SIGNALING PATHWAYS REGULATING CELL DIFFERENTIATION IN GERMLINE AND SOMATIC CELLS OF DROSOPHILA MELANOGASTER

by

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(Under the Direction of Cordula Schulz)

ABSTRACT

Communication between cells is a highly regulated process that requires selective control of signaling events to ensure proper development of tissues. Within all metazoans there exist conserved signaling pathways between different cell types that eventually give rise to specialized cells. While the mechanisms of these signaling pathways have been well characterized through the use of model organisms, how these signaling events define specific cellular contexts still requires further study. The *Drosophila melanogaster* gonad is a well-studied model and provides a genetically tractable platform to ask basic developmental questions using genetic, molecular, and biochemical tools. In both male and female gonads, germline and somatic cells are closely associated with one another and must employ various signaling events in order to eventually form sperm and eggs respectively. Two such signaling events that regulate cell maintenance in the germline and somatic cell lineages are the focus of this study.

Chapter 1 reviews one of the most highly conserved signaling pathways: Notch (N), Drosophila gonadogenesis, and current understanding of N in the gonads. Chapter 2 discusses the novel role N serves in the *Drosophila* testes in modulating germline-soma interactions. Chapter 3 looks at how the COP9 signalosome (CSN) acts through the Cullin RING ubiquitin ligase (CRL) complexes to regulate the germline microenvironment.

INDEX WORDS: *Drosophila melanogaster,* Notch, CSN, spermatogenesis, cell differentiation, germline, soma

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The ability of cells to communicate through signal transduction pathways is a hallmark of multicellularity and a requirement for development. One such pathway, N signaling, is evolutionary conserved and essential for regulating cell fate, cell proliferation, and cell death. The involvement of N signaling in many different cellular processes despite its simplistic mechanism of signal transduction speaks to its importance in development but also its implicated role in many cancers (S. J. Bray, 2006).

Due to the requirement of N in all metazoans, it is essential to understand its function in different cellular contexts. One model we can use to study the role of N signaling in development is the *Drosophila melanogaster* testis. Testes are composed of germline and somatic stem cells and differentiated daughters and the spatio-temporal regulation between cells is essential for spermatogenesis. While N has been studied fairly well in the *Drosophila* female gonads, its function in the *male gonads* is still not well understood. In this chapter I review the N Signaling Pathway, *Drosophila melanogaster* spermatogenesis, oogenesis, and the current understanding of N signaling in gametogenesis.

N Signaling

The canonical N signaling pathway is defined by a relatively simple activation mechanism with binding of two transmembrane proteins: the receptor and ligand. In *Drosophila melanogaster* there is one N receptor and two ligands, Delta (Dl) and Serrate

(Ser). Mammals have four N paralogs (NOTCH1-4) with ligands from the Delta-like (DLL1, DLL3, and DLL4) and Jagged (JAG1 and JAG2) protein families. Receptor-ligand interaction results in susceptibility of the receptor to proteolytic cleavage by the ADAM protease (Fortini, 2002). The resulting intermediate, the N extracellular truncation, is cleaved by γ-secretase releasing the N intracellular domain from the membrane. NICD translocates to the nucleus and associates with DNA-binding transcription factor CSL {C promoter-binding factor (CBF-1). CSL is called recombination signal binding protein for immunoglobulin kappa J region in mice (RBP-J_k), Suppressor of Hairless in flies (Su(H)), and Longevity-assurance gene-1 in *C. elegans* (Lag-1)}. In cooperation with the co-activator Mastermind (Mam), CSL initiates transcription of target genes (S. J. Bray, 2006; Kopan & Ilagan, 2009) (Fig. 1.1). The most well known N targets are the basic helix-loop-helix transcriptional repressors Hairy/Enhancer of split and Hairy-related protein families (Iso et al., 2003).

Versatility of N

The first description of *N* was made by Thomas Hunt Morgan over a 100 years ago in the observation of notched wings in the fly. Since then, our understanding of the signaling pathway has greatly expanded through discoveries in many model organisms. N is responsible for a plethora of functions dependent on both distinct spatial and temporal cell contexts. For example, during *Drosophila* neural development, N signaling specifies neuroblasts and sensory organ precursors from a equipotent pool of ectoderm cells in the central nervous system and peripheral nervous systems respectively (Schweisguth & Posakony, 1992). In Zebrafish, N controls the cell cycle state of neural stem cells (NSC)

from quiescent to proliferative. High levels of N correspond to NSCs maintaining quiescence, while N inhibition leads to increased NSC cell division and differentiation (Chapouton et al., 2010). N in mice drives muscle progenitor satellite cells out of quiescence with aberrant loss of RBP-J_k in adults leading to premature differentiation (Bjornson et al., 2012). These studies demonstrate that N can function to regulate differentiation and fate choice.

Drosophila Spermatogenesis

The *Drosophila* male has a pair of testes that are tubular structures approximately 2 mm-long (Fig. 1.2A). A group of terminally differentiated somatic cells called the hub is situated at the apical tip of the testis. Associated with the hub are 6-9 germline stem cells (GSCs) (Boyle & DiNardo, 1995). Each GSC undergoes asymmetric division to produce a cell still attached to the hub that maintains stem cell identity and a daughter cell that is displaced away from the hub, called the gonialblast. Associated with the GSCs are a pair of CySCs that send out cellular extensions to surround the GSC at the hub. Together, the hub and CySC form the cellular microenvironment for the GSCs, called the stem cell niche (de Cuevas & Matunis, 2011). The CySC daughters enclose and co-differentiate with the GSC daughters to become cyst cells and the gonialblast respectively. The gonialblast then undergoes 4 rounds of transit amplification divisions with incomplete cytokinesis to produce clusters of 16 spermatogonia. The spermatogonia remain connected by cytoplasmic bridges as they develop from 2-cell to 16-cell spermatogonia.

The 16-cell spermatogonia develop into spermatocytes as they grow in size and eventually divide through meiosis and differentiate into 64 spermatids (Hardy, Tokuyasu, Lindsley, & Garavito, 1979; Fuller, 1993 Schulz, Wood, Jones, Tazuke, & Fuller, 2002) (Fig. 1.2B).

Drosophila Oogenesis

The *Drosophila* female possesses a pair of ovaries that contain on average 16 ovarioles (Spradling & C., 1993). Within each ovariole are up to 14 different stages of egg chambers, or follicles, starting with the germarium at the anterior of the ovariole and the egg chamber with the mature egg at the posterior end (Fig. 1.3). The apical end of the germarium contains terminal filament (TF) cells that are followed by somatic cap cells (CpCs). The CpC along with another group of somatic cells, escort stem cells (ESCs), surround 2-3 GSCs to form the female GSC niche (Kirilly & Xie, 2007). Like the *Drosophila* testis, a GSC divides asymmetrically producing one new stem cell and another daughter cell, the cystoblast (CB), that is displaced from the niche. While enclosed by the ESCs, the CB undergoes four rounds of incomplete mitotic divisions, producing 16 interconnected cystocytes (Margolis & Spradling, 1995; Xie & Spradling, 2000). One germline cell becomes the oocyte while the other 15 cells become nurse cells. The 16 cells also become surrounded by a layer of epithelial follicle cells that originate from precursor follicle cells in the germarium. Together, the germline cells and follicle cells eventually form an egg chamber that becomes released from the germarium. The other precursor follicle cells develop into polar and stalk cells that play essential roles for the development of the egg chamber (J. Xu & Gridley, 2012).

N is required for several steps of gonadogenesis.

In the *Drosophila* testes, the somatic cells originate from somatic gonadal precursor cells (SGPs), which are specified in parasegments 10-12 during embryogenesis. Together with the germline cells, they migrate anteriorly through parasegments 12 and 11 to form the gonad at parasegment 10. The SGPs undergo differentiation into somatic cells of the hub and the somatic cyst stem cells (CySCs) (Boyle and DiNardo, 1995). N signaling regulates hub cell specification in the developing gonad. It is opposed by Epidermal Growth factor and Sevenless signaling throughout the gonad except at the anterior end. The SGPs at the anterior tip where only N signaling is active then assume hub cell fate while all other SGPs are specified as CySCs (Kitadate & Kobayashi, 2010; Okegbe & DiNardo, 2011). N is also a negative regulator of the Maf transcription factor Traffic Jam (Tj), a repressor of markers for hub cell fate in the early gonad. Subsequent to N activation in hub cells, Tj is downregulated, relieving repression of the Jak/STAT ligand Unpaired (Upd) from hub cells, which acts on CySCs to maintain them as stem cells (Flaherty et al., 2010; Leatherman & Di Nardo, 2008; Wingert & DiNardo, 2015).

The *Drosophila* female germline stem cell niche is also regulated by N signaling. Dl and Ser from the GSCs induce N signaling in the somatic cells. When N is activated, the stem cell niche grows and the number of cap cells increase. Inhibiting N in the somatic cells causes the cap cell population to decrease and the niche to become smaller in size (Song et al., 2007; Ward, Zhou, et al., 2006).

In subsequent steps of oogenesis, polar cells and stalk cells require N signaling to specify their differentiation from the precursor follicle cells. Loss of Delta in the germline or loss of N in the encompassing follicle cells leads to enlarged egg chambers that are

missing polar cells. Conversely, constitutively activating N in the egg chambers results in more polar cells and longer stalks between the follicles (Grammont & Irvine, 2001).

The layer of follicle cells that surround the germline cells undergo cell cycle programs that are directly regulated by N signaling. In stages 1-6 of oogenesis, the follicle cells undergo a normal mitotic cycle. Following stage 6, follicle cells initiate three rounds of endocycle through replicating their genomic DNA without dividing. This mitotic/endocycle (M/E) switch is induced by the germline cells signaling through Delta to N on the surrounding follicle cells (Deng et al., 2001; López-Schier & St Johnston, 2001). N activation of the zinc-finger protein Hindsight (Hnt) suppresses expression of the homeobox gene *cut* that mediates the M/E switch (Sun & Deng, 2007). Finally, at stage 10B, the posterior follicle cells that surround the oocyte transition from the endocycle to amplification of genes encoding eggshell proteins required for late oogenesis. This process requires downregulation of N to allow for the upregulation of Ecdysone and subsequently the expression of Tramtrack. High levels of Tramtrack halts the endocycle such that follicle cells can enter the amplification stage (Sun et al., 2008).

The role of N in the mouse testis is complicated by inconsistent findings. N signaling components are expressed in the mammalian testis (Dirami et al., 2001; Mori et al., 2003), but loss of *Notch1* and other N signaling components in mammalian germ cells had no distinguishable phenotype in regards to both testes size and fertility. (Batista et al., 2012; Hasegawa et al., 2012). NOTCH1 gain-of-function experiments increased apoptosis in the germline and reduced sperm counts (Huang et al., 2013). Surprisingly, the N-acetylglucosaminyltransferase I, responsible for synthesis of essential N-glycans on the N receptor, is required for spermatogenesis (Batista et al., 2012).

While the role of N in spermatogenesis is still not well understood in the mouse model, we can harness the studies done in the *Drosophila* ovary and apply them to the male gonad. Using this simpler model system will allow a better understanding of the role of this highly conserved pathway in regulating cells in the testes. With only one receptor and two ligands, the N signaling pathway is easily manipulable and controlled in the fly compared to the diverse signaling outcomes afforded to the mouse sysem with four Notch receptors and five canonical Notch ligands. Furthermore, there are established tools in the *Drosophila* field that afford numerous advantages for investigating N signaling in the testes of flies.

Figure 1.1: The canonical N signaling pathway.

Cartoon showing the basic steps in N signaling. N ligand expressed on the signaling cell interacts with the N receptor on the signal-receiving cell. N activation leads to consecutive proteolytic cleavage events on the N receptor by the metalloprotease ADAM (S2) followed by γ-secretase cleavage (S3). Release of the NICD to the nucleus and subsequent recruitment of co-activators (CoA) to the CSL complex culminates in transcriptional activation of N target genes. Based on (Kume, 2009).

