Occluding junctions regulate germline differentiation and stem cell niche homeostasis in the *Drosophila melanogaster* testis

by

Michael John Fairchild

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Abstract

Spermatogenesis is a reiterative process wherein sperm are continually produced from a small group of adult stem cells that are found in a unique niche. In *Drosophila melanogaster* this stem cell niche is made up of a cluster of cells, called the hub, which maintains both germline stem cells and somatic cyst stem cells using a combination of cell adhesion proteins and secreted signalling molecules. As each germ cell leaves the stem cell niche, it is surrounded and encapsulated by two somatic cyst cells, which are required for the germline to differentiate. To better understand how interactions between the cyst cells and germ cells control spermatogenesis I disrupted cytoskeletal and junctional proteins specifically in the somatic cyst cells and investigated the resulting changes in cellular architecture and cell-cell signalling pathways.

This thesis describes the identification of a somatic permeability barrier that separates germ cells from their environment. This barrier is analogous to the mammalian blood-testis barrier and has not been previously identified in *Drosophila*. The permeability barrier is established by occluding septate junctions that seal together the two cyst cells that encapsulate the germline. If this barrier is disrupted the germline fails to differentiate resulting in infertility. This thesis presents data suggesting that the barrier controls differentiation by blocking the ability of germ cells to access signalling molecules that promote stem cell fate. These findings represent a novel role for occluding junctions in controlling stem cell differentiation by isolating them from their niche.

While characterizing stem cell niche signalling it was observed that disrupting occluding junctions in differentiating cyst cells led to progressive growth of the stem cell niche in adult testes. This growth was due to cyst cells converting into hub cells and recruiting more stem cells over time. The results presented in this thesis suggest that the conversion of cyst cells to hub cells was due to changes in the EGFR and Notch signalling pathways that regulate somatic differentiation. This represents an unexpected role for occluding junctions in maintaining stem cell niche homeostasis.

Preface

CHAPTER 2. A version of this work has been published as:

> Michael J. Fairchild, Christopher M. Smendziuk, and Guy Tanentzapf. (2015) A somatic permeability barrier around the germline is essential for *Drosophila* spermatogenesis. **Development** *142*, 268–281.

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For this publication I received help with quantification of cell numbers and proliferation from Christopher Smendziuk. I also received feedback and assistance from Christopher Smendziuk and Fayeza Islam. Guy Tanentzapf and I designed experiments and co-wrote the paper.

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For this publication I received help with quantification of cell numbers from Lulu Yang and help with automated measurement from Katharine Goodwin. I also received help on a pilot project from Enzo Casal. Guy Tanentzapf and I designed experiments and co-wrote the paper.

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List of abbreviations

abd-A	abdominal-A
Abd-B	Abdominal-B
Arm	Armadillo / β-Catenin
Bam	Bag-of-marbles
BMP	Bone morphogenetic protein
BTB	Blood-testis-barrier
Chic	Chickadee / Profilin
Cora	Coracle
CySC	Cyst stem cell
DEcad	DE-Cadherin / Shotgun
Dl	Delta
DNcad	DN-Cadherin / Cadherin-N
Dome	Domeless
DPCI	Days post-clone induction
DPE	Days post eclosion
dpERK	Di-phosphorylated ERK
Dpp	Decapentaplegic
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFR-CA	Constitutively activated EGF receptor / λTop
ERK	Extracellular regulated kinase / MAPK / Rolled
Eya	Eyes absent
FasIII	Fasciclin-III
Gal80ts	Temperature-sensitive Gal80

Gbb	Glass-bottomed boat
GDNF	Glial-cell-line derived neurotrophic factor
GFP.nls	EGFP::nuclear localization signal
GSC	Germline stem cell
HFSC	Hair follicle stem cell
Hh	Hedgehog
MAPK	Mitogen activated protein kinase / ERK / Rolled
mGFP	mCD8::EGFP
mRFP	mCD8::Tomato
Notch-DN	Dominant negative Notch / Notch-ECN
Nrx-IV	Neurexin-IV
PGC	Primordial germ cell
pH3	phospho-Histone H3
pMad	phosphorylated-Mad
RA	Retinoic acid
RFP.nls	DsRed::nuclear localization signal
SCF	Sertoli cell factor / Kit-Ligand
SGP	Somatic gonadal precursor
STAT	Signal transducers and activators of transcription
TGF-β	Transforming growth factor beta
Tj	Traffic jam
Upd	Unpaired-1
Zfh1	Zinc finger homeodomain 1
β2tub	Beta-Tubulin 85D
β3tub	Beta-Tubulin 60D

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And a tip of the hat to the millions of flies that lived in vials so that I could mess around with their cells.

Dedication

This thesis is dedicated to my uncle, Michael Robinson, who inspired me to be a scientist when he told me that I already knew more about how cells work in high school than most of his university students. He also threatened to hit me over the head with a wooden spoon.

CHAPTER ONE: Introduction

1.1 The germline and sexual reproduction

Sexual reproduction is the predominant form of reproduction in multicellular animals and is typically anisogamous, meaning it involves two different types of gametes, sperm and ova (or eggs) [1]. Sperm are the smaller of the two gametes and are produced in testes, while ova are larger and produced in ovaries. The creation of these two different types of gametes, or gametogenesis, requires two different developmental processes: spermatogenesis to form sperm, and oogenesis to form ova. Specialized cells, collectively known as the germline, produce these gametes. During gametogenesis germ cells undergo meiosis to give rise to haploid sperm and ova. It is these sperm and ova which fuse together to form a single diploid zygote in the process of fertilization [1]. As the zygote grows to become a multicellular animal the germline is specified and becomes distinct from all other cells of the body, collectively referred to as the soma [2].

The germline is specified via two possible mechanisms that take place during the early stages of animal development [3]. In the first mechanism the germline is specified by localized factors in the zygoyte termed 'germ plasm'. In the second mechanism the germline is specified from the soma by an inductive interaction between cells early in embryogenesis [3]. In both mechanisms the ability to induce the formation of germ cells from the soma is typically present for only a brief window during development. Even in certain invertebrates that retain the ability to form new germ cells throughout life, the germline is typically specified only once during early development [2,4,5]. Thus once the germline is formed it must be protected and supported by the somatic cells of the body to

ensure successful gametogenesis and fertility.

Embryonic germ cells, termed primordial germ cells (PGCs), generally congregate in specific anatomical locations that will become the gonads [6]. The formation of gonads usually involves the PGCs pairing with specialized somatic cells termed somatic gonadal precursors (SGPs). The SGPs intimately associate with the PGCs often surrounding them completely as they coalesce into the gonad [7,8]. These SGPs are critical for gametogenesis and eliminating them leads to the failure of germline development and sterility in range of animal phyla [9-13].

This thesis explores the ways in which the somatic cells of the gonad guide development of the germline during spermatogenesis in the *Drosophila* testis.

1.2 Spermatogenesis

Spermatogenesis can be divided into three distinct developmental stages: The spermatogonial stage, characterized by mitotic germ cells; The spermatocyte stage, characterized by meiotic germ cells; and the spermatid stage, characterized by the morphological changes that create sperm [8].

The spermatogonial population is sustained by a subset of cells termed germline stem cells (GSCs) [14]. GSCs are able to divide asymmetrically forming both GSCs as well as spermatogonial cells that will proceed through spermatogenesis. To ensure continued spermatogenesis the GSC population must be maintained while still allowing the spermatogonia they produce to differentiate. A specialized stem cell niche typically mediates this balance by providing a physical structure and signalling molecules that control GSC behaviour [14].

Spermatogonia that initiate a program of differentiation undergo transit-amplifying mitotic divisions, which form a large number of germ cells for every GSC division. These divisions are incomplete and all the resulting germ cells retain small cytoplasmic bridges connecting them in a clonal syncytium [8].

Spermatocyte development begins once transit-amplifying mitotic divisions are complete. This stage is a point-of-no-return during spermatogenesis as the genome is irreversibly altered and reduced from diploid to haploid by meiosis. The differentiation of each spermatogonial syncytium into spermatocytes thus represents the beginning of terminal germ cell differentiation [8].

Spermatid development begins once meiotic divisions are complete. The genome is compacted into a small high-density nucleus and the cellular machinery required for fertilization and motility are formed. Finally, the spermatids remove excess cytoplasm and sever their intercellular bridges before being released as individual sperm [8].

1.3 Soma-germline interactions during spermatogenesis

The hypothesis that soma-germline interactions play an essential role in spermatogenesis can be traced to 1859 when Enrico Sertoli observed that germ cells were completely enveloped by a specialized layer of somatic cells as they underwent spermatogenesis in mammals [15]. Similar specialized cells were later identified across many other animal phyla (reviewed in [7,8]). Even anatomically simple animals such as sponges were found to have somatic cells that envelop each germ cell during spermatogenesis [16] (**Figure 1.1**). The ubiquity of this soma-germline relationship suggested that somatic cells play a fundamental and conserved role during spermatogenesis. Consistent with this idea, experimental ablation of somatic cells in

worm, fly, and mammal testes all lead to the failure of germ cell differentiation or survival [17-19].

Although spermatogenesis takes place in the fully developed organism soma-germline interactions are a model for the sort of morphogenetic tissue interactions that take place during embryogenesis. During spermatogenesis, as in embryonic morphogenetic processes, the soma and germline interact to shape their mutual development; both physically through cell-cell adhesions and by signalling to each other using multiple cell-cell signalling pathways [13]. The study of soma-germline interactions during spermatogenesis can therefore provide general clues about the type of processes that underpin embryonic morphogenesis. The ongoing nature of spermatogenesis also allows developmental processes to be studied continuously in an adult organism. This reiterative aspect of spermatogenesis requires an active adult stem cell system that can continually replenish the supply of germ cells in adults [14]. Together this positions the study of soma-germline interactions as a nexus for the study of stem cells, development, and reproduction in animals.

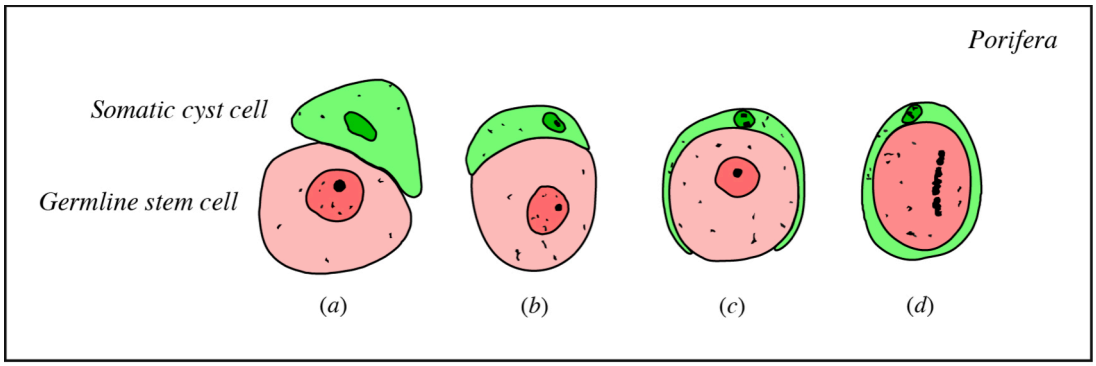

Figure 1.1 - Somatic cells surround germ cells during animal spermatogenesis
Germline stem cell being surrounded by a somatic cyst cell in *Spongilla*. In (*d*) a cyst has been established and the first spermatogonial division is taking place. Figure adapted from Roosen-Runge EC. The process of spermatogenesis in animals. Cambridge University Press, 1977; after the original study from Wilhelm Görich. (1904). *Z. Wiss. Zool.* **76**, 522-43.

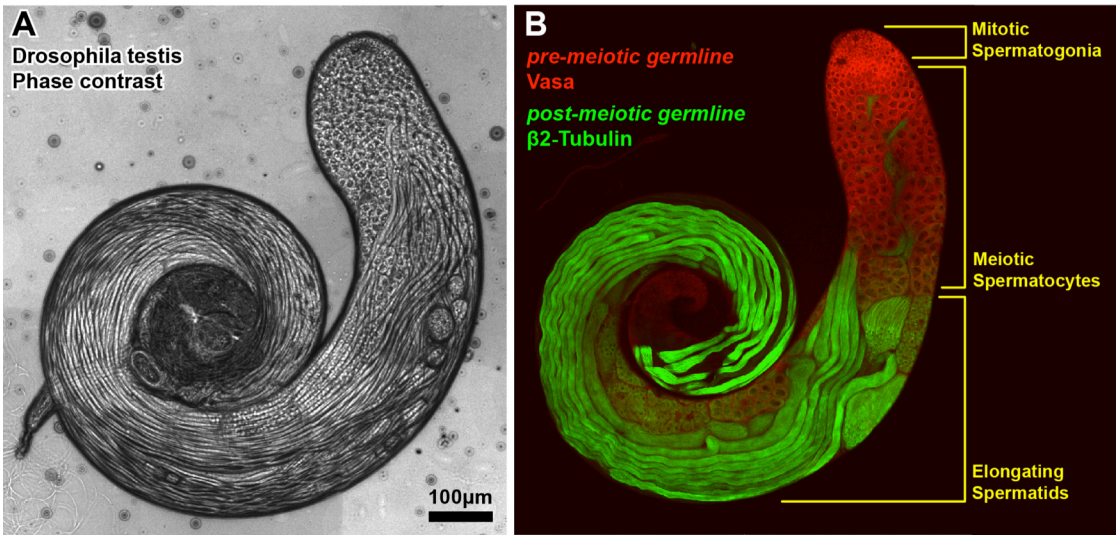

Figure 1.2 – The *Drosophila* testis and stages of germline differentiation.
(A) Phase contrast image of a *Drosophila* testis. (B) Germ cells stained for the pre-meiotic gene Vasa and the post-meiotic gene β2-Tubulin, with the stages of spermatogenesis indicated.

1.4 *Drosophila melanogaster*: A model for spermatogenesis

Drosophila testes are a pair of coiled tubular organs located in the abdomen of the male (**Figure 1.2**). A sheath of extra-cellular matrix, pigment cells, and smooth muscle cells covers each testis. The early stages of spermatogenesis occur at the closed anterior end of the testis, known as the 'apical tip'. Progressively more advanced stages of spermatogenesis are found down the length of the testis, with mature sperm being extruded from the basal end of the testis into a seminal vesicle for storage [20].

The apical tip of each testis holds a stem cell niche known as the hub. The hub is made up of tightly clustered somatic cells attached to a dense accumulation of extra-cellular matrix [21]. The hub maintains both germline stem cells (GSCs) and somatic cyst stem cells (CySCs) using a combination of secreted signalling molecules and cell-cell adhesion [22-25]. Both GSCs and CySCs orient their centrosomes perpendicular to the hub during

mitosis, resulting in predominantly asymmetrical divisions, with one cell retaining contact with the hub, and the other being displaced from it [26,27].

Displaced GSC daughter cells differentiate into gonialblasts that will go on to become sperm, while displaced CySC daughter cells differentiate into cyst cells that will support the germ cells throughout spermatogenesis. Cyst cells support the germline in part by wrapping around each gonialblast as it leaves the niche in process termed encapsulation [10]. During encapsulation exactly two cyst cells wrap each gonialblast forming a spermatocyst. All further germ cell differentiation takes place within the spermatocyst, inside the lumen formed between the two encapsulating cyst cells [10] (**Figure 1.3**).

Each encapsulated gonialblast proceeds through four rounds of transit-amplifying spermatogonial divisions resulting in 2-cell, 4-cell, 8-cell, and finally 16-cell germline syncytia. Spermatogonia then differentiate into spermatocytes and undergo massive growth expanding approximately twenty-five times in volume as they move towards the basal end of the testis. The spermatocytes go through meiosis I and II mid-way down the testis forming sixty-four round spermatids. These spermatids then elongate with their nuclei continuing to move basally, while their flagella grow back towards the apical tip. Once their excess cytoplasm is stripped away and their intercellular bridges are severed, the spermatids become individualized sperm [20].

The two cyst cells encapsulating the germline also differentiate during spermatogenesis. The cyst cells undergo dramatic changes in gene expression, grow in size, and become two distinct types of cyst cells – head and tail cyst cells [10]. When the head cyst cell surrounding the spermatid nuclei reaches the end of the testis it attaches to

a layer of somatic cells called the terminal epithelium. The individualized sperm within the spermatocyst are then coiled at the base of the testis before being threaded tail first through a narrow duct into the seminal vesicle [28].

The entire process of spermatogenesis requires cooperation between cyst cells and germ cells to be successfully completed. Eliminating cyst cells or disrupting their ability to encapsulate both result in the failure of germ cells to differentiate past the early spermatogonial stage [17,29,30]. Why encapsulation is necessary for germline differentiation is not fully understood, but analogous processes across the animal kingdom suggest a conserved role in regulating spermatogenesis.

This thesis examines the structural basis for encapsulation and its impact on the cell-cell signalling pathways active between the soma and germline.

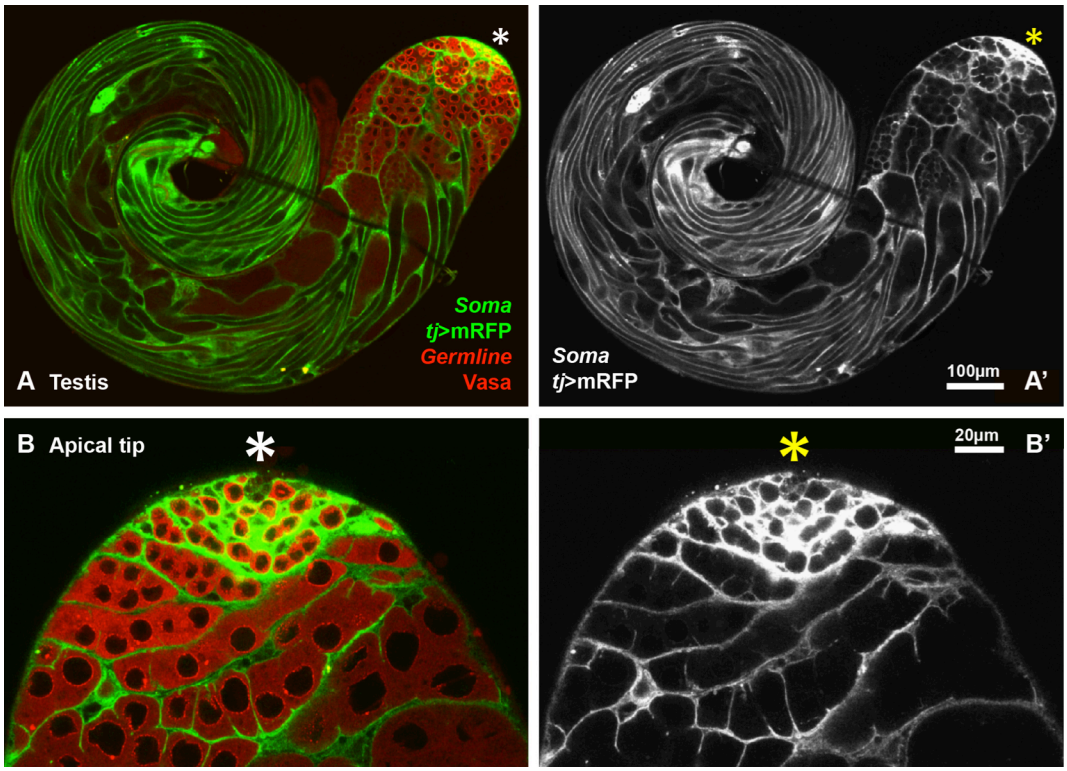

Figure 1.3 – Somatic cyst cells encapsulate the germline throughout spermatogenesis
(A) The *Drosophila* testis with somatic cells labelled by membrane bound RFP (*tj*>mRFP), and the germline labelled by Vasa::GFP. (B) The apical tip of the testis, with the hub indicated by an asterisk.

1.5 Formation of the stem cell niche

Drosophila testes form when migrating primordial germ cells (PGCs) reach the somatic gonadal precursors (SGPs) during stage 11 of embryogenesis [31-33]. The SGPs are specified in several clusters from the lateral mesoderm through the action of the Hox genes abdominal-A (abd-A) and Abdominal-B (Abd-B). These clusters of SGPs migrate out of the mesoderm, surround the PGCs, and then coalesce and compact into the gonad. The anterior-most SGPs, which express abd-A, are fated to form cyst cells, unless they receive an inductive Notch signal that promotes the formation of hub cells. While the posterior SGPs, which do not express abd-A, are fated to form structures such as the terminal epithelium at the base of the testis [32-35].

The specification of the hub occurs during embryonic stages 11-12 when the SGPs transiently contact the neighbouring endoderm. The endoderm expresses the Notch ligand Delta and activates Notch signalling in a subset of SGPs promoting the formation of hub cells [34]. The SGPs are only sensitive to Notch signalling during this time window in embryonic development, at all other stages no hub cells are induced regardless of Notch activity [34]. Hub formation is repressed by the PGCs, which secrete the ligands Spitz and Sevenless to activate Epidermal Growth Factor Receptor (EGFR) signalling in the surrounding SGPs [36,37]. Disrupting EGFR signalling in SGPs leads to the increased formation of hub cells at the expense of cyst cells. This demonstrates that EGFR signalling can repress the formation of hub cells induced by Notch signalling [36,37]. The combination of inductive Notch signalling from the endoderm and repressive EGFR signalling from the germline thus modulate the number of hub cells and cyst cells formed by the SGPs.

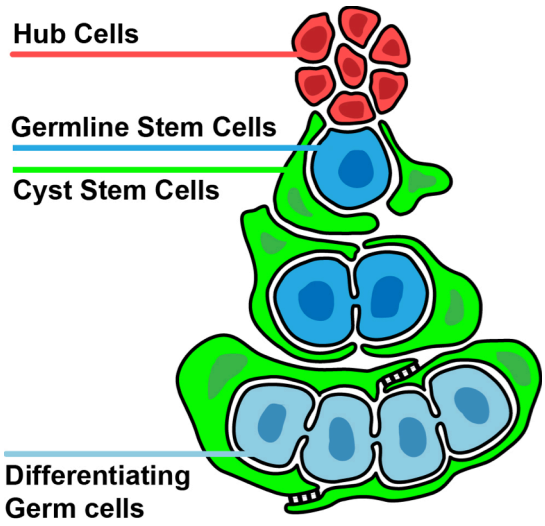

Figure 1.4 - Diagram of the stem cell niche
The lineal descendants representing one GSC and two flanking CySCs are shown for clarity.

The stem cell niche becomes apparent by embryonic stage 15 when a subset of the anterior-most SGPs express high levels of cell-cell adhesion proteins such as DE-Cadherin, DN-Cadherin, and Fasciclin-III. These SGPs then undergo a process similar to mesenchymal-epithelial transition, adhering preferentially to each other and forming a tightly packed epithelia-like spheroid of cells [32]. The resulting hub attaches to the extra-cellular matrix at the apical end of the testis using integrin-based adhesion [38]. The hub also attaches to the neighbouring PGCs and SGPs via cadherin-based adherens junctions recruiting the presumptive GSCs and CySCs [23,25,39].

The surface area of the hub is critical in determining the number of stem cells as all GSCs and CySCs maintain contact with it [21,40]. The round GSCs directly abut the surface of the hub and attach to it with a wide area of contact, while the thin CySCs wrap around the GSCs and contact the hub by extending lamellipodia between the GSCs [21]. The complete structure of the stem cell niche including hub cells, GSCs, and CySCs is in place by the end of embryogenesis, at late stage 17 [41,42] (**Figure 1.4**).

