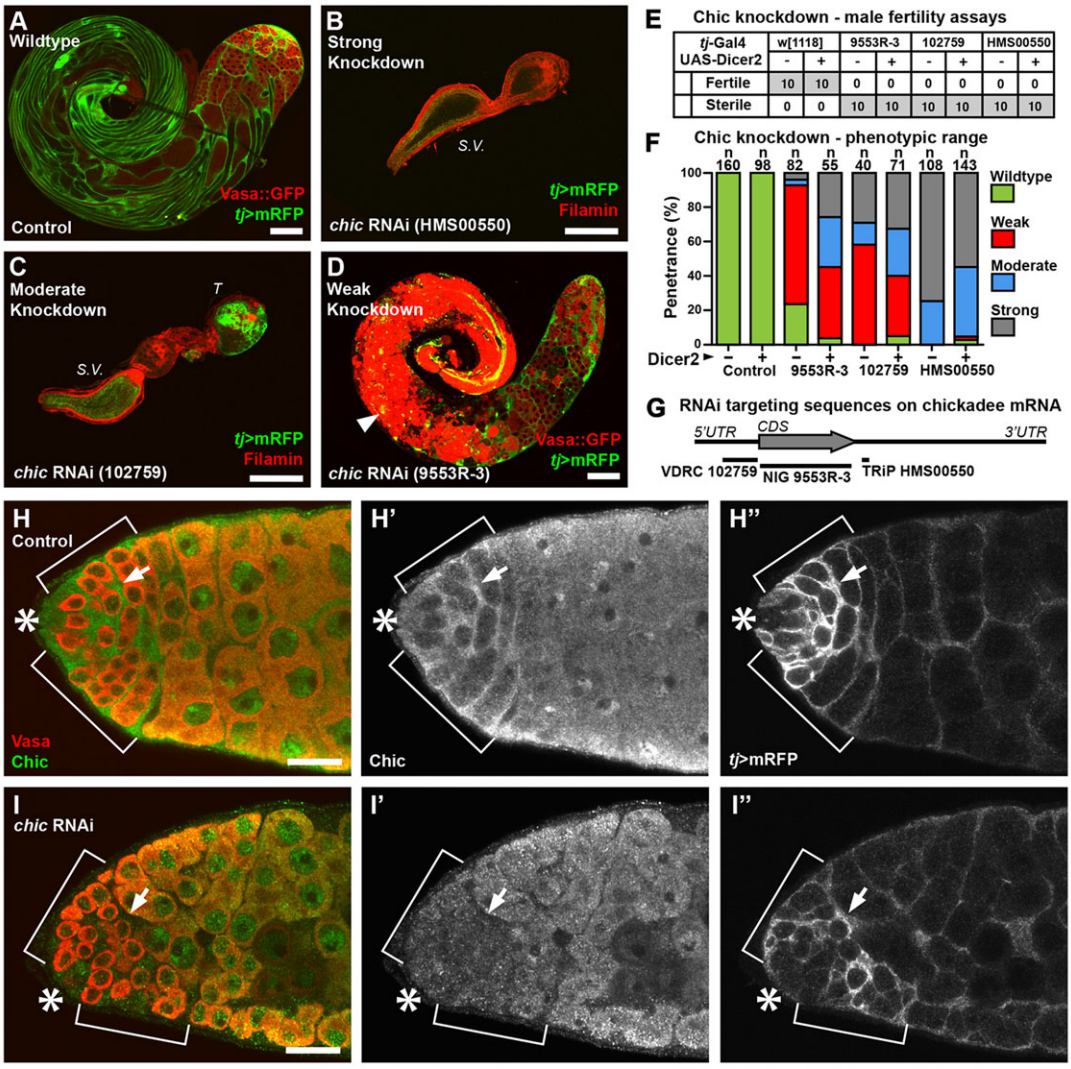

Fig. 1. Chic (*Drosophila* Profilin) is essential in the soma for spermatogenesis and fertility. (A) Wild-type testis. (B-D) Classes of testis phenotype generated by somatic Chic knockdown with different *chic* RNAi lines using *tj*-Gal4. (B) Strong Chic knockdown phenotype is characterized by an ablation of somatic cells from the testis (*S.V.*, seminal vesicle). (C) Moderate Chic knockdown phenotype is characterized by small, underdeveloped testis (*T*). (D) Weak Chic knockdown phenotype is characterized by germline tumors that fill the testis (arrowhead). (E) Male fertility assays showing sterility for all *chic* RNAi lines tested, with or without the co-expression of Dicer2. Numbers indicate the results of fertility assays per genotype; '10' indicates that 10 out of 10 assays were fertile (for controls) or steril (for *chic* knockdowns) (see materials and methods). (F) Quantification of phenotypic classes shown in B-D for each RNAi line. *n*, number testes examined. (G) *chic* mRNA showing regions targeted by each RNAi line. (H-I″) RNAi-mediated knockdown efficiently depletes Chic protein from the soma. (H-H″) In wild-type testes, early somatic cells (brackets) near the hub (asterisk) were enriched for Chic (arrow). (I-I″), By contrast, testes expressing *chic* RNAi 9553R-3 showed a strong reduction of Chic in early somatic cells (arrow). Soma is labeled with *tj*>mRFP (A-D,H,I), germline with Vasa::GFP (A,D,H,I) and overall genital architecture highlighted with Filamin (B,C). Scale bars: 100 µm in A-D; 20 µm in H,I.

encapsulation was defective upon somatic knockdown of Chic. We observed that somatic cells were tightly associated with germ cells throughout control testes (Fig. 3A). By comparison, somatic knockdown of Chic disrupted the characteristic production of encapsulated cysts, leaving voids around some spermatocyte stage cysts due to a lack of associated somatic cells (Fig. 3B, arrowhead). These observations suggest that Chic is required in the soma for encapsulation, in agreement with recent work by Shields et al. (2014).

To obtain further insight into how the encapsulation defect develops, temperature-sensitive Gal80 (*tub*-Gal80^{ts}) was used to conditionally repress *tj*-Gal4 such that RNAi-mediated Chic knockdown was induced only in adult testes (Fig. 3C-F). Flies were grown at the restrictive temperature and shifted upon eclosion to the permissive temperature to induce RNAi expression. Chic knockdown in the adult resulted in a failure to maintain encapsulation around some cysts, as some germ cells were no longer surrounded by somatic cells (Fig. 3F, arrow). These results, taken together with the previous analysis by Shields et al. (2014), show that Chic is required to maintain germline encapsulation in the testis.

A permeability assay for the *Drosophila* testis

As Chic is required in the soma for germline encapsulation, we sought a method to characterize this defect in more detail. Light microscopy cannot always resolve the thin somatic membranes, and electron microscopy requires harsh treatments that can disrupt membranes. We therefore developed a non-invasive assay to assess whether the germline is isolated from the outside environment by the surrounding somatic cells. This assay uses a fluorescently conjugated 10 kDa dextran dye added to the medium surrounding

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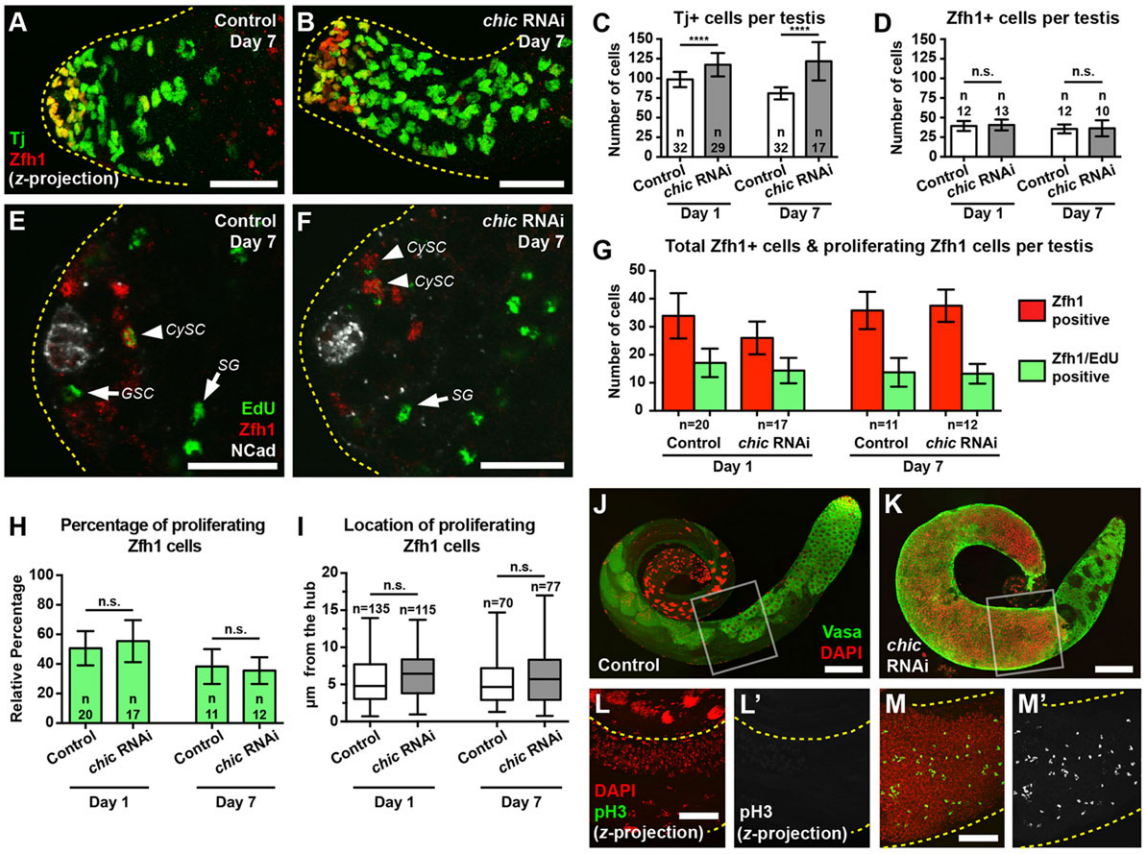