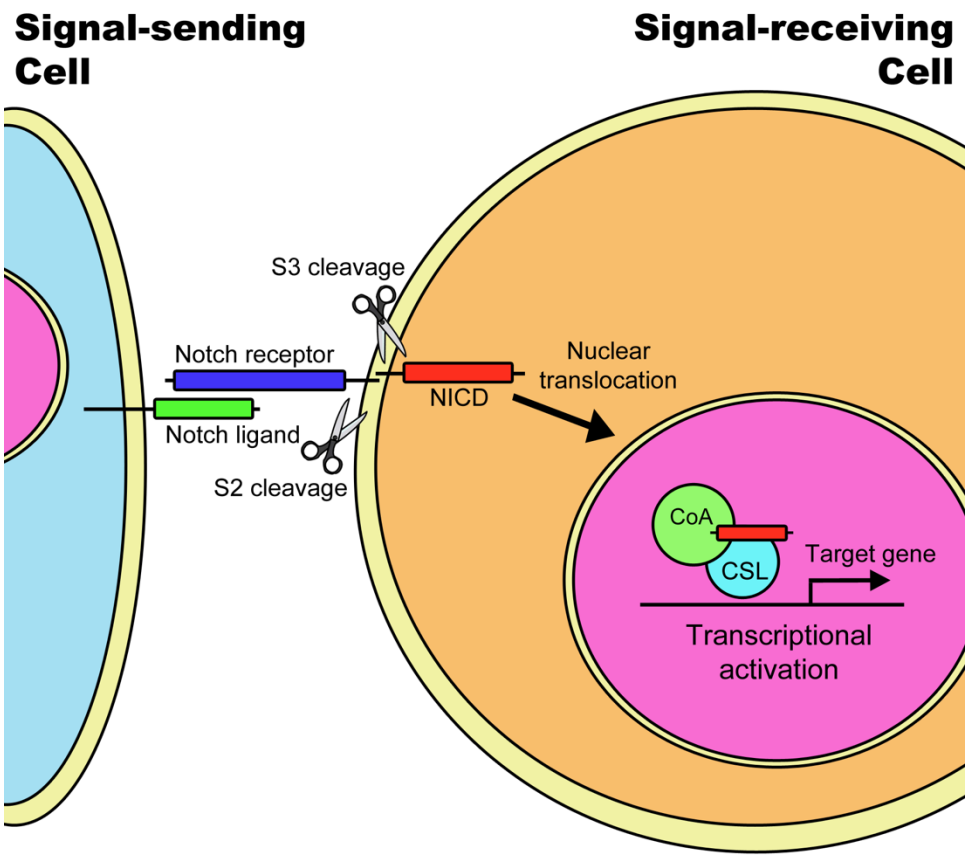

Signal-sending Cell
Signal-receiving Cell
S3 cleavage
Notch receptor
Notch ligand
S2 cleavage
NICD
Nuclear translocation
CoA
CSL
Target gene
Transcriptional activation

Figure 1.2: The *Drosophila* testes.

(A) Diagram of the adult male Drosophila testis. The testis is connected to the ejaculatory duct of the reproductive system via the seminal vesicle (arrow). Adapted from (Kuckwa et al., 2016).

(B) An illustration depicting organization and development of the germline cysts along the apical (left) to basal (right) axis of the testis. GSCs attached at the hub divide to form daughter cells that differentiate into spermatocytes and eventually mature spermatids. CySC: cyst stem cell; GSC: germline stem cell.

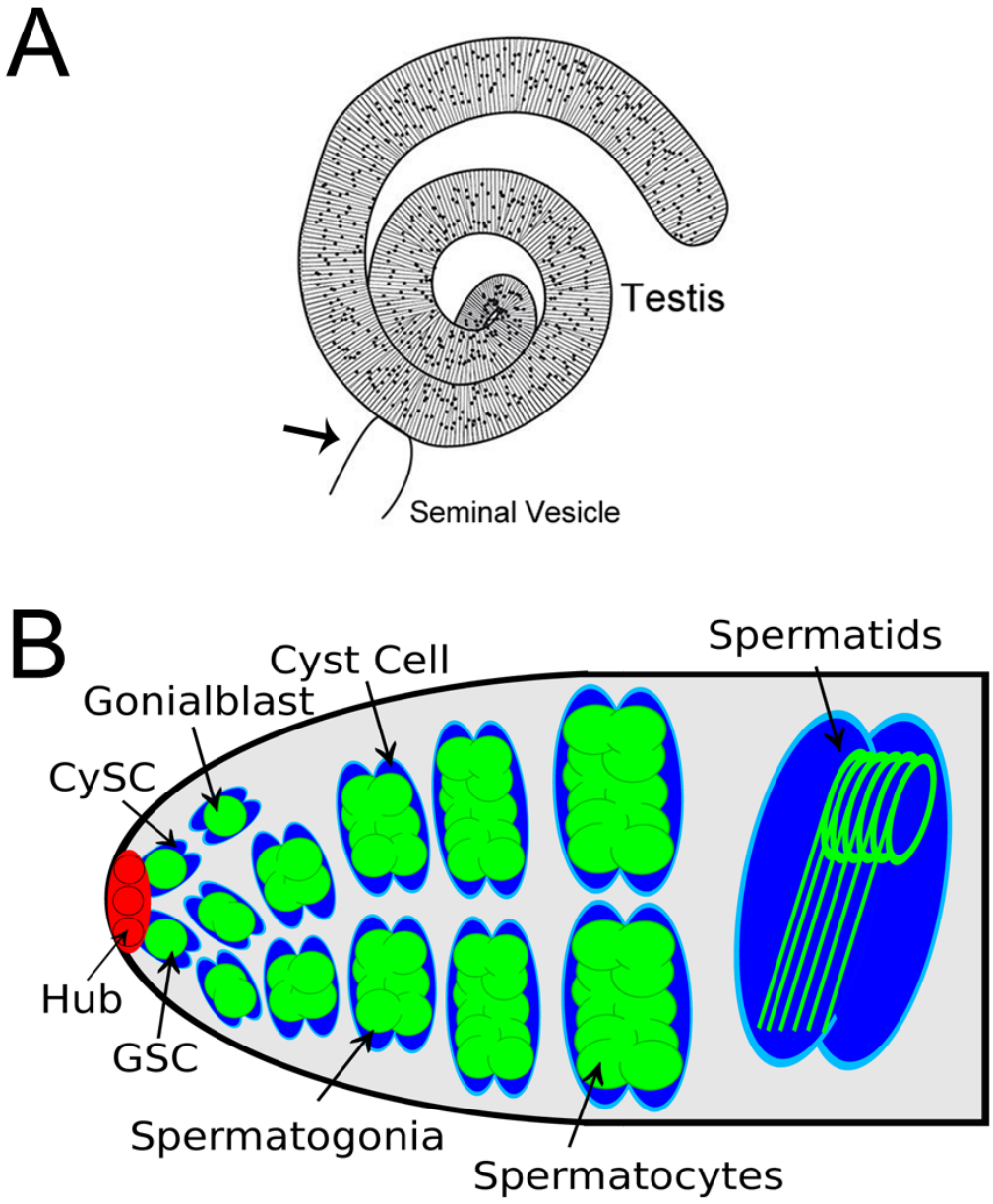
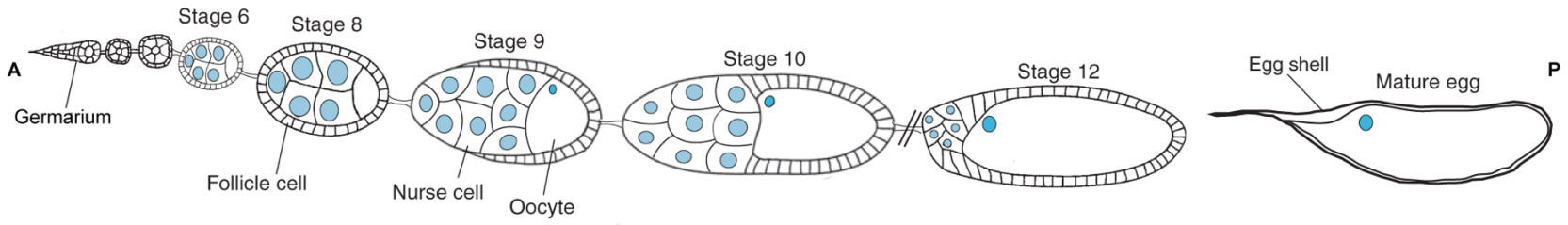

A
Testis
Seminal Vesicle
B
Spermatids
Cyst Cell
Gonialblast
CySC
Hub
GSC
Spermatogonia
Spermatocytes

Figure 1.3: The *Drosophila* ovary.

An illustration depicting the development and organization of the female gonads with the germarium at the anterior (A) and a fully developed egg posterior (P). The egg chambers are connected by stalk cells. Adapted from (Becalska & Gavis, 2009).


A
Germarium
Stage 6
Stage 8
Follicle cell
Stage 9
Nurse cell
Oocyte
Stage 10
Stage 12
Egg shell
Mature egg
P

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CHAPTER 2

NOTCH SIGNALING REGULATES DIFFERENTIATION AND MAINTENANCE OF GERMLINE-SOMA CYSTS IN TESTES OF DROSOPHILA MELANOGASTER[1]

[1] Ng C. and Schulz C. (2018). To be submitted to *PLOS Genetics*

Abstract

Notch (N) signaling is a highly conserved pathway responsible for a myriad of developmental programs across all metazoans. Proper communication between cells is required to orchestrate the temporal and spatial processes involved in cell differentiation and cell fate. In *Drosophila melanogaster* ovaries, N has been well described in regulating some of these cellular interactions between germline and somatic cells, but its role in the adult testis is still not well understood. In the male gonad, germline cells are completely enclosed by the cellular extensions of somatic cyst cells. Components of the N signaling pathway are present in testes and were detectable by immunofluorescence. Loss of the N ligand Delta (Dl) from the germline and knockout of N in the somatic cells both revealed a requirement for N signaling in germline-soma cyst maintenance. Activation of the N pathway disrupted germline and somatic differentiation. We propose that Notch signaling is required in testes for cyst maintenance and is sufficient to mediate cyst cell differentiation.

Introduction

N Signaling is involved in a variety of developmental contexts such as nervous system formation, cardiac patterning, and sensory hair formation (Guruharsha et al., 2012; Jain et al., 2010; MacGrogan et al., 2010). Its mechanism of signal transduction requires direct cell-to-cell contact. Such intimate cell contact is found in *Drosophila* testes, where communication between two groups of cells drives successful production of sperm. However, the role of N has not been well studied in this model.

The testis contains a group of terminally differentiated somatic cells at the tip called the hub. Attached to the hub are two populations of stem cells: germline cells and somatic cyst cells. GSCs attach to the hub in a rosette and are associated with a pair of cyst stem cells (CySCs). Both stem cell populations undergo divisions to self-renew and to produce daughter cells, the gonialblasts and cyst cells respectively. The gonialblast is enclosed by two cyst cells and together the cells are referred to as a cyst. Through subsequent transit amplifying mitotic divisions, clusters of early-stage germline called spermatogonia develop from the gonialblasts. The spermatogonia become spermatocytes, grow in size, and eventually differentiate into spermatids. Concurrent with the differentiation of the germline, the surrounding cyst cells also grow in size and differentiate with the germline. The intimate cellular contact formed by the germline and encompassing cyst cells is maintained throughout spermatogenesis (Fuller, 1993; Hardy et al., 1979; Sarkar et al., 2007).

Here we present the expression of N signaling molecules in the adult testes and a functional analysis of loss and gain-of-function phenotypes. We discovered that loss of Dl in the germline or loss of N from the soma led to a failure of the testes to maintain

germline cysts. Overactivation of the N pathway in the soma led to cell differentiation defects in both the germline and somatic stem cell lineages.

Material and Methods

Fly husbandry

Flies were maintained on a standard cornmeal-agar diet. The following stocks were used: UAS-Cas9 (kind gift from Ting Xie), N gRNA constructs (Gao et al. 2014), UAS-Su(H)VP16 (kind gift from Patricia Estes). Other stocks were obtained from the Bloomington Drosophila Stock Center (BDSC) (Table 1).

UAS-Gal4 Expression studies

Crosses were set up and progeny collected on apple juice-agar plates. Animals were then transferred into food bottles, raised at 18^{o}C and shifted to 29^{o}C as adults. Timeline experiments were performed by shifting flies as adults for an extended period between 0-14 days.

N CRISPR

gRNA constructs were injected into w^{1118} flies by the company The Best Gene. These stocks were crossed with UAS-Cas9 and then the progeny mated with Gal4 transactivators to specifically express N CRISPR in the wing margin or the soma of the testes in the next generation.

Clonal analysis

Mitotic clones were produced using the FLP/FRT technique of site-specific recombination and GFP as a negative marker (T Xu & Rubin, 1993). One to three-day-old adult flies were heat shocked for one hour at 37°C for three consecutive days. FLP/FRT lines FRT82B Dl^{RevF10}, Ser^{Rx106} and FRT82B^{rosy} were crossed to hs-flp; FRT82B-GFP. Males were raised at 25°C and testes were dissected seven days after heat shock.