The stem cell niche formed during embryogenesis remains stable throughout subsequent development and into adulthood. In the adult the number of hub cells remains relatively constant while the number CySCs and GSCs vary, but remain in an

approximate ratio of between 1:1 and 2:1 [21,43]. The two stem cell populations are both mitotically active and individual CySCs and GSCs can be lost and replaced [43,44]. By contrast hub cells are lost only infrequently [45,46] and do not undergo mitosis under normal conditions [21,35,39,47,48]. The hub thus provides a consistent anchor for the more dynamic stem cells surrounding them. This ensures the mutual interaction of hub cells, CySCs, and GSCs in the adult stem cell niche.

1.6 Stem cell maintenance

The stem cell niche is defined by both the signals and structures that promote stem cell maintenance [49]. The hub physically attaches to both populations of stem cells keeping them in close proximity to both itself and each other [21]. The hub also secretes signalling ligands that repress stem cell differentiation or increase the retention of stem cells in the niche [22-24]. Cells displaced from the hub by asymmetric stem cell divisions lose direct contact with the hub and may experience lower concentrations of hub cell-produced signalling molecules [26,27]. The displaced cells are then poised to differentiate and undergo spermatogenesis without disrupting the stem cell niche. The architecture of the stem cell niche can thus guide both stem cell maintenance and differentiation.

1.6.1 Germline stem cell maintenance

Differentiation of GSCs is repressed by Bone Morphogenic Protein (BMP) signalling. Hub cells secrete the BMP ligands Decapentaplegic (Dpp) and Glass-bottomed boat (Gbb) that bind the receptors Thickveins and Punt on germ cells to activate BMP signalling. BMP signalling through Mad directly represses expression of the gene Bag-of-marbles (Bam) which is both necessary and sufficient for germ cells to differentiate from spermatogonia into spermatocytes [50-57].

The levels of BMP signalling must be carefully regulated to ensure GSC maintenance. If BMP signalling is eliminated, GSCs will express Bam and differentiate. Over-activation of BMP signalling also results in defective spermatogenesis. If BMP signalling is ectopically activated or if Bam expression is reduced, spermatogonia fail to differentiate into spermatocytes. These spermatogonia instead go through multiple extra rounds of transit-amplifying divisions, forming large clusters of syncytial spermatogonia [51,54-56,58]. This suggests that the germline may require additional signals within the niche to remain as single-celled GSCs. Alternatively, it may only be the privileged position enjoyed by GSCs near the source of BMP ligands, and/or the severing of the connection between dividing GSC-gonialblast pairs, that allow these cells to develop independently [59].

The interface between the hub cells and the GSCs has been shown to have high levels of BMP signalling. This may be due to expression of the proteoglycan Dally-like on hub cells, which can bind to BMP ligands and slow their diffusion [60]. Alternatively, high levels of BMP signalling may be maintained by a synapse-like signalling domain or cytoneme-like projections from the GSCs into the hub [61,62]. The hub cell-GSC interface is maintained by prominent cell-cell adhesions between the two cell types. These cell-cell adhesions are in turn the product of STAT signalling [23] . The STAT ligand Unpaired-1 (Upd) is released by hub cells and binds the receptor Domeless (Dome) on germ cells. Activation of STAT signalling increases expression of the adherens junction protein DE-Cadherin in GSCs, ensuring they remain attached to the hub [23]. These DE-Cadherin based junctions also control the asymmetric division of GSCs by positioning their centrosomes during mitosis [63]. STAT signalling from the hub

thus ensures both the retention of GSCs and the displacement of gonialblasts from the hub where they receive less STAT and BMP signalling, positioning them for differentiation.

1.6.2 Cyst stem cell maintenance

CySCs are also maintained by signals they receive from the hub. Specifically, STAT signalling is both necessary and sufficient for CySC maintenance, in part by driving expression of the transcription factors Zfh1 and Chinmo [64,65]. If STAT signalling is lost CySCs differentiate while constitutively activating STAT causes all cyst cells throughout the testis to act as functional CySCs [64,66,67]. The hub also secretes the ligand Hedgehog (Hh) that binds the receptor Patched in CySCs to activate Hh signalling. Hh signalling, much like STAT signalling, prevents CySC differentiation though likely through a separate mechanism. Unlike STAT signalling, Hh signalling is not sufficient to induce CySC identity and ectopically expressing Hh only delays the differentiation of cyst cells [22,68,69]. The hub is the only source of the STAT and Hh ligands in the apical tip of the testis and only CySCs and those cyst cells nearest the hub activate these signalling pathways. This pattern suggests that distance from the hub may limit access to these signals and could be one of the controlling factors in CySC differentiation.

Similar to GSCs, CySCs are also retained in the niche by cadherin-based adherens junctions and possibly by integrin-based adhesions as well [25,39,70,71]. Expression of DE-Cadherin in CySCs is regulated in part by the receptor Robo2 when activated by the ligand Slit expressed on the hub [25]. It is unclear whether these junctions guide the asymmetrical divisions of CySCs as they do in GSCs [27]. Interestingly, while CySC divisions are typically asymmetric they are not as tightly regulated as GSC divisions.

This results in CySCs being lost from the niche and replaced more often than GSCs suggesting that CySCs may be a more dynamic stem cell population [27,43].

1.6.3 Stem cells form part of the niche

While the hub physically adheres to the GSCs and CySCs and secretes signalling ligands that maintain them, each stem cell population may also form part of the functional niche. Recent findings suggest that signals from GSCs can enhance CySC maintenance and conversely signals from CySCs can enhance GSC maintenance. This may balance competition between the two populations for stem cell niche occupancy and ensure the ratio of CySCs to GSCs remains relatively consistent [72,73].

GSCs may enhance CySC maintenance through EGFR signalling. All early germ cells secrete the EGF ligand Spitz that activates EGFR signalling in cyst cells [29,30]. EGFR signalling acts through the MAPK pathway to mediate the wrapping of germ cells during encapsulation [74]. EGFR signalling also increases the likelihood that CySCs are retained in the niche [71,75]. Production of the EGF ligand Spitz by GSCs can thereby ensure that neighbouring CySCs are less likely to be lost and replaced. However CySCs also produce another EGFR ligand, Vein, complicating analysis of the role EGFR signalling plays in the stem cell niche [30,71].

CySCs may also enhance GSC maintenance through BMP signalling. CySCs secrete the ligands Dpp and Gbb similar to hub cells [54,56]. If cyst cell differentiation is disrupted the ectopic CySCs remain associated with GSCs that show signs of active BMP signalling [64,76]. This illustrates that BMP signals originating from CySCs are sufficient to repress GSC differentiation even at a distance from the hub. However, if CySC are completely lost, the hub is still able to retain GSCs and the remaining germ cells still

show signs of active BMP signalling [17]. Therefore CySCs are sufficient for GSC maintenance but not necessary for it.

Overall, the architecture of the stem cell niche facilitates the interactions between hub cells, GSCs, and CySCs. Attachment of both stem cell populations to the hub retains them near maintenance signals produced by the hub cells. It also ensures the mutual interaction of both stem cell populations, which may also regulate their maintenance (**Figure 1.5**).

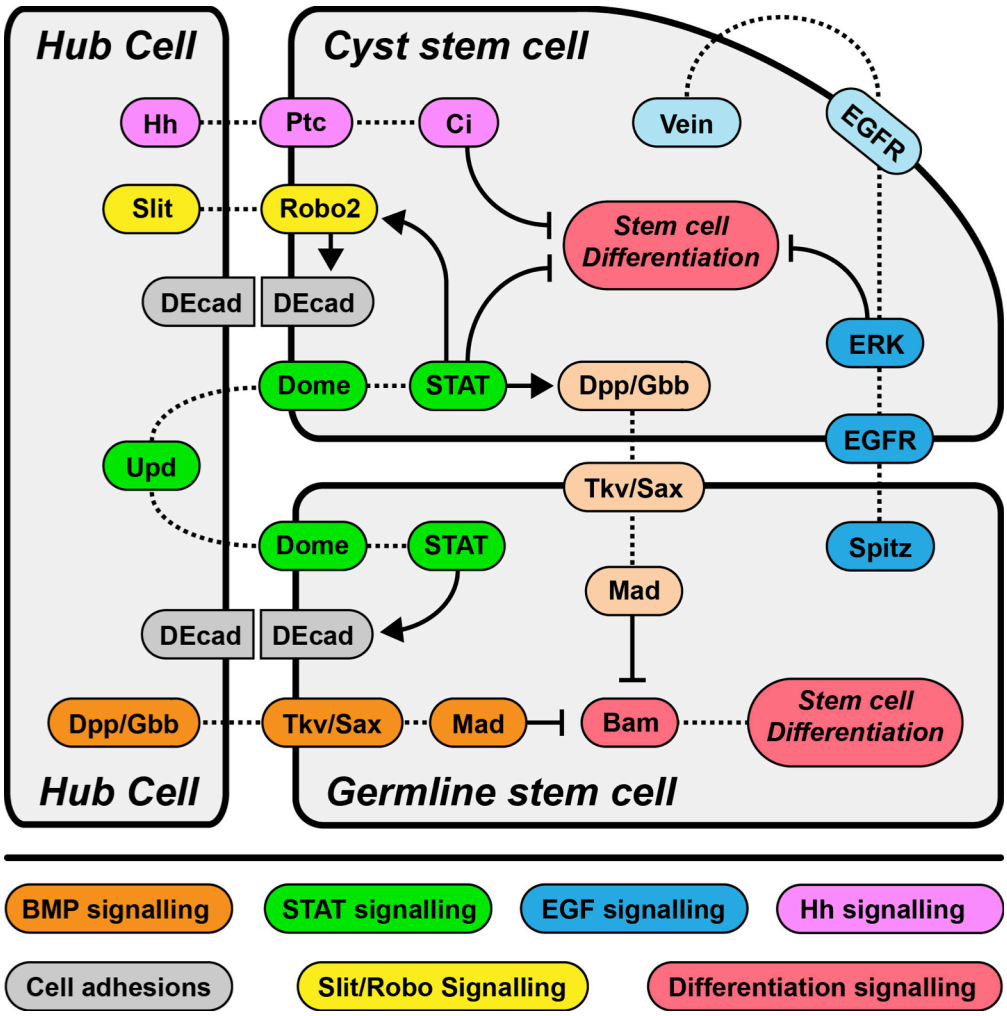

Figure 1.5 - Cell-cell signalling pathways active in the stem cell niche
Diagram of the stem cell niche depicting the signalling pathways active between hub cells, GSCs, and CySCs. Only ligands, receptors and secondary messengers are shown for clarity. Signalling pathways suppress differentiation by maintaining stem cell identity or increasing the retention of stem cells in the niche.

1.7 Stem cell differentiation

1.7.1 Bag of marbles and germline differentiation

When GSCs divide asymmetrically the resulting gonialblasts are displaced from the hub. As the gonialblasts undergo transit-amplifying spermatogonial divisions they move further from the stem cell niche where a reduced level of BMP signalling allows the expression of Bag of marbles (Bam) [50-57]. When Bam expression reaches a critical threshold it induces spermatogonia to differentiate into spermatocytes [58]. Bam expression is first detected in the 4-cell spermatogonial stage, peaks in the 8-cell spermatogonial stage, and is then reduced as 16-cell spermatogonia form spermatocytes [58]. A delay or reduction in Bam expression results in spermatogonia continuing through extra rounds of transit-amplifying divisions giving rise to large clusters of syncytial spermatogonia [58]. Similarly, precocious expression of Bam in early spermatogonia causes them to go through fewer rounds of transit-amplifying divisions producing spermatocytes earlier with fewer cells [58,77]. The onset and extent of Bam expression thus acts as a master regulator controlling both the number of spermatogonial divisions and their differentiation into spermatocytes.

It is unclear how Bam regulates germline differentiation, however it is known to bind the proteins Tut and Bgcn to form a complex that represses translation of mRNAs [78]. This complex controls expression of Mei-P26 that can in turn repress translation of Bam mRNA, leading to a feedback loop that controls the extent of Bam expression [58]. Other proteins such as How and microRNAs such as *miR-275* and *miR-306* can also repress the translation of Bam mRNA, though it is less clear how they are regulated [77,79]. The complexity of the regulatory mechanisms that have developed to control the

translation of Bam mRNA illustrates the precision with which germ cells must control when and how Bam is expressed.

How Bam expression is triggered precisely at the 4-cell spermatogonial stage is not fully understood [58]. Transcription of Bam is directly repressed by BMP signalling which is highest in the GSCs contacting the hub [61,62]. Despite this, BMP signalling also extends to germ cells not contacting the hub. For instance, BMPs produced by CySCs are sufficient for GSC maintenance even without the hub [23,64]. Furthermore, in larval testes BMP signalling can be measured directly using phosphorylated-Mad (pMad) and is active in GSCs, gonialblasts, and 2-cell stage spermatogonia [80]. Differentiating cyst cells encapsulating transit-amplifying spermatogonia also show signs of active BMP signalling even at quite a distance from the hub [80-82]. While BMP signals extending further from the stem cell niche could repress the expression of Bam in early spermatogonia, it still does not explain how BMP signalling is abruptly shut off between the 2-cell and 4-cell spermatogonial stage [58,80].

The onset of Bam expression must be carefully orchestrated as it controls the number of transit-amplifying divisions spermatogonia will go through before forming spermatocytes [58]. Even small perturbations in the expression of Bam can lead to a large increase in the size of each germline syncytium as the germ cells divide in parallel to grow exponentially in number [83]. The expression of Bam at the 4-cell spermatogonial stage is therefore critical to ensure that each germline syncytium can develop within the confines of the testis [58]. While germline differentiation is directly controlled by the expression of Bam, it also requires the somatic cyst cells.

1.7.2 Encapsulation by differentiating cyst cells

Germline differentiation is dependent on encapsulation by cyst cells [29,30,84]. Germ cells coordinate encapsulation by secreting the EGF ligand Spitz, which activates EGFR signalling in the surrounding cyst cells. EGFR signalling acts through the MAPK pathway to modulate the actin cytoskeleton and mediate the extension of cyst cell lamellipodia around the gonialblast [29,30,74,84,85]. EGFR signalling in cyst cells also regulates the severing of the cytoplasmic bridge connecting GSCs and gonialblasts, allowing these two cells to develop independently [59].

When EGFR signalling is disrupted encapsulation fails and spermatogonia are unable to develop into spermatocytes [29,30,84]. Spermatogonia that are not encapsulated go through multiple extra rounds of transit-amplifying divisions, similar to what is observed when BMP signalling is ectopically activated or Bam expression is reduced in germ cells [51,54-58]. Intriguingly, disrupting encapsulation also delays the expression of Bam in the spermatogonia near the stem cell niche [29]. However, spermatogonia that are not encapsulated further from the hub eventually express low levels of Bam but still fail to differentiate into spermatocytes [29]. Together this suggests that encapsulation may control germline differentiation in part by regulating the onset of Bam expression.

One mechanism by which encapsulation could control germline differentiation is by inhibiting the access of germ cells to BMP signals emanating from the stem cell niche. However encapsulation occurs as gonialblasts leave the stem cell niche [21,59] whereas BMP signalling remains active in germ cells up to the 2-cell spermatogonial stage [58,80]. This suggests that encapsulation is not sufficient in and of itself to stop BMP signalling in germ cells. Additional mechanisms may also be required for encapsulating cyst cells to stop BMP signalling or otherwise promote germline differentiation.

1.7.3 Signalling in the spermatocyst

Once the two cyst cells have encapsulated the germline and formed the spermatocyst, they must receive at least two additional signals to facilitate differentiation of the enclosed germ cells. The first is mediated by an elevated level of EGFR signalling in the encapsulating cyst cells [86]. The second is activation of cyst cell specific Activin signalling through the receptors Punt, Sax, and Baboon acting on the downstream effector Smox [81,82]. If cyst cells do not receive either of these signals the germline fails to differentiate despite being properly encapsulated [81,82,86]. It remains unknown how these two signals are regulated or how they allow the encapsulating cyst cells to support germline differentiation.

Distinct changes in the morphology and signalling environment of both the soma and germline are required for spermatogenesis. Germ cells must activate EGFR signalling in cyst cells in order to be encapsulated; while the encapsulating cyst cells must receive an elevated level of EGFR signalling. Germ cells must also receive less BMP signalling in order to express Bam; while cyst cells must activate the related Activin signalling pathway. These events allow encapsulated spermatogonia to form spermatocytes that are then committed to undergoing meiosis and proceeding through spermatogenesis. It remains an open area of inquiry as to how these signals relate to each other in the context of the spermatocyst. To understand these processes in more detail I undertook a study of somatic cyst cell structure and how it shapes the signalling environment during spermatogenesis. This investigation makes up Chapters 2 and 3 of this thesis.

1.8 Other models of spermatogenesis

Although there are overall similarities in spermatogenesis between different animal species the structure of the testis varies significantly. Two common themes seen throughout the animal kingdom are the presence of a GSC niche and the envelopment of differentiating germ cells by specialized somatic cells [7,16]. An examination of the structures and signals that control stem cell maintenance and differentiation in other animals provides a useful comparison to *Drosophila*. These soma-germline interactions are structurally diverse but fall into two broad categories [87] (**Figure 1.6**):

(1) Cystic spermatogenesis, observed in insects such as *Drosophila melanogaster* [20,88]. Characterized by somatic cells wrapping around the germ cells followed by both cell types differentiating together as a cyst. Somatic cells associate with a single generation of germ cells and differentiate with them in unison. Cystic spermatogenesis is also observed in sponges, fish, and amphibians [16,87].

(2) Acystic spermatogenesis, observed in mammals such as mice, rats, and humans [89]. Characterized by a single layer of somatic epithelial cells that can pair with multiple generations of germ cells, which differentiate as they transit from the basal to the apical side of the epithelium. These somatic cells are each spatially differentiated along their apical-basal axis and undergo repeated cycles of differentiation during each round of spermatogenesis. This mode of soma-germline interaction is also observed in cnidarians, gastropods, reptiles, and birds [87,90,91].

The extensive research on acystic spermatogenesis in mammals allows for a detailed comparison to cystic spermatogenesis in *Drosophila*. This comparison can help

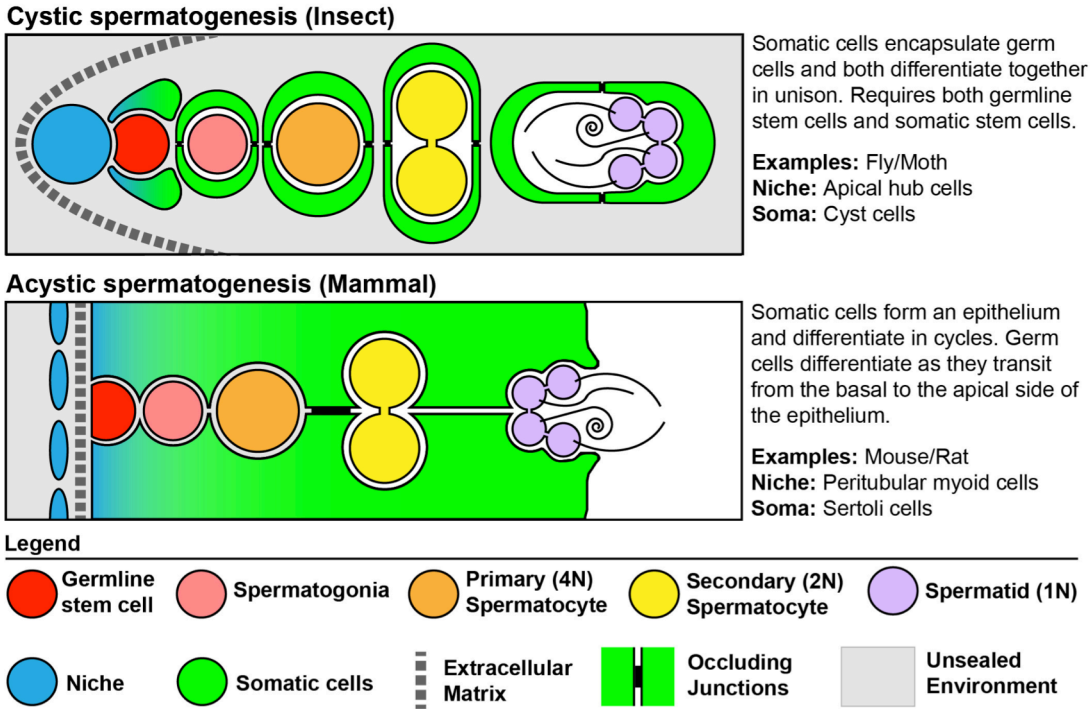

Figure 1.6 – Types of soma-germline interaction during spermatogenesis
Simplified diagrams depicting cystic and acystic spermatogenesis in insects and mammals respectively. Diagrams represent a single germline stem cell and spermatogonium developing without transit-amplifying mitotic divisions.

identify evolutionarily conserved features of soma-germline structure and signalling and informed the research presented in this thesis.

1.8.1 Mammalian spermatogenesis

In mammals spermatogenesis occurs in a tubular structure known as the seminiferous epithelium made up of somatic Sertoli cells [9]. Sertoli cells are attached to a basal lamina that is surrounded by a layer of peritubular myoid cells [9]. GSCs are attached to the inner side of the basal lamina and are in direct contact with the basal side of the Sertoli cells. As GSCs proliferate some will form transit-amplifying spermatogonia that also remain attached to the basal lamina. As these spermatogonia differentiate into spermatocytes they detach from the basal lamina and translocate apically through

junction complexes between adjacent Sertoli cells and become sequestered in an adluminal compartment above the junctions. The spermatocytes then complete meiosis, forming spermatids that morphologically differentiate and are released into the tubule lumen as sperm [9]. This process is analogous to encapsulation during *Drosophila* spermatogenesis as in both cases germ cells are enclosed within a compartment created by somatic cells as they differentiate into sperm.

Mammalian GSCs are maintained by multiple signalling pathway ligands produced by Sertoli cells, peritubular myoid cells, Leydig cells, and other neighbouring cells types [92]. The most critical of these is Glial-cell-line Derived Neurotrophic Factor (GDNF) binding to the receptors Ret and GFRα1 on germ cells to activate PI3K/Akt and MAPK signalling [93]. Similar to BMP signalling in *Drosophila*, loss of GDNF signalling leads to GSC differentiation, while over-expression of GDNF leads to the failure of germline differentiation, resulting in spermatogonia-like tumours [94]. Both Sertoli cells and peritubular myoid cells produce GDNF. However, Sertoli cell-derived GDNF is insufficient for GSC maintenance *in vivo* if GDNF is genetically knocked-out in the peritubular myoid cells [95]. This indicates that GDNF produced by the peritubular myoid cells forms a critical part of the mammalian testis stem cell niche. The expression of GDNF by peritubular myoid cells is controlled by testosterone secreted by groups of neighbouring interstitial Leydig cells [96]. Furthermore GSCs are predominantly found in regions of the seminiferous tubule nearest these interstitial Leydig cells [97]. Together this suggests that Leydig cells cooperate with peritubular myoid cells to maintain the mammalian GSC population. The localized production of stem cell maintenance signals by somatic cells neighbouring the GSCs is analogous to the stem cell niche in

Drosophila. While in mammals these stem cell maintenance signals are produced by somatic cells surrounding the outside of the seminiferous tubules, in *Drosophila* they are produced by a single cluster of somatic hub cells.