Fig. 2. Chic is required in the soma to regulate somatic differentiation and germline proliferation. (A,B) *z*-projections of the apical tip of control and Chic knockdown testes 7 days post-eclosion (DPE) stained for the somatic markers Tj and Zfh1. (C) Quantification of Tj-positive cells shows that Chic knockdown testes have significantly more Tj-positive cells than controls at 1 and 7 DPE. (D) There are similar numbers of Zfh1-positive cells in control and Chic knockdown testes at 1 and 7 DPE. (E,F) S-phase CySCs labeled for Zfh1 and with EdU (arrowheads). S-phase GSCs and spermatogonia (*SG*) are indicated by arrows, DN-Cadherin marks the hub. (G) Quantification of Zfh1-positive and Zfh1/EdU double-positive cells in control and Chic knockdown testes shows no significant differences. (H) Normalization of Zfh1/EdU double-positive cells relative to total Zfh1-positive cells per testis confirms that there is no significant change in CySC proliferation upon somatic Chic knockdown. (I) The distance of somatic proliferation events from the hub reveals no significant difference between control and Chic knockdown testes at 1 and 7 DPE. (J) Control testes show a progression from intensely Vasa-positive, DAPI-stained spermatogonia near the apical tip of the testes, through to weaker Vasa and DAPI staining spermatocytes in basal regions of the testis. (K) Chic knockdown testes contained large spermatogonia-like tumors filling basal regions of the testis, while also retaining spermatocyte stage germ cells. (L-M′) *z*-projection from mid region of the testis (similar to the boxed areas in J,K) stained for the mitotic marker phospho-Histone H3 (pH3), showing that spermatogonia-like tumors contain many pH3-positive cells (M,M′). *n* refers to number of testes (C,D,G,H) or proliferative events (I) examined. ****P<0.0001; n.s., not significant. Scale bars: 30 µm in A,B; 15 µm in E,F; 100 µm in J,K; 50 µm in L,M.

ex vivo cultured testes. The ability of dye to access the surface of the germ cells at different stages of development can then be analyzed using confocal light microscopy. In wild-type testes, the dye readily penetrated past cells of the testis sheath (Hardy et al., 1979; Susic-Jung et al., 2012) and could be found between all cells within the stem cell niche (Fig. 4A). Surprisingly, dye was able to access gonialblasts and 2-cell stage spermatogonia, even though encapsulation is believed to occur at the gonialblast stage (Hardy et al., 1979). In early stages, the dye appeared to penetrate between germ cells and colocalized with somatic membrane markers (*tj*>mGFP, *tj*>mRFP). Since 10 kDa dextran does not penetrate the plasma membrane of intact healthy cells (Lamb et al., 1998), this colocalization is likely to reflect the thin nature of somatic cells at this stage and the resolution limit of light microscopy.

Importantly, starting at the 4-cell spermatogonial stage, access of dye to the germline was increasingly restricted, consistent with the establishment of a permeability barrier by the somatic cells encapsulating the germline (Fig. 4D). Nonetheless, in some cysts the germline was still accessible to small amounts of dye after the 4-cell spermatogonial stage (Fig. 4B, arrows). However, by the late spermatocyte stage, the germline was consistently isolated from dye by the permeability barrier (Fig. 4C, arrows).

Overall, these observations identify two distinct steps in cyst formation by somatic cells: germline encapsulation, followed by the formation of a permeability barrier that isolates the germline from the surrounding environment.

Chic is required in the soma for the formation of a permeability barrier and for stem cell maintenance

Since data from Chic knockdown testes are consistent with a disruption in encapsulation, we examined whether somatic Chic knockdown also perturbed the permeability barrier around the germline (Fig. 5A,B). In the stem cell niche, gonialblasts and early spermatogonial stages, dye was able to access the germline in both control and Chic knockdown testes. However, whereas control testes exhibited reduced dye accessibility in late spermatocyte stages (Fig. 5A), this did not occur in Chic knockdown testes, as both spermatogonia and spermatocytes were still accessible to dye (Fig. 5B, arrowhead). In some spermatocytes, dye was even found within the cytoplasm (Fig. 5B, arrow), indicative of failures in

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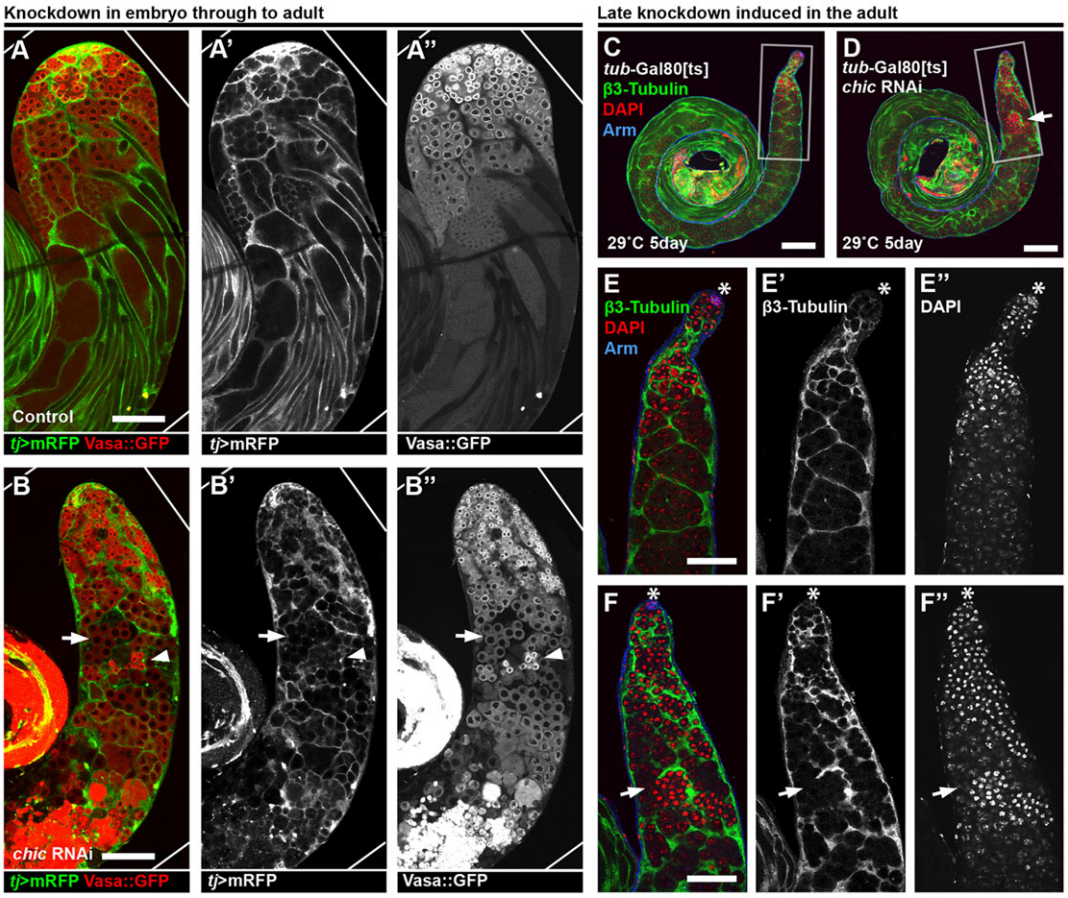