Immunofluorescence and image analysis

Testes were isolated as described previously (Parrott et al., 2012). Testes were stained by a combination of different antibodies. The following antibodies were used: mouse anti-Eyes Absent (1:10), rat anti-Vasa antibody (1:10), mouse anti-NICD (1:10), mouse anti-NECD (1:10) (C458.2H), mouse anti-Hindsight (1:10), mouse anti-spectrin (1:10) were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, rabbit anti-Armadillo (Arm) antibody (1:200) (Santa Cruz Biotechnology Inc.), goat anti Vasa (Santa Cruz Biotechnology Inc.) (1:500), rabbit anti-phosphorylated Histone-H3 (pHH3) (Santa Cruz Biotechnology Inc.) (1:500), rabbit anti-GFP antibody (1:250) (Life Technologies), guinea pig-anti-Traffic Jam antibody (1:5000) was a gift from Dorothea Godt, rabbit anti-Zfh-1 (1:100) was a gift from Ruth Lehman, Alexa-488, 568, and 647-conjugateed secondary antibodies (Invitrogen) (1:1000). Images were taken using a Zeiss Axiophot with a digital camera and apotome and processed using

Axiovision Rel. software. Images were analyzed using ImageJ and processed with Adobe Photoshop. Tj-positive cells were quantified in ImageJ.

Apoptosis detection

We used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect cells in apoptosis. Testes were fixed as previously described (Parrott et al., 2012) and then labeled according to the manufacturer's instructions using the Apoptag Red In Situ Kit (S1765 Milipore). Statistical analysis was performed using the Student's T-test in GraphPad software.

Western Blotting

Testes were dissected in 1x Tissue Isolation Buffer (1x TIB: 183 mM KCl, 47 mM NaCl, 10 mM Tris, pH 6.9), homogenized in Laemmli sample buffer (Bio-Rad) with 5% 2-Mercaptoethanol and 1% Halt Protease Inhibitor Cocktail (Thermo Scientific), and then incubated for five minutes in boiling water. The protein mixture was centrifuged at 13,000 rpm for 20 minutes at 4^{o}C. The supernatant was fractionated on 10% SDS-PAGE at 120V for 1 hour. The gel was then transferred onto Amersham Hybond ELC Nitrocellulose Membrane (GE Healthcare) at 10V for 30 minutes using a semi-dry transfer cell. The membrane blots were incubated in 1x TBS-T blocking solution (5% BSA in Tris-buffered saline and 0.1% Tween 20) at 4^{o}C overnight. Membranes were incubated with primary antibodies mouse anti-Hindsight (DSHB) (1:100) or mouse anti-Delta (DSHB) (1:100) at 4^{o}C overnight. Primary antibody was detected by peroxidase

conjugated secondary antibody (1:10,000) (Sigma Aldrich). Visualization of protein was done by Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Molecular Techniques

Single wings or testes were taken from males and genomic DNA was extracted by incubating tissue with squishing buffer (10 mM Tris-Cl pH 8.2, 1mM EDTA, 25 mM NaCl) at 37^{o}C for 20 minutes, followed by an incubation with 20 mg/ml Proteinase K and 1 ug/ml RNAse (Life Technologies) at 95^{o}C for 2 min. Standard PCR Reactions were set up and the products were run on 15% Acrylamide gel. The functionality of the N gRNA constructs were verified by crossing them to the *C96*-Gal4 driver and observing defects along the wing margin.

The following primers were used in this study:

Notch Total F1: 5'-ATTTTCTTGAACGTTCGGCCGC-3'

Notch Total R1: 5'-ATTGATTTGACATCGTGCGCCC-3'

Notch Total F2: 5'- GCGAGGGAGATAGAGCGCTTAG-3'

Notch Total R2: 5'-ATCATCCAGGCAAGATCCCTCG-3'

Results

N is localized to the somatic lineage in Drosophila testes

N signaling components such as N and Dl were detected through RNA expression analysis of adult male gonads (Kudyba et al., unpublished data). N expression was confirmed using immunofluorescence experiments. In wildtype testes, the germline marker Vasa is expressed in the cytoplasm of all germline cells. These germline cells are surrounded by the cytoplasmic extensions of the encompassing cyst cells that express high levels of the adherens junction protein Armadillo. We observed high levels of expression of the Notch Intracellular Domain (NICD) as first reported by (Tian Xu & Artavanis-Tsakonas, 1990) that colocalized with Armadillo at the membranes of the cyst cells (Fig. 2.1A-A'', arrowheads). Consistent with co-localization of NICD with Armadillo, the Notch Extracellular Domain (NECD) was also highly expressed at the cyst cell membranes (Fig. 2.1B-B''). Although we were not able to observe specific expression of Dl within the testes through immunofluorescence, we did detect a signal through Western blot corresponding to the expected protein size ~ 62 kDa (Fig. 2.1C-C'') (Klueg et al., 1998).

To investigate at which stage N is active, we utilized a N reporter, Notch Response Element enhanced GFP (NRE-EGFP), as a readout for N activity (Bray & Furriols, 2001; Saj et al., 2010). This reporter contains two pairs of Suppressor of Hairless [Su(H)] binding sites as part of its enhancer element upstream of EGFP. Su(H) is a DNA-binding protein that normally forms a complex with NICD to initiate transcription of target genes (Mumm & Kopan, 2000). When NICD is not present at the NRE-EGFP promoter, Su(H) functions as a repressor and no GFP is expressed. However, in the

presence of NICD, the complex is formed and initiates transcription of target genes which corresponds to expression of GFP. We observed Su(H)-dependent GFP expression in the somatic lineage corresponding to the transition zone. This area is defined by the germline and somatic cells undergoing specific morphological and molecular changes. At this point, spermatogonia have reached the final two stages of transit amplifying divisions and thereafter will develop into spermatocytes. Additionally, cyst cells change the expression of two transcription factors: Traffic-Jam (Tj) and Eyes Absent (Eya). Tj is expressed at high levels in cyst cells that surround GSCs, gonialblasts, and the spermatogonia (early-stage cyst cells) (Fig. 2.2A, A''). By the time cyst cells have fully developed and surround the spermatocytes (late-stage cyst cells) Tj expression is downregulated. Eya is expressed at very low levels in the early-stage cyst cells but at high levels in the late-stage cyst cells (Fabrizio et al., 2003) (Fig. 2.2A, A'''). Thus, the transition zone is defined by a reduction in Tj and an increase in Eya expression. Even though we observed high expression of NICD we did not detect high levels of GFP expression near the hub (Fig. 2.2B-B''). High levels of GFP were expressed in the cyst cells of the transition zone and colocalized with Eya expression, continuing on towards the basal end of the testes (data not shown). We conclude that N becomes active in the transition zone in testes.

Expression of dl-RNAi in the germline leads to loss of cells in the transition zone

To investigate the role for N activation, we first knocked down Dl in the germline. As *dl* mutations are embryonic lethal, we employed the FRT/Flp system to generate negatively GFP-marked clusters of cells (Theodosiou & Xu, 1998). In this system, all cells express ubiquitin GFP and upon heat shock, flp induces recombination to induce GFP-negtive

clonal cells. We had difficulties identifiying GFP-negative GSCs or their immediate daughers because the GFP-signal was too weak. Therefore we foucsed on later-stage clusters of germline cells that were clearly GFP-negative in the remainder of the testes where the differentiated spermatocytes were located. In control animals, 39.1% of testes (n=46) testes contained one or more GFP-negative clusters of germline cells (Fig. 2.3A-A''). In the experimental animals however, only 3.8% of testes (n=80) had a single cluster of GFP-negative, *dl* mutant germline clone (Fig. 2.3B-B''). We also never detected more than one GFP-negative clone from each testes of the experimental group. We conclude that *dl* mutant germline clones are either rarely formed or rarely survive to the point where they develop into spermatocytes.

In order to address why germline is reduced upon loss of Dl, we used the UAS-Gal4 tissue-specific expression system (Duffy, 2002). To specifically express *dl*-RNAi in the germline, we used the *nanos*-Gal4 (NG4) transactivator and to express targets in the soma we used the *tj*-Gal4 transactivator. Reducing *dl* in the soma had no observable phenotype, but knockdown of *dl* in the germline caused a dramatic loss of germline cells. Upon 4 days after the shift to the restrictive temperature of 29ºC, NG4/*dl*-RNA*i* (NG4/*dl-i*) animals had similar morphology to those kept at the permissive temperature of 18ºC (n > 20, Fig. 2.4A-B'). When animals were shifted to the restrictive temperature for 6-14 days, testes appeared long and thin and displayed a range of germline loss (n > 20, Fig. 2.4C-G'). While only some testes displayed loss of all germline cells from the apical tip and had an accumulation of Tj-positive somatic cells (Fig. 2.4F, F'), the majority of testes still had GSCs and some early-stage germline cells adjacent to the hub, but showed differing levels of loss of later-stage germline cells. Some of the cysts contained

degenerating germline and/or single spermatogonia or spermatocyte (Fig. 2.4G-G'). These testes also had no or few sperm bundles, suggesting a failure of terminal differentiation (Fig. 2.4H') compared to controls (Fig. 2.4I'). Further analysis using somatic markers showed that Eya-positive cells were still present in NG4/*dl-i* testes, but a significant amount of cells appeared lost from these testes (Fig. 2.4H-H''') compared to controls (Fig. 2.4I-I'''). To confirm that NICD is expressed in the soma, we labeled NG4/*dl-i* testes raised at 29ºC for 14 days with anti-NICD antibody and discovered that in testes with reduced germline, NICD was still clearly present in the soma (Fig. 2.5A-A''').

In order to address if cells in NG4/*dl-i* testes were lost due to apoptosis, we used TUNEL and observed that testes indeed contained increased numbers of TUNEL-positive spots compared to controls (Fig. 2.6A, B). We observed the most cell death at five to eight days after the temperature shift and within the transition zone (Fig. 2.6C). This is consistent to the observation of testes lacking germline cells in NG4/*dl-i* testes. We conclude that N signaling is required for cyst survival.

To examine if *dl* is required specifically in the germline within the transition zone we expressed *dl*-RNA*i* using the spermatogonia-specific *bag-of-marbles* Gal4 transactivator (*bam*-Gal4) which is strongly expressed in 8 and 16-cell stage spermatogonia. *bam*-Gal4/*dl-i* produced very few testes with a phenotype similar to NG4/*dl-i* ($n > 20$ Fig. 2.7C, C'). Most testes resembled control testes with no loss of germline and containing fully differentiated sperm (Fig.2.7B, B'). This observation suggests that either Dl is required earlier than the bam-Gal4 is expressed or that the expression level of this transactivator is not high enough to produce a reliable phenotype.

N is required for cyst maintenance

We next investigated if N is required in the soma. N mutant alleles and expression of N-RNA*i* in adult testes did not produce a phenotype in our hands (Table 1). Therefore, we utilized the UAS-Gal4 system in conjunction with CRISPR-Cas9 to specifically express Cas9 in *Drosophila* testes to mediate N gRNA targeting of the *N* gene (Fig. 2.8). We first verified gene knockout by expressing Cas9 in the wing margin where N is known to be expressed using the *C96*-Gal4 transactivator (Fig. 2.9B, C) (Xue et al., 2014). All progeny observed possessed wing phenotypes demonstrating the high penetrance of the construct and targeting specificity (Fig. 2.9A). Driving expression of N CRISPR using *tj*-Gal4 transactivator in the testes produced a phenotype similar to NG4/*dl-i* where germline cells were not visible in the transition zone (Fig. 2.10B, B') as compared to controls (Fig. 2.10A, A'). TUNEL analysis at 14 days after temperature shift revealed that testes contained significantly more TUNEL-positive spots than controls (Fig. 2.10C-E). We conclude that N is required in the somatic lineage to maintain germline-somatic cysts.

Activation of N caused accumulation of early-stage cyst cells with CySC characteristics.

To explore if overactivation of N in testes had an effect, we first overexpressed Dl ligand in the germline using NG4. We observed a significant increase in Tj-positive cyst cells in NG4/UAS-*dl* testes (Fig. 2.11B, B') compared to controls (Fig. 2.11A, A'). A careful analysis of flies shifted to restrictive temperature over 12 days revealed that testes with overexpression of Dl had significantly increased numbers of Tj-positive cells compared to controls (Fig. 2.11C). In wildtype testes, only cyst stem cells are mitotic while cyst

cells are post-mitotic based on labeling with the mitotic marker pHH3 and Tj (Fig. 2.11 D-D'"). Dl overexpression testes had pHH3- and Tj-positive cyst cells in positions far away from the hub (Fig. 2.11E-E'''). We also observed that within the transition zone, there appeared to be more early-stage germline based on germline markers Vasa and anti-Spectrin. Spectrin labels the cytoplasmic fusome that connects the interconnected germline cells. As the germline cells grow the fusome becomes larger and more branched (Fig. 2.11F, F''). When Dl is overactivated in the germline, we observed a cell-autonomous effect where many of the germline cells have less branched fusomes reflective of an early-stage germline state (Fig. 2.11G-G''). These data suggest that expression of Dl in the germline leads to cells maintaining early-stage activity.