As mammalian spermatogonia differentiate into spermatocytes they are engulfed between Sertoli cells before proceeding through meiosis [98]. Similar to *Drosophila*, mammals have a putative homolog of Bag of marbles (Bam) known as Gm114/Kizuna that is expressed in differentiating germ cells and appears to be repressed by GDNF signalling [99]. However a partial deletion of the Gm114/Kizuna protein coding sequence does not disrupt spermatogenesis [99]. Instead cyclical waves of retinoic acid (RA) signalling stimulate spermatogonia to differentiate into spermatocytes and undergo meiosis [98]. RA signalling acts on both germ cells and Sertoli cells to promote spermatogenesis. In germ cells RA signalling induces expression of the receptor tyrosine kinase c-Kit [100]. In Sertoli cells RA signalling induces expression of the ligand SCF which activates c-Kit signalling in germ cells, driving the differentiation of spermatogonia into spermatocytes [101]. RA signalling in Sertoli cells also regulates the expression of other genes required for germline differentiation including Occludin which is a structural component of tight junctions [102].

Tight junctions are the predominant occluding junctions found in vertebrates and prevent ions, proteins, and other molecules from diffusing past them [103]. Tight junctions between the Sertoli cells seal the inner, adluminal side of the seminiferous tubule from the outer, basal side [104]. Tight junctions thus form a permeability barrier between Sertoli cells that prevents substances in the blood from reaching germ cells within the seminiferous tubule. The resulting structure was therefore named the

Blood-Testis-Barrier (BTB) [104]. The BTB is required for the formation of meiotic spermatocytes and disrupting it physically, chemically, or genetically all lead to the failure of spermatogenesis [105-107]. How this structural feature of the seminiferous epithelium supports germline differentiation is an area of active research [108].

1.8.2 The blood-testis-barrier

Germline differentiation in mammals requires spermatocytes to travel from the outer basal side of the seminiferous epithelium to the inner adluminal side, which is sealed from the surrounding environment by the presence of the BTB. During this process tight junctions form below spermatocytes before dissolving above them, transitioning the spermatocytes into the adluminal section where they complete meiosis [109]. Furthermore the establishment of the BTB coincides with the onset of meiosis during juvenile development [110]. Chemically disrupting the BTB also prevents spermatogonia from differentiating into spermatocytes and re-establishment of the BTB corresponds with the resumption of spermatocyte development and meiosis [107,111].

Genetic disruption of tight junctions in Sertoli cells *in vivo* has confirmed that they are essential for the formation and maintenance of the BTB [112]. Genetic knock-out of the tight junction protein Claudin-11 in Sertoli cells leads to loss of the permeability barrier and a failure of spermatocytes to survive and complete meiosis [105]. Claudin-11 knock-out also results in a subset of Sertoli cells detaching from the basal lamina and forming tightly packed cell clusters within the seminiferous tubules, possibly due to a loss of epithelial polarity [105]. The Claudin-11 based tight junctions that help form the BTB are therefore critical both for germline differentiation and for maintaining Sertoli cell morphology.

How the BTB regulates germline differentiation during spermatogenesis is a subject of much speculation but there are four biological roles that have been ascribed to it:

(1) The BTB isolates differentiating germ cells from glucose and other metabolites, forcing them to depend on the surrounding somatic cells for nutrients [113]. This may allow somatic cells to eliminate germ cells by simply ceasing to provide energy sources such as lactate [114]. The BTB could thus protect against germline mutations that would allow haploid germ cells to selfishly exploit the body's resources [115].

(2) The BTB isolates haploid germ cells from immune cells and antibodies. In vertebrates, haploid germ cells are highly immunogenic as they are only formed after self-tolerance has been established. The BTB thus protects haploid germ cells from the immune system that would otherwise attack them as foreign cells, leading to infertility [116].

(3) The BTB maintains a unique chemical environment around the germ cells that is necessary for their development. The fluid within seminiferous tubules has different concentrations of ions compared to the blood [113]. Sertoli cells are hypothesized to form this ionic gradient to power a range of membrane transporters that maintain the composition of the seminiferous tubule fluid [113]. Inhibition of potassium channels in Sertoli cells also disrupts the final stages of spermatogenesis, suggesting a role for this ionic environment in the maturation of spermatids [117].

(4) The BTB provides polarity to the Sertoli cells surrounding the germline. Occluding junctions form molecular fences that stop membrane protein diffusion and also interact with evolutionarily conserved sets of protein modules that mediate cell polarity

[118,119]. Disruption of the Par3 polarity module in Sertoli cells leads to a transient loss of BTB integrity and miss-oriented spermatids [120]. Whereas disrupting the Scribbled polarity module leads to a strengthening of BTB integrity [121]. Whether cell polarity regulates spermatogenesis in addition to its roles in BTB integrity is unknown but could involve the endosomal trafficking of proteins required for spermatogenesis [122].

While these biological functions of the BTB are intriguing they do not necessarily explain how the BTB controls germline differentiation. One hypothesis that could explain this is that the BTB blocks the movement of diffusible signalling molecules that mediate germline differentiation. The BTB could regulate the signalling environment of the germline in two ways: by locally concentrating signalling molecules inside of the barrier that support germline differentiation or by blocking signalling molecules from outside of the barrier that repress germline differentiation. Whether either of these mechanisms function during mammalian spermatogenesis remains unknown. Studies in a more genetically tractable model organism will help investigate this hypothesis and make up chapters 2 and 3 of this thesis.

1.8.3 Other soma-germline barriers

Somatic cell based permeability barriers that isolate the germline similar to the mammalian BTB have been identified throughout the animal kingdom [123]. These soma-germline barriers have been identified in other vertebrates including birds [124], reptiles [125], amphibians [126], and fish [127]. They have also been identified in many invertebrates including cnidarians [128], flatworms [129], nematodes [130], crustaceans [131], gastropods [90], and insects [132].

The ubiquity of soma-germline barriers across much of the animal kingdom suggests a fundamental role in spermatogenesis. Unfortunately, direct studies of the biological functions of soma-germline barriers have been done almost exclusively on the mammalian BTB [123]. One exception is the study of soma-germline barriers in insects, such as locusts and moths [132,133]. Research on spermatogenesis using insect models has benefited from the small size of their organs, enabling the observation of spermatogenesis in whole *ex vivo* cultured testes [134-137]. Additionally, because each spermatocyst contains only a single generation of germ cells experiments can more easily resolve how somatic cells support specific stages of germline differentiation.

Early evidence for a soma-germline barrier in insects was derived from moth spermatocysts cultured *in vitro*, which demonstrated that cyst cells regulate the osmolality of the encapsulated germ cells [138]. The soma-germline barrier was later identified in both moths and locusts when membrane impermeable dyes were shown to be unable to enter the lumen of their spermatocysts; blocked by septate junctions between the encapsulating cyst cells [132,133]. Septate junctions are the predominant occluding junctions used by invertebrates and, similar to tight junctions, are able to block the paracellular passage of ions, proteins and other molecules [139]. Septate junctions between insect cyst cells are therefore functionally homologous to tight junctions between mammalian Sertoli cells, as both isolate the differentiating germ cells.

Similar to the mammalian BTB, the locust soma-germline barrier forms during development at a time that is coincident with the initial onset of meiosis [140]. The locust soma-germline barrier also isolates germ cells during the meiotic spermatocyte stage, again similar to the mammalian BTB [141]. By contrast the moth soma-germline barrier

isolates germ cells during the earlier transit-amplifying spermatogonial stage. This leaves only GSCs and those spermatogonia directly adjacent to the stem cell niche free of any somatic permeability barrier [141].

It is not currently known if the insect soma-germline barrier regulates germline differentiation similar to the mammalian BTB. Investigation of the soma-germline barrier in a more genetically tractable insect model would help elucidate this and other general principals of these barriers. No soma-germline barrier has been previously identified in the genetic model *Drosophila melanogaster*; identification and characterization this barrier makes up Chapter 2 of this thesis.

1.9 Aims and scope of thesis

The aim of this thesis is to provide insight into the mechanisms by which somatic cells support the maintenance and differentiation of the germline during spermatogenesis. Specifically, I focus my analysis on how *Drosophila* cyst cells form structures such as the stem cell niche and the soma-germline barrier to regulate germ cell behavior.

First, I identify and characterize the soma-germline barrier in *Drosophila* and determine its role in controlling germline differentiation (Chapter Two). Secondly, I explore the role of the soma-germline barrier in maintaining stem cell niche homeostasis (Chapter Three). Overall, I demonstrate how the cellular architecture of a developing system can control the activity of signalling pathways that mediate stem cell maintenance and differentiation.

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

2.1 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 [10-12,89]. The soma provides support and instructive cues for the germline, including such roles as forming the stem cell niche that regulates germline stem cells (GSCs) [67,94,142,143], providing signals that instruct the germline during gamete differentiation [29,30,144,145], supplying nutrients to the developing germline [113], and maintaining the tissue architecture required for gamete production [38,146]. 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 [7,8]. 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 [123] of both mammals [104,147] and insects [132,148]. 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 environment during differentiation [123,149,150]. Additionally, it is thought that the soma-germline barrier protects the

germline from cytotoxic materials [151] and from the immune system in vertebrates [116].

The mammalian soma-germline barrier, called the blood-testes barrier (BTB), has been extensively studied [108]. 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 [104]. Disruption of the BTB in mammals leads to a failure in germline differentiation, and consequently to sterility [108].

The testis of *Drosophila melanogaster* is an important model for studying soma-germline interactions [10,13]. The fly testes are coiled tubular organs that contain a stem cell niche in their apical region, known as the hub [21]. 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 post-mitotic spermatocytes that grow, undergo meiosis, and form 64 spermatids [20]. 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 [10,13,21]. During encapsulation, germ cells activate EGFR and MAPK signalling in the soma, which act on the actin cytoskeletal regulators Rac and Rho to coordinate the somatic envelopment of the germline.

Disruption of EGF signalling inhibits encapsulation and leads to defects in germline differentiation resulting in spermatogonia-like tumours [29,30,74,84].

In this study, I sought to identify genes that act in the soma to regulate soma-germline interactions during spermatogenesis, specifically during encapsulation. My 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, I developed a novel assay to assess the formation and maintenance of a somatic permeability barrier around the germline. Using this assay, I demonstrate that encapsulation and the formation of a permeability barrier are separate processes, both of which require Chic. Moreover, I show that the formation of the permeability barrier correlates with the expression of septate junction components, which are functionally analogous to vertebrate tight junctions [139]. In addition, knockdown of septate junction proteins disrupts the formation of the permeability barrier. Finally, I provide evidence that the permeability barrier is required to restrict the range of niche-derived BMP signalling. Together, my 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 signalling molecules from the outside environment.

2.2 Results

2.2.1 Chic is required in the soma for fertility

In order to identify genes required in the somatic cyst cells for germline development I carried out an RNAi-based fertility screen with the soma-specific driver *tj*-Gal4 [152] to target gene knockdown in early somatic cyst cells of the fly testis. This

screen identified Chic, the fly homolog of the actin-polymerizing protein Profilin [153-155], as being required in the somatic cyst cells for spermatogenesis. Three RNAi lines were used that target independent regions of the *chic* mRNA to confirm that sterility was specifically due to *chic* knockdown (**Figure 2.1G**). Expression of any of these *chic* RNAi lines in cyst cells led to sterility, with or without the co-expression of Dicer2, which has been shown to enhance RNAi knockdown phenotypes [156] (**Figure 2.1E**). Morphological analysis of *chic* knockdown testes revealed a phenotypic range, which was categorized as strong, moderate or weak (**Figure 2.1A-F**). Strong phenotypes were defined as a lack of testis structures or the presence of testes lacking detectable somatic cells (**Figure 2.1B**). Moderate phenotypes were defined as rudimentary testes that lacked differentiated cyst cells or germ cells (**Figure 2.1C**). Weak phenotypes were defined as testes that contained large germline tumours (**Figure 2.1D**).

Subsequent analysis focused on the *chic* RNAi line 9553R-3, which predominantly yielded weaker phenotypes (**Figure 2.1F**). 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 cyst cells, where it is enriched in wild-type controls (**Figure 2.1H,I**). In summary, these results show that somatic RNAi knockdown of *chic* leads to depletion of the protein and results in severe defects during spermatogenesis.

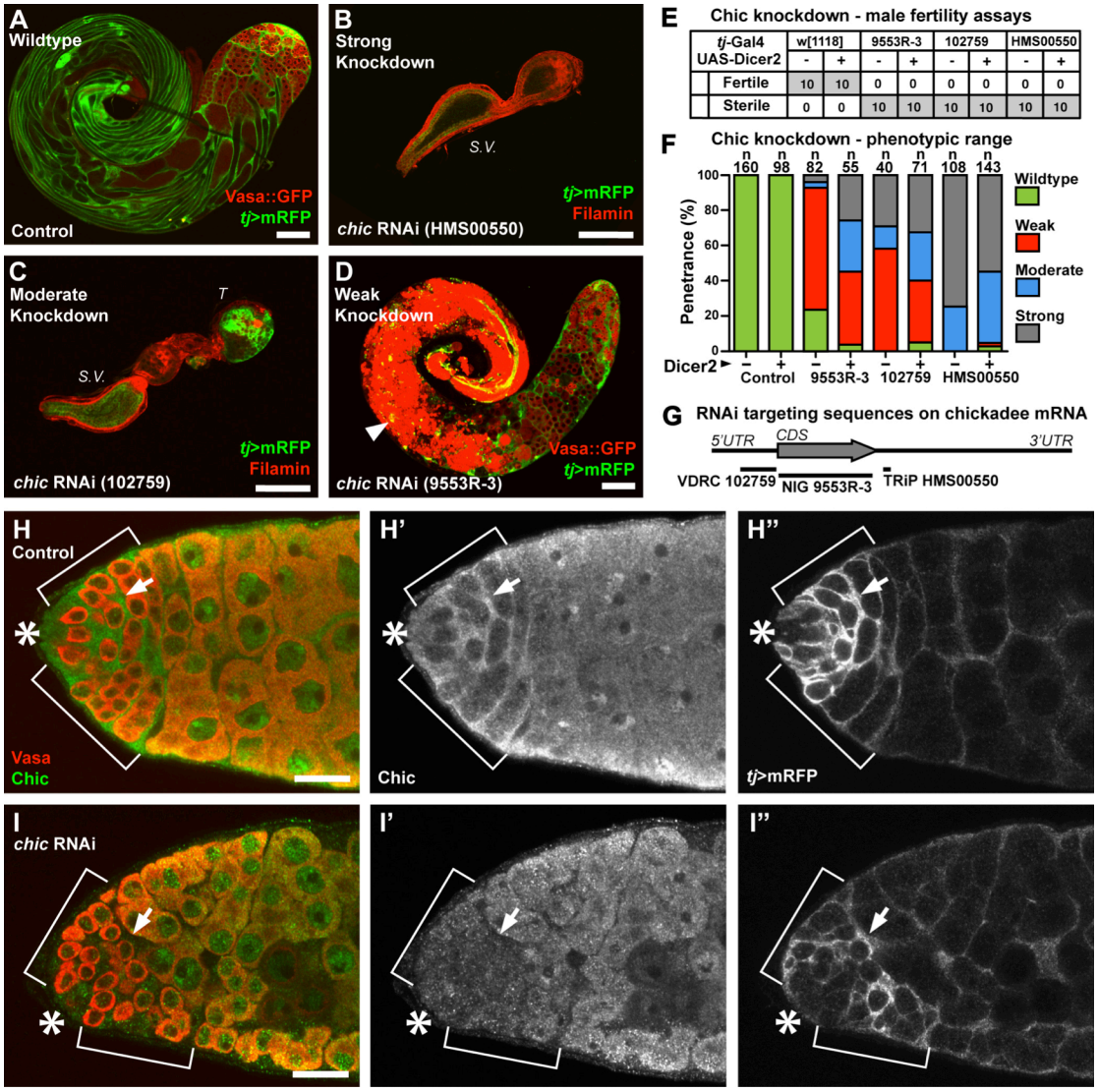

Figure 2.1 - Chic (*Drosophila* Profilin) is essential in the soma for spermatogenesis and fertility. (A) Wild-type testis. (B-D) Classes of testis phenotype generated by somatic *chic* knockdown with different *chic* RNAi lines using *tj*-Gal4. (B) Strong *chic* knockdown phenotype is characterized by a loss of all cyst 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 tumours 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 sterile (for chic knockdowns). (F) Quantification of phenotypic classes shown in B-D for each RNAi line; n indicates the number of testes. (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 cyst cells (arrow). Cyst cells labeled with *tj*>mRFP (A-D,H,I), germline with Vasa::GFP (A,D,H,I) and

2.2.2 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, cyst cell numbers were quantified using Tj as a marker of early cyst cells, and Zfh1 as a marker of CySCs (**Figure 2.2A,B**). Control testes maintained consistent numbers of Tj-positive cyst 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 (**Figure 2.2C**). To further characterize this expansion in the Tj-positive cell population, the size of the cyst cell population expressing Zfh1, a marker for CySCs and early daughter cells, was quantified [64]. This analysis revealed that this population was nearly the same size in control and *chic* knockdown testes at both 1 and 7 DPE (**Figure 2.2D**). These 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 the use of a different Gal-4 driver and UAS-RNAi line to obtain a weaker knockdown of *chic* in this study. This data therefore suggest that the expansion in the Tj-positive population was due to an increase in CySC proliferation or a delay in somatic differentiation.

To determine whether or not the expansion in the Tj-positive population was due to increased proliferation or delayed differentiation, the population of proliferating cyst cells was quantified by labelling S-phase cells with a 30-min pulse of EdU and staining for early cyst cells with Zfh1 (**Figure 2.2E,F**). This analysis showed that the absolute and relative number of cells that co-stained with both markers was not significantly different

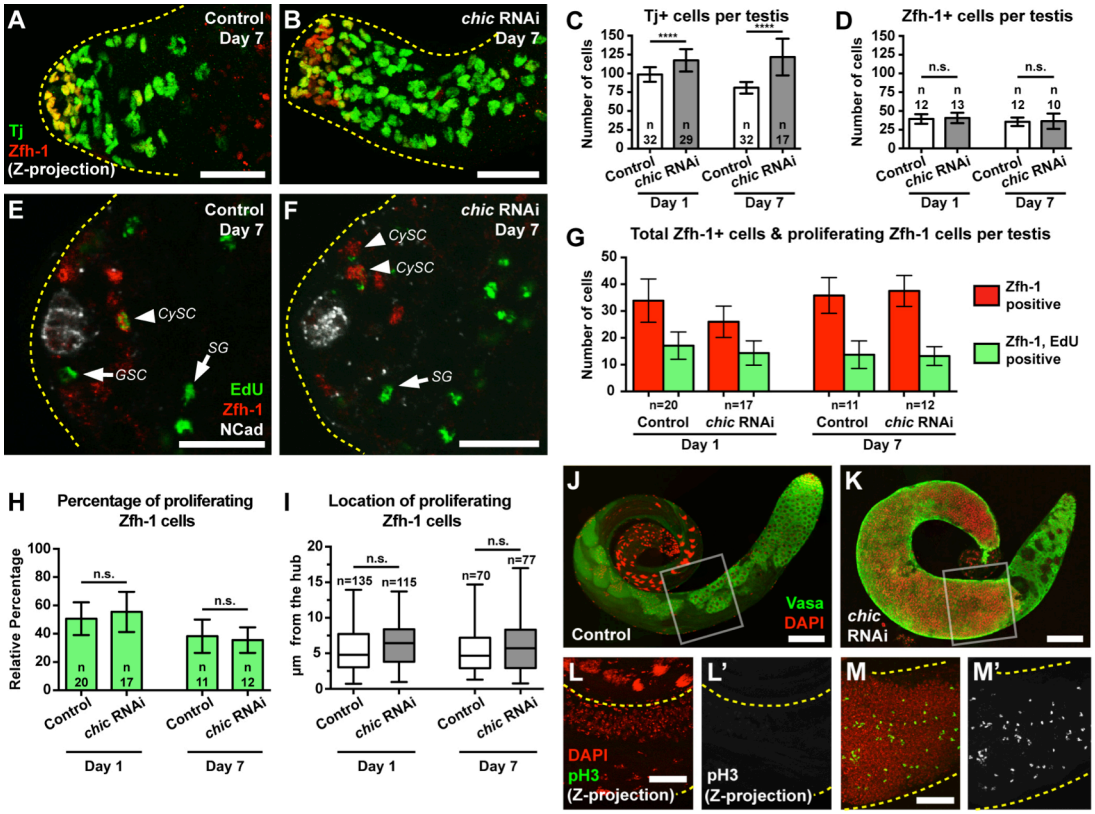

Figure 2.2 - Chic is required to regulate cyst cell differentiation and germline proliferation. (A,B) *z*-projections of the apical tip of control and *chic* knockdown testes 7 days post-eclosion (DPE) stained for the somatic markers Tj and Zfh1. (C) Quantification of Tj-positive cells shows that *chic* 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 tumours 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 tumours contain many pH3-positive cells (M,M'). n refers to the number of testes (C,D,G,H) or proliferative events (I) examined. Scale bars: 30µm in A,B; 15µm in E,F; 100µm in J,K; 50µm in L,M.

between control and *chic* knockdown testes at 1 or 7 DPE (**Figure 2.2G,H**). Moreover, because only CySCs proliferate in wild-type testes [27,42], cyst cells were examined for abnormal proliferation upon *chic* knockdown. To this end, the relative position at which cyst cell proliferation occurred in control and *chic* knockdown testes was determined by staining testes for Zfh1 and for EdU and measuring the distance of proliferative events from the hub. This analysis found that cell proliferation occurred a similar distance from the hub in control and *chic* knockdown testes (**Figure 2.2I**). These results suggest that there are no major differences in cyst cell proliferation rates or location upon somatic *chic* knockdown. Overall, this data supports a model whereby expansion in the Tj-positive population is due to delayed cyst cell differentiation.

In comparison, knockdown of *chic* in the cyst cells caused a germline proliferation defect, resulting in large spermatogonia-like tumours (**Figure 2.2J,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 (**Figure 2.2J**). In *chic* knockdown testes, large numbers of spermatogonia-like germ cells filled the basal regions of the testis (**Figure 2.2K**) and were confirmed to be mitotically active using the marker phospho-Histone H3 (pH3) (**Figure 2.2L,M**). In a recent study, Shields *et al*. (2014) obtained results consistent with those that I describe here; supporting my findings that somatic *chic* knockdown leads to defects in cyst cell differentiation and germline proliferation.