Fig. 3. Chic is required to maintain encapsulation. (A-A″) Somatic cells (*tj*>mRFP) closely wrap the germline (Vasa::GFP) throughout all stages of spermatogenesis in control testes. (B-B″) In Chic knockdown testes, somatic cells do not fully wrap the germline (arrow) and spermatogonia are found throughout the testis (arrowhead). (C,D) Control (C) and Chic knockdown (D) testes 5 days post-RNAi induction in adult flies; DAPI-stained nuclei of spermatogonial stages were detected basal to their normal position (arrow). (E-F″) Enlargement of boxed regions from C,D. In the Chic knockdown (F-F″) spermatogonia-like cells compose germline cysts where encapsulation by the soma (labeled with β3-Tubulin) has failed (arrow). Hub is marked by Armadillo (Arm; asterisk). Scale bars: 100 µm in A-D; 50 µm in E,F.

membrane integrity (Nishimura and Lemasters, 2001; Carter et al., 1994). It is known that germ cells in both the ovary (Jenkins et al., 2013) and testis (Yacobi-Sharon et al., 2013) can undergo necrosis, which involves loss of plasma membrane integrity (McCall, 2010). Taken together, these data show that depletion of Chic leads to failure of the somatic permeability barrier.

To determine whether the defect in the establishment of a permeability barrier is cell-autonomous, clonal analysis in somatic cells was undertaken (Fig. 6). Using the MARCM technique (Lee and Luo, 2001) with a somatic cell-specific driver (c587-Gal4), positively labeled clones were induced in the testis. Clones were either wild-type controls, homozygous for the null mutation $chic^{221}$, or expressed the *chic* RNAi line 9553R-3. At 3 days post-clone induction (DPCI), cells homozygous for $chic^{221}$ or expressing the *chic* RNAi line showed a marked reduction in Chic protein levels, as determined by immunostaining (Fig. 6B-D).

Next, the MARCM clones were analyzed using the permeability assay (Fig. 6G,H). This experiment illustrated that the permeability barrier was established normally in control clones (Fig. 6G, arrowheads). However, somatic cells that clonally express *chic* RNAi showed a defective permeability barrier, which resulted in dye permeating the spaces between germline and somatic cells (Fig. 6H, arrow). Attempts to confirm this result using $chic^{221}$ clones were unsuccessful as such clones were rapidly lost. This prompted a closer examination of the maintenance of *chic* null somatic cells.

The number of $chic^{221}$ and *chic* RNAi clones per testis that expressed Tj at 3 and 7 DPCI was quantified (Fig. 6A). At 3 DPCI, equivalent numbers of Tj-positive control and *chic* RNAi clones were maintained (14.3±0.97 and 17.7±1.49, respectively), whereas $chic^{221}$ Tj-positive clones were significantly reduced (6.7±0.97). At 7 DPCI, there were similar numbers of Tj-positive control clones as at 3 DPCI (15.7±3.27), whereas both *chic* RNAi and $chic^{221}$ Tj-positive clones (5.62±2.28 and 0.3±0.10, respectively) were significantly reduced compared with the 7 DPCI controls. This could result from a defect in maintaining $chic^{221}$ or *chic* RNAi CySC clones in the niche. Consistent with this hypothesis, *chic* RNAi clones near the niche did not have the thin cytoplasmic extensions to the hub that are characteristic of CySCs (Hardy et al., 1979) (Fig. 6E,F), similar to observations made by Shields et al. (2014). These results show that Chic is required cell-autonomously in the soma for the establishment of the permeability barrier and for CySC maintenance in the niche.

The permeability barrier is dependent on the function of septate junction proteins

To better characterize how the soma forms the permeability barrier, we investigated the expression and localization of septate junction

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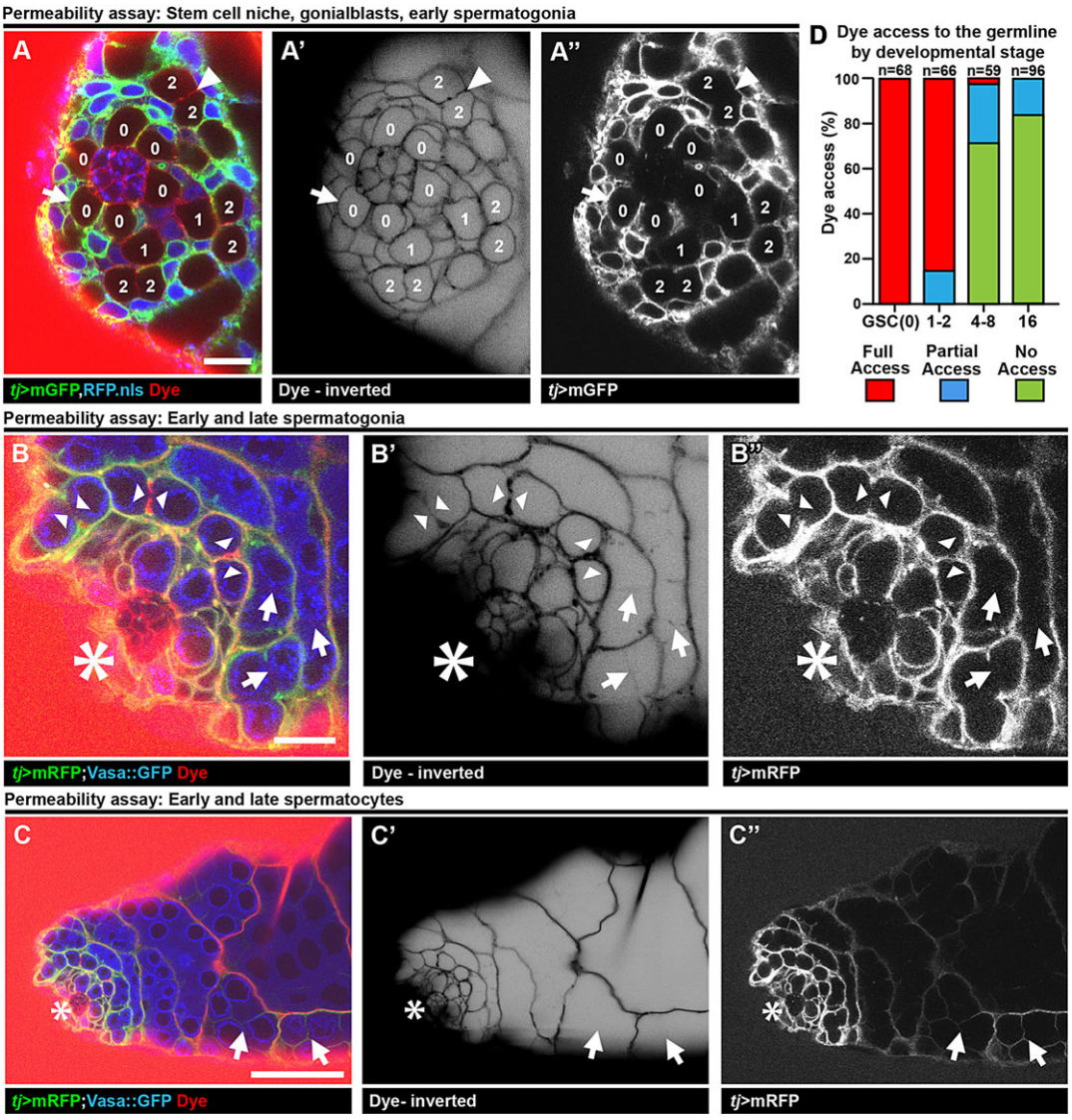

Fig. 4. A permeability assay for the *Drosophila* testis. (A-A″) Results of permeability assays in the stem cell niche of wild-type testes reveal no permeability barriers, as assessed by the ability to block dye access to germ cell surfaces. Soma is labeled with *tj*>mGFP,RFP.nls; numbers in germ cells indicate developmental stage: 0, GSCs (arrow); 1, gonialblasts; 2, 2-cell spermatogonia (arrowhead). (B-B″) Permeability assays on 2- to 8-cell spermatogonial cysts with the soma indicated by *tj*>mRFP and the germline by Vasa::GFP. At the 2-cell spermatogonial stage, dye can access the germline (arrowheads); subsequent spermatogonial stages are less accessible to dye (arrows). (C-C″) By late spermatocyte stages, dye cannot access the germline (arrows). Asterisks indicate the hub. (D) Quantification of the ability of dye to access cysts by developmental stage. Full access, strong staining around germ cells; partial access, weak or incomplete staining around germ cells; no access, no staining around germ cells. Germline indicated by stage: GSCs; gonialblasts and 2-cell spermatogonia (1-2); 4- and 8-cell spermatogonia (4-8); 16-cell spermatocytes (16). *n* refers to number of cysts examined. Scale bars: 10 µm in A,B; 50 µm in C.