We next ectopically activated Su(H) and N using two constructs: Su(H)VP16 and UAS-*Dl::N.ΔECN* (UAS-NICD). Su(H)VP16 is a fusion of Su(H) with the viral activation domain VP16. Su(H)VP16 has been shown previously to closely phenocopy Notch activation effects (Cooper et al., 2000). UAS-NICD contains a fusion of the *dl* start and membrane transport signal sequence fused to the coding region of the N transmembrane and intracellular domains (Baker & Schubiger, 1996). Both constructs are constitutively active and ligand independent. Testes with overactivated Su(H) in the soma exhibited accumulation of cells that expressed markers for cyst stem cells and early stage-cyst cells respectively: anti-Zinc finger homeodomain-1 (Zfh-1) and anti-Tj but lacked expression of the later-stage cyst cell marker Eya (Fig. 2.12B''') compared to controls (Fig 2.12A'''). Furthermore we detected pHH3- and Tj-positive cyst cells in positions far away from the hub (Fig. 2.12D-D''') compared to controls that only have mitotically active somatic cells adjacent to the hub (Fig. 2.12C-C'''). We also labeled

testes with NICD and found that compared to controls (Fig. 2.12E-E''), Su(H) activated testes exhibited expanded NICD and Tj expression near the apical tip of the testes (Fig. 2.12F-F''). We conclude that Su(H) overactivation in the soma leads to cyst cells maintaining cyst stem cell-like activity.

Overactivation of N in the soma using UAS-NICD had a slightly weaker phenotype compared to Su(H)VP16. Testes stained with the DNA-binding fluorescent dye, 4', 6-diamidino-2-phenylindole (DAPI) reveals germline and somatic cell nuclei. In wildtype testes, the DAPI-stained nuclei are seen as small puncta at the apical end of the testes and the signal becomes more diffuse in nuclei further basal down the testes (Fig. 2.13A, A'). With N overactivation the testes were filled with small puncta marked by DAPI suggesting that most of the cells were in early-stages (Fig. 2.13B, B'). Using somatic markers revealed that N overactivated testes had persistent Tj expression even in cyst cells far from the apical end that also expressed Eya (Fig. 2.13B'', B''') as compared to controls (Fig. 2.13A'', A'''). We conclude that N overactivation in the soma maintains early cyst cell identity.

N overactivation in the soma also led to germline differentiation defects where germline cells throughout the testes resembled early-stage germline cells. When N is overactivated in the soma, we observe a nonautonomous effect where most of the germline cells have less branched fusomes reflective of an early-stage germline state (Fig. 2.13D'', D''') compared to controls (Fig. 2.13C'', C'''). Germline cells stained with Vasa and DAPI also resemble early-stage cells (Fig. 2.13D, D') seen at the apical tip of wildtype testes (Fig 2.13C, C'). Thus, we conclude that activation of N signaling in the

soma is sufficient to induce cells to retain early-stage identity in both germline and somatic lineages.

N target Hnt is active in testes

The N target Pebbled/Hindsight (Hnt) is well characterized in the *Drosophila* ovary and is responsible for mediating the switch between mitosis and the endocycle in the somatic follicle cells where cells undergo DNA synthesis without divisions (Sun & Deng, 2005). Through Western Blotting, Hnt is detected in testes extracts (Fig. 2.14A, and Anti-Hnt expression colocalized with NRE-EGFP-positive cells in the transition zone (Fig. 2.14B-B''). We reasoned that since we detected Hnt in the testes, perhaps testes have an endocycle similar to ovaries. However, we did not detect somatic cells positive for S-phase marker Edu displaced far from the stem cell niche in wildtype testes (data not shown). This suggests Hnt does not play a parallel role in testes. Interestingly, Hnt expression is also detected in the germline within the late-stage spermatogonia. Hnt expression is most pronounced in germline and soma in transition zone (Fig. 2.14C-C'''). In testes where Dl was overexpressed in the germline, we observed an expansion of Hnt-positive cells in both the germline and somatic lineage (Fig. 2.15B-B'') compared to controls (Fig. 2.15A-A''). However, Hnt RNAi studies fail to reveal a phenotype similar to those observed with loss of Dl or N expression (data not shown). Based on our data, Hnt expression may be mediated by N signaling but serves an endocycle-independent role in testes.

Discussion

Previous studies revealed a role of N during embryogenesis (Boyle & DiNardo, 1995; Kitadate & Kobayashi, 2010; Okegbe & DiNardo, 2011). Here we show that N signaling components are essential for germline and somatic cell maintenance and differentiation in the adult *Drosophila* testes. We discovered that N is expressed and required in the somatic lineage and its ligand Dl is required in the germline. We detected NICD on the cell membrane of somatic cells and not in the nucleus most likely because of lack of specificity of the anti-NICD antibody to the cleaved N (Zacharioudaki & Bray, 2014). Furthermore, the activity of the NRE-EGFP reporter in the somatic lineage provides strong evidence for a somatic role for N.

NICD is strongly expressed at the apical region of the testes and the NRE-EGFP reporter becomes active in the transition zone. Our data show that N is required for differentiation and survival of the germline-soma cysts in the transition zone. We also show that when N signaling is attenuated either through loss of Dl in the germline or through loss of N in the soma, dramatic cell death is observed. Previous observations showed that proper communicatoin from the germline to the soma is essential for cell differentiation, potentially through a signal feedback signal from the soma to the germline (Griswold, 1998; Hudson et al., 2013). Similarly, we observed that germline maintenance is compromised when N signaling is attenuated. This adds further evidence to the proposed existence of a feedback mechanism from the encompassing somatic cells to the germline. It is possible that without Dl signal from the germline, somatic cells are unable to either recognize the enclosed germline cells or properly differentiate together with the germline, ultimately leading to germline-cyst apoptosis. It is well established

that N receptor-ligand interaction provides a strong adhesive force between two communicating cells (Murata & Hayashi, 2016). Failure to maintain cell adhesion may contribute to the inability for cysts to properly differentiate in unison and eventual cell death.

An apoptotic phenotype was also reported in *eya* mutant cyst cells. While depletion of *eya* did not affect spermatogonial development, loss of spermatocytes is pronounced (Fabrizio et al., 2003). However, *eya* mutants used in this study were not null for *eya* function so the residual Eya protein might prevent earlier defects. In ovaries, Eya functions downstream of N through Extra macrochaetae protein to regulate somatic cell fate specification and differentiation (Adam, 2004). It is possible that Eya serves as an effector of N signaling in testes, regulating the differentiation of cyst cells from early to late stage in the transition zone.

In addition to exploring the requirement of N signaling in cyst maintenance, we also looked at the effect of ectopic N activity. Overactivation of N led to an excessive increase in the numbers of somatic cells, however overexpression of Dl in the germline had a less dramatic effect. Most likely the binary nature of binding Dl to the N receptor limits the potential activation of the N pathway with Dl overexpression in the germline as compared to the direct ligand-independent activity of the N and Su(H) activation constructs. Both constructs that stimulated N signaling in the soma caused an increase in the expression of Tj in cyst cells. However based on our findings, we hypothesize that N hyperactivity promotes cyst stem cell identity in the soma. It is possible that we created a situation that mimics the interactions between hub cells and cyst stem cells either during gonad formation or in the adult. During the initial stages of gonadogenesis, N is required

for differentiation of the somatic gonadal precursor cells into the somatic hub cells and the somatic CySCs (Boyle & DiNardo, 1995).

N signaling may also play a role in the niche of adult testes. This idea is supported by several observations: 1) Dl lac-Z is expressed in the hub of adult testes (Fairchild et al., 2016); 2) The NRE-EGFP reporter also is expressed, albeit not consistently, in the hub (data not shown); 3) Neuralized (Neu), an E3 ubiquitin ligase associated with the endocytosis of Dl (Le Borgne et al., 2005), was shown to be required in the GSC and spermatogonia for germline maintenance (Terry et al., 2006). However, there are cases where Neuralized acts in a different cell type than Dl, suggesting that Neuralized also acts in other signaling pathways. This is the case in the ovary, the wing vein and the wing margin (Lai & Rubin, 2001; López-Schier & St Johnston, 2001). Interestingly, a widely used LacZ reporter for *neuralized* is expressed in testes in the early-stage germline (data not shown) at the apical tip where N is not activated, suggesting that *neuralized* does not play a role in N signaling within the transition zone.

Another aspect of N signaling that requires further study in the testes is the role of cis-interactions between Notch and its ligands. While canonical N signaling involves binding of N receptor to ligand to initiate N signaling, non-canonical signaling that is ligand-independent can also lead to N activation (Del Álamo et al., 2011; Micchelli et al., 1997). A previous study in ovaries showed that ligands on the same cell as Notch can inhibit the ligand-independent activity of Notch signaling (Palmer et al., 2014). It may be possible that a ligand-independent Notch effect can influence cell maintenance and differentiation in the testes.

Hnt may be regulated through N in the Drosophila testes

Direct targets of N have been identified such as Hnt and Su(H) (Jia et al., 2015; Krejcí et al., 2009). Hnt is involved in numerous developmental processes outside of the gonads in *Drosophila.* During embryonic development, *hnt* is required for maintenance of the amnioserosa and germ band retraction (Yip et al., 1997). Hnt is also required for neuronal morphogenesis in adults (Pickup et al., 2002). Hnt is described as being regulated by N but surprisingly, *hnt* expression may regulate Dl in the *Drosophila* eye as Hnt binding sites are found upstream of the *dl* gene (Pickup et al., 2009).

Hnt is also responsible for the M/E switch in ovaries (Sun & Deng, 2007). Hnt expression in ovarian follicle cells colocalizes with NRE-EGFP (Palmer et al., 2014). While there are many other examples of developmentally programmed endocycles in multicellular organisms (Edgar et al., 2014), there is no study that has established endocycles in the Drosophila testes. We detected Hnt in testes within the transition zone and throughout late-stage somatic cells. Hnt expression expands with the overexpression of Dl in the germline. Hnt colocalization with NRE-EGFP in the somatic cells is similar to Hnt expression in ovaries where late-stage somatic cells express Hnt. However, Hnt expression in follicle cells is downregulated during late oogenesis and then re-upregulated facilitating ovulation of fertilizable oocytes (Deady et al., 2015, 2017). Hnt expression was not observed to be downregulated and then upregulated again in the late-stage cyst cells in the testes (data not shown).

Conservation of N in spermatogenesis

N signaling is highly conserved across all metazoans. We have shown evidence that N is expressed and required in *Drosophila* testes. However, there are conflicting reports on the role of N in the mouse testes. Previous studies have shown that mammalian Notch components and its multiple ligands are present in testes (Dirami et al., 2001; Mori et al., 2003). However, there is also evidence that loss of individual N components do not result in a visible phenotype (Batista et al., 2012; Hasegawa et al., 2012). These N-deficiency studies relied on a conditional deletion in the *Pofut1* gene. *Pofut1* encodes a protein responsible for transfer of O-fucose to the N receptor that modulates the ability of N to respond to ligands (Wang et al., 2001). The mammalian endoplasmic reticulum alpha-glucosidase I can compensate for Pofut 1 function (Stahl et al., 2008). Furthermore, *N* mouse studies are complicated by the possibility of redundancy between the N receptors (Kitamoto et al., 2005). Interestingly, N-acetylglucosaminyltransferase I, responsible for synthesis of essential N-glycans on the N receptor, is required for spermatogenesis (Batista et al., 2012). Further studies are needed to confirm a N requirement in mouse testes and if loss of N function can be compensated by multiple receptors.

N activation studies also do not offer conclusive evidence on the role of N in mouse spermatogenesis. One study found that constitutive activation of Notch1 in testes did not affect expression levels of *Notch2, Notch3, and Notch4* (Huang et al., 2013). In testes where N signaling is activated in Sertoli cells, the somatic cells experienced premature differentiation and the germline migrated prematurely leading to apoptosis (Garcia et al., 2013). Our further studies on N may shed light on the various roles it could serve in spermatogenesis.

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Table 1: List of stocks from the Bloomington Drosophila Stock Center used to modify N signaling components in both the germline and soma.

Each stock listed includes stock numbers (BL#), description, genotype, and if phenotype was detected when crossed to Gal4 transactivator(s): germline (NG4, *bam*-Gal4) or soma (*tj*-Gal4, *eya*-Gal4, *C784*-Gal4).