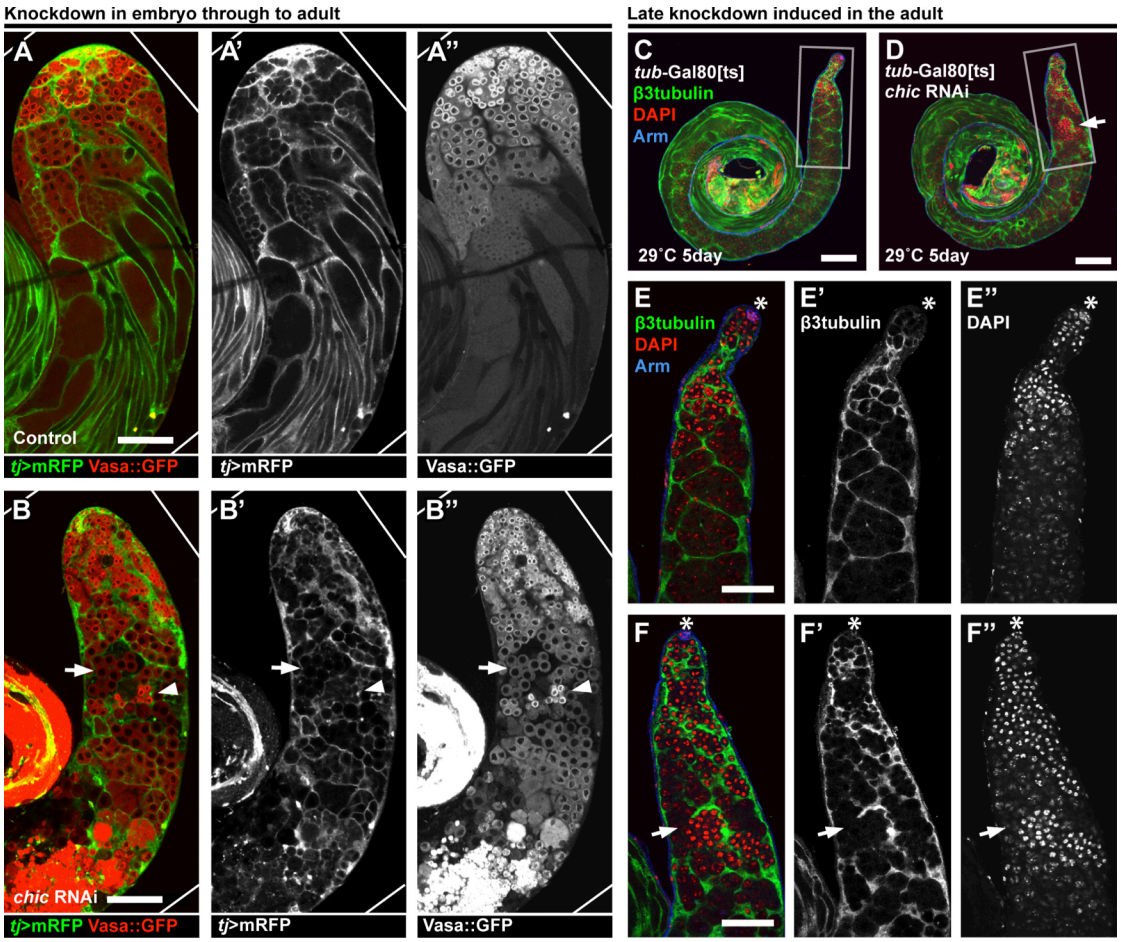

Figure 2.3 - Chic is required to maintain encapsulation.
(A-A'') Cyst cells (tj>mRFP) closely wrap the germline (Vasa::GFP) throughout all stages of spermatogenesis in control testes. (B-B'') In *chic* knockdown testes, cyst cells do not fully wrap the germline (arrow) and spermatogonia are found throughout the testis (arrowhead). (C,D) Control (C) and *chic* knockdown (D) testes after 5 days of 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 marked by Armadillo (Arm; asterisk). Scale bars: 100μm in A-D; 50μm in E,F.

2.2.3 Chic knockdown in the soma leads to defects in encapsulation

As germline tumours have previously been associated with failures in encapsulation [29,30,74,84,85] I investigated whether encapsulation was defective upon the knockdown of *chic* in cyst cells. Cyst cells were tightly associated with germ cells throughout control testes (**Figure 2.3A**). By comparison, cyst cell knockdown of *chic* disrupted the characteristic production of encapsulated cysts, leaving voids around some

spermatocyte stage cysts due to a lack of associated cyst cells (**Figure 2.3B**, arrowhead). These observations suggest that Chic is required in the cyst cells 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 (**Figure 2.3C-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, as some germ cells were no longer surrounded by cyst cells (**Figure 2.3**F, 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.

2.2.4 A permeability assay for the Drosophila testis

As Chic is required in the cyst cells for germline encapsulation, I sought a method to characterize this defect in more detail. Light microscopy cannot always resolve the thin cyst cell membranes, and electron microscopy requires harsh treatments that can disrupt membranes. I therefore developed a non-invasive assay to assess whether the germline is isolated from the outside environment by the surrounding cyst cells. This assay uses a fluorescently conjugated 10 kDa dextran dye added to the medium surrounding *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 [21] and could be found between all cells within the stem cell niche (**Figure 2.4A**).

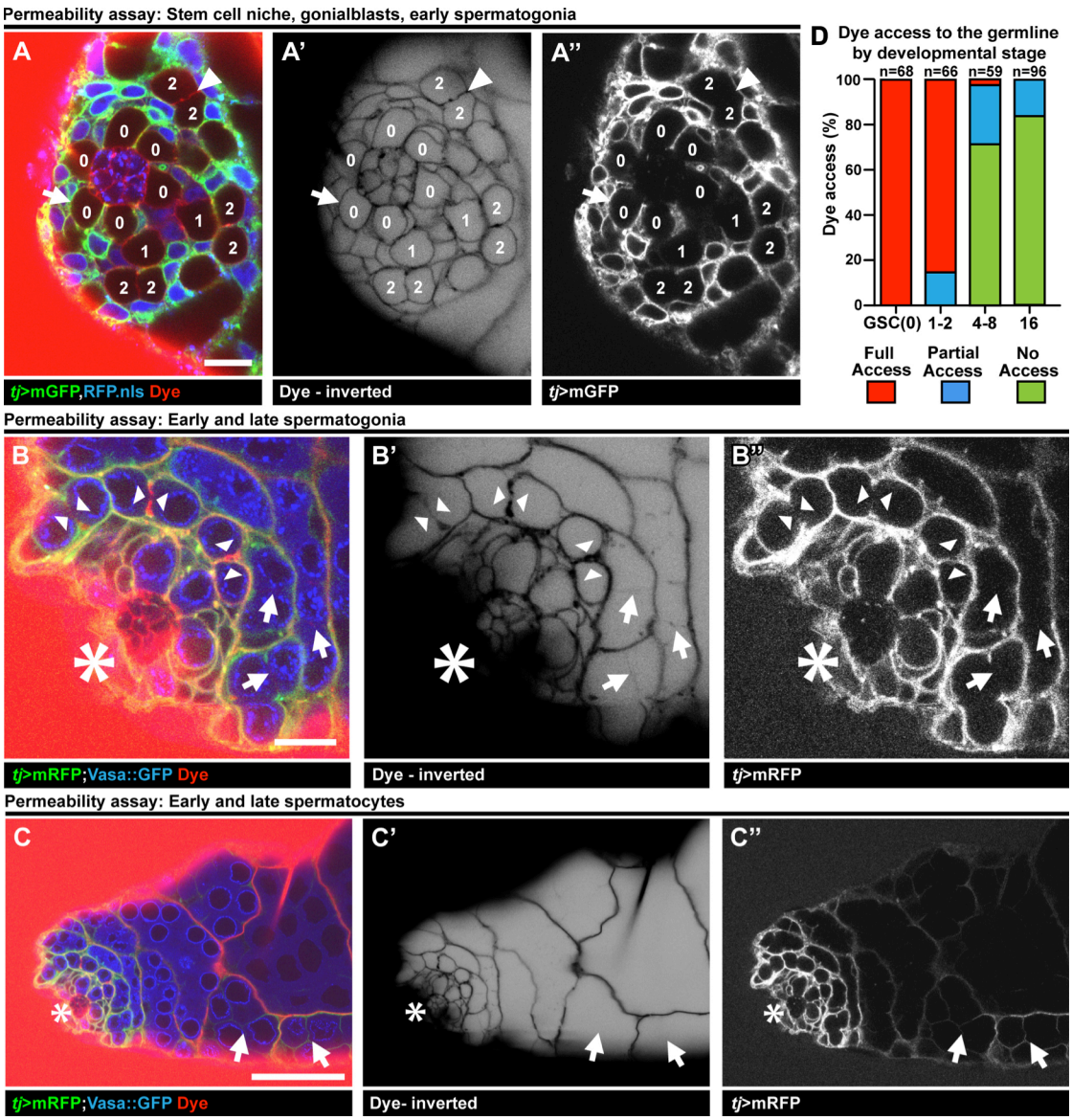

Figure 2.4 - A permeability assay for the Drosophila testis.
(A-A'') Results of permeability assays in the stem cell niche of wild-type testes reveal no permeability barriers, as assessed by the ability to block dye access to germ cell surfaces. Cyst cells labeled with *tj*>mGFP,RFP.nls; numbers in germ cells indicate developmental stage: 0, GSCs (arrow); 1, gonialblasts; 2, 2-cell spermatogonia (arrowhead). (B-B'') Permeability assays on 2-cell and 4-cell spermatogonial stage with the cyst cells 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-cell and 8-cell spermatogonia (4-8); 16-cell spermatocytes (16). n refers to the number of cysts examined. Scale bars: 10μm in A,B; 50μm in C.

Surprisingly, dye was able to access gonialblasts and 2-cell stage spermatogonia, even though encapsulation is believed to occur at the gonialblast stage [21]. In early stages, the dye appeared to penetrate between germ cells and colocalized with a cyst cell membrane marker (*tj*>mGFP). Since 10 kDa dextran does not penetrate the plasma membrane of intact healthy cells [157], this colocalization is likely to reflect the thin nature of cyst 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 cyst cells encapsulating the germline (**Figure 2.4D**). Nonetheless some germ cells were still accessible to small amounts of dye after the 4-cell spermatogonial stage (**Figure 2.4B**, arrows). However, by the late spermatocyte stage, the germline was consistently isolated from dye by the somatic permeability barrier (**Figure 2.4C**, arrows).

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

2.2.5 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, I examined whether the knockdown of *chic* in cyst cells perturbed the permeability barrier around the germline (**Figure 2.5A,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 (**Figure 2.5A**), this did not occur in *chic*

knockdown testes, as both spermatogonia and spermatocytes were still accessible to dye (**Figure 2.5B**, arrowhead). In some spermatocytes, dye was even found within the cytoplasm (**Figure 2.5B**, arrow), indicative of failures in membrane integrity [158,159]. It is known that germ cells in both the ovary [160] and testis [161] can undergo necrosis, which involves loss of plasma membrane integrity [162]. Taken together, these data show that depletion of Chic leads to failure of the somatic permeability barrier.

To determine whether or not the defect in the establishment of a permeability barrier is cell-autonomous, clonal analysis in cyst cells was undertaken (**Figure 2.6**). Using the MARCM technique [163] with a cyst cell-specific driver (c587-Gal4), positively labelled 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

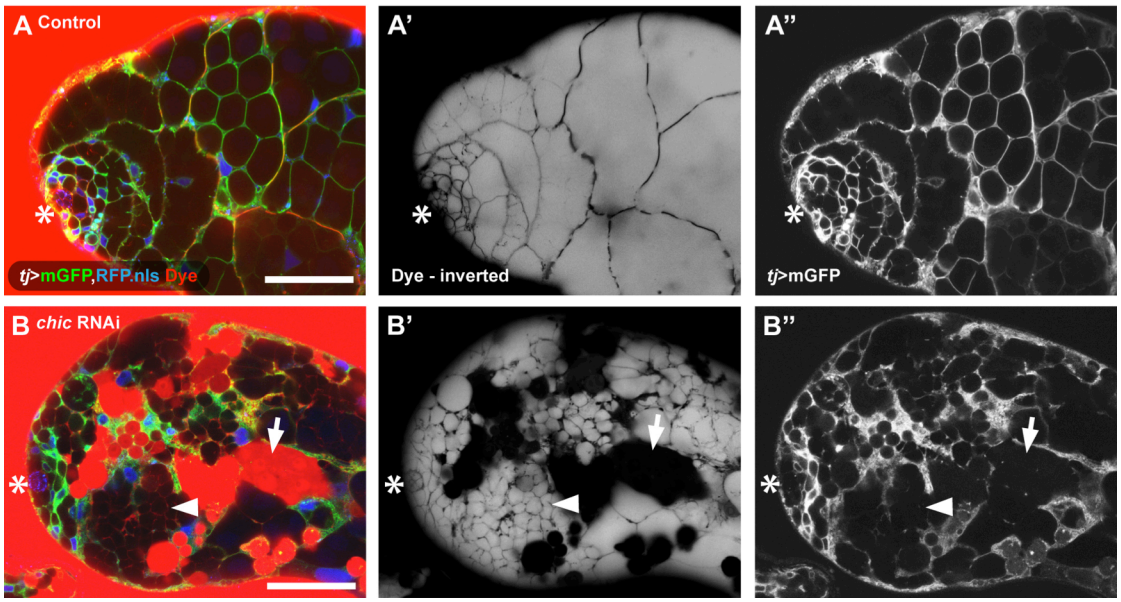

Figure 2.5 - Chic is required in cyst cells to form a permeability barrier around the germline. (A-A'') In control testes, early spermatogonial stages are accessible to dye, whereas late spermatogonial and early spermatocyte cysts show progressive reduction in accessibility. By late spermatocyte stages, 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. Cyst cells indicated by *tj*>mGFP,RFP.nls. Scale bars: 50μm.

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 (**Figure 2.6B-D**).

Next, the MARCM clones were analyzed using the permeability assay (**Figure 2.6G,H**). This experiment illustrated that the permeability barrier was established normally in control clones (**Figure 2.6G**, arrowheads). However, cyst cells that clonally express *chic* RNAi showed a defective permeability barrier, which resulted in dye permeating the spaces between germline and cyst cells (**Figure 2.6H**, 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 cyst cells. The number of *chic*^{221} and *chic* RNAi clones per testis that expressed Tj at 3 and 7 DPCI was quantified (**Figure 2.6A**). 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 [21] (**Figure 2.6E,F**), similar to observations made by Shields *et al*. (2014). These results show that Chic is required cell-autonomously in cyst cells for the establishment of the permeability barrier and for CySC maintenance.

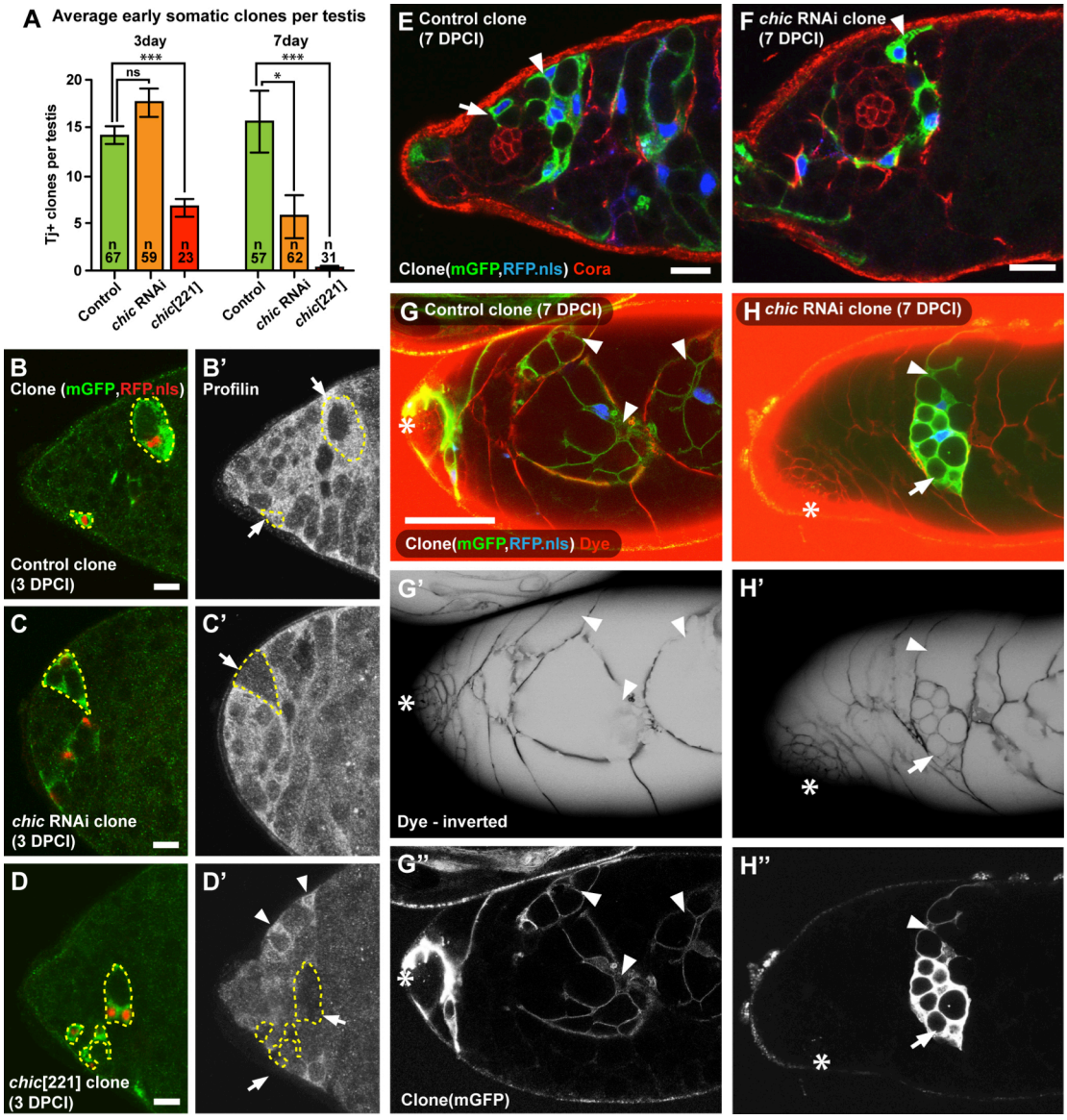

Figure 2.6 - Clonal analysis of Chic in the soma shows that it is required for CySC maintenance and permeability barrier function.
Somatic MARCM clones (mGFP,RFP.nls) were generated that were either wild-type control, expressed chic RNAi, or were homozygous for the null allele *chic*^{221}. (A) Average number of early somatic cell (Tj-positive) clones per testis at 3 and 7 days post-clone induction (DPCI). n indicates the number of testes examined. (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: 10μm in B-F; 50μm in G,H.

2.2.6 The permeability barrier is dependent on septate junction proteins

To better characterize how the soma forms the permeability barrier, I investigated the expression and localization of septate junction proteins, the main occluding junctions in flies [139]. The core septate junction components Neurexin-IV (Nrx-IV) and Coracle (Cora) [164] both localized around the germline throughout spermatogenesis (**Figure 2.7A**). 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 (**Figure 2.7F**). To explore the localization of septate junction markers in greater detail, one of the two cyst cells in a spermatocyst was clonally labelled using MARCM and stained for Cora. This analysis showed that septate junction markers concentrated at the sites of contact between the two cyst cells that encapsulate the germline (**Figure 2.7B,C**). Moreover, by isolating, culturing and imaging spermatocysts 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 cyst cells (**Figure 2.7D,E**). These circumferential belts of junctional proteins were also visible in intact testes (**Figure 2.7F**). Together this data shows that septate junction proteins form a belt at the interface between the two cyst cells that encapsulate the germline, consistent with the idea that septate junctions help the cyst cells to isolate the germline.

Next, the possible role of septate junction components in spermatogenesis and specifically in permeability barrier function was tested directly using cyst cell-specific RNAi knockdown of *Nrx-IV* or *cora* and the permeability assay. First, it was found that

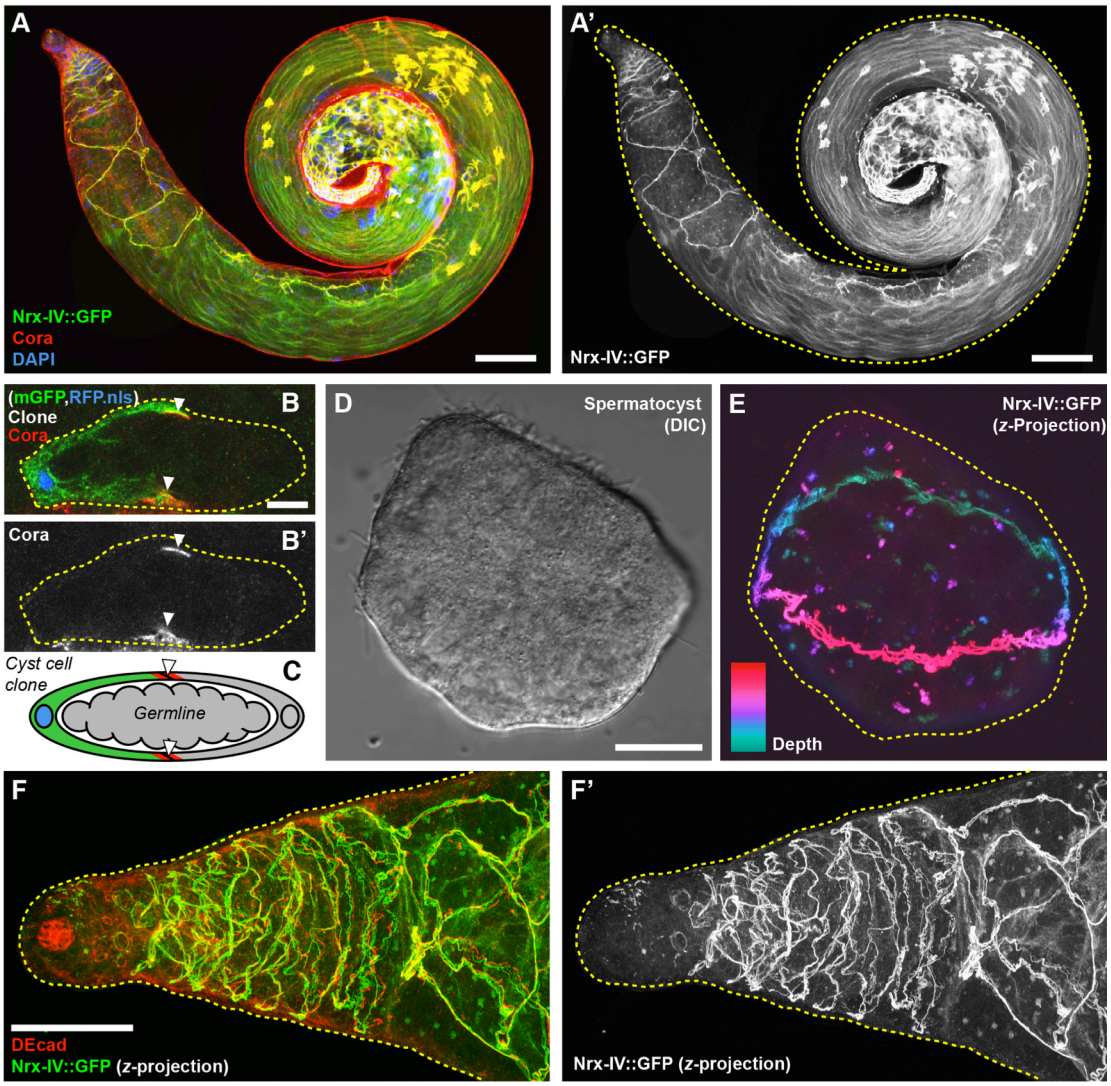

Figure 2.7 - Septate junctions form between the cyst cells encapsulating the germline.
(A,A') Wild-type testis stained for the septate junction markers Cora and Nrx-IV (Nrx-IV::GFP), which colocalize surrounding the germline. (B,B') Single germline cyst with one of two encapsulating cyst cells positively 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 spermatocyst (spermatocyte stage) 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 50µm; 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: 100µm in A,A'; 10µm in B; 20µm in D; 50µm in F.