proteins, the main occluding junctions in flies (Banerjee et al., 2006). We found that the core septate junction components Neurexin-IV (Nrx-IV) and Coracle (Cora) (Oshima and Fehon, 2011) both localized around the germline throughout spermatogenesis (Fig. 7A). Specifically, septate junction proteins encircled the germline beginning several cell lengths from the hub, and colocalized with markers that highlight germline encapsulation, such as DE-Cadherin (Shotgun – FlyBase) (Fig. 7F). To explore the localization of septate junction markers in greater detail, one of the two somatic cells in a spermatogonial stage cyst was clonally labeled using MARCM and stained for Cora. This analysis showed that septate junction markers concentrated at the sites of contact between the two somatic cells that encapsulate the germline (Fig. 7B,C). Moreover, by isolating, culturing and imaging spermatocyte stage cysts expressing a genomic GFP-tagged Nrx-IV (Nrx-IV::GFP), it was revealed that Nrx-IV::GFP localized as a belt that spanned the circumference of the interface between the two somatic cells of the cyst (Fig. 7D,E). These circumferential belts of junctional proteins were also visible in intact testes (Fig. 7F). We therefore conclude that septate junction proteins form a belt at the interface between the two somatic cells that encapsulate the germline, consistent with the idea that septate junctions help the soma to insulate the germline.

Next, the possible role of septate junction components in spermatogenesis and specifically in permeability barrier function was tested directly using soma-specific RNAi knockdown of Nrx-IV or Cora and the permeability assay. First, it was found that knockdown of the septate junctions components Nrx-IV or Cora gave rise to small rudimentary testes (Fig. 8A-C). Using a range of RNAi lines targeting *cora* and *Nrx-IV* a phenotypic series of germline arrest was generated, using Vasa and DAPI to distinguish

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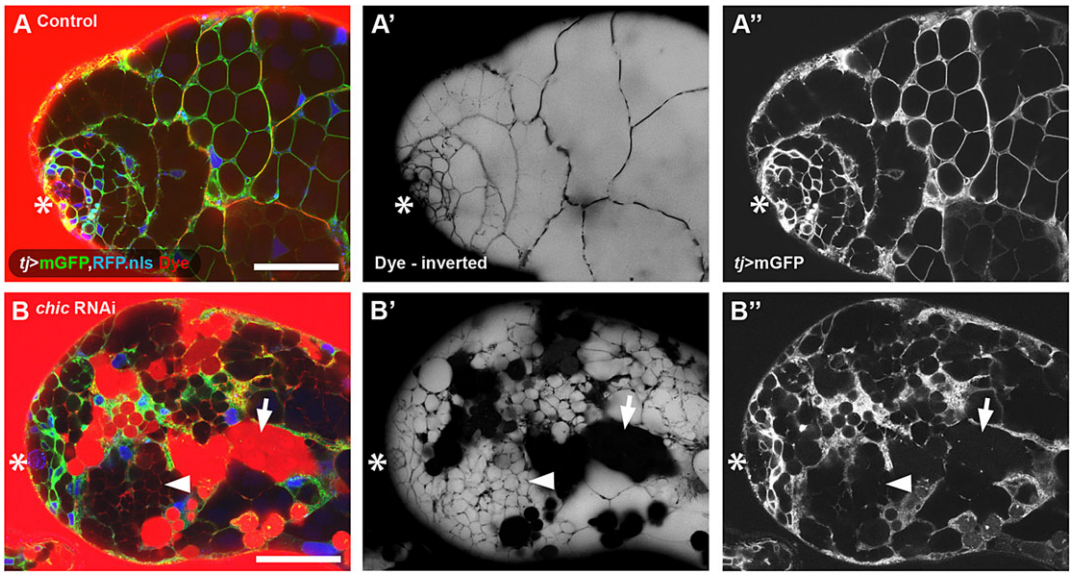

Fig. 5. Chic is required in the soma to form a permeability barrier around the germline. (A-A″) In control testes, early spermatogonial stages are accessible to dye, whereas late spermatogonial and early spermatocyte cysts show progressive reduction in accessibility. By late spermatocyte stages, dye cannot access the germline. (B-B″) By comparison, in Chic knockdown testes, dye can access the germline throughout the testis (arrowhead). In some spermatocytes, dye penetrates the germline cytoplasm (arrow). Asterisks indicate the hub. Soma indicated by *tj*>mGFP,RFP.nls. Scale bars: 50 µm.

spermatocyte stages and Boule to distinguish meiotic stages (Eberhart et al., 1996). This analysis showed that knockdown of Nrx-IV or Cora resulted in a germline differentiation block and that an increased severity of the phenotype correlated with an earlier block (Fig. 8D). The resulting germline phenotype was analyzed further by examining the pattern of spermatogonial mitotic events. In Nrx-IV or Cora knockdown testes, we observed pH3-positive spermatogonia much further away from the hub than with control testes: up to 300 µm versus 35 µm from the hub, respectively (Fig. 8E-I). Consistent with this, upon septate junction component knockdown there was a significant increase in both 1-cell and 2-cell stage pH3-positive spermatogonia (Fig. 8E-I). Both of these results are consistent with a delay in germline differentiation.

Importantly, using the permeability assay we showed that Nrx-IV and Cora are both essential for a functional permeability barrier (Fig. 8J-L). Upon knockdown of either component, dye was able to access the germline in spermatogonial stage cysts throughout the testis. Strikingly, and in contrast to Chic, knockdown of Cora or Nrx-IV disrupted the permeability barrier but did not affect encapsulation, as somatic cells still surrounded the germline (Fig. 8M-O).

The permeability barrier regulates the accessibility of the germline to signals from the niche

As germline differentiation was blocked or delayed when the permeability barrier was disrupted, and signals that emanate from the stem cell niche regulate germline differentiation, we hypothesized that this phenotype resulted from abnormal germline signaling. To test this, expression of the germline differentiation factor *bag of marbles* (*bam*) was examined using the reporter *bam*-GFP (Chen and McKearin, 2003b). In wild-type testes, *bam* expression is directly repressed by BMP signaling ligands secreted from the hub and CySCs (Kawase et al., 2004; Shivdasani and Ingham, 2003). In control testes, *bam* expression is repressed in GSCs and early spermatogonia near the niche, limiting expression to late spermatogonial stages (Fig. 9A,A″,G). By contrast, in Nrx-IV or Cora knockdown testes, *bam*-GFP appeared to be repressed further from the niche. (Fig. 9B,B″,C,C″). This was illustrated by obtaining intensity profiles for *bam*-GFP expression across the length of the testis, starting at the hub (Fig. 9D-F). These showed that in control testes there was a sharp peak of *bam*-GFP expression in spermatogonia near the hub which then declined (Fig. 9D). In comparison, Nrx-IV or Cora knockdown resulted in a more gradual increase in *bam*-GFP intensity, which peaked much further from the hub (Fig. 9E,F). This defect was quantified by measuring the distance of peak *bam*-GFP intensity from the hub, which was approximately 18 µm in controls and over 120 µm upon knockdown of Nrx-IV or Cora (Fig. 9I). These results suggested a link between *bam* expression and the permeability barrier.

To investigate this link directly, *bam*-GFP-expressing testes from control and Nrx-IV or Cora knockdown flies were colabeled with the dye (Fig. 9A-C,G). Intriguingly, there appeared to be a positive correlation between the presence of the barrier and *bam*-GFP expression. To quantify this correlation in wild-type testes, relative *bam*-GFP intensity was measured in spermatogonia that were either accessible or inaccessible to dye (Fig. 9G,H). This showed that there was a significant increase of nearly 3-fold in relative *bam*-GFP intensity when the permeability barrier was formed. These data suggest that one function of the soma-germline barrier is to shape the signaling environment experienced by the germline, restricting access to BMP signals emanating from the stem cell niche.