BL#	Description	Genotype	Phenotype Observed
5830	UAS-constitutively active Notch	*y1 w*; P{UAS-Dl::N.ΔECN}B2a2*	Yes
26674	UAS-constitutively active Notch	*y1 w*; P{UAS-Dl::N.ΔECN}B2a3*	Yes
26820	UAS-Notch full length	*w*; P{UAS-Nfull}3*	No
26872	UAS-Enhancer of split	*w*; P{UAS-E(spl)m8-HLH.T}3*	No
9319	UAS-Delta	*y1 w*; P{UAS-dl.H}2*	No
26695	UAS-Delta	*w*; P{UAS-Dl.J}3*	Yes
26696	UAS-Delta with C-terminal RFP tag	*w*; P{UAS-Dl-mRFP}3/TM6B, Tb+*	Yes
26697	UAS-dominant negative Delta intracellular domain	*w*; P{UAS-Dl.DN}TJ2/CyO*	No
26698	UAS-dominant negative Delta intracellular domain	*w*; P{UAS-Dl.DN}TJ3*	No
6300	FRT82B Dl, Serrate	*Dl[RevF10] e[*] Ser[RX82] P{ry[+t7.2]=neoFRT}82B/TM6B, Tb[1]*	Yes
36784	UAS-Delta RNAi	*y1 sc* v1; P{TRiP.GL00520}attP40*	No
34322	UAS-Delta RNAi	*y1 sc* v1; P{TRiP.HMS01309}attP2*	Yes
28032	UAS-Delta RNAi	*y1 v1; P{TRiP.JF02867}attP2*	No
33616	UAS-Notch RNAi	*y1 v1; P{TRiP.HMS00009}attP2*	No
7078	UAS-Notch RNAi	*P{UAS-N.dsRNA.P}14E, w**	No
7077	UAS-Notch RNAi	*w*; P{UAS-N.dsRNA.P}9G*	No
7076	UAS-Notch RNAi		No
8611	UAS-Delta with GFP inserted in extracellular domain	*w[*]; P{w[+mC]=UAS-Dl::GFP}DA55*	No
28713	UAS-Ser RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03140}attP2*	No
31502	UAS-Notch RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01043}attP2*	No

28981	UAS-Notch RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01637}attP2*	No
31503	UAS-Notch RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01053}attP2*	No
36784	UAS-Notch RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00520}attP40*	No
27988	UAS-Notch RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02959}attP2*	No
31180	UAS-Notch RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01693}attP2*	No
6578	Dominant Negative Kuzbanian	*w[*]; P{w[+mC]=UAS-kuz.DN}2*	No
35640	UAS-Notch RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLV21004}attP2*	No
33967	UAS-Cut RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00924}attP2*	No
29625	UAS-Cut RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03304}attP2*	No
33901	UAS-Hnt RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00843}attP2*	No
33943	UAS-Hnt RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00894}attP2/TM3, Sb[1]*	No
28735	UAS-Hnt RNAi	*w[1118]; Mi{ET1}MB10256*	No
31182	UAS-Numb RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01695}attP2*	No
35045	UAS-Numb RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01459}attP2*	No
28046	UAS-mastermind-RNAI	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02881}attP2*	No
35412	neuralized RNAi	*y[1] ssc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00334}attP2*	No
26023	neuralized RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02048}attP2*	No
28900	UAS-Su(H) RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05110}attP2/TM3, Sb[1]*	No
27498	Nicastrin RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02648}attP2*	No
27681	Presenilin RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02760}attP2/TM3, Sb[1]*	No
4369	Neuralized-lacZ	*P{ry[+t7.2]=lArB}neur[A101] ry[506]/TM3, ry[RK] Sb[1] Ser[1]*	-

26872	UAS-E(spl)m8	*w[*]; P{w[+mC]=UAS-E(spl)m8-HLH.T}3*	-
28713	UAS-Ser RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03140}attP2*	No
34700	UAS-Ser RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01179}attP2*	No
35045	UAS-numb RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01459}attP2*	No
28735	UAS-Hnt RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03162}attP2*	No
33943	UAS-Hnt RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00894}attP2/TM3, Sb[1]*	No
33901	UAS-Hnt RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00894}attP2/TM3, Sb[1]*	No
29625	UAS-Cut RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03304}attP2*	No
33967	UAS-Cut RNAi	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00924}attP2*	No
26679	UAS-E(spl)m4	*w[*]; P{w[+mC]=UAS-E(spl)m4-BFM.A}15.5*	No
26322	UAS-E(spl)m8 RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02096}attP2*	No
26675	UAS-E(spl)mbeta	*y[1] w[*]; P{w[+mC]=UAS-E(spl)mbeta}48.1*	No
30727	Express EGFP under control of NRE	*w[1118]; P{w[+m*]=NRE-EGFP.S}5A*	-
30728	Express EGFP under control of NRE	*w[1118]; P{w[+m*]=NRE-EGFP.S}1*	-
26873	E(spl)m8 lacZ	*w[*]; P{w[+mC]=UAS-E(spl)m8-HLH.N}8602/CyO, P{ry[+t*]=elav-lacZ.H}YH2*	-
29856	UAS-Notch truncated, ligand non-responsive	*P{w[+mC]=UAS-N.DeltaEGF.LV}1, w[1118]*	No
27179	UAS-E(spl)alpha	*y[1] w[*]; P{w[+mC]=UAS-E(spl)malpha-BFM.A}A17.4*	No
5815	UAS-Serrate	*w[*]; P{w[+mC]=UAS-Ser.mg5603}SS1*	No
27681	UAS-Presenilin RNAi	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02760}attP2/TM3, Sb[1]*	No

Figure 2.1: N and Dl are expressed in testes.

(A-C') Immunofluorescence labeling of the apical regions of wildtype testes using antibodies against N signaling components. Scale bars = 20 μm. Staining as indicated. Asterisks denote apical tip of the testes.

(A-B'') NICD and NECD expression co-localized with Armadillo. Note that Armadillo marks both the hub and cytoplasmic extensions of the somatic cells that encompass the germline cells (arrowheads).

(C, C') Vasa but not expression of the extracellular domain of Dl was detected in testes through immunofluorescence.

(C") Dl is detected in wildtype testes extract analyzed by Western blot with antibody against the Dl extracellular domain.

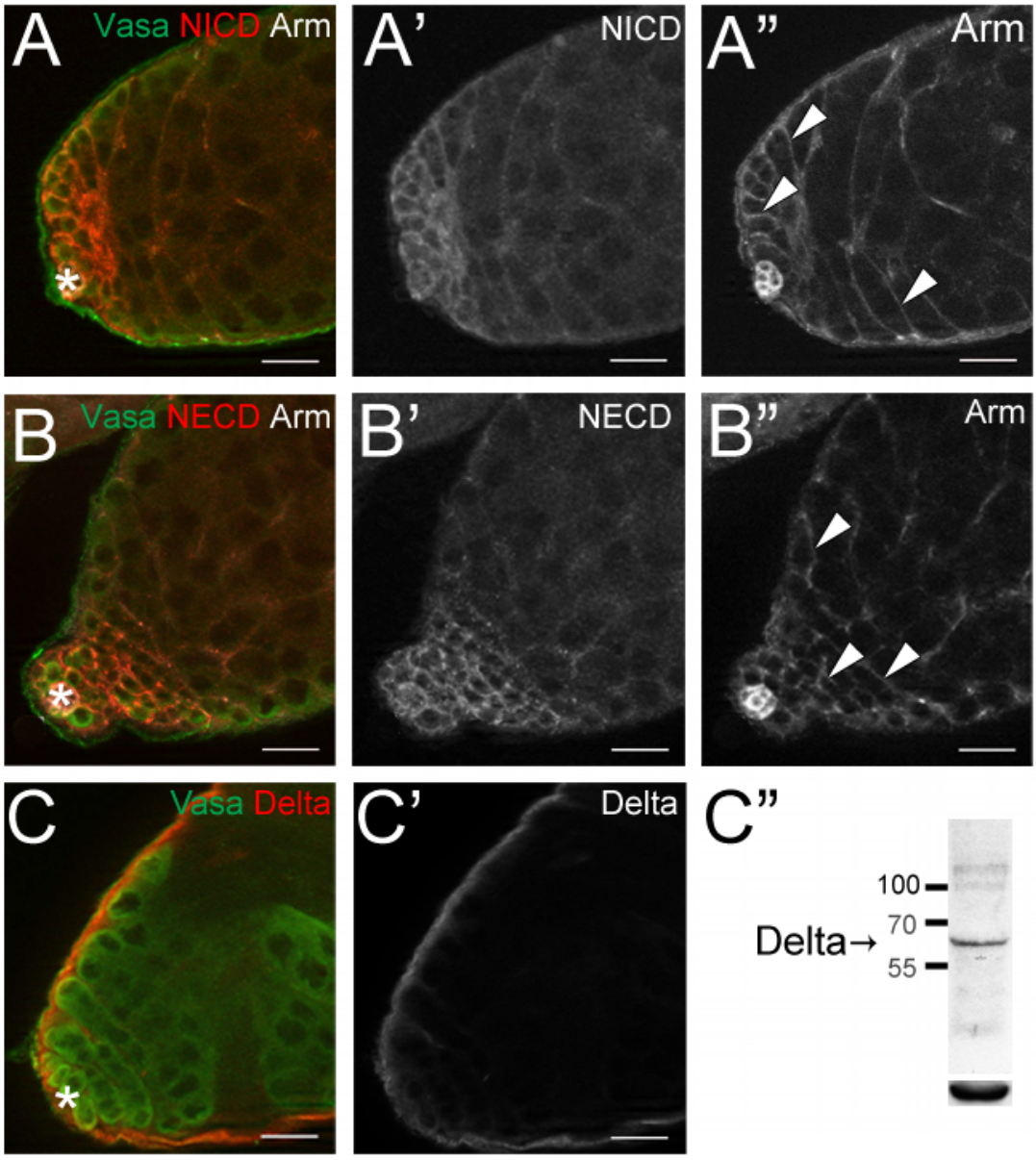

A
Vasa NICD Arm
A'
NICD
A"
Arm
B
Vasa NECD Arm
B'
NECD
B"
Arm
C
Vasa Delta
C'
Delta
C"
100
70
55
Delta→

Figure 2.2: The NRE-EGFP reporter localizes to the somatic lineage in the transition zone.

(A-B'') Depicted are immunofluorescent labeling of the apical regions of testes using molecular markers as indicated. Scale bars = 20 μm. Asterisks denotes apical tip.

(A-A''') Expression of somatic markers for early- (Tj) and late-stage (Eya) cyst cells within the NRE-EGFP reporter. Strong NRE-EGFP expression localized in the transition zone marking cyst cells positive for both Tj and Eya (arrowhead).

(B-B') NICD is strongly expressed in early-stage cells at the apical tip of the testes.

(B'') Expression of NRE-EGFP in the transition zone but not the apical tip.

Scale bars = 20 μm.

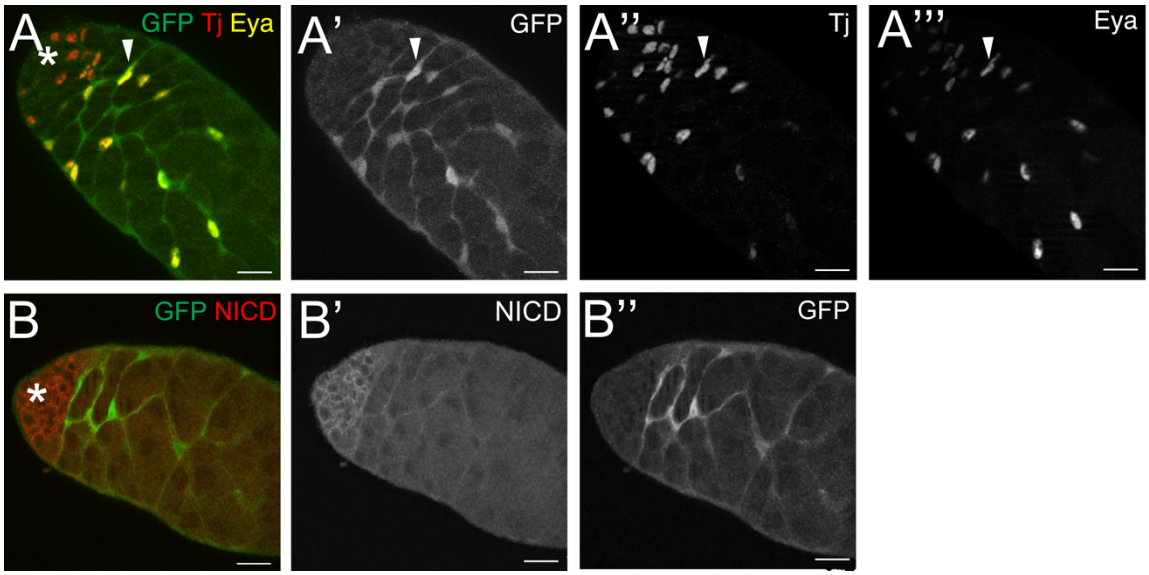

A
GFP Tj Eya
A'
GFP
A''
Tj
A'''
Eya
B
GFP NICD
B'
NICD
B''
GFP

Figure 2.3: Dl germline clones are greatly outnumbered by control clones.