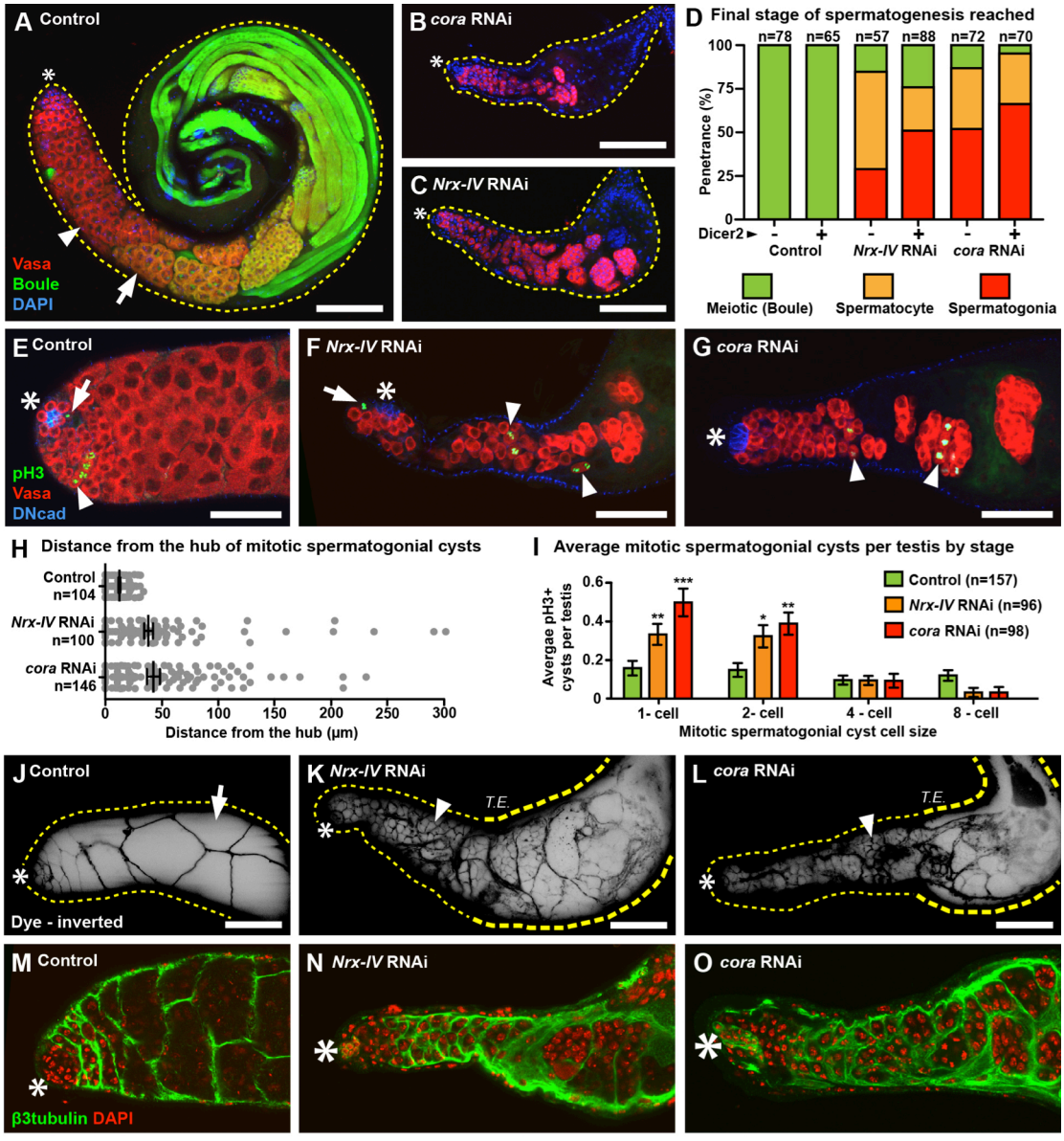

Figure 2.8 - Septate junction components are required for a functional permeability barrier and for germline differentiation.

(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) Cyst cell 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. 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 the number of cysts (H) or testes (D,I). Scale bars: 100μm in A-C; 50μm in E-G,J-O.

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knockdown of the septate junctions components *Nrx-IV* or *cora* gave rise to small rudimentary testes (**Figure 2.8A-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 spermatocyte stages and Boule to distinguish meiotic stages [165]. 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 (**Figure 2.8D**). The resulting germline phenotype was analyzed further by examining the pattern of spermatogonial mitotic events. In *Nrx-IV* or *cora* knockdown testes pH3-positive spermatogonia were much further away from the hub than in control testes: up to 300 μ m versus 35 μ m from the hub, respectively (**Figure 2.8E-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 (**Figure 2.8E-I**). Both of these results are consistent with a delay in germline differentiation.

Importantly, the permeability assay showed that Nrx-IV and Cora are both essential for a functional permeability barrier (**Figure 2.8J-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 *Nrx-IV* or *cora* disrupted the permeability barrier but did not affect encapsulation, as cyst cells still surrounded the germline (**Figure 2.8M-O**).

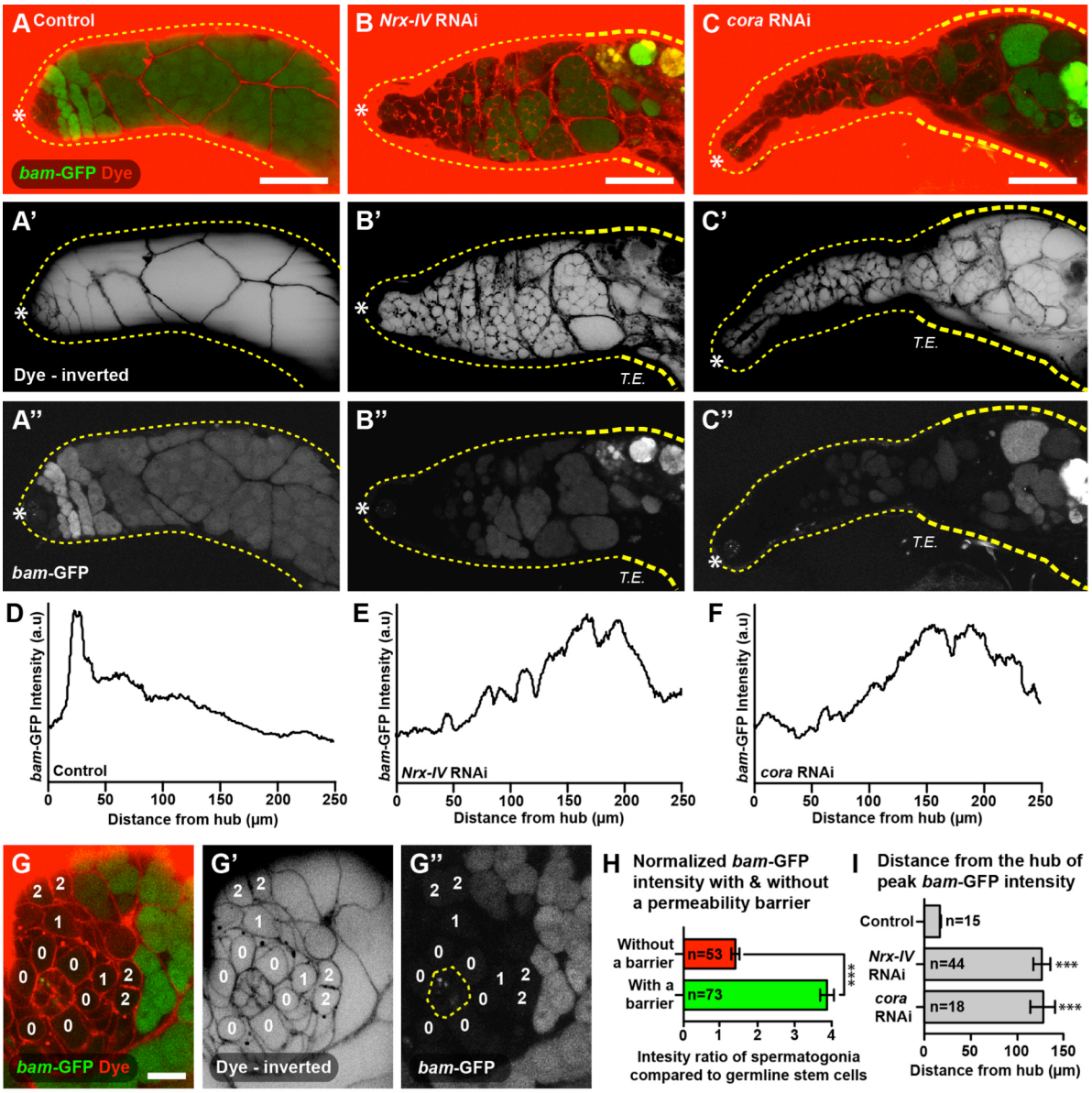

Figure 2.9 - The somatic permeability barrier limits the range of niche-derived signals.
(A-C'') Permeability assays performed on *bam*-GFP-expressing control and *Nrx-IV* or *cora* knockdown testes. In control testes (A-A''), BMPs in the stem cell niche repress expression of *bam*-GFP, which then peaks in 4- to 8-cell stage cysts near the hub before declining. Knockdown of *Nrx-I*V (B-B'') or *cora* (C-C'') in the cyst cells 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±1.2μm from the hub, whereas in *Nrx-IV* or *cora* knockdown testes peak intensity occurs much further from the hub (126±9.1μm and 127±13.3μm, respectively). n refers to the number of cysts (H) or testes (I). Scale bars: 50μm in A-C; 10μm in G.

2.2.7 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, I hypothesized that this phenotype resulted from abnormal germline signalling. To test this, expression of the germline differentiation factor bag-of-marbles (*bam*) was examined using the reporter *bam*-GFP [52,53]. In wild-type testes, *bam* expression is directly repressed by BMP signalling ligands secreted from the hub and CySCs [54,56]. In control testes, *bam* expression is repressed in GSCs and early spermatogonia near the niche, limiting expression to late spermatogonial stages (**Figure 2.9A,A'',G**). By contrast, in *Nrx-IV* or *cora* knockdown testes, *bam*-GFP appeared to be repressed further from the niche. (**Figure 2.9B,B'',C,C''**). This was illustrated by obtaining intensity profiles for *bam*-GFP expression across the length of the testis, starting at the hub (Figure 2.9D-F). These showed that in control testes there was a sharp peak of *bam*-GFP expression in spermatogonia near the hub, which then declined (**Figure 2.9D**). In comparison, *Nrx-IV* or *cora* knockdown resulted in a more gradual increase in *bam*-GFP intensity, which peaked much further from the hub (**Figure 2.9E,F**). This defect was quantified by measuring the distance of peak *bam*-GFP intensity from the hub, which was approximately 18μm in controls and over 120μm upon knockdown of *Nrx-IV* or *cora* (**Figure 2.9**I). 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 colabelled with the dye (**Figure 2.9A-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 (**Figure 2.9G,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 somatic permeability barrier is to shape the signalling environment experienced by the germline, restricting access to BMP signals emanating from the stem cell niche.

2.3 Discussion

Here I sought to characterize the genetic control of soma-germline interactions in the adult fly testis. This work defines two distinct phases of soma-germline interaction during early spermatogenesis: an encapsulation/enclosure stage, when the cyst cells wrap 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, I 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 place starting at the gonialblast stage [21]. Second, I provide evidence suggesting that 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.

2.3.1 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 [85]. In the germline, Chic is required to maintain DE-Cadherin at the GSC-hub interface and is therefore required for GSC maintenance [85]. My experiments using *chic* null clones, and previous work [85], 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, I was able to dissect a broader range of phenotypes as cyst cells persisted. Earlier analysis had shown that RNAi-mediated somatic knockdown of *chic* results in encapsulation defects and the formation of germline tumours [85]. My work supports this earlier analysis, and additionally demonstrates that reduced Chic levels may lead to delayed cyst cell differentiation. Moreover, I utilize *chic* knockdown to illustrate the requirement for proper encapsulation prior to formation of the permeability barrier. This 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.

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

This 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,

as both form between surrounding somatic cells and occlude the differentiating germline from the rest of the organism. This work shows that the development of this permeability barrier in the fly is gradual, beginning at the 4-cell spermatogonial stage, and is completed in all spermatocysts by the late spermatocyte stage. Moreover, this 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 [139]. Tight junctions are an integral component of the BTB and are essential for its ability to restrict the movement of small molecules [149]. Electron microscopy studies have shown that septate junctions exists between the two cyst cells encapsulating late spermatid stages in *Drosophila* [166]. Studies in other insects have identified septate junctions between cyst cells and shown that these junctions form an occluding barrier between cyst cells [132,133,167,168]. Finally, Discs large 1, a septate junction-associated protein [164], is expressed in a similar pattern to that described for Nrx-IV and Cora, and its loss resembles the loss Nrx-IV or Cora [169]. These studies support my 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.

2.3.3 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 [116]. 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 [112,170]. Especially interesting is the fact that the establishment of the BTB corresponds to a major transition

during germline differentiation as spermatocytes enter meiosis [123,150]. 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 [171-173].

In flies, TGF-β signalling 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 [53,54,56,174]. This work provides 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 signalling environment. For example, it has been shown that the maintenance and proliferation of GSCs in the fly testis are regulated by diet [175] and long-range insulin signalling [176,177]. Other systemic signals, such as the hormone ecdysone, also regulate fly spermatogenesis [178]. The germline itself may additionally release factors that regulate distant somatic tissues of the body, modulating these same systemic signalling pathways [179,180]. It is intriguing to speculate that, by establishing a permeability barrier around the differentiating germline, the cyst 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 signalling, responding only to signals mediated by the surrounding cyst cells.

Overall, this data fits with a model whereby actin modulators and junctional proteins act in the somatic cyst cells to establish an isolated microenvironment around the developing germline. This might be a conserved feature of spermatogenesis across multiple animal phyla. Moreover, this work establishes the *Drosophila* testis as a model for analyzing the formation and maintenance of soma-germline 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 identify other genes that are involved in its establishment, maintenance and regulation.

2.4 Materials and methods

2.4.1 Genetics

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, $chic^{22}1$, *hs*-Flp and *bam*-GFP (all obtained from the Bloomington *Drosophila* Stock Center); and Neurexin-IV::GFP from FlyTrap (CA06597) [181].

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.

2.4.2 Staining and immunohistochemistry

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).

2.4.3 Data collection

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 or pupae were present. Phenotypic scoring for *chic*, *Nrx-IV*, and *cora* knockdowns was performed with males at 1-3 DPE.

Somatic cell counts used males at 1 or 7 DPE. S-phase cells were labelled 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.

Intensity measurements of *bam*-GFP 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 assays were performed on testes 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 0.2µg/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 were performed using Prism (Graphpad). All student t-tests were two-tailed and applied Whelch's correction, asterisks represent P-values : <0.001=*** ; <0.01=** ; <0.05=* ; >0.05=ns (non significant).

CHAPTER THREE: Occluding junctions maintain stem cell niche homeostasis in the fly testes.

3.1 Introduction

Stem cells can be controlled by their local microenvironment, known as the stem cell niche. The *Drosophila* testes contain a morphologically distinct niche called the hub, composed of a cluster of between 8-20 hub cells that contact and regulate germline stem cells (GSC) and somatic cyst stem cells (CySC). Both hub cells and CySCs originate from somatic gonadal precursor cells during embryogenesis, but while hub cells, once specified, cease all mitotic activity, CySCs remain mitotic into adulthood [32,42]. Cyst cells, derived from the CySCs, first encapsulate the germline and then, using occluding junctions, form an isolating permeability barrier [182]. This barrier promotes germline differentiation by excluding niche-derived stem cell maintenance factors.

The hub regulates stem cell behavior in multiple ways. First, the hub physically anchors the stem cells by forming an adhesive contact with both GSCs and CySCs. The hub thus provides a physical cue that orients centrosomes such that stem cells predominantly divide asymmetrically, perpendicular to the hub [26,27]. Following asymmetric stem cell division, one daughter cell remains attached to the hub and retains stem cell identity while the other is displaced from the hub and differentiates. Second, hub cells produce signals including the STAT ligand Unpaired-1 (Upd), Hedgehog (Hh), and the BMP ligands Decapentaplegic (Dpp) and Glass bottomed boat (Gbb) that signal to the adjacent stem cells to maintain their identity [22-24]. As germ cells leave the stem cell niche, two somatic cyst cells surround and encapsulate them to form a spermatocyst. As spermatocysts move from the apical to the basal end of the testis both somatic cyst

cells and germ cells undergo a coordinated program of differentiation [10,13]. Previously I showed that differentiation of encapsulated germ cells requires their isolation behind a somatic occluding junction-based permeability barrier [182]. Specifically I identified a role for septate junctions, which are functionally equivalent to vertebrate tight junctions [139], in establishing and maintaining a permeability barrier for each individual spermatocyst [182].

Here, I show that the somatic permeability barrier is also required to regulate stem cell niche homeostasis. Loss of septate junction components in the somatic cells results in hub overgrowth. Enlarged hubs are active and recruit more GSCs and CySCs to the niche. Surprisingly, hub growth results from depletion of septate junction components in differentiating cyst cells, not from depletion in the hub cells themselves. Moreover, hub growth is caused by incorporation of cells that previously expressed markers for cyst cells and not by hub cell proliferation. Importantly, depletion of septate junctions disrupts Notch and MAPK signalling, and hub overgrowth defects are partially rescued by modulation of either signalling pathway. Overall these data show that septate junctions shape the signalling environment between the soma and the germline in order to maintain niche homeostasis.

3.2 Results

3.2.1 Loss of occluding junctions in cyst cells leads to growth of the hub

During analysis of septate junction protein localization it was observed that some, notably Coracle, were expressed in both the hub and the differentiating cyst cells (**Figure 3.1A**). Moreover, knockdown of septate junction components in the somatic cells of the

gonad resulted in enlarged hubs (**Figure 3.1B-C**). Based on these results the role of septate junction components in regulating the number of hub cells was explored in detail.

To this end RNAi was used to knock down the expression of the core septate junction components Neurexin-IV (Nrx-IV) and Coracle (Cora) [164] in both the hub and cyst cell populations, and the number of hub cells counted in testes from newly eclosed and 7-day-old adults. RNAi was expressed using three tissue specific drivers: *upd*-Gal4, expressed in hub cells; *tj*-Gal4, expressed weakly in hub cells, and strongly in both CySC and early differentiating cyst cells; and *eyaA3*-Gal4, expressed strongly in all differentiating cyst cells, weakly in CySC, and at negligible levels in the hub (**Figure 3.1D-F**) [32,64,152,183]. To visualize hub cells they were stained for multiple established hub markers including *upd*-Gal4, *upd*-lacZ, Fasciclin-III (FasIII), and DN-Cadherin (DNcad) [32,42]. Surprisingly, knockdown of *Nrx-IV* or *cora* driven by *upd*-Gal4 gave rise to normal hubs (**Figure 3.2A-F**). In comparison knockdown of *Nrx-IV* or *cora* using *tj*-Gal4 or *eyaA3*-Gal4 led to large increases in the number of the hub cells (**Figure 3.2G-L,M-R**). Hub growth was not uniform and varied between testes but median hub cells numbers in *Nrx-IV* and *cora* knockdown testes grew by 30% and 55% respectively, between 1 and 7 days-post-eclosion (DPE) (**Figure 3.2L)**. However, in extreme cases hubs contained up to five times the number of cells found in age-matched control testes (**Figure 3.2K**). This result was confirmed using a series of controls that discounted the possibility that hub overgrowth was due to temperature or leaky expression of the RNAi lines (**Figure 3.7A-**B). These results suggested that hub growth occurred as a result of knockdown of septate junction proteins in cyst cells rather than the hub. This was further supported using another somatic driver that is not thought to be

expressed in the hub, c587-Gal4 [27,56] (**Figure 3.3A-B**). However analysis of c587-Gal4 was complicated by the fact this driver severely impacted fly viability when combined with *Nrx-IV* or *cora* RNAi lines.

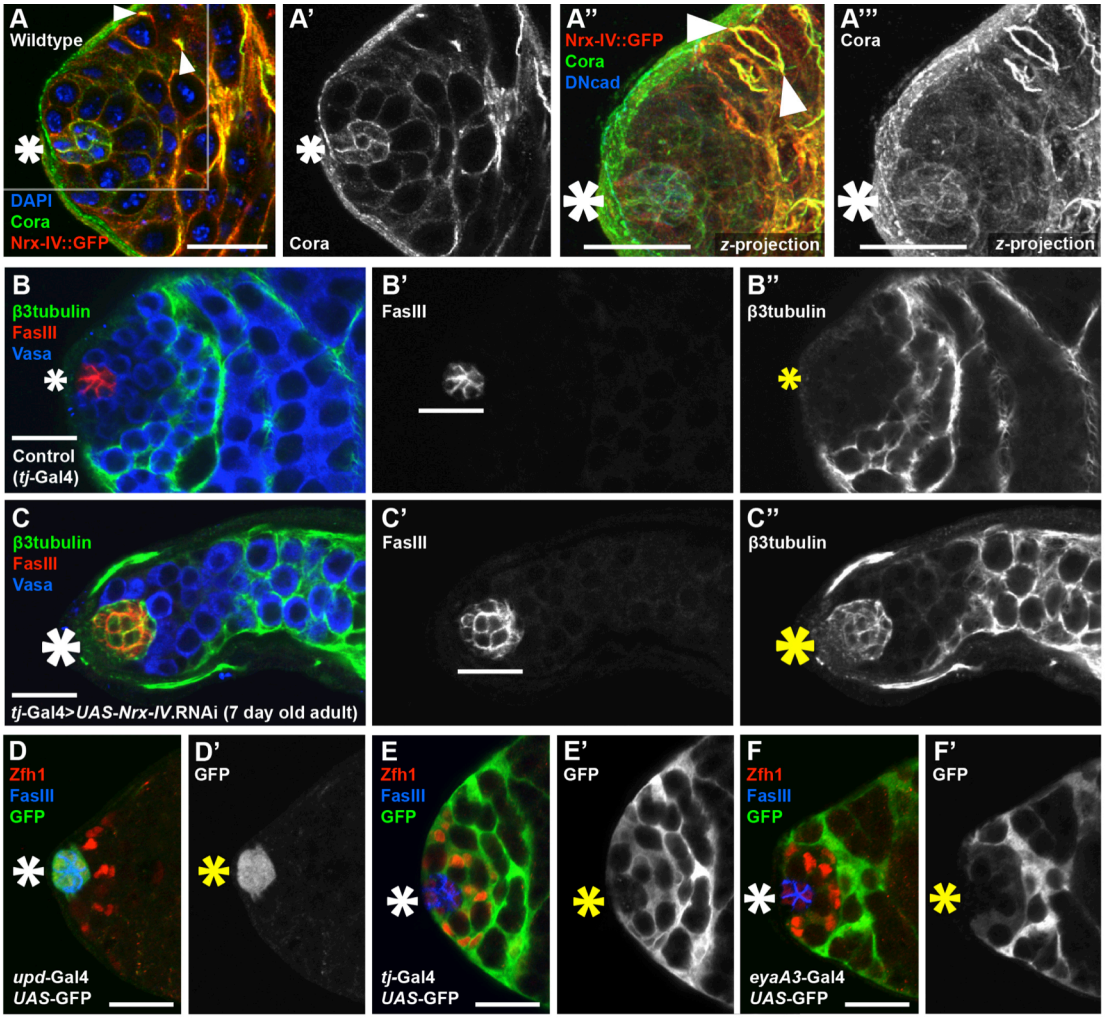

Figure 3.1 – Expression pattern of septate junctions, hub cell markers, cyst cell markers, and Gal4 drivers.
(A) Septate junctions proteins Neurexin-IV (Nrx-IV::GFP) and Coracle (Cora) are expressed in hub cells (asterisk) and differentiating somatic cyst cells (arrowheads). (A''-A''') *z*-projection of additional focal planes from boxed area in panel A reveal a circumferential ring of septate junctions connecting the two somatic cells that surround the germline (arrowheads). (B-C) The hub, labeled by FasIII, is enlarged in 7-day-old adult testes when septate junctions are disrupted in the somatic cells of the testis (*tj*-Gal4 driving expression of *UAS*-RNAi targeting *Nrx-IV*). The enlarged hubs express β3-tubulin, a marker of differentiating somatic cyst cells (B,B'',C,C''). (D-F) Tissue specific drivers expressed in distinct somatic cell populations. FasIII labels hub cells, Zfh1 labels CySCs, and GFP indicates Gal4 expression. (D) *upd*-Gal4 is expressed in hub cells; (E) *tj*-Gal4 is expressed at high levels in CySC and early differentiating cyst cells; (F) and *eyaA3*-Gal4, is expressed at high levels in differentiating cyst cells, and low levels in CySC. Scale bars represent 20μm; asterisks indicate the hub.