DISCUSSION

Here we sought to characterize the genetic control of soma-germline interactions in the adult fly testis. Our work defines two distinct phases of soma-germline interaction during early spermatogenesis: an encapsulation/enclosure stage, when the soma wraps around the germline; and an ‘occlusion’ phase, during which a permeability barrier around the germline is established. This distinction is based on two lines of evidence. First, we show that a permeability barrier that excludes the passage of small molecules forms around the 4-cell spermatogonial stage, while it is believed that encapsulation takes places starting at the gonialblast stage (Hardy et al., 1979). Second, we provide evidence suggesting that

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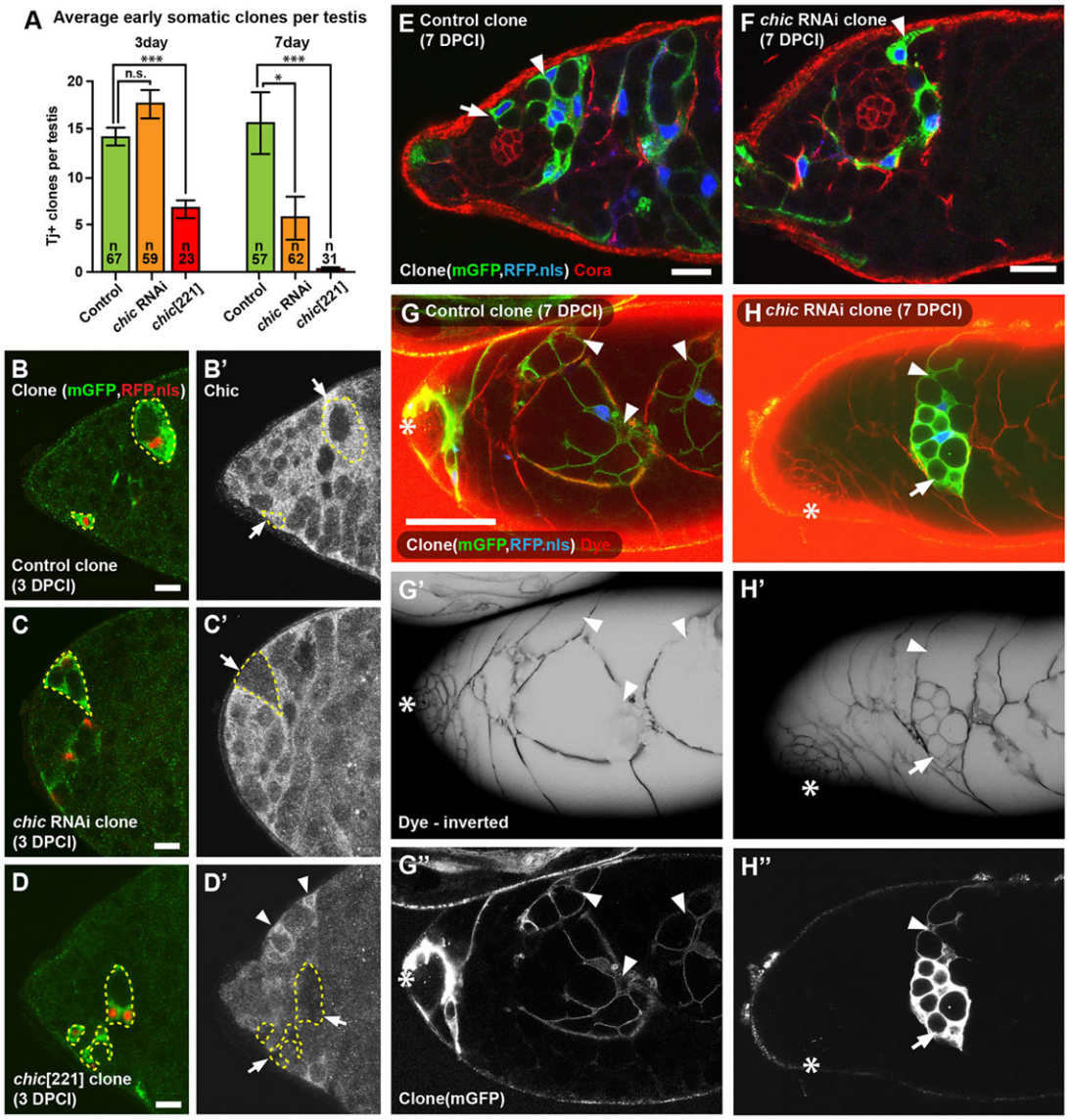

Fig. 6. Clonal analysis of Chic in the soma shows that it is required for CySC maintenance and permeability barrier function. Somatic MARCM clones (mGFP,RFP.nls) were generated that were either wild-type control, expressed *chic* RNAi, or were homozygous for the null allele $chic^{221}$. (A) Average number of early somatic cell (Tj-positive) clones per testis at 3 and 7 days post-clone induction (DPCI). *n*, number of testes examined. **P*<0.05, ****P*<0.001; n.s., not significant. (B-D′) Somatic cell clones (outlined, arrows) stained for Chic at 3 DPCI. Control clones (B,B′, arrows) show similar levels of Chic as neighboring somatic cells, whereas both *chic* RNAi (C,C′) and $chic^{221}$ (D,D′) clones show reduced Chic levels compared with neighboring somatic cells. In $chic^{221}$ clones, Chic levels are lower in all cells due to the heterozygous mutant background (arrowheads in D′ indicate clone spots). (E,F) Control and *chic* RNAi clones near the hub, labeled for Cora at 7 DPCI. Control clones maintain thin extensions towards the hub (E, arrow), whereas knockdown clones do not (F). (G-H″) Permeability assay performed on control and *chic* RNAi clones at 7 DPCI. Knockdown with *chic* RNAi can disrupt the permeability barrier (H-H″, arrow). Asterisks indicate hub. Arrowheads (E-H″) indicate cyst cell clones with intact permeability barriers. Scale bars: 10 µm in B-F; 50 µm in G,H.

knockdown of septate junction components does not interfere with encapsulation but does disrupt the permeability barrier. Failure in either encapsulation or permeability barrier function has catastrophic effects on spermatogenesis, resulting in sterility. Thus, the formation of the permeability barrier is not only subsequent to encapsulation but dependent on it for proper completion.

Chic and germline encapsulation

Recent work has greatly clarified the role of Chic (*Drosophila* Profilin) in the germline and provided hints as to its somatic role (Shields et al., 2014). In the germline, Chic is required to maintain DE-Cadherin at the GSC-hub interface and is therefore required for GSC maintenance (Shields et al., 2014). Our experiments using *chic* null clones, and previous work by Shields et al. (2014), suggest that Chic is also required to maintain somatic CySCs in the testis. This might be because Chic is required for cell viability and null mutant cells die or Chic is required to maintain CySCs in the niche. However, using a hypomorphic RNAi-mediated Chic knockdown, we were able to dissect a broader range of phenotypes as somatic cells persisted. Earlier analysis had shown that RNAi-mediated somatic knockdown of Chic results in encapsulation defects and the formation of germline tumors (Shields et al., 2014). Our work supports this earlier analysis, and additionally demonstrates that reduced Chic levels may lead to delayed somatic cell differentiation. Moreover, we utilize Chic knockdown to illustrate the requirement