(A-B'') FRT/Flp germline clones identified in the transition zone of testes. Staining as indicated. Scale bars = 20 μm. Asterisks denotes apical tip.

(A-A'') Multiples clusters of GFP-negative germline clones were generated in controls and marked by Vasa.

(B, B') Representative image of single FRT82B Dl germline clone cluster that is GFP-negative.

(B, B'') Vasa confirms GFP-negative cells are germline.

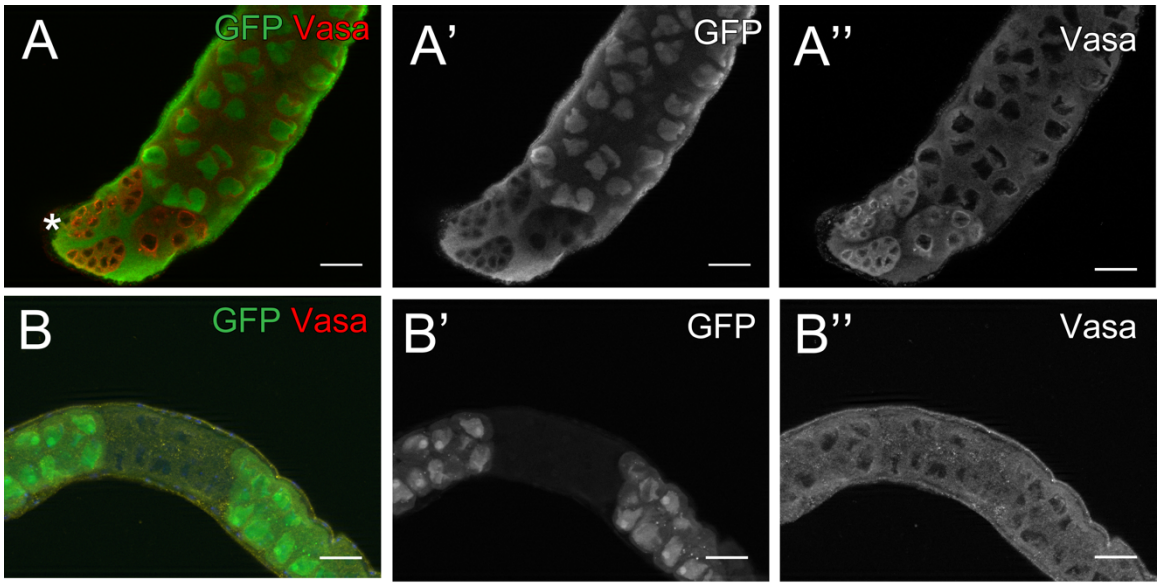

A
GFP Vasa
*
A'
GFP
A''
Vasa
B
GFP Vasa
B'
GFP
B''
Vasa

Figure 2.4: Knockdown of *dl* in the germline induced severe germline defects.

(A-I’’’) Timeline experiment performed on testes using the germline transactivator NG4. Scale bars = 20 μm. Staining as indicated. Arrows depict the apical tip of testes.

(A-A’) NG4/*dl*-i testes raised at the permissive temperature of 18^{o}C appeared wildtype.

(B-B’) Knockdown of *dl* in germline by shifting males to the restrictive temperature of 29^{o}C for only 4 days did not produce an observable phenotype.

(C, C’) At 6d, the testes lacked large clusters of spermatogonia and spermatocytes.

(D, D’) At 8d, some GSCs and most spermatogonia were frequently gone.

(E, E’) At 10d, testes had large regions with no germline.

(F-G’) When animals were shifted to 29^{o}C for 12d or 14d, the germline was absent in most of the testes except some early-stage germline near the tip.

(H, H’) Presence of differentiated spermatids was not observed for NG4/*dl-i* testes.

(H’’) Tj-positive cells observed at the apical tip.

(H’’’) Eya-marked cells span length of testes but do not appear associated with any germline cells (arrow).

(I-I’’’) Control testes raised at permissive temperature for 14 days stained with antibodies labeling cyst cells.

(I’) Dapi-labeled nuclei are apparent in the region with early-stage cells and also at the basal end of the testes marking differentiated sperm bundles.

(I’’) Tj-positive cells localized at the apical tip.

(I’’’) Eya-positive cells span the length of the testes.

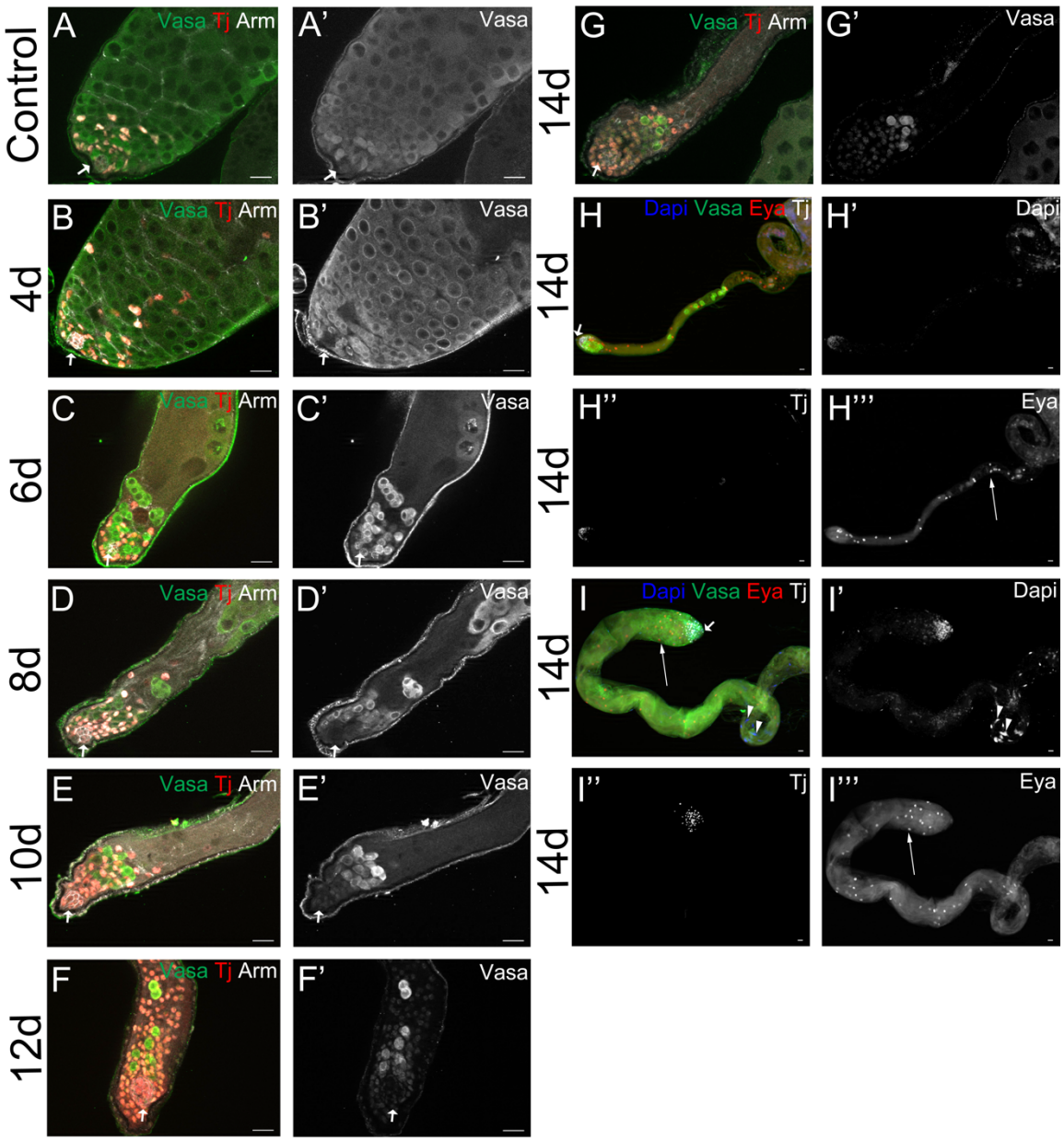

Control
A
Vasa Tj Arm
A'
Vasa
4d
B
Vasa Tj Arm
B'
Vasa
6d
C
Vasa Tj Arm
C'
Vasa
8d
D
Vasa Tj Arm
D'
Vasa
10d
E
Vasa Tj Arm
E'
Vasa
12d
F
Vasa Tj Arm
F'
Vasa
14d
G
Vasa Tj Arm
G'
Vasa
14d
H
Dapi Vasa Eya Tj
H'
Dapi
14d
H''
Tj
H'''
Eya
14d
I
Dapi Vasa Eya Tj
I'
Dapi
14d
I''
Tj
I'''
Eya

Figure 2.5: NICD expression was persistent in NG4/*dl-i* testes.

(A-A’’’) Somatic and germline markers are still identifiable in NG4/*dl-i* testes raised at the restrictive temperature of 29^{o}C for 14 days. Scale bars = 20 μm. Staining as indicated. Arrows denote the apical tip of testes.

(A, A’) A few Vasa-positive cells are present near the apical tip of NG4/*dl-i* testes.

(A, A’’) Despite knockdown of *dl* in the germline, NICD expression is still present in the apical region of the testes.

(A, A’’’) Armadillo expression colocalizes with expression of NICD near the apical tip even in regions with no germline present.

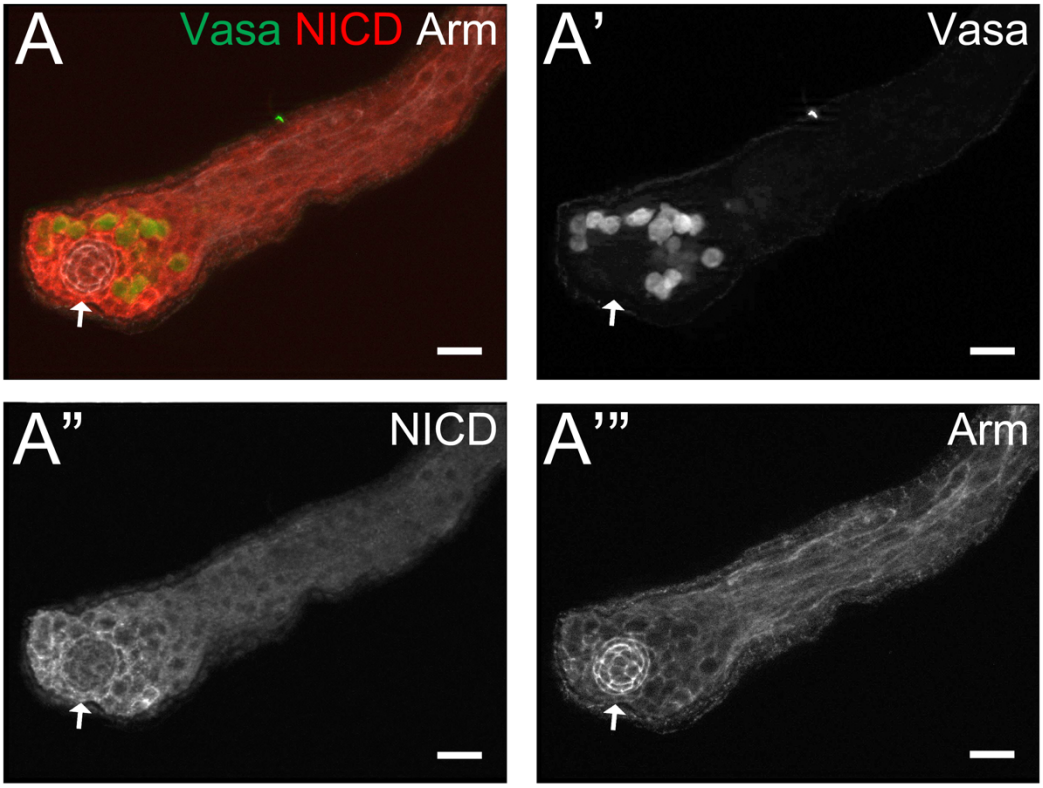

A
Vasa NICD Arm
A'
Vasa
A"
NICD
A'"
Arm

Figure 2.6: TUNEL detects higher levels of apoptosis in NG4/*dl-i* testes.

(A-B) TUNEL-positive spots (arrowhead) observed within the apical region in testis. Scale bars = 20 μm. Staining as indicated. Asterisks denote the apical tips of testes.

(A) A rare TUNEL-positive spot in a control testis.

(B) TUNEL-positive spots observed within the transition zone in NG4/dl-*i* testis shifted for 10 days at 29^{o}C.

(C) Diagram showing numbers of TUNEL positive spots in testes 3 to 8 days after temperature shift to restrictive temperature. ***: $P < 0.05$.