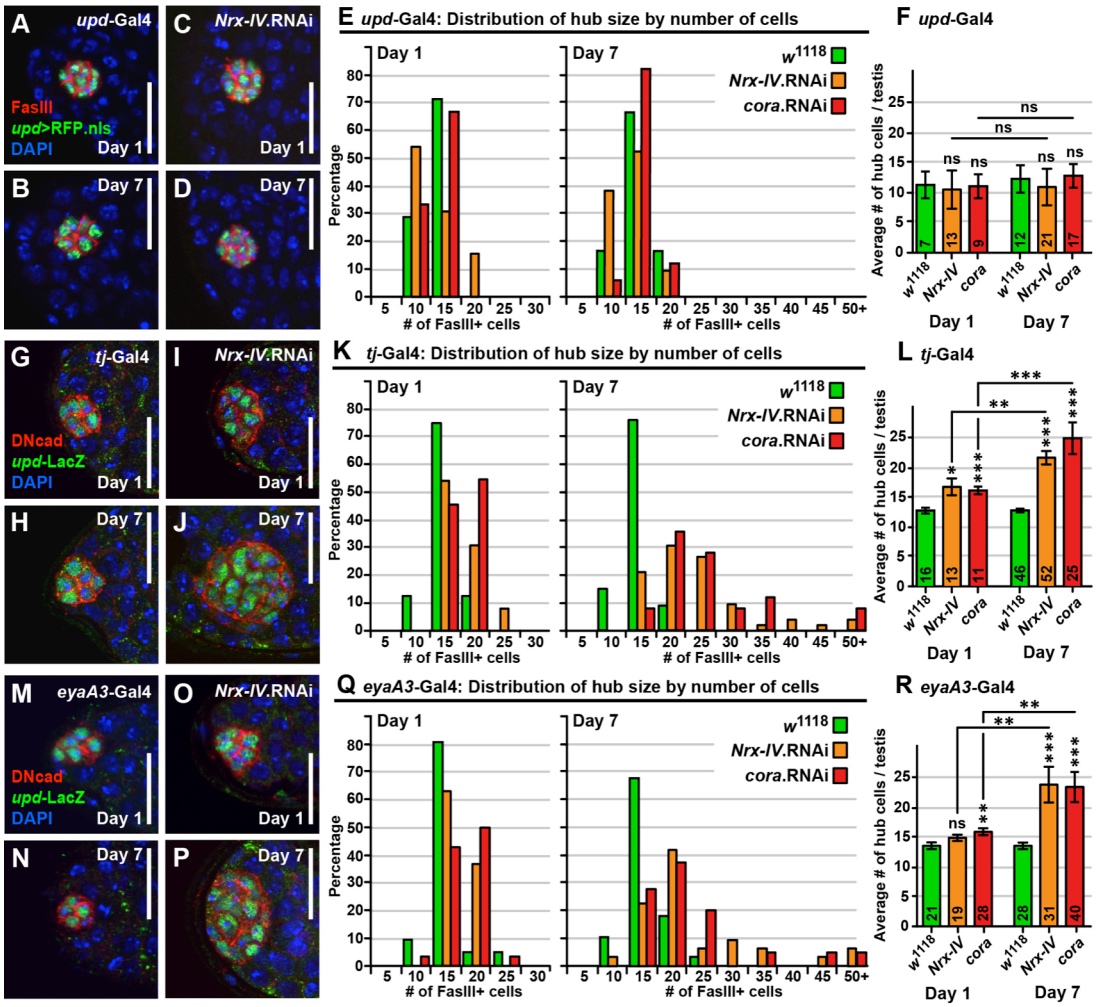

Figure 3.2 – Disruption of cyst cell septate junctions leads to increasing numbers of hub cells in adult testes

Number of hub cells measured 1 and 7 days post eclosion (DPE). Septate junctions disrupted using UAS-RNAi mediated knockdown of *Nrx-IV* or *cora*, expressed using *upd*-Gal4 (A-F); *tj*-Gal4 (G-L); or *eyaA3*-Gal4 (M-R). (A-D) Hub cells labelled by *upd*>RFP.nls and FasIII. (G-J,M-P) Hub cells labelled by *upd*-LacZ and DNcad. Strong examples of hub growth are shown in panel J and P. (E,K,Q) Histograms of FasIII-positive cells per testis. X-axis numbers represent the largest hub per group. (F,L,R) Average number of FasIII-positive cells. Mean±SEM, N displayed on bars, asterisks represent student t-tests compared to age- matched controls or between samples. Controls are the indicated driver crossed to w^{1118}. Scale bars represent 20µm.

Intriguingly, hub growth largely occurred after adult flies eclosed and not in earlier developmental stages. For example, when the driver *eyaA3*-Gal4 was used to knock down *Nrx-IV* or *cora*, hubs from 1-day-old adults were not larger than controls but hubs from 7-day-old adults were significantly larger (**Figure 3.2M-R**). Moreover,

overgrowth phenotypes were recapitulated when temperature-sensitive Gal80 was used to delay induction of *eyaA3*-Gal4 mediated *Nrx-IV* knockdown until after eclosion (**Figure 3.3C-D**). Hub growth manifested both in a higher mean number of hub cells per testis (**Figure 3.3C**) and by a shift in the distribution of hub cells per testis upwards, towards larger hubs sizes (**Figure 3.3D**). This distribution suggested a gradual, stochastic process of hub growth, resulting in a population of testes containing a range of hub sizes (**Figure 3.2K,Q**; **Figure 3.3D**). These results reveal progressive hub growth in adults upon knockdown of septate junction components in cyst cells and suggest that this growth is not driven by events occurring in the hub itself but rather by events occurring in cyst cells.

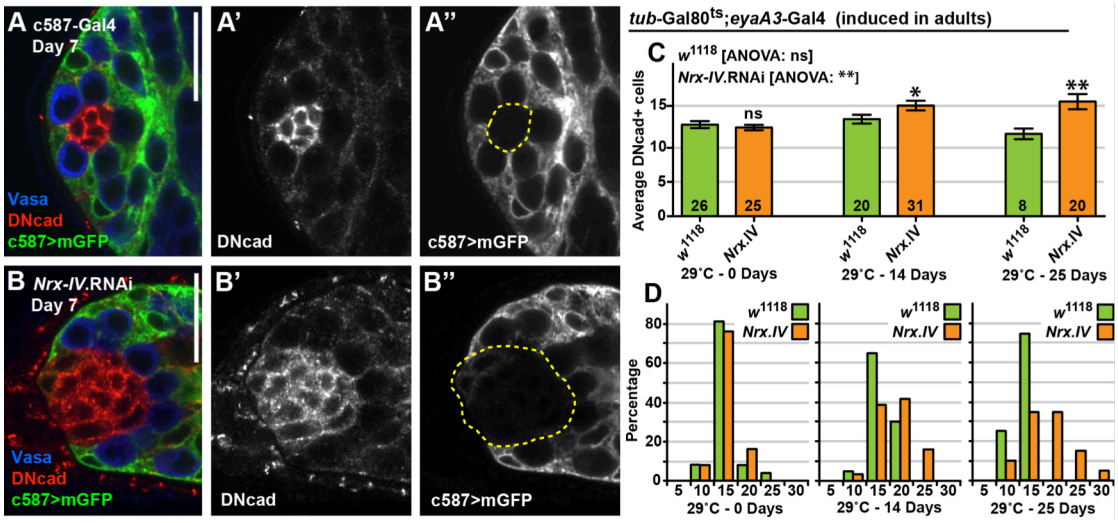

Figure 3.3 - Hub growth is specifically due to disruption of cyst cell septate junctions in the adult
(A-B) Expression of the cyst cell specific driver c587-Gal4 marked by membrane bound GFP (c587>mGFP), hub cells marked by DN-Cadherin (DNcad), and germ cells by Vasa. c587-Gal4 driving expression of *Nrx-IV* RNAi leads to growth of the hub, and a subset of hub cells that express low levels of mGFP. Controls are c587-Gal4 crossed to w^{1118}. Scale bars represent 20μm. (C-D) Disruption of septate junctions specifically in the adult testes using *tub*-Gal80^{ts}; *eyaA*3-Gal4 crossed to *Nrx-IV* RNAi. Gal4 repressed by temperature-sensitive Gal80 at 18˚C until eclosion, adults then shifted to 29˚C to induce RNAi expression for 0, 14, or 25 days. (C) Average number of hub cells in control and *Nrx-IV* knockdowns after 0, 14 and 25 days of RNAi expression. Control testes show no change in average hub size, whereas *Nrx-IV* knockdown show an increase between 0, 14, and 25 days (ANOVA testing). Mean±SEM displayed, number of testes listed on bars, asterisks represent student t-tests compared to age matched controls, or ANOVA testing. (D) Histogram of hub sizes at 0, 14, and 25 days of RNAi induction. X-axis numbers represent the largest number of hub cells per testis within the group.

3.2.2 Larger hubs maintain more germline stem cells and cyst stem cells

Niche size has been shown in various tissues, including vertebrate hematopoietic stem cells and somatic stem cells in the fly ovary, to be an important factor in regulating the number of stem cells that the niche can support [184,185]. In the fly testes it has been shown that older males or mutants having few hub cells could nonetheless maintain a large population of GSCs [40,46]. To determine how a larger hub, containing more cells, affects niche function, the number of GSCs and CySCs was monitored following knockdown of septate junction components in cyst cells. Overall, the average number of germ cells contacting the hub grew substantially in *Nrx-IV* or *cora* knockdown testes between 1 and 7 DPE (**Figure 3.4**).

To confirm that the germ cells contacting the hub were indeed GSCs spectrosome morphology was studied and found it to be consistent with that seen in wild-type GSCs (**Figure 3.4I-J**) [186]. Moreover, in individual testes there was a positive correlation between the number of hub cells and the number of GSCs (**Figure 3.4B-D**). Similar growth was also observed in the number of CySCs, defined as cyst cells expressing Zfh1 but not the hub cell marker DNcad. Control testes (from *tj*-Gal4 x w^{1118} progeny) had on average 34.3±1.30 CySCs while *Nrx-IV* and *cora* knockdown testes had 53.4±3.18 and 50.2±4.92 CySCs, respectively (P-values: <0.001 and <0.05; mean±SEM, N=10 per sample; measured 7 DPE). These results show the importance of maintaining a stable stem cell niche size as enlarged hubs were active and could support additional stem cells, which may result in the excess production of both germ cells and cyst cells.

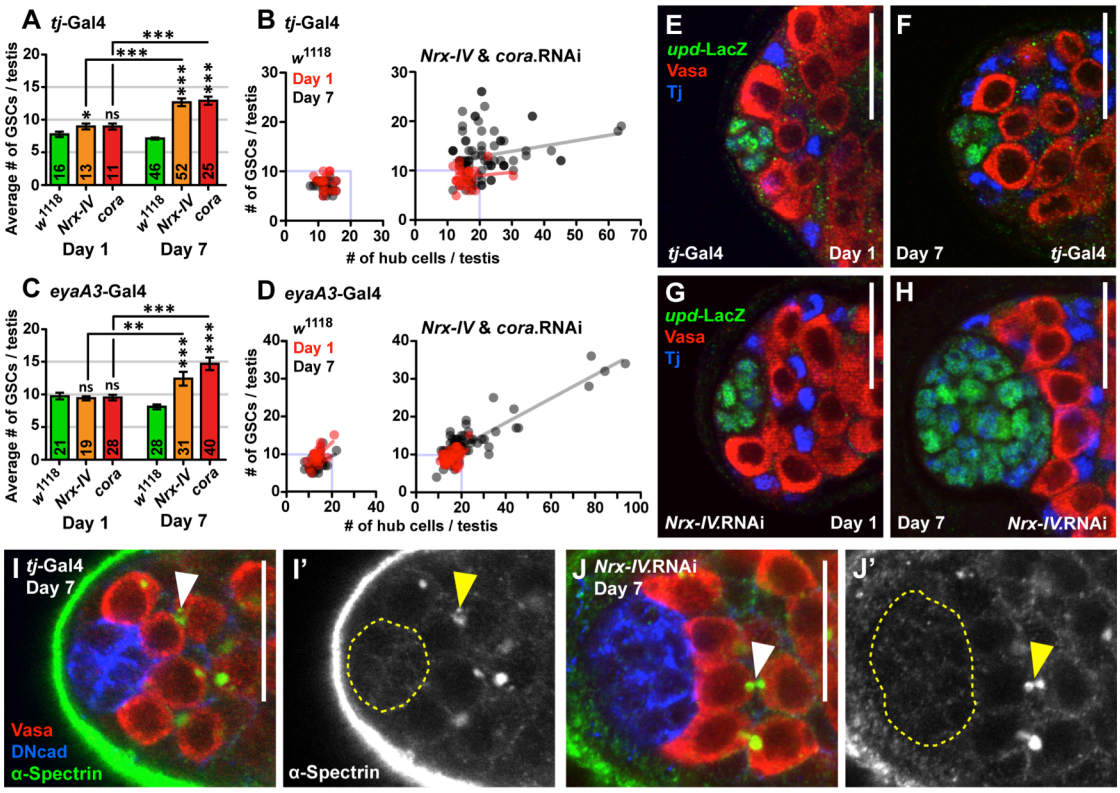

Figure 3.4 - Enlarged hubs maintain more germline stem cells
(A,C) Average number of GSCs. (B,D) Scatter plots of GSC and hub cell numbers for each testis. *Nrx-IV* and *cora* RNAi data is combined from separate experiments. (E-H) Hub cells labelled by *upd*-LacZ, germ cells labelled by Vasa, and cyst cells labelled by Tj. A strong example of stem cell niche growth is shown in panel H. (I-J) Spectrosomes, labelled by α-Spectrin, in germ cells contacting the hub were not found connecting more then two cells (arrowheads), confirming only GSCs and dividing GSCs occupy the niche. Mean±SEM, N displayed on bars, asterisks represent student t-tests compared to age-matched controls or between samples. Controls are the indicated driver crossed to w^{1118}. Scale bars represent 20 μm.

3.2.3 Cyst cells form new hub cells when occluding junctions are disrupted

Next, I wished to determine the mechanism driving hub growth in adult flies upon knockdown of septate junction components in cyst cells. One possible mechanism that can explain this growth is hub cell proliferation. However a defining feature of hub cells is that they are not mitotically active [32,42]. Consistent with this, a large number of testes (Control N=124, *Nrx-IV* and *cora* RNAi N=286) were stained for the mitotic marker phospho-Histone H3 (pH3) and no cells were observed where *upd*-LacZ and pH3

were detected simultaneously (**Figure 3.5A-C**). These results argue that division of hub cells is unlikely to explain hub growth in the adult *Nrx-IV* and *cora* knockdown testes.

To determine the origin of the extra hub cells the lineage of *eyaA3* expressing cells was traced using G-TRACE [187]. The driver *eyaA3*-Gal4 was chosen as both the expression pattern of septate junctions and the *Nrx-IV* or *cora* knockdown results suggested that hub growth involved differentiating *eyaA3*-positive cyst cells. The *eyaA3*-Gal4 driver utilizes a promoter region of the *eya* gene, which is required for somatic cyst cell differentiation, and is expressed at very low levels in CySCs and at high levels in differentiating cyst cells [64,183,188] (**Figure 3.1F**). Using G-TRACE allows us to identify both cells that previously expressed *eyaA3*-Gal4 (marked with GFP) and cells currently expressing *eyaA3*-Gal4 (marked with RFP), additionally, the hub was identified using expression of *upd*-LacZ and FasIII (**Figure 3.5D-E**). In control experiments at both 1 and 7 DPE there were few GFP-positive cells in the hub (0.4±0.13 and 0.6±0.14 respectively; mean±SEM) (**Figure 3.5F-J**). Those few GFP-positive cells could be explained by the transient expression of *eya* in the embryonic somatic gonadal precursor cells that form both hub and cyst cell lineages [32], or extremely low levels of expression in adult hub cells. When G-TRACE was combined with knockdown of *Nrx-IV* the results were strikingly different. Initially, 1 DPE, hubs were only slightly larger then controls (13.3±0.39 vs, 11.6±0.36; a 14% difference) and few GFP-positive hub cells were observed (0.4±0.10) (**Figure 3.5F-J**). In comparison, 7 DPE hubs contained on average more than twice as many cells compared to controls (24.3±2.46 vs. 11.5±0.36; a 111% difference). Importantly, hub growth in *Nrx-IV* knockdowns was largely attributable to the incorporation of GFP-positive cells (8.4±1.8) (**Figure 3.5F-J**).

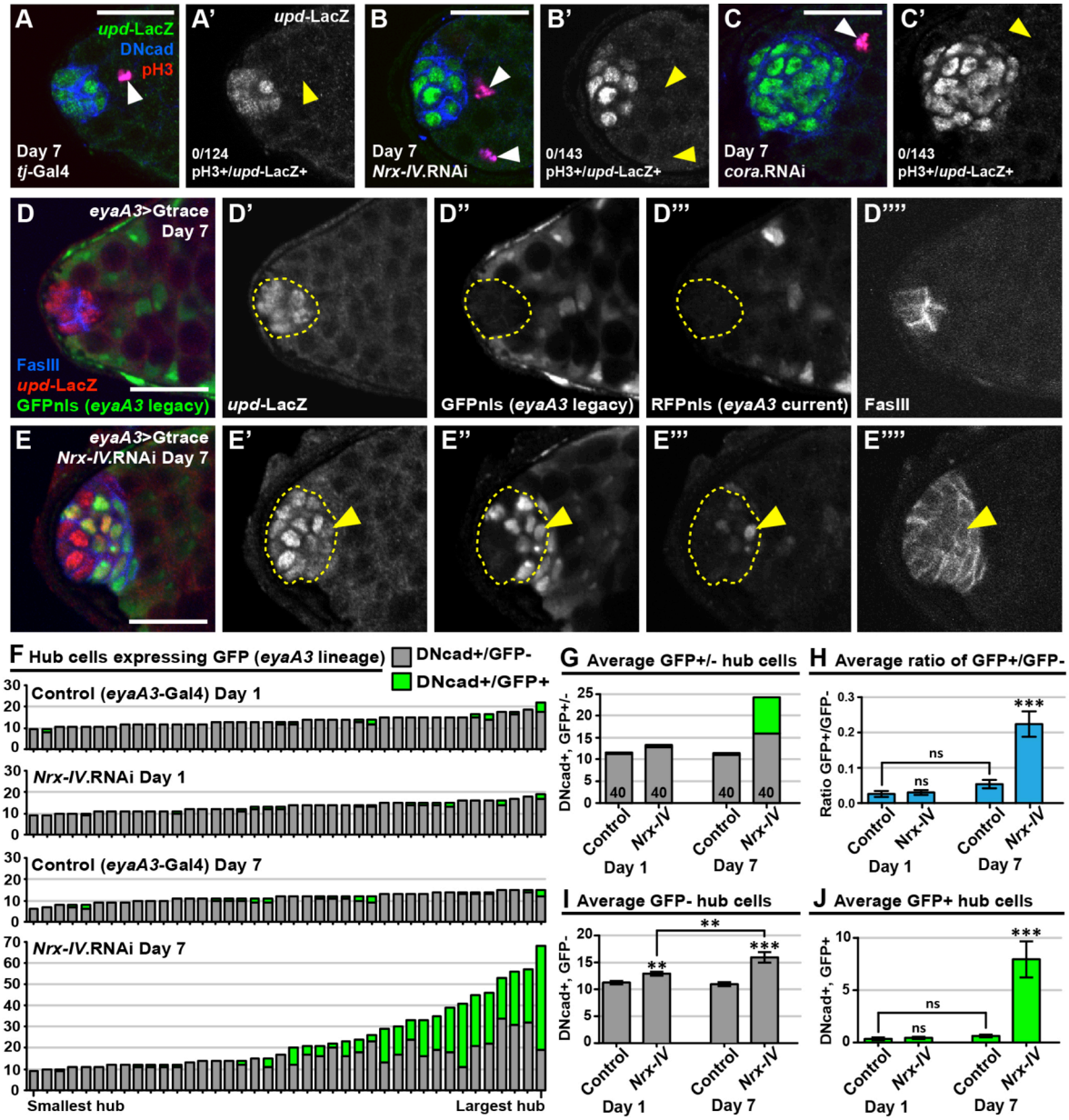

Figure 3.5 - New hub cells derive from the cyst cell population
(A-C) Hub cells labelled by upd-LacZ never underwent mitosis (labelled by pH3 staining). Stem cell divisions indicated (arrowheads). (D-J) Lineage tracing of *eyaA3*-Gal4 expressing cyst cells using G-TRACE. (D-E) Hub cells labelled by *upd*-LacZ and FasIII, cells that expressed *eyaA3*-Gal4 labelled by GFP.nls (*eyaA3* legacy), cells that currently or recently expressed *eyaA3*- Gal4 labelled by RFP.nls (*eyaA3* current). (F) GFP-negative (grey) and GFP-positive (green) hub cells per testis at 1 and 7 DPE; bars represent individual testes.(G) Average number of GFP-positive and GFP-negative hub cells.(H) Average ratio of GFP-positive to GFP-negative hub cells. (I) Average number of GFP-negative hub cells. (J) Average number of GFP-positive hub cells. Mean±SEM; N displayed on bars; asterisks represent student t-tests compared to age- matched controls or between samples. Controls are the indicated driver crossed to w^{1118} (A), or G-TRACE (D-J). Scale bars represent 20 μm.

Moreover, a population of *upd*-LacZ labelled cells that were also RFP-positive was observed consistent with ongoing or recent expression of *eyaA3*-Gal4 in hub cells (**Figure 3.5E'''**; arrowhead). These results suggest that knockdown of *Nrx-IV* or *cora* leads cyst cells to adopt hallmarks of hub cell identity and express hub cell specific genes.

3.2.4 Cyst cell to hub cell conversion is modulated by Notch and EGFR signalling

To learn more about the differentiation state of non-endogenous hub cells in *Nrx-IV* and *cora* knockdown testes various markers were used to label the stem cell niche. This analysis showed normal expression of hub cell markers such as Upd, FasIII, DNcad, as well as Hedgehog (*hh*-LacZ), Armadillo (Arm), and DE-Cadherin (DEcad) (**Figure 3.6A-F**). To identify how cells that were previously, and in some instances were still, *eyaA3*-positive could express multiple hub-cell fate markers the signalling mechanisms that determine hub fate in *Nrx-IV* and *cora* knockdown testes were investigated.