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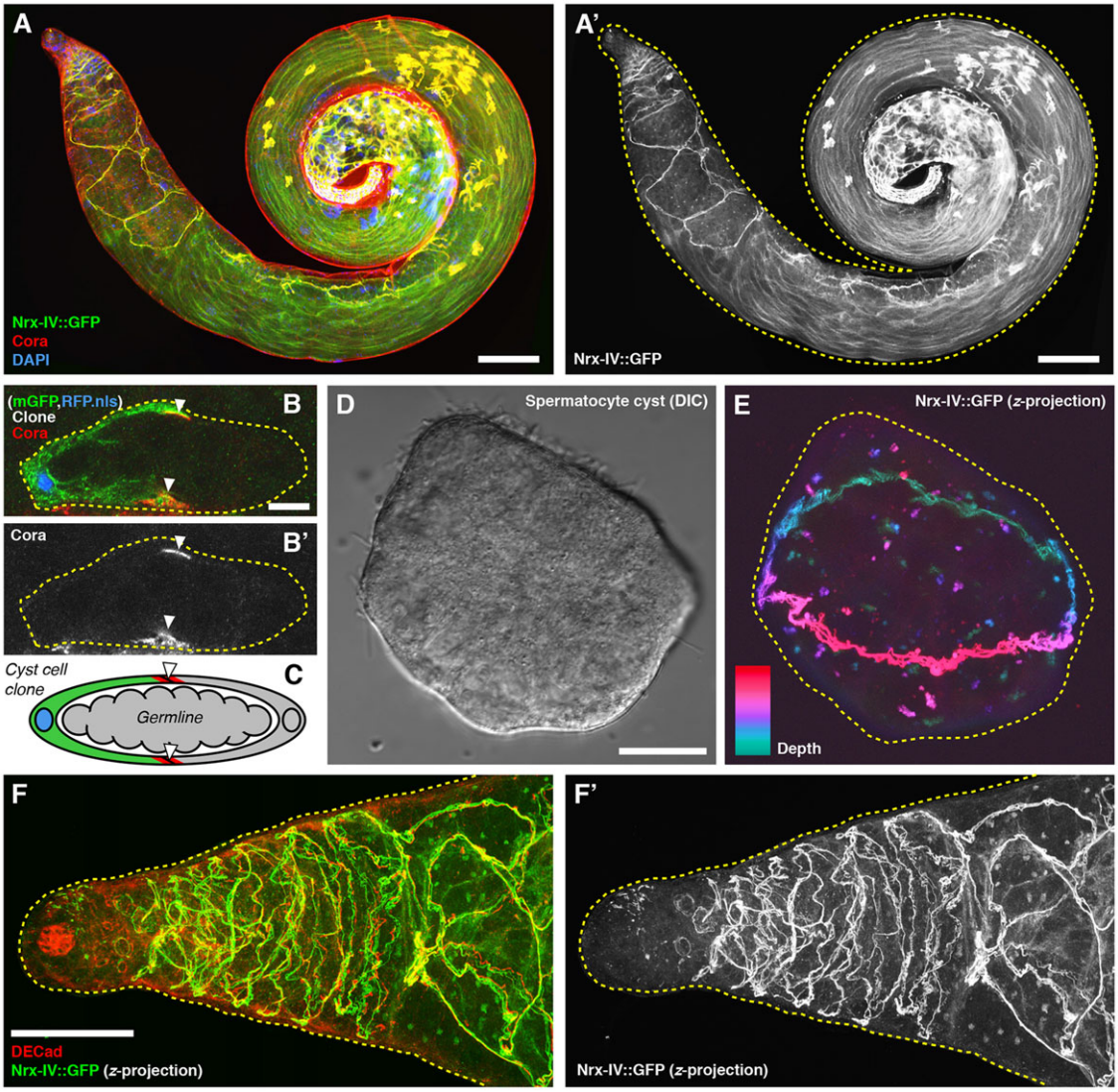

Fig. 7. Septate junctions form between the cyst cells encapsulating the germline. (A,A′) Wild-type testis stained for the septate junction markers Cora and Nrx-IV (Nrx-IV::GFP), which colocalize surrounding the germline. (B,B′) Single germline cyst with one of two encapsulating cyst cells positively labeled using MARCM (mGFP,RFP.nls), showing that Cora is localized at the cyst cell-cyst cell boundary (arrowheads). (C) Schematic of cyst shown in B. (D,E) Single spermatocyte stage cyst cultured *ex vivo* and shown in differential interference contrast (D) and as a depth-cued *z*-projection of the septate junction marker Nrx-IV::GFP (E). Nrx-IV::GFP localizes in a circumferential belt between the somatic cells. *z*-projection depth is 50 µm; red indicates proximity to the imaging surface. (F,F′) The septate junction protein Nrx-IV::GFP and the adherens junction protein DE-Cadherin (DECad) colocalize and form a belt several cell lengths from the hub. Scale bars: 100 µm in A,A′; 10 µm in B; 20 µm in D; 50 µm in F.

for proper encapsulation prior to formation of the permeability barrier. Our work does not illustrate a direct role for Chic in the formation and maintenance of the barrier, but rather serves to illustrate that proper somatic encapsulation of the germline is required for the formation of the permeability barrier.

Characterization of a soma-germline permeability barrier formed by septate junctions

Our study describes and characterizes the presence of a somatic permeability barrier around the germline in the fly testis and identifies proteins that contribute to its formation and maintenance. This barrier is functionally analogous to the vertebrate BTB (or SCB), as both form between surrounding somatic cells and occlude the differentiating germline from the rest of the organism. We show that the development of this permeability barrier in the fly is gradual, beginning at the 4-cell spermatogonial stage, and is completed in all cysts by the late spermatocyte stage. Moreover, our work implicates septate junctions, which are the functional analog of tight junctions, in establishment of the occluding function of the barrier.

Septate junctions perform the same function in invertebrates that tight junctions perform in vertebrates (Banerjee et al., 2006). Tight junctions are an integral component of the BTB and are essential for its ability to restrict the movement of small molecules (Cheng and Mruk, 2012). Electron microscopy studies have shown that septate junctions exists between the two encapsulating somatic cells in late spermatid stages in *Drosophila* (Tokuyasu et al., 1972). Studies in other insects have identified septate junctions between somatic cells and shown that these junctions form an occluding barrier between somatic cells (Szöllösi and Marcaillou, 1977; Szöllösi et al., 1980; Toshimori et al., 1979; Miranda and Cavicchia, 1986). Finally, Discs large 1, a septate junction-associated protein (Oshima and

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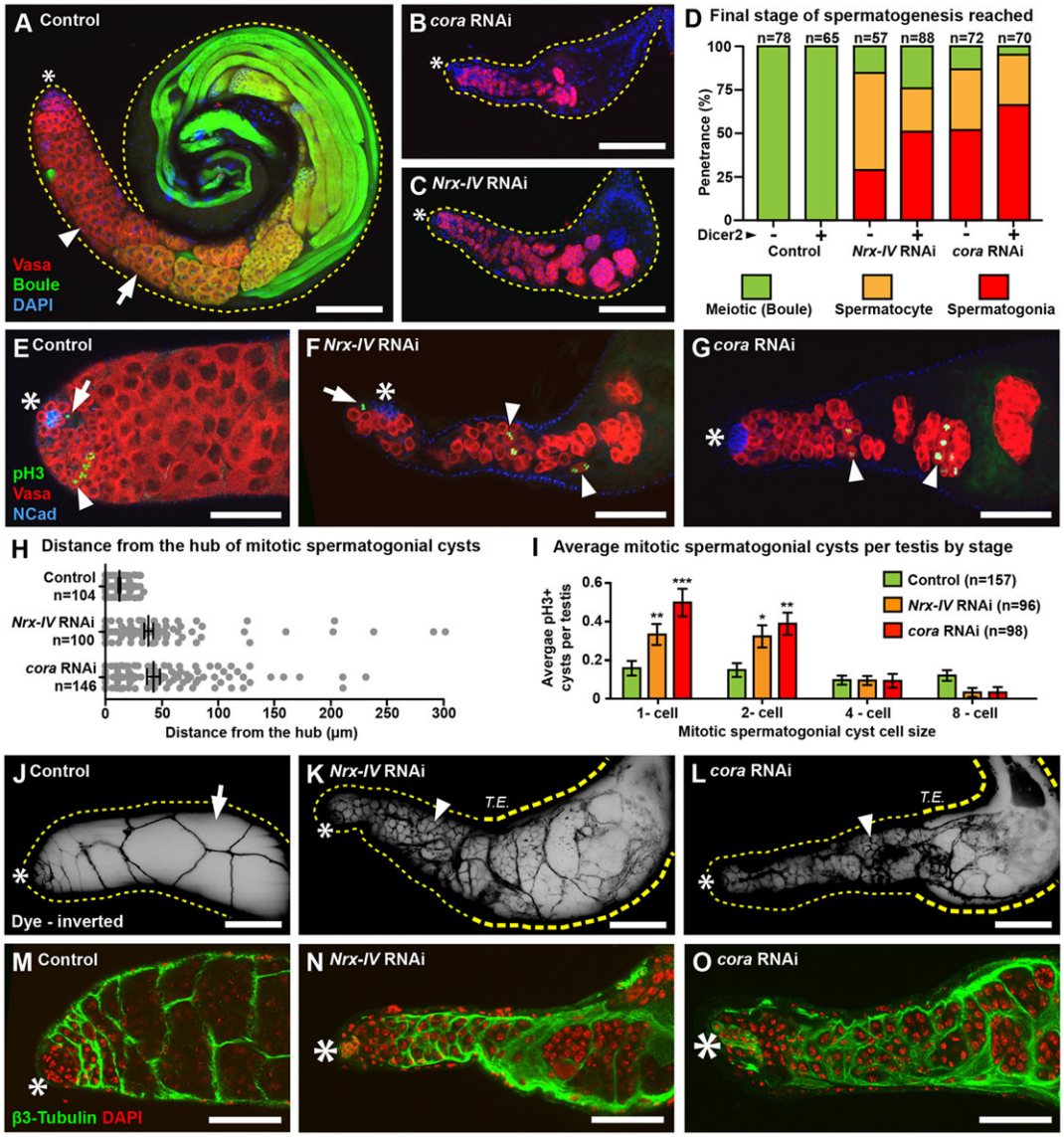