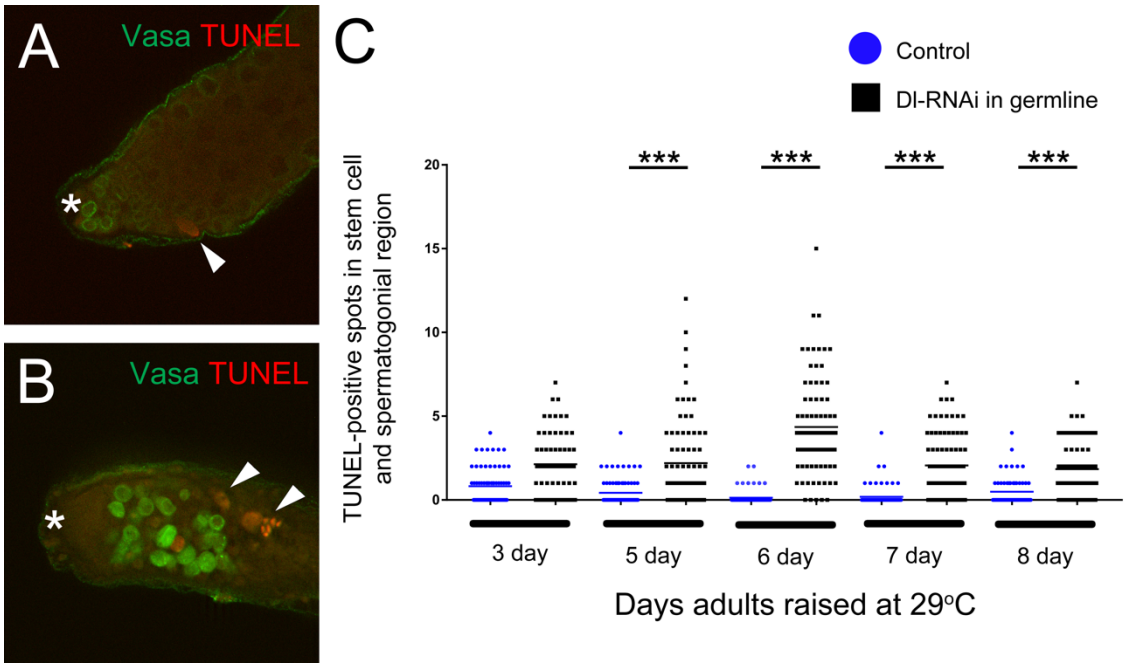

A
Vasa TUNEL
B
Vasa TUNEL
C
Control
DI-RNAi in germline
TUNEL-positive spots in stem cell and spermatogonial region
***
***
***
***
20
15
10
5
0
3 day
5 day
6 day
7 day
8 day
Days adults raised at 29°C

Figure 2.7: *bam*-Gal4/*dl-i* rarely produced a phenotype.

(A-C') Immunofluorescence of the apical end of wildtype of *bam*-Gal4/*dl-i* testes. Scale bars = 20 μm. Staining as indicated. Asterisks denote the apical tips of testes.

(A, A') *bam*-Gal4 is expressed primarily in the 8- and 16-cell stage spermatogonia as evident through GFP expression when crossed with UAS-GFP. GFP colocalizes with Vasa in the transition zone.

(B, B') Most testes of *bam*-Gal4/*dl-i* of flies raised at the restrictive temperature are similar to controls and have no visible phenotype.

(C-C') A few *bam*-Gal4/*dl-i* testes bear resemblance to NG4/*dl-i* testes with loss of germline (arrow).

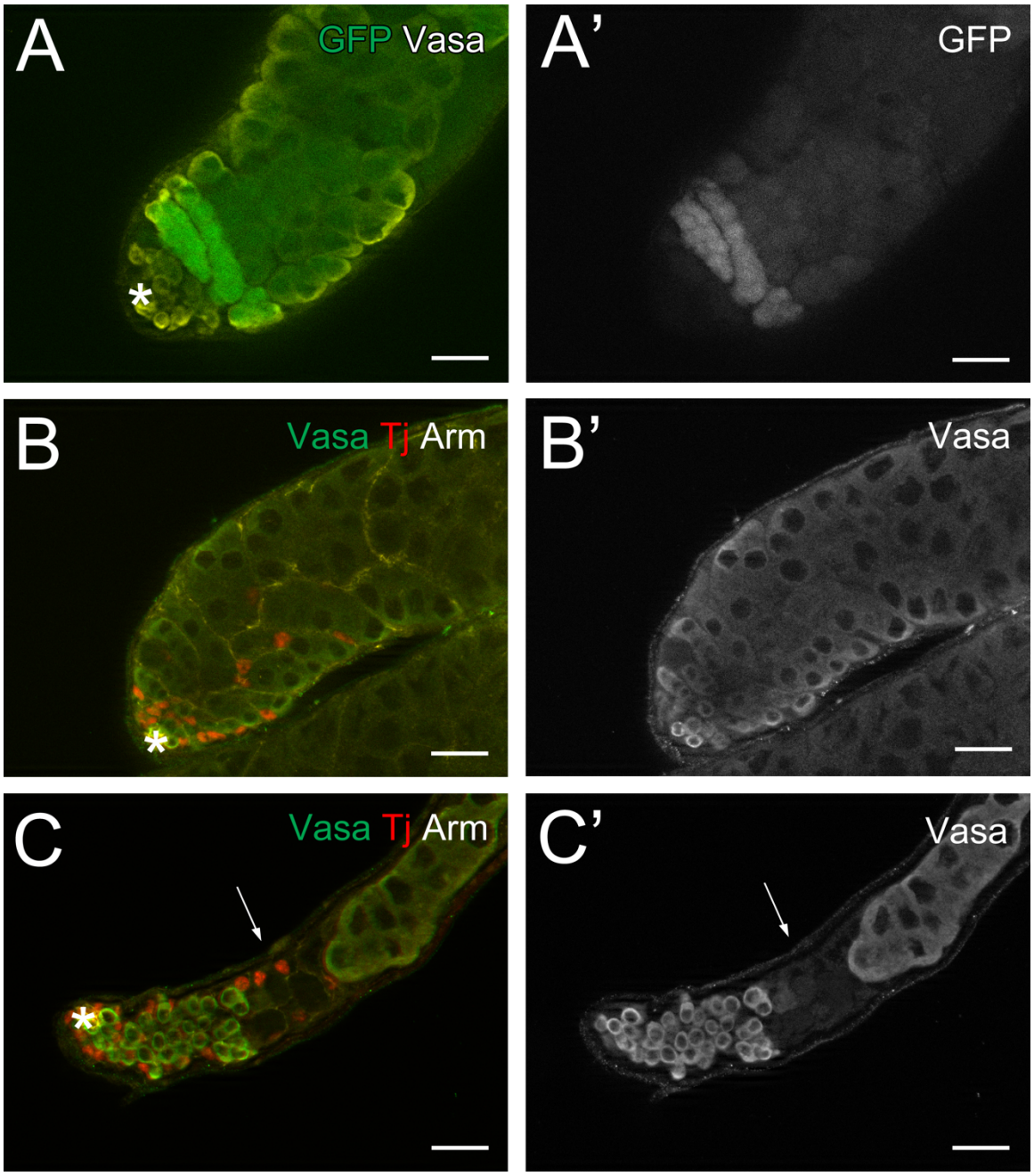

A
GFP Vasa
A'
GFP
B
Vasa Tj Arm
B'
Vasa
C
Vasa Tj Arm
C'
Vasa

Figure 2.8: Crossing scheme for CRISPR/Cas9 mediated conditional mutagenesis. N gRNA construct expresses RFP marker in the eye. This stock is mated with Cas9 stock under UAS control that expresses GFP in the eye. Progeny from G_0 cross were mated with the w^+; Gal4 virgin flies to drive expression of Cas9 in specific tissues such as the wing margin and testes. The resulting progeny that carry all constructs exhibit red eyes, GFP, and RFP.

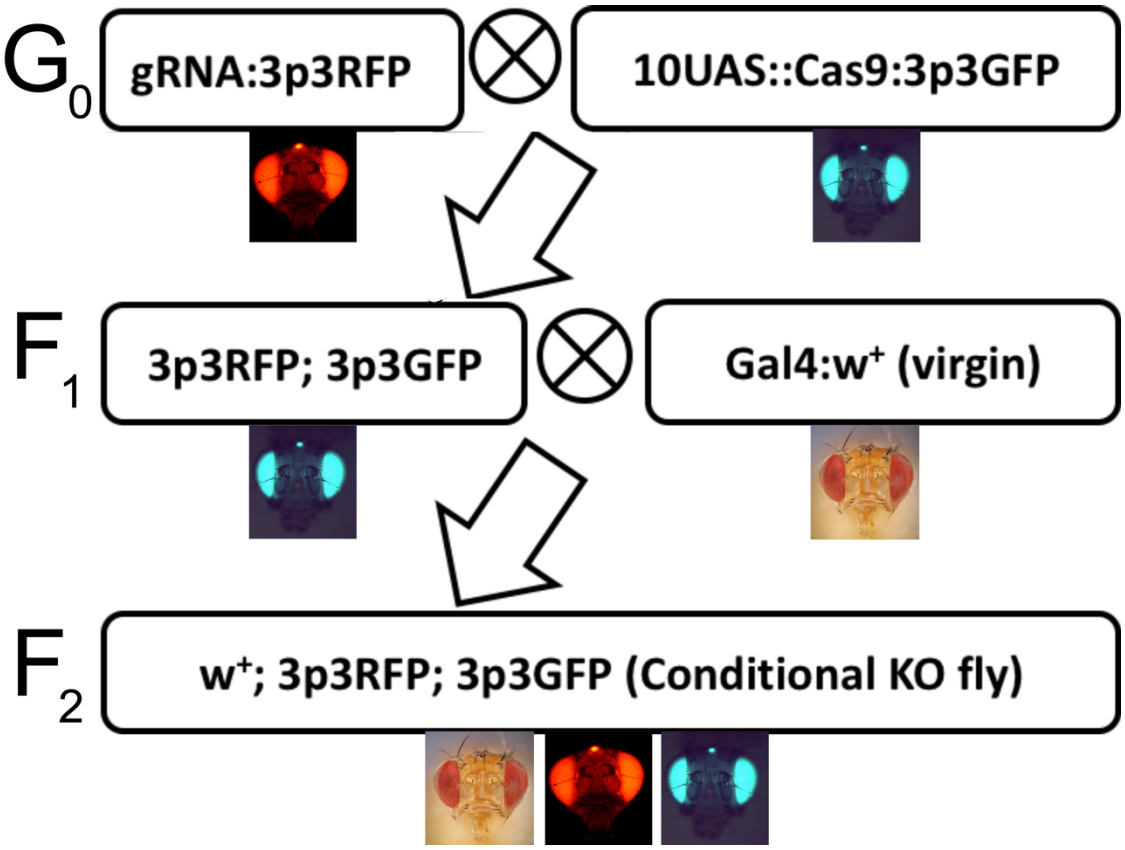

G0
gRNA:3p3RFP
10UAS::Cas9:3p3GFP
F1
3p3RFP; 3p3GFP
Gal4:w+ (virgin)
F2
w+; 3p3RFP; 3p3GFP (Conditional KO fly)

Figure 2.9: N CRISPR verified in wings.

(A) Representative classes of wing defects through conditional mutation of *N*. Classes 1-4 have progressively more severe notching of wings and loss of bristles.

Modified from (Xue et al., 2014).

(B) Representative class 2 wing of a fly expressing N gRNA specifically in the wing margin using *C96*-Gal4 transactivator.

(C) 15% Polyacrylamide gel of PCR products using primers specific for *N* exon 6 from wings of N CRISPR flies and wildtype flies. Expected PCR product size of 959 bp is contrasted by the shorter fragments produced by N CRISPR in notched wings (arrow). Lane 1 is the DNA ladder. Lane 2-4 are PCR products from Notched wings genomic DNA extracts and Lane 5 is the PCR product from wildtype wings.