Hub growth phenotypes similar to those produced by *Nrx-IV* and *cora* knockdown have been described previously, most notably in agametic testes that lack germ cells, suggesting that the germline regulates the formation of hub cells [47]. One specific germline derived signal shown to regulate hub fate is the Epidermal Growth Factor (EGF) ligand Spitz [30,84]. In embryonic testes somatic cells express the EGF Receptor (EGFR) which, when activated, represses hub formation [37]. EGFR induced mitogen-activated protein kinase (MAPK) signalling, visualized by staining for di-phosphorylated-ERK (dpERK), was active in CySCs and spermatogonial stage cyst cells (**Figure 3.6M**). Quantifying dpERK-staining intensity in cyst cell nuclei showed that

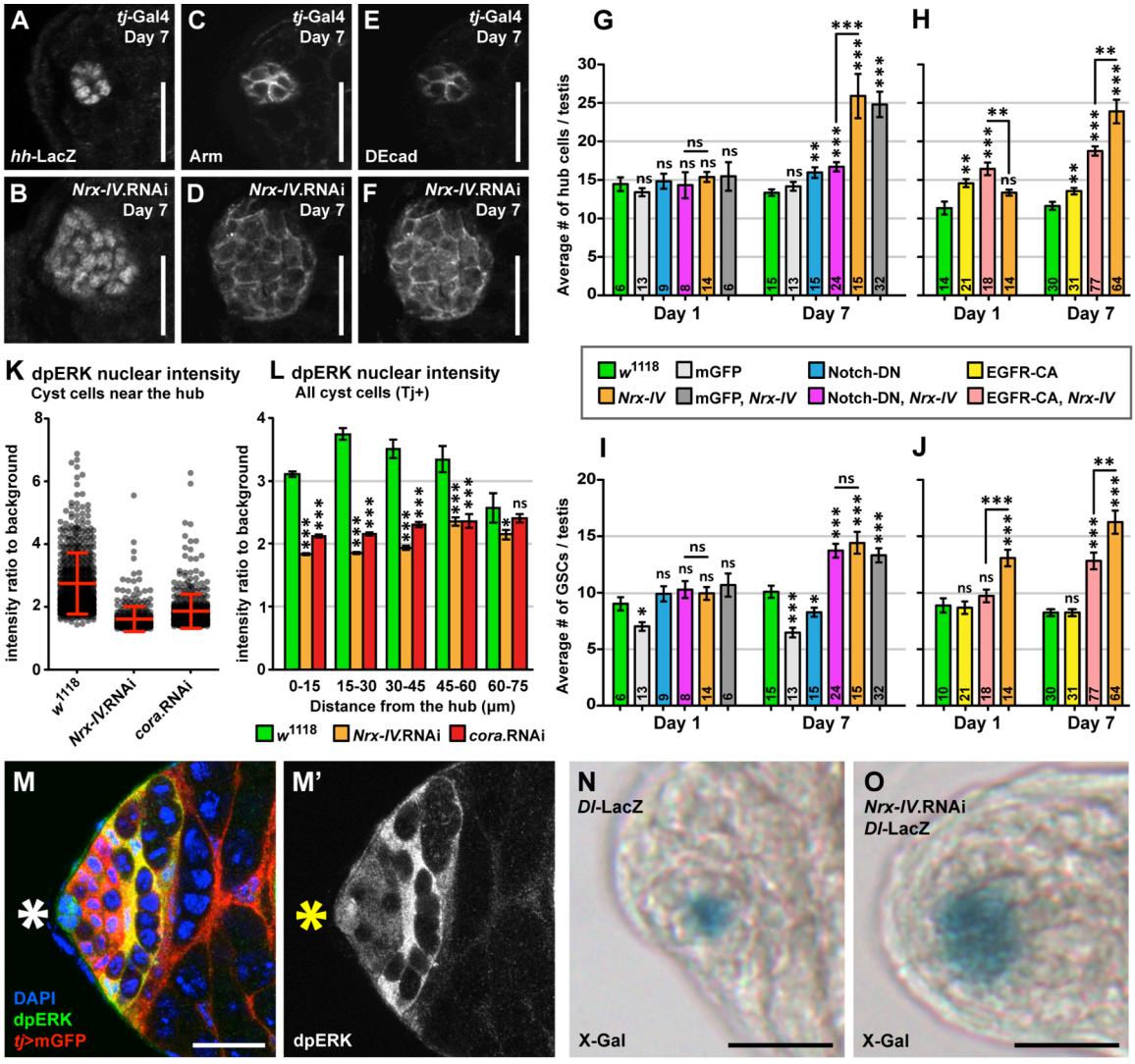

Figure 3.6 – Notch and EGFR signalling modulate hub growth in the adult testis
(A-F) Testes stained for the hub cell markers Hedgehog (*hh*-LacZ), Armadillo (Arm), and DE-Cadherin (DEcad).(G-J) Average number of hub cells (G-H) and GSCs (I-J) from the progeny of *tj*-Gal4 crossed to the indicated UAS-transgenes (boxed legend). (G-H) Co-expression of dominant negative Notch (Notch-DN, panel G) or constitutively active EGFR (EGFR-CA, panel H) reduces the growth of the hub compared to *Nrx-IV* RNAi alone.(I-J) Co-expression of EGFR-CA (panel J), but not Notch-DN (panel I), reduces the growth of the GSC population compared to *Nrx-IV* RNAi alone. (G,I) Co-expression of UAS-mCD8::GFP (mGFP) does not significantly alter the growth of the hub or GSC populations compared to expression of *Nrx-IV* RNAi alone. (K-L) MAPK signalling measured with di-phosphorylated ERK (dpERK) in *tj*-Gal4 mediated knockdown and control testes 7 DPE. Represents results from 46 w^{1118}, 36 *Nrx-IV*.RNAi, and 26 *cora*.RNAi testes.(K) Nuclear dpERK intensity of cyst cells within 15 µm of the hub; mean±SD overlaid in red.(L) Average dpERK intensity in cyst cell nuclei grouped by their distance from the hub.(M) MAPK signalling (dpERK) is active in the cyst cell population (*tj*>mGFP) surrounding the hub (asterisk).(N-O) X-Gal staining detects a reporter for the Notch ligand Delta (*Dl*-lacZ) in hub cells 7 DPE.Mean±SEM; N displayed on bars; asterisks represent student t-tests compared to age- matched controls or between samples. Controls are *tj*-Gal4 crossed to w^{1118} (A,C,E), or *Dl*-LacZ (N). Scale bars represent 20 µm.

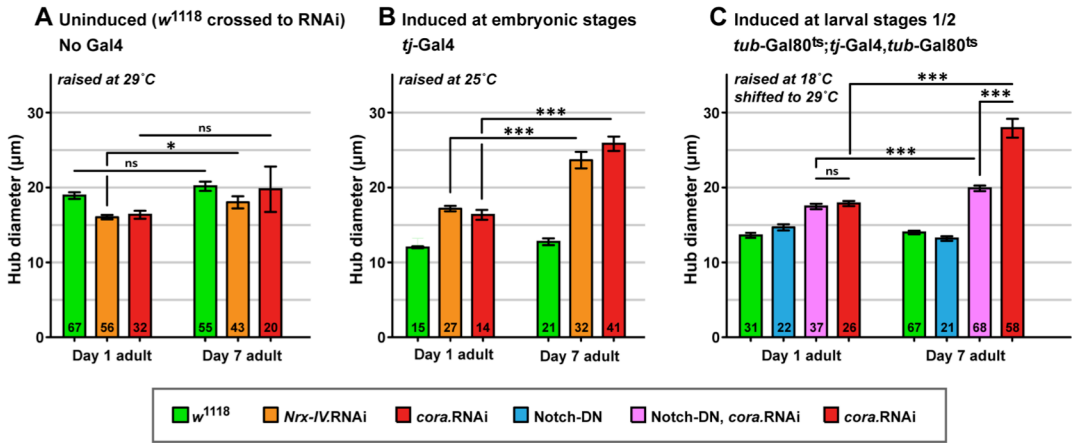

Figure 3.7 - Hub growth in the adult is due to Gal4-mediated RNAi expression and requires post-embryonic Notch signalling.
(A) Average hub size in the progeny of w^{1118} crossed to UAS-RNAi and control (w^{1118}) flies without any Gal4 driving expression of RNAi (parents and progeny raised at 29°C). (B) Average hub size in the progeny of *tj*-Gal4 crossed to UAS-RNAi and control (w^{1118}) flies (progeny raised at 25°C). (C) Average hub size in the progeny of *tub*-Gal80^{ts}; *tj*-Gal4,*tub*-Gal80^{ts} crossed to control (w^{1118}), UAS- Notch-DN, UAS-RNAi, and combined UAS-Notch-DN and UAS-RNAi transgenes. At 18°C two copies of temperature sensitive Gal80 inhibit Gal4 during embryogenesis, shifting progeny to 29˚C induces Gal4 mediated expression specifically in 1st and 2nd instar larva. (A-C) Hub sizes all measured as maximum diameter of FasIII positive cells in µm, mean±SEM, N displayed on bars, asterisks represent student t-tests.

MAPK activity was lower in CySC following knockdown of *Nrx-IV* or *cora* suggesting reduced EGFR signalling (**Figure 3.6K**). Moreover, the effect of *Nrx-IV* or *cora* knockdown on MAPK signalling was not restricted to CySC as lower dpERK staining was observed at a distance from the hub (**Figure 3.6L**).

To see if disruption of EGFR signalling could underlie hub defects in *Nrx-IV* and *cora* knockdown testes, a rescue of these phenotypes by increasing EGF signalling was attempted. When a constitutively activated EGF receptor (EGFR-CA) [189] was co-expressed in cyst cells along with *Nrx-IV* RNAi hub growth was attenuated, resulting in a reduction in the average number of hub cells compared to expressing only *Nrx-IV* RNAi (an increase of 14%, P<0.05, compared to 79%, P<0.001, between 1 and 7 DPE; **Figure**

3.6H). Similar results were also observed in the growth of the GSC population (**Figure 3.6J**), suggesting that reduced EGFR activation in cyst cells contributes to the overall growth of the stem cell niche caused by the knockdown of *Nrx-IV* or *cora*. Surprisingly, analysis of testes with loss of function mutations in the EGFR/MAPK pathway reveals different phenotypes than those observed in this study: encapsulation is disrupted, CySC are lost, but hub size is largely unaffected [74,75,86]. This result shows that the partial reduction in EGFR/MAPK signalling seen in *Nrx-IV* and *cora* knockdown testes results in distinct phenotypes and highlights the complexity of EGFR signalling in the fly testis.

Another pathway that is documented to regulate hub cell fate is Notch signalling. Notch plays important roles in hub specification in embryos [37]. The notch ligand Delta is produced by the embryonic endoderm and acts to promote hub cell specification in the anterior-most somatic gonadal precursor cells [34]. While it has been suggested that Notch acts in the adult to regulate hub fate such a role has not been clearly demonstrated. A reporter for the Notch ligand Delta (*Dl*-lacZ) was observed in hub cells of both control and *Nrx-IV* knockdown testes (**Figure 3.6N-O**). Intriguingly, reducing Notch signalling efficiently rescued the hub overgrowth seen in adult *Nrx-IV* knockdown testes. When a dominant negative Notch (Notch-DN) [190] was co-expressed in the somatic cells, along with *Nrx-IV* RNAi, the growth of the hub was reduced compared to the expression of *Nrx-IV* RNAi alone (an increase of 16%, $P>0.1$, compared to 67%, $P<0.01$, between 1 and 7 DPE; **Figure 3.6G**). Growth in the GSC population was not significantly reduced by co-expression of Notch-DN (**Figure 3.6I**), suggesting that the Notch pathways may modulate hub growth through a different mechanism compared to the EGFR pathway. Since Notch is well established to regulate hub growth in the embryo, temperature-

sensitive Gal80 was used to delay expression of Notch-DN and confirm that the reduction in hub cells was due to disruption of post-embryonic Notch signalling (**Figure 3.7C**). These results suggest that Notch signalling in cyst cells may contribute to the hub overgrowth phenotypes caused by septate junction knockdown in the adult testes.

3.3 Discussion

3.3.1 Hub cell fate is actively repressed in cyst cells

In addition to Notch and EGFR, other signalling pathways that regulate hub size may contribute to the hub growth seen upon somatic knockdown of septate junction components. For example I previously showed that the range of BMP signalling is expanded following *Nrx-IV* or *cora* knockdown in cyst cells [182]. Constitutive activation of BMP signalling in the germline was shown to increase the size of the hub and the number of GSCs [55]. Additionally, the relative expression levels of the genes *drm, lines,* and *bowl* regulate hub size in the adult [35]. In particular, it is known that *lines* maintains a "steady-state" in the testes by repressing expression of a subset of hub genes in the cyst cell population [35]. Unlike *lines* mutants, *Nrx-IV* or *cora* knockdowns generally lack ectopic hubs. This may reflect the more gradual hub growth seen in septate junction knockdowns or, alternatively, highlight key mechanistic differences in how hub growth is achieved in each respective genetic background.

This work is consistent with the model whereby occluding junctions are required for proper soma-germline signalling in the fly testes. This signalling maintains stem cell niche homeostasis by preventing somatic cyst cells from adopting hub cell fate, which would lead to niche overgrowth. It is well established that in embryonic testes hub fate is

both positively and negatively regulated by signals from the germline and the endoderm [34,37,47]. The results presented in this work, and recent findings about the genes *lines* [35] and *traffic jam* [191], argue that in the adult testes hub fate is actively repressed in the cyst cell lineage. Failure to repress hub fate allows cyst cells to exhibit features of hub cells and act as a functional stem cell niche. However, these cyst cell derived hub cells are distinct from the true endogenous hub cells, in that they show non-hub cell features including expression of the differentiating cyst cell markers *eyaA3*-Gal4 (**Figure 3.5E'''**) and β3-tubulin (**Figure 3.1C,C''**). The data presented here suggests that following disruption of septate-junctions proteins, the signalling environment surrounding the somatic cells is altered such that cyst cells gradually begin expressing hub cell markers.

3.3.2 Somatic cell structures and signalling pathways mediate niche homeostasis

One major outstanding question is how *eyaA3*-Gal4 expressing cyst cells become incorporated into the endogenous hub. Previously I showed that a septate-junction mediated permeability barrier forms by the 4-cell spermatogonial-stage spermatocyst. The hub growth phenotypes induced by *Nrx-IV* and *cora* knockdowns may occur due to defects in cell-cell signalling, possibly involving EGFR and Notch, that manifest in these later spermatocysts; however, this model requires an explanation for how these cyst cells translocate back to and join the hub. Alternatively, signalling defects in these later spermatocysts may somehow be instructing earlier cyst cells such as CySCs to join the hub. It is easier to envisage the latter model as early cyst cells are spatially much closer to the hub, but the sequence of signalling events in such a case will likely be complex and require further elucidation.

The ability of CySCs to convert into hub cells in wild-type testes is a controversial subject [35,39]. The incorporation of CySC cells into the hub may not necessitate complete conversion into hub cells, but could rather involve simple de-repression or activation of genes that confer hub cell function including regulators of the cell cycle and hub cell specific signalling ligands. Notably, the transition between CySC and hub cell fate is linked to the cell cycle [48].

Why would loss of the septate-junction mediated somatic permeability barrier result in disruption of signalling between the soma and germline? There are many possible answers to this question, but I can speculate about two such mechanisms that could explain hub overgrowth. One possibility is that germline differentiation, which is dependent on the permeability barrier, is required for the release of signals that maintain stem cell niche homeostasis. Another possibility is that the permeability barrier locally concentrates germline-derived signals that repress hub cell fate by trapping them in the luminal space between the encapsulating cyst cells and the germline. The latter scenario could explain my observation that activated EGFR signalling partially rescues hub overgrowth. In this model, septate-junctions allow localized build-up of the EGF ligand Spitz ensuring that sufficient signalling is available to repress hub fate. It is more difficult to draw strong conclusions about how Notch signalling is altered when septate junctions are disrupted; particularly as the Notch ligand Delta appears restricted to the hub.

In this study, I find an unexpected role for an occluding-junction based permeability barrier in the testis – that of mediating stem cell niche homeostasis. Also, this work highlights how the architecture of the stem cell-niche system in the fly testis,

which is highly regular and contains a reproducible number of stem cells and niche cells, is in fact the result of an active and dynamic signalling environment.

3.4 Materials and methods

3.4.1 Genetics

Fly lines included *upd*-Gal4 (courtesy of Christian Bökel, Center for Regenerative Therapeutics Dresden, Germany), *tj*-Gal4 (P{GawB}NP1624; *Drosophila* Genetic Resource Center), *eyaA3*-Gal4 (courtesy of Steven DiNardo, University of Pennsylvania, USA), c587-Gal4 (courtesy of Leanne Jones, University of California, Los Angeles, USA), *upd*-LacZ (courtesy of David Bilder, University of California, Berkeley, USA), and EGFR-CA (*UAS*-λTop ; courtesy of Bruce Edgar, University of Heidelberg Alliance, Germany). Additional lines from the Bloomington *Drosophila* Resource Center included Nrx-IV::GFP (CA06597), *hh*-LacZ (hh^{P30}), *Dl*-LacZ (Dl^{05151}), mGFP (*UAS*-mCD8::EGFP), GFP (*UAS*-2xEGFP), GTRACE (*UAS*-RedStinger,*UAS*-FLP,*ubi-p63E*{*FRT.STOP*}Stinger), and Notch-DN (UAS-NECN). RNAi lines included *UAS*-HM05144 (*cora*.RNAi) and UAS-JF01342 (*Nrx-IV*.RNAi) from the Transgenic RNAi Resource Project used in all experiments with the exception of those with *Dl*-LacZ or Notch-DN (Figure 3.6G,I,N-O, Figure 3.7C) which used UAS-GD2436 (*Nrx-IV*.RNAi) or UAS-KK109444 (*cora*.RNAi) from the Vienna *Drosophila* Resource Center. UAS-Dicer2 was included for quantifications in Figure 3.2 and Figure 3.4. *tub*-$Gal80^{ts}$ was included for quantifications in Figure 3.3C-D and Figure 3.7C.

3.4.2 Staining and immunohistochemistry

Testes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and

washed with 0.2% bovine serum albumin and 0.3% Triton-X in PBS, with the exception of dpERK stainings done using testis buffer supplemented with phosphatase inhibitor cocktail 2 (1:100, P5726, Sigma) [192]. Primary antibodies included: rabbit-anti-β3-tubulin (courtesy of R. Renkawitz-Pohl, Philipps-Universität Marburg, Germany), rabbit-anti-Zfh1 (courtesy of R. Lehmann, New York University, USA), guinea-pig-anti-Zfh1 (courtesy of J. Skeath, Washington University in St Louis, USA), guinea-pig-anti-Tj (courtesy of courtesy of D. Godt, University of Toronto, Canada), goat-anti-Vasa (dC-13, Santa Cruz), chicken-anti-GFP (ab13970, Abcam), chicken-anti-LacZ (ab9361, Abcam), mouse-anti-pH3 (6HH3-2C5, Millipore), rat-anti-dsRed (5f8, Chromotek), rabbit-anti-dpERK (4370P, Cell Signaling). Additional antibodies from the Developmental Studies Hybridoma Bank included: mouse-anti-Cora (C566.9/C615.16), mouse-anti-FasIII (7G10), rat-anti- DNcad (DN-Ex #8), rat-anti-DEcad (DCAD2), mouse-anti-LacZ (40-1A), mouse-anti-α-Spectrin (3A9). Secondary antibodies used were conjugated to Alexafluor-488, Cy3, Cy5, or Pacific-orange and were obtained from ThermoFisher Scientific and Cederlane. X-Gal stains were fixed similar to other samples prior to incubation in X-Gal staining solution for 3 days at 20˚C [192].

3.4.3 Data collection

Crosses to induce RNAi knockdown used Gal4 female flies with the exception of Figure 3.6H‚J which used Gal4 male flies. Parents were raised at 20˚C and progeny raised at 25˚C (0-2 days after egg laying), with the following exceptions: *upd*-Gal4 progeny were raised at 29˚C (Figure 3.2A-F), uninduced control cross parents and progeny were raised at 29˚C (Figure 3.7A), and Gal80^{ts} crosses where parents and progeny were raised at 18˚C until shifted to 29˚C (1-3 days post eclosion for Figure 3.3C-D; 3-5 days after

egg laying for Figure 3.7C).

dpERK nuclear intensity was measured on a single image per testis selected to contain the hub and most intense dpERK staining cyst cell nuclei. Nuclei were identified automatically in Matlab (Mathworks) and the mean dpERK intensity measured and normalized to intensity outside of the testes.

Statistics were performed using Prism (Graphpad) or Matlab (Mathworks). All student t-tests were two- tailed and applied Whelch's correction, asterisks represent P-values : <0.001=*** ; <0.01=** ; <0.05=* ; >0.05=ns (non significant).

CHAPTER FOUR: Discussion

4.1 Overview of findings

The goal of the work described in this thesis was to identify how somatic cells regulate spermatogenesis in the *Drosophila* testis. Specifically, it focused on the formation of occluding junctions between the somatic cyst cells that form the spermatocyst. The research presented here demonstrates a role for these junctions in germline differentiation and stem cell niche homeostasis. Importantly, this thesis provides evidence that occluding junctions shape the activation of the cell-cell signalling pathways that mediate these developmental processes. The novel findings of this work are summarized as follows:

(1) Septate junctions form between cyst cells establishing an occluding permeability barrier that isolates the encapsulated germ cells. This permeability barrier is required for germline differentiation and regulates the expression of Bam. Since Bam is also required for germline differentiation and is repressed by BMP signalling, this suggests that the permeability barrier restricts the range of BMP signalling in the testis.

(2) Septate junctions between the differentiating cyst cells maintain stem cell niche homeostasis by repressing the growth of the hub. When septate junctions are disrupted the size of the stem cell niche increases due to cyst cells forming new hub cells, which in turn recruit more stem cells. Inhibiting Notch signalling or activating EGFR signalling reduces the growth of the hub, which suggests these pathways are altered by the disruption of septate junctions.

Overall this work explores the structure of the stem cell niche in the context of the signalling pathways that mediate stem cell maintenance and differentiation. The research presented here unifies the study of encapsulation with the formation of an evolutionally conserved soma-germline permeability barrier. This research also uncovers unexpected connections between the soma-germline barrier, germline differentiation, and stem cell niche homeostasis. This work can serve to inform and guide future studies of how somatic cell structures regulate the signalling pathways required for spermatogenesis.

4.2 Emergent themes and implications

4.2.1 Soma-germline barriers are required for spermatogonial differentiation

The somatic cell-based permeability barrier described in Chapter 2 of this thesis is analogous to other soma-germline barriers including the mammalian blood-testis barrier (BTB) [123]. The study of this barrier in *Drosophila* represents the first genetic characterization of a soma-germline barrier in a non-vertebrate animal. Furthermore this barrier is required for germline differentiation in *Drosophila* making it functionally similar to the mammalian BTB. This suggests it may play a conserved role in regulating spermatogenesis. The study of the soma-germline barrier in *Drosophila* may thus shed light on the fundamental mechanisms regulating spermatogenesis in animals.

In *Drosophila* the soma-germline barrier is formed in two stages. Cyst cells first encapsulate the germ cells using actin-based cellular protrusions mediated by Chickadee, the *Drosophila* homologue of Profilin. Cyst cells then seal together utilizing septate junctions consisting of the proteins Neurexin-IV and Coracle. Both of these events are required to establish the permeability barrier that isolates differentiating germ cells;

however, there are salient differences between the two stages. For instance, when encapsulation is disrupted, spermatogonia fail to differentiate and continue to grow, filling the testis with mitotic spermatogonia that resemble a germline tumour. By contrast, when encapsulation proceeds normally, but septate junctions are disrupted, spermatogonia fail to differentiate and form small isolated clusters of spermatogonia. This suggests that when septate junctions are disrupted, the undifferentiated spermatogonia are eliminated before they can grow to fill the testis. The observation of a decrease in mitotic spermatogonia past the 4-cell stage supports this hypothesis.

Drosophila cyst cells are known to kill damaged germ cells during the mitotic spermatogonial stages by undergoing apoptosis themselves [193]. In many other animals somatic cells eliminate damaged germ cells by phagocytosis [7,8]. This indicates that encapsulation, like phagocytosis, may be required for cyst cells to eliminate aberrant or damaged spermatogonia. Therefore, the primary difference between disrupting encapsulation and disrupting septate junctions is that undifferentiated spermatogonia that are also not encapsulated are likely to survive longer to continue through more mitotic divisions.