Fig. 8. Septate junction components are required for a functional permeability barrier and for germline differentiation. (A-C) Testes were stained for Vasa and with DAPI to distinguish spermatocyte stages and for Boule to highlight meiotic stages. (A) Control testes contain both spermatocyte (arrowhead) and meiotic (arrow) stage cysts. (B,C) Somatic knockdown of Nrx-IV or Cora results in rudimentary testes containing mostly spermatogonia. (D) Quantification of the proportion of testes in which the germline reached spermatocyte or meiotic stages. (E-G) In control testes (E), mitotic spermatogonia (Vasa/pH3 double positive) are detected near the hub (labeled with DN-Cadherin), whereas in Nrx-IV (F) or Cora (G) knockdown testes, mitotic spermatogonia were observed much further from the hub. Arrowheads indicate germline mitosis; arrow indicates somatic mitosis. (H) Quantification of distance from the hub of mitotic spermatogonia in control and knockdown testes. (I) Quantification of average number of mitotic spermatogonial cysts per testis by stage. **P*<0.05, ***P*<0.01, ****P*<0.001. (J-L) Knockdown of Nrx-IV or Cora in the soma disrupts the permeability barrier, as cysts remain permeable to dye throughout the testis (arrowheads). (M-O) The germline (indicated by DAPI staining) is surrounded by somatic cells (labeled with β3-Tubulin) in Nrx-IV or Cora knockdown testes, indicating they are still encapsulated. Asterisks indicate hub. Thicker dashed lines indicate terminal epithelium (*T.E.*). *n* refers to number of cysts (H) or testes (D,I) examined. Scale bars: 100 µm in A-C; 50 µm in E-G,J-O.

Fehon, 2011), is expressed in a similar pattern to that we describe for Nrx-IV and Cora, and its loss resembles the Nrx-IV and Cora knockdown phenotypes (Papagiannouli and Mechler, 2009). These studies support our assertion that septate junctions between the somatic cyst cells of the fly testis form an occluding barrier that is essential for spermatogenesis, similar to the BTB.

Possible roles of the soma-germline permeability barrier in *Drosophila*

The predominant role assigned to the BTB in vertebrates is as a barrier that protects germ cells from the immune system (Kaur et al., 2014). Additional work has shown that the BTB is required for the maintenance of spermatogenesis by helping to provide a microenvironment that supports proper germline differentiation (Cheng and Mruk, 2012; França et al., 2012; Lui et al., 2003b). Especially interesting is the fact that the establishment of the BTB corresponds to a major transition during germline differentiation as spermatocytes enter meiosis (Cheng and Mruk, 2012; França et al., 2012; Lui et al., 2003b). During this process, both remodeling of the BTB and differentiation of the germline are regulated by cytokines, particularly by members of the TGF-β family (Li et al., 2009; Lui et al., 2003a; Loveland and Hime, 2005).

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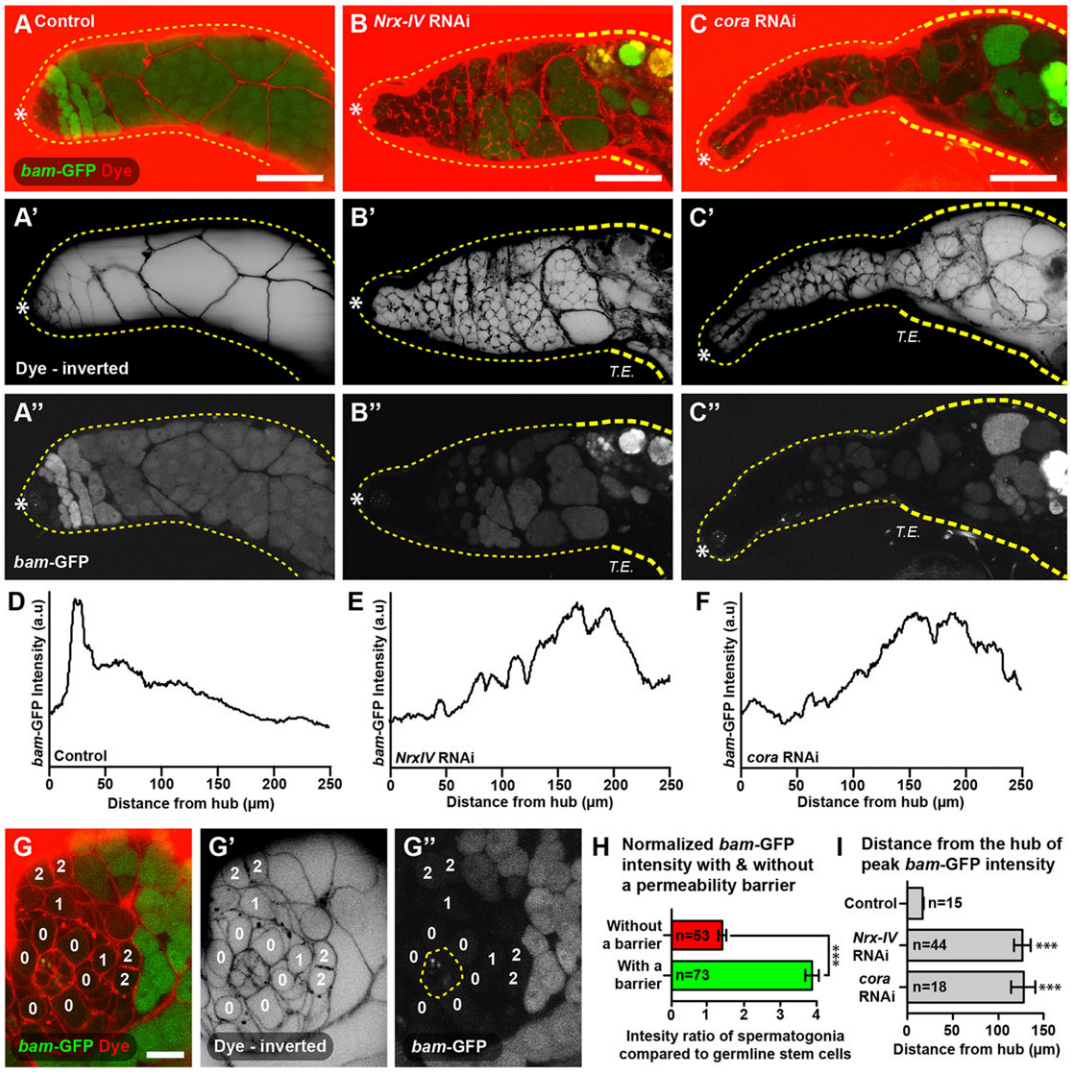

Fig. 9. The somatic permeability barrier limits the range of niche-derived signals. (A-C″) Permeability assays performed on *bam*-GFP-expressing control and Nrx-IV or Cora knockdown testes. In control testes (A-A″), BMPs in the stem cell niche repress expression of *bam*-GFP, which then peaks in 4- to 8-cell stage cysts near the hub before declining. Knockdown of Nrx-IV (B-B″) or Cora (C-C″) in the soma delays *bam*-GFP expression, which is found further from the hub. Asterisks indicate hub. Thicker dashed lines indicate terminal epithelium (*T.E.*). (D-F) Representative *bam*-GFP intensity profiles along the length of control, Nrx-IV or Cora knockdown testis illustrating peak reporter expression (0 is at hub). Intensity is in arbitrary units (a.u). (G-G″) In wild-type testes, expression of *bam*-GFP correlates with formation of the permeability barrier. Expression of *bam*-GFP is highest in 4-cell and 8-cell spermatogonia with an established permeability barrier. 0, GSCs; 1, gonialblasts; 2, 2-cell spermatogonia. (H) The relative intensity of *bam*-GFP was quantified in spermatogonial stage cysts with and without a permeability barrier. Spermatogonia isolated by a permeability barrier have significantly higher *bam*-GFP intensity than those without (3.9±0.17 versus 1.4±0.10). (I) Quantification of average distance from the hub to peak *bam*-GFP intensity. In control testes, peak intensity occurs 17±1.2 μm from the hub, whereas in Nrx-IV or Cora knockdown testes peak intensity occurs much further from the hub (126±9.1 μm and 127±13.3 μm, respectively). *n* refers to number of cysts (H) or testes (I) examined. ***P<0.001. Scale bars: 50 μm in A-C; 10 μm in G.