When either encapsulation or septate junctions are disrupted, the somatic permeability barrier is not established, the expression of Bam is delayed, and the germline fails to differentiate. As Bam is required for germline differentiation and is repressed by BMP signalling, this implies that the permeability barrier limits the range of BMP signalling. Hub cells and CySCs secrete BMP ligands within the stem cell niche, while the neighboring cyst cells surround and isolate germ cells as they leave the stem cell niche. A simple model for how the permeability barrier regulates germline

differentiation would be that septate junctions between the cyst cells block niche-derived BMP ligands from reaching the encapsulated germ cells. This would lead to lower BMP signalling, allowing the germ cells to express Bam and differentiate. This model implies that the specific expression of Bam at the 4-cell spermatogonial stage is the direct result of cyst cells establishing the permeability barrier and blocking BMP signals (**Figure 4.1**).

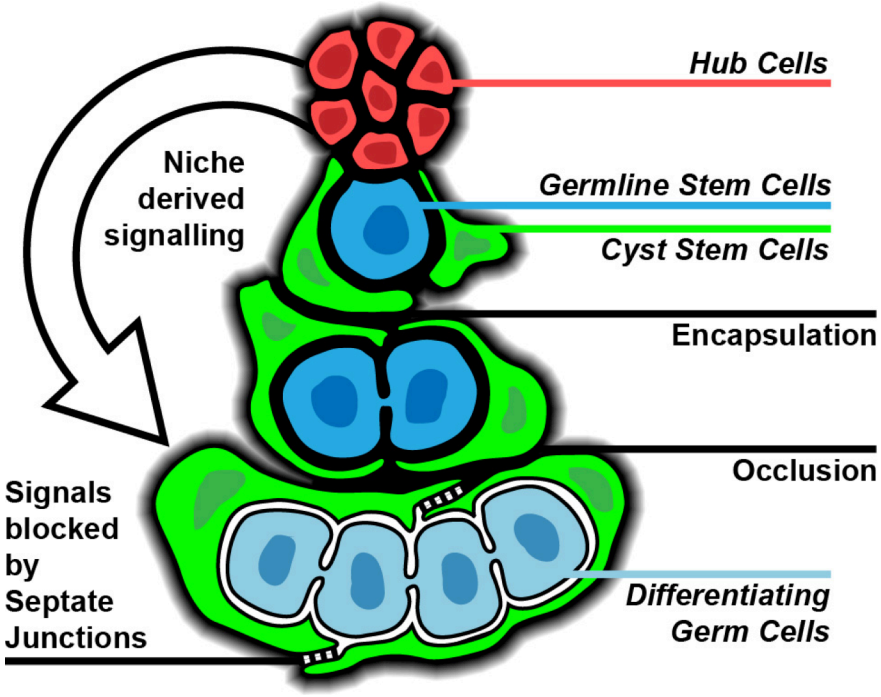

Figure 4.1 – The Drosophila soma-germline barrier and germline differentiation
Model of the stem cell niche, highlighting the encapsulation and occlusion of germ cells by cyst cells. Septate junctions seal the spermatocyst and limit the range of niche-derived signalling.

Both *Drosophila* and mammalian GSCs are maintained by signals from the TGF-β superfamily of ligands. While *Drosophila* utilizes the BMP class ligands Gbb and Dpp, mammals utilize the unique ligand GDNF [24,93]. In *Drosophila* I identified a novel role for the soma-germline barrier in restricting BMP signalling. It is not known whether the mammalian BTB also restricts GDNF signalling to control germline differentiation [108]. Both Sertoli cells and peritubular myoid cells express GDNF [95]. While localizing extra-cellular GDNF is difficult it has been observed in patches associated with the GSCs on the basal side of the seminiferous tubule [194]. GDNF signalling is maintained in GSCs by a positive feedback loop that increases expression of its own receptor, GFRα1 [195]. As spermatogonia undergo transit-amplifying divisions they reduce expression of GFRα1, becoming less sensitive to GDNF [196]. These spermatogonia then differentiate into spermatocytes before they are engulfed between Sertoli cells and pass through the BTB [109]. Together, these observations suggest that the mammalian BTB is unlikely to control germline differentiation by directly restricting access to GDNF.

As mammalian spermatogonia undergo transit-amplifying divisions and become less sensitive to GDNF signalling, they also become more sensitive to RA signalling due to expression of the receptor RAR-γ [196]. When RA is expressed in the seminiferous tubule it induces both spermatogonial differentiation and the restructuring of the BTB to isolate the resulting spermatocytes [98,100,102]. While RA signalling controls both germline differentiation and the BTB, the mechanism by which disruption of the BTB interferes with germline differentiation remains unknown [98].

In contrast to the mammalian BTB, the *Drosophila* soma-germline barrier appears to regulate germline differentiation by directly restricting BMP signalling. This suggests

that the mammalian BTB and the *Drosophila* soma-germline barrier regulate spermatogenesis using different mechanisms, or that there is a more fundamental and conserved mechanism that has not yet been identified. Regardless of any differences, both the mammalian BTB and the *Drosophila* soma-germline barrier are required for the same developmental process, the differentiation of spermatogonia into spermatocytes.

The convergence of different regulatory mechanisms to control the same developmental process suggests that both the *Drosophila* and mammalian barriers may have evolved primarily for isolating the meiotic and post-meiotic germ cells. Support for this view comes from a comparison of spermatogenesis with oogenesis. In mammals, both male and female germ cells depend on RA signalling to drive differentiation [98], however mammalian ovaries lack any tissue barrier analogous to the male BTB [197]. Similarly, in *Drosophila* both male and female germ cells depend on Bam to drive differentiation [50], however, a somatic cell-based permeability barrier has not been detected isolating differentiating germ cells in the ovaries of *Drosophila* or other insect species [198-201]. This demonstrates that in the ovaries both of *Drosophila* and of mammals the germline can differentiate without a somatic cell-based permeability barrier. Critically, while meiosis begins during oogenesis, it is not completed until the ovum (or egg) is fertilized [202]. Thus, unlike testes, ovaries do not contain any post-meiotic germ cells. The lack of any soma-germline barrier in females, but its strong developmental link with meiosis in males, suggests an evolutionarily conserved role for the barrier in isolating meiotic or post-meiotic germ cells.

Seen from this perspective, spermatogonial differentiation may depend on the formation of the barrier to ensure the isolation of the resulting meiotic spermatocytes.

The mechanisms by which the barrier controls germline differentiation would therefore have evolved to suit the developmental constraints of spermatogenesis in each species. Regardless of their differences, the similarities between *Drosophila* and mammalian somatic cell barriers demonstrate their conserved role in germline differentiation. Study of the *Drosophila* barrier may thus provide insights into the fundamental roles that the soma-germline barrier plays during spermatogenesis generally in animals.

4.2.2 The testis stem cell niche is in homeostatic balance

When septate junctions are disrupted, as described in Chapter 3 cyst cells convert into hub cells. The increased number of hub cells recruits more stem cells progressively enlarging the size of the stem cell niche (**Figure 4.2**). Cyst cells may partially convert into hub cells due to reduced levels of EGFR signalling. The formation of new hub cells also requires Notch signalling, which may be activated by the ligand Delta expressed by hub cells. This suggests that disrupting septate junctions leads to lower EGFR signalling in cyst cells, and also makes them competent to respond to Notch signalling from the hub.

Curiously, stem cell niche homeostasis appears to be dependent on septate junctions only in the differentiating cyst cells, not in CySCs or the hub. This suggests that the changes to the stem cell niche might be caused by disruption of the differentiated cells outside of the niche itself. This was further supported by the fact that the growth of the stem cell niche primarily occurred in the adult testis and not during embryonic or early larval stages when spermatogenesis has yet to fully begin. This suggests that the failure of germline differentiation when septate junctions are disrupted could be a driving factor in the growth of the stem cell niche. Such a model is in line with other research

where a similar pattern of stem cell niche growth was found when BMP signalling was activated in the germline [55]. In this case, the germline also fails to differentiate past the spermatogonial stage, indicating that the presence of differentiating germ cells may somehow regulate stem cell niche homeostasis. This may explain the slower growth of the hub when septate junctions are disrupted specifically in adult testes. In those experiments, differentiated germ cells are already present and may temporarily suppress the growth of the hub while they persist.

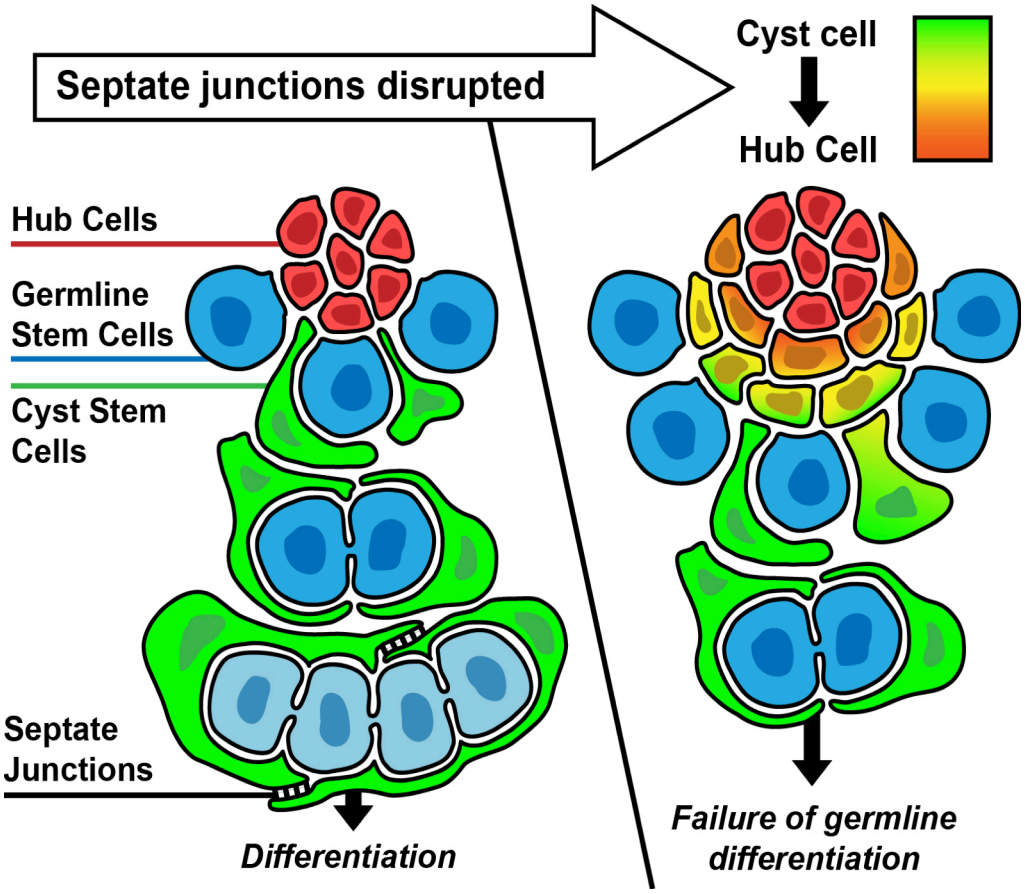

Figure 4.2 – Disruption of stem cell niche homeostasis
Diagram of the stem cell niche in wild-type testis (Left), compared to testis that lack septate junctions (Right). Disrupting occluding junctions leads to the failure of germline differentiation, and the growth of the stem cell niche by cyst cell to hub cell conversion.

How germ cells regulate the conversion of cyst cells into hub cells is not clear, but likely involves changes in EGFR signalling as this pathways is known to repress the formation of hub cells in the embryo [37]. Moreover, when germline differentiation is disrupted in the *Drosophila* ovary the neighbouring somatic cells also show a reduction in the level of EGFR signalling [203]. This suggests that the failure of germline differentiation may affect the expression of EGFR ligands or make somatic cells less competent to respond to EGFR ligands. In the testis, septate junctions seal the germ cells within the lumen of the spermatocyst, which may increase the local concentration of germ cell derived EGFR ligands. Disruption of septate junctions could therefore lead to reduced EGFR signalling by allowing EGF ligands to diffuse out of the spermatocyst. This would only be predicted to regulate EGFR signalling in the differentiating cyst cells, however, the data showed a general reduction in EGFR signalling in all cyst cells including CySCs. This indicates that any mechanism by which septate junctions regulate EGFR signalling is likely to be more complicated then these simple models predict.

Although reduced EGFR signalling may partly explain the conversion of cyst cells into hub cells, it is unlikely to be the only factor. For example, if EGFR signalling is disrupted directly, encapsulation is defective and the germline fails to differentiate, but the hub does not grow in size [75,86]. One explanation for this incongruity is that EGFR signalling also leads to reduced CySC maintenance [75]. This implies that EGFR signalling may retain CySCs in the niche, while simultaneously inhibiting them from forming new hub cells. The reduced EGFR signalling when septate junctions are disrupted may be enough to allow the formation of hub cells, while still retaining CySCs.

While EGFR signalling represses the formation of hub cells, Notch signalling is required for it. This is true in the embryo and in the adult, as activating genes downstream of Notch signalling can cause adult cyst cells to take on some of the features of hub cells [34,35,191]. The identification of the Notch ligand Delta in hub cells suggests that contact with the hub may be a prerequisite for cyst cells to convert into hub cells. This strongly implies that when septate junctions are disrupted it is the CySCs that form the new hub cells. However, there is at least one piece of evidence that the cyst cells outside of the stem cell niche may actually be the origin of these new hub cells. New hub cells express *eyaA3*-Gal4 and β3-tubulin, both of which are markers for the differentiating cyst cells that form septate junctions.

It is difficult to account for how cyst cells outside of the niche could re-enter it to join the hub. Nonetheless, I would propose the following speculative model. When septate junctions are disrupted many of the undifferentiated germ cells die, leaving a free population of cyst cells outside the niche. These cyst cells may stochastically re-enter the stem cell niche, partially de-differentiating into CySCs as they contact the hub. Notch signalling from the hub could then induce the expression of hub specific genes in these cyst cell-derived CySCs. The stochastic nature of such a process could explain why the size of the hub varies so drastically between testes. Such a model would also predict that differentiated cyst cells are more likely then CySCs to convert into new hub cells. This might be due to the differentiating cyst cells being competent to respond to Notch signalling while CySCs are not. This could also explain why endogenous CySCs in wild-type testes do not convert into hub cells despite their continuous contact with the hub.

The possible ability of cyst cells to switch fate and create more hub cells could thus act as a homeostatic mechanism during spermatogenesis. For instance when PGCs are reduced in the embryo a larger hub is formed which can recruit more of the remaining PGCs as GSCs [37]. The hub can also grow in size when GSCs are reduced in the adult testis [47,79,204-207]. This illustrates that hub growth may be a homeostatic response to increase the number of GSCs. While the loss of GSCs is known to cause the growth of the hub, the data presented here suggests that it is more specifically the absence of differentiated germ cells that drives this phenotype. Thus when germline differentiation is disrupted, but GSCs are retained, the hub still grows to a similar extent as when GSCs are absent. Larger hubs can therefore result in a higher number of active stem cells within the niche and could be a general homeostatic response to increase the chances of fertility when spermatogenesis is disrupted.

The ability of a stem cell niche to reorganize and change in response to signals from differentiated progeny is a common theme in stem cell biology [208,209]. In many niches this enables stem cells to respond dynamically to the needs of the tissue [208,209]. For example, the mammalian hair follicle stem cell (HFSC) niche produces an epithelial layer of follicle cells that create each hair [210]. Once a hair is fully-grown it shifts position and brings a subset of follicle cells back in contact with the HFSC niche. These neighbouring follicle cells then supress HFSC activity by inducing a state of quiescence, which prevents the growth of the next hair until after the prior hair has been lost [210]. This illustrates how the presence of cellular structures and signals produced by differentiated cells can directly impact the activity of their stem cell niche. Unlike the mammalian HFSCs, the Drosophila GSCs are continually active and make sperm

throughout adulthood. Therefore, the presence of differentiated germ cells in the testis merely maintains the current activity of the stem cell niche, instead of inducing quiescence. However, if germline differentiation is disrupted, the stem cell niche responds by growing larger as this is one of the few mechanisms that can increase the output of an otherwise continuously active stem cell niche. In this way the homeostatic mechanisms at work in the Drosophila testis stem cell niche are similar to those in many other tissues where the stem niche is regulated by the differentiated cells it produces. Studying how cyst cells transform into hub cells in the *Drosophila* testis can thus illustrate mechanisms that other stem cell niches might use to regulate their activity.

4.3 Limitations and future directions

Although the models I have proposed can explain many of my observations, a number of alternatives mechanisms could explain the phenotypes described in this thesis; these are discussed in the following sections.

4.3.1 Expression of signalling molecules

It is clear that the formation of the somatic permeability barrier regulates the range of BMP signalling in the *Drosophila* testis. However, BMP signalling can also be regulated by other independent mechanisms. For instance, in the *Drosophila* ovary GSCs are attached to cap cells that produce BMP ligands, performing a function similar to hub cells in the testis. In contrast to males where BMP signalling extends to germ cells not contacting the hub, in females BMP signalling is only active in the GSCs contacting the cap cells [24,211,212]. Thus, in females Bam is expressed immediately as each individual germ cell leaves the stem cell niche. Many mechanisms have been postulated for how BMP signalling is restricted to female GSCs. These include the expression of

proteoglycans and collagen molecules that inhibit BMP diffusion, and germ cell autonomous changes in BMP receptors or their responsiveness [212]. Some of these mechanisms also function in the testis [60,80].

There also is evidence in the ovary that a specialized subset of somatic cells can restrict the range of BMP signalling in the ovary. These somatic cells, named escort cells, temporarily envelope each germ cell as it leaves the niche in a process similar to encapsulation. If the EGFR pathway or the actin cytoskeleton regulator Rho are disrupted in escort cells they fail to envelope the germ cells which consequently fail to express Bam or differentiate [203]. This is similar to what occurs when the permeability barrier is disrupted in the testis. However, the ovary does not have a somatic cell-based permeability barrier isolating their germ cells. Together, this suggests that female escort cells and male cyst cells regulate BMP signalling and germline differentiation somewhat differently.

Further comparison with the ovary provides alternative explanations to account for the defective BMP signalling observed when septate junctions are disrupted in the testis. Specifically, STAT signalling induces the expression of BMP ligands in the escort cells, but are only produced at low levels due to repression by a combination of Hh and Wnt signalling [213,214]. If any of these cell-signalling pathways are disrupted, the escort cells show altered expression of BMP ligands and the germ cells fail to differentiate [213,214]. Similar to escort cells, the male CySCs produce BMP ligands under the control of STAT signalling [64]. It is possible that disrupting encapsulation or septate junctions also leads to increased BMP expression by CySCs or the early cyst cell population. An increased expression of BMP ligands could result in the increased range of BMP

signalling observed; however, this interpretation is obfuscated by the accompanying increase in the size of the hub, which could also contribute to any increased expression of BMPs.

A key goal of future studies would be to obtain a more comprehensive understanding of the regulation of BMP signalling in the testes and in particular the role of occluding junctions in this regulation. Specifically, it would be useful if future studies directly visualized the distribution of BMP ligands upon disruption of the somatic permeability barrier.

4.3.2 Cell polarity and signalling

Another mechanism to explain the defective BMP signalling observed when septate junctions are disrupted is a loss of cyst cell polarization. Once cyst cells encapsulate the germline and form the spermatocyst they become inherently polarized with an adluminal side facing the germline and an abluminal side facing the environment. This could allow cyst cells to express BMP ligands but only secrete them on the abluminal side of the spermatocyst, where they would not interact with the encapsulated germ cells. This requires a mechanism for polarized trafficking that would likely be dependent on a functional cell-polarity system. It is known that encapsulating cyst cells require the Scribbled polarity module, suggesting they have at least a rudimentary cell-polarity [169]. It is not known whether or not any additional cell-polarity modules are required in the cyst cells for spermatogenesis, but their expression could indicate stage specific changes in cell-cell signalling activity.

In epithelia it is common to observe occluding junctions and cell polarity modules cooperating to control cell-cell signalling pathways by segregating or sequestering

ligands from their receptors [215]. This mechanism is known to regulate EGFR signalling in both mammalian and *Drosophila* epithelia [216-218]. Apical EGF ligands are segregated from the EGF receptor that is sequestered on the basolateral side of the epithelial cells. Thus, if epithelial integrity is compromised the EGF ligand and receptor can interact leading to localized EGFR/MAPK signalling that reseals the epithelial sheet [215,217,219,220]. This mechanism may also regulate TGF-β signalling, in some epithelia TGF-β ligands are segregated from TGF-β receptors by polarized sorting to the membrane [221-223]. Thus if epithelial integrity is compromised, TGF-β signalling is activated, which can disrupt occluding junctions and promote an epithelial-mesenchymal transition [224,225]. This contrasts with many BMP class receptors, which are polarized but not kept segregated from their BMP ligands, leading to sustained BMP signalling that strengthens occluding junctions and helps maintain epithelial integrity [224,226-228]. Together this illustrates the possible relationships between occluding junctions and signalling pathways that are dependent on cell polarity. Similar mechanisms may also control the changes in EGFR and BMP signalling required during *Drosophila* spermatogenesis.

Future studies should systematically characterize how cyst cells establish cell polarity and distinguish their adluminal and abluminal domains. This analysis may help to elucidate how occluding junctions control germline differentiation.

4.3.2 Origin of new hub cells

The data presented in chapter 3 indicates that cyst cells convert into hub cells when septate junctions are disrupted, however it remains possible that other somatic cells contribute to this process. For example, new hub cells could originate indirectly from the

hub itself. While hub cells do not undergo mitosis, they are able to convert into CySCs when forced to undergo mitosis [48]. Therefore, in principal, hub cells could partially convert into CySCs, undergo mitosis to grow in number, and then convert back into hub cells. If this process involved large changes in the expression of hub cell markers and differentiated cyst cell markers it could lead to the observed results. New hub cells could also be derived from somatic cells that already express markers of both the hub and cyst cell lineages. For instance the SGP-derived terminal epithelium cells at the base of the testis express Unpaired and Fasciclin-III like hub cells, while also expressing *eyaA3*-Gal4 and β3-tubulin like cyst cells. When septate junctions are disrupted the hub and the terminal epithelium are also in close proximity due to the smaller size of the testis. This might allow terminal epithelium cells to migrate apically, express additional markers of both the cyst cell and hub cell lineages, before attaching to the hub and joining it. While both of these alternative explanations for the growth of the hub are possible, they both require multiple steps involving complex changes in somatic cell gene expression. This suggests that the relatively simple conversion of cyst cells directly into hub cells is more likely to be the mechanism by which the hub gains new cells.

Future work should investigate the process by which somatic cells join the hub and begin to function as part of the stem cell niche. A close examination of their transition into hub cells may reveal more details about their origin and the mechanisms driving growth of the stem cell niche.

4.4 Conclusions

Overall my work illustrates the value of the *Drosophila* testis as a model for investigating fundamental mechanisms in spermatogenesis and stem cell biology. This research reveals an evolutionarily conserved process that links the differentiation of the germline with the establishment of an isolating permeability barrier during animal spermatogenesis. This research also demonstrates that the testis stem cell niche is dynamic and can increase in size when spermatogenesis is disrupted, revealing a homeostatic mechanism to increase the number of active stem cells. Finally I conclude that occluding junctions mediate germline differentiation and stem cell niche homeostasis by regulating the activation of cell-cell signalling pathways; underscoring the importance of occluding junctions in shaping signalling activity within the complex architecture of a stem cell niche.

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