In flies, TGF-β signaling also serves a regulatory role during spermatogenesis; in particular, BMP ligands released from the stem cell niche function in GSC maintenance by repressing *bam*, the expression of which is both necessary and sufficient for germline differentiation (Chen and McKearin, 2003a; Kawase et al., 2004; Shivdasani and Ingham, 2003; Song et al., 2004). We provide evidence indicating that disruption of the permeability barrier extends the range of *bam* repression in the testis, consistent with an extension of the range of niche-derived BMP signals. This result is in accordance with a role of the permeability barrier in limiting access of the differentiating germline to signals regulating GSCs in the niche.

Another possible role for the permeability barrier in fly testis is to limit accessibility of the germline to the systemic signaling environment. For example, it has been shown that the maintenance and proliferation of GSCs in the fly testis are regulated by diet (McLeod et al., 2010) and long-range insulin signaling (Ueishi et al., 2009; Wang et al., 2011b). Other systemic signals, such as the hormone ecdysone, also regulate fly spermatogenesis (Wang et al., 2011a; Li et al., 2014). The germline itself may additionally release factors that regulate distant somatic tissues of the body, modulating these same systemic signaling pathways (Flatt et al., 2008; Drummond-Barbosa, 2008). It is intriguing to speculate that, by establishing a permeability barrier around the differentiating germline, the somatic cells prevent the germline from exchanging signals with the rest of the organism. The permeability barrier may thus ensure that, past a critical point, the germline becomes independent of both local paracrine and systemic hormonal

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signaling, responding only to signals mediated by the surrounding somatic cells.

Overall, our data fit with a model whereby actin modulators and junctional proteins act in the soma to establish an isolated microenvironment around the developing germline. This might be a conserved feature of spermatogenesis across multiple animal phyla. Moreover, our work establishes the *Drosophila* testis as a model for analyzing the formation and maintenance of permeability barriers such as the BTB during spermatogenesis. Using the dextran dye assay as a functional readout for the effectiveness of the permeability barrier in the testis, it will now be possible to systematically identify genes that are involved in its establishment, maintenance and regulation.

MATERIALS AND METHODS

Fly stocks

Fly lines used: somatic cyst cell drivers *tj*-Gal4 and c587-Gal4; UAS-anti-*chic* RNAi lines 9553R-3 (National Institutes of Genetics, Japan), 102759 (Vienna Drosophila Resource Center) and HMS00550 [Transgenic RNAi Resource Project, Harvard (TRiP)]; UAS-anti-*cora* RNAi line HM05144 (TRiP); and UAS-anti-*Nrx-IV* RNAi line JF01342 (TRiP); w^{1118}, Vasa::GFP, UAS-mCD8::GFP (mGFP), UAS-Dcr2, UAS-mCD8::Tomato (mRFP), UAS-RedStinger (RFP.nls), *tub*-Gal80^{ts}, FRT40a, *chic*^{221}, *hs*-Flp and *bam*-GFP (all obtained from the Bloomington Drosophila Stock Center); and Neurexin-IV::GFP from FlyTrap (CA06597) (Buszczak et al., 2007).

Genetics

RNAi knockdowns, unless otherwise stated, were performed using *tj*-Gal4 crossed to UAS-9553R-3 (for *chic*), UAS-HM05144 (for *cora*) and UAS-JF01342 (for *Nrx-IV*). Controls were *tj*-Gal4 crossed to w^{1118}. All experiments were at 25°C unless otherwise noted. Late induction of knockdown used *tj*-Gal4,*tub*-Gal80^{ts}. Progeny were raised at 18°C, males were collected at 1-3 DPE and kept at 29°C for 5 days. Clones were *hs*-Flp,c587-Gal4,UAS-mCD8:GFP,UAS-Redstinger;*tub*-Gal80^{ts},FRT40a crossed to FRT40a for control clones, FRT40a;UAS-9553R-3 for RNAi clones or *chic*^{221},FRT40a for mutant clones. Progeny were raised at 20°C, clones were induced in males at 1-3 DPE using two 1-h heat shocks at 37°C, 1.5 h apart, then aged 3-7 days at 20°C.

Immunostaining

All stained flies were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) or Testes Buffer (TB) (Henderson, 2004), and washed and incubated in PBS or TB plus 0.3% Triton X-100 and 0.2% BSA. Primary antibodies used were: mouse anti-Chic [Developmental Studies Hybridoma Bank (DSHB), chi 1J; 1:1], guinea pig anti-Traffic jam (D. Godt, University of Toronto, Canada; 1:2500), rabbit anti-Zfh1 (R. Lehmann, New York University, USA; 1:1000), guinea pig anti-Zfh1 (J. Skeath, Washington University in St Louis, MO, USA; 1:500), rabbit anti-Vasa (P. Lasko, McGill University, Montreal, Canada; 1:5000), mouse anti-Coracle (DSHB, C566.9 and C615.16; 1:500), rat anti-DN-Cadherin (DSHB, DN-Ex #8; 1:50), rat anti-DE-Cadherin (DSHB, DCAD2; 1:20), rabbit anti-β3-Tubulin (R. Renkawitz-Pohl, Philipps-Universität Marburg, Germany; 1:4000), rat anti-N-Filamin (L. Cooley, Yale University, New Haven, CT, USA; 1:1000), rabbit anti-Boule (S. Wasserman, University of California, San Diego, CA, USA; 1:1000), goat anti-Vasa (Santa Cruz, dC-13; 1:200), mouse anti-phospho-Histone H3 (Millipore, 6HH3-2C5; 1:1000), rabbit anti-GFP (Invitrogen, A6455; 1:1000), mouse anti-GFP (Invitrogen, A11120; 1:1000), rat anti-dsRed (Chromotek, 5f8; 1:1000) and mouse anti-Armadillo (DSHB, N2 7A1; 1:1000).

Fertility assays and phenotype scoring

Fertility assays were performed using single males at 1-5 DPE crossed to three virgin w^{1118} females, aged for 14 days, and then scored as sterile if no larvae/pupae were present. Phenotypic scoring for Chic, Nrx-IV and Cora knockdowns was performed with males at 1-3 DPE.

Proliferation assays

Somatic cell counts used males at 1 or 7 DPE. S-phase cells were labeled by incubating vivisected testes for 30 min with EdU in TB and stained using Click-iT EdU (Life Technologies). Distance measurements of S-phase cells were recorded as the linear distance from the edge of the hub to the nearest edge of EdU/Zfh1 double-positive nuclei. Proliferation assays using pH3 in Nrx-IV and Cora knockdowns used males at 1-5 DPE with UAS-Dicer2. Mitotic spermatogonia were defined as pH3/Vasa double-positive cells replicating in clusters of one, two, four or eight cells. Distance measurements of mitotic spermatogonia were recorded as the linear distance from the edge of the hub to the nearest edge of the pH3/Vasa double-positive cells.

bam-GFP intensity measurements

bam-GFP intensity profiles were created in ImageJ (NIH) using the RGB Profiler Macro, via a rectangular selection encompassing the testis starting from the edge of the hub. Distance to peak *bam*-GFP intensity was measured from the edge of the hub to the edge of Vasa-positive cells with the highest *bam*-GFP intensity. Wild-type *bam*-GFP intensity ratios were calculated by normalizing *bam*-GFP intensity in spermatogonia to the average *bam*-GFP intensity of two to four GSCs per testis.

Permeability assay

Testes were vivisected into Schneider's *Drosophila* Medium (Gibco). Testes were transferred to medium containing 10 kDa dextran conjugated to Alexa Fluor 647 (Invitrogen) at a final concentration of 0.2 µg/µl. Testes were imaged within 60 min of dye addition. Images were acquired from near the imaging surface to minimize out-of-plane fluorescence from dye in the medium. Comparable detection thresholds were ensured by setting the exposure level in the medium outside the testes to saturation level for image acquisition.

Statistics

Mean and s.e.m. are shown. Significance testing was performed with Prism (GraphPad) using unpaired *t*-tests with Welch's correction.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.J.F. designed and performed all experiments, with the exception of experiments by C.M.S. relating to somatic cell proliferation. M.J.F., C.M.S. and G.T. interpreted data and prepared the manuscript. G.T. conceived of initial project, designed experiments and supervised the project.

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