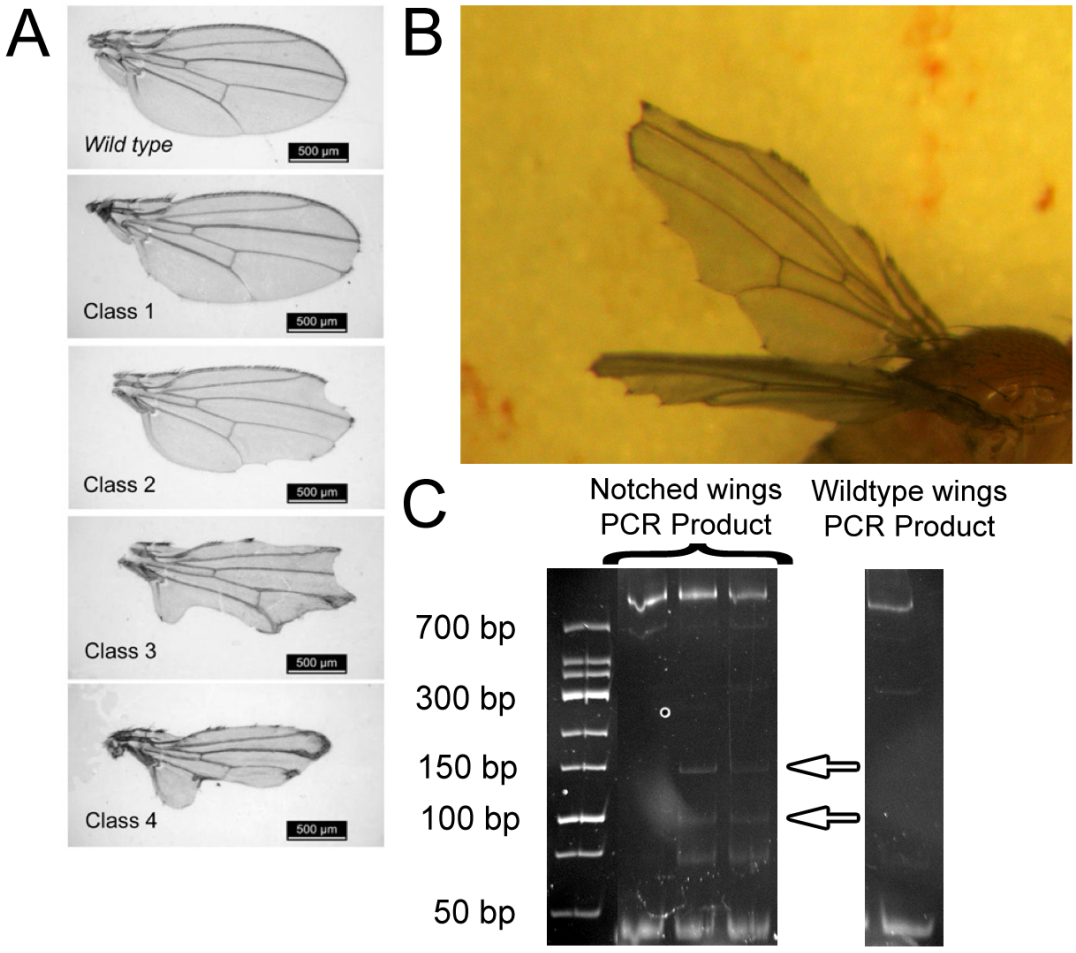

A
Wild type
500 µm
Class 1
500 µm
Class 2
500 µm
Class 3
500 µm
Class 4
500 µm
B
C
Notched wings
PCR Product
Wildtype wings
PCR Product
700 bp
300 bp
150 bp
100 bp
50 bp

Figure 2.10: Testes with N CRISPR expressed in soma have increased cell death.

(A-D) Immunofluorescence of the apical end of *tj*-Gal4/N CRISPR and control testes. Scale bars = 20 μm. Staining as indicated. Asterisks denote the apical tips of testes.

(A, A') Control testes are full of germline cysts.

(B, B') Visible loss of germline in the transition zone in *tj*-Gal4/N CRISPR testes (arrow).

(C) TUNEL-positive spots observed in control testis.

(D) TUNEL-positive spots observed within the transition zone in *tj*-Gal4/N CRISPR testis shifted for 14 days at 29ºC.

(E) Diagram showing numbers of TUNEL-positive spots in testes 14 days after temperature shift. More spots were observed for *tj*-Gal4/N CRISPR compared to controls.

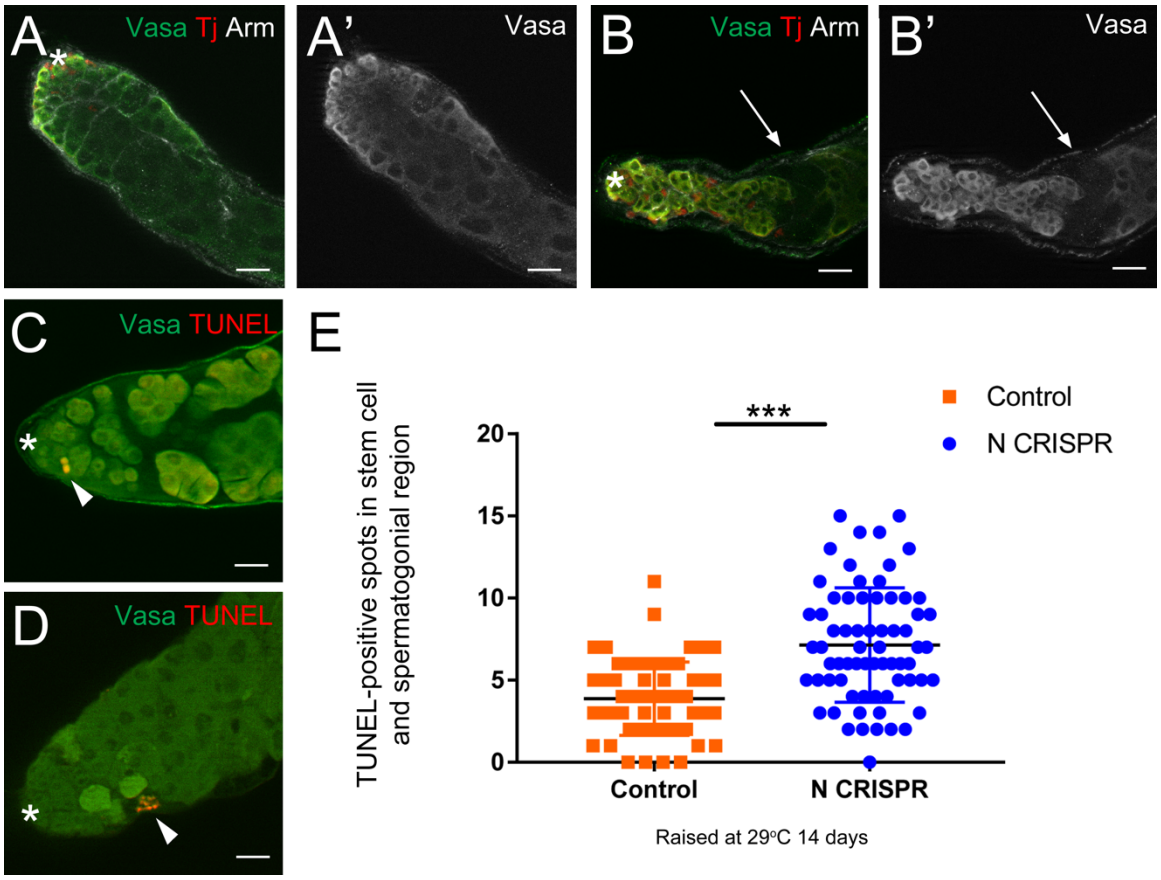

A
Vasa Tj Arm
A'
Vasa
B
Vasa Tj Arm
B'
Vasa
C
Vasa TUNEL
D
Vasa TUNEL
E
TUNEL-positive spots in stem cell and spermatogonial region
***
Control
N CRISPR
20
15
10
5
0
Control
N CRISPR
Raised at 29°C 14 days

Figure 2.11: Overexpression of *dl* in the germline is sufficient to increase cyst cell numbers and clusters of early germline.

(A-B', D-G''') Immunofluorescent labeling of testes. Scale bars = 20 μm. Staining as indicated. Asterisks denotes the apical tip of testes.

(A, A') Wildtype testis with localization of Tj-positive cyst cells at apical tip.

(B, B') Overexpression of Dl in the germline leads to an increase in the number of Tj-positive cells many of which are not associated with germline..

(C) Diagram showing numbers of Tj-positive cells for testes of animals raised at 29^{o}C for 4-12 days. Counts of Tj-positive cells for animals overexpressing Dl in germline is significantly higher compared to wildtype controls.

**: $p < 0.01$; ***: $p < 0.001$.

(D-D''') In control testes, Tj-positive cyst cells close to the hub are in mitosis.

(E-E''') Cyst cells weakly positive for Tj and pHH3 are observed far from the apical tip of the testes in testes with Dl overexpression in germline

(F-F''') Branching fusomes (arrowhead) connect germline and becomes more branched as they differentiate.

(G-G''') More clusters of slightly branched fusomes (arrowheads) indicative of early-stage cells accumulate in Dl overexpression testes.

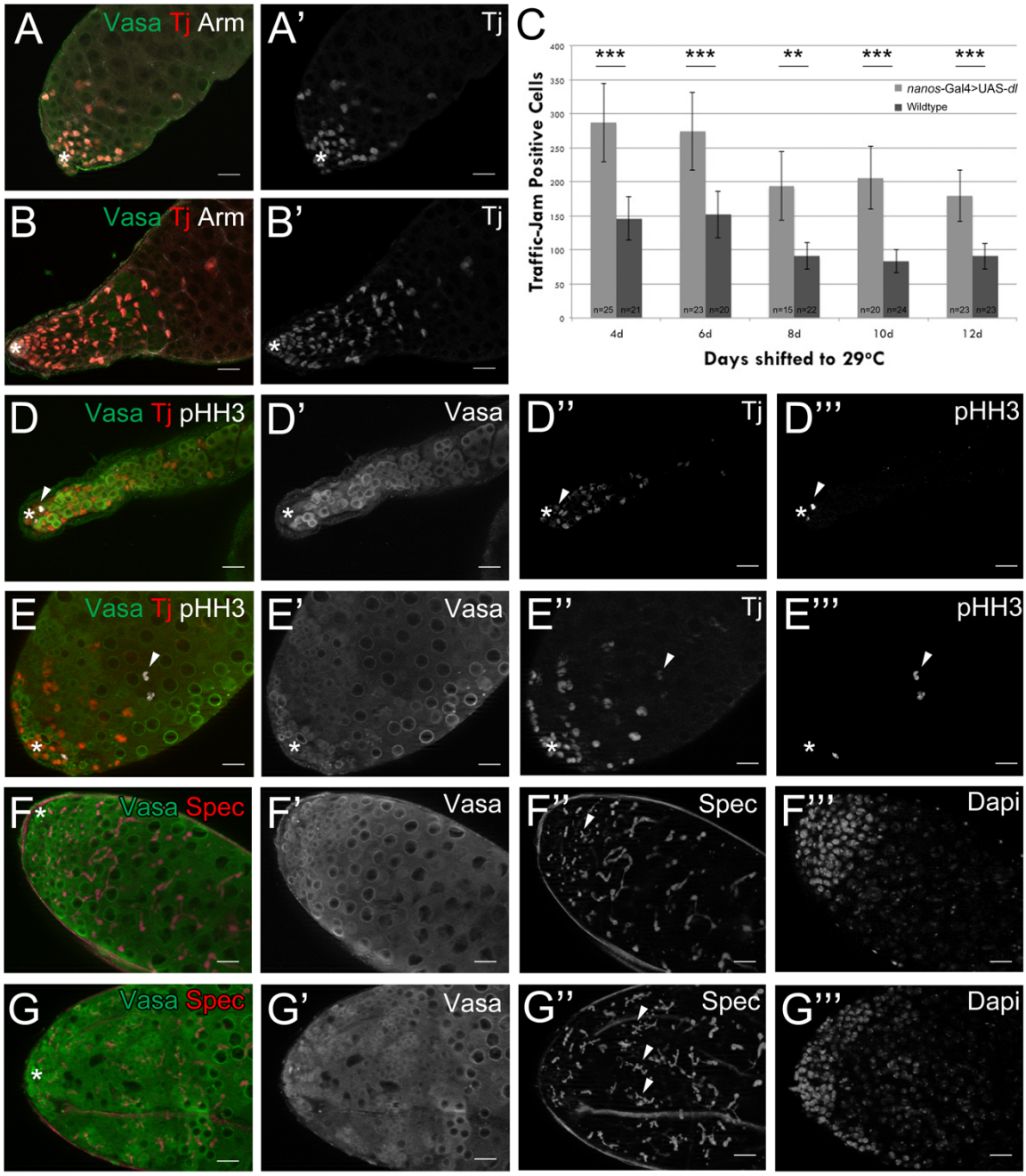

A
Vasa Tj Arm
A'
Tj
B
Vasa Tj Arm
B'
Tj
C
***
***
**
***
***
nanos-Gal4>UAS-dl
Wildtype
Traffic-Jam Positive Cells
400
350
300
250
200
150
100
50
0
n=25 n=21
n=23 n=20
n=15 n=22
n=20 n=24
n=23 n=23
4d
6d
8d
10d
12d
Days shifted to 29°C
D
Vasa Tj pHH3
D'
Vasa
D''
Tj
D'''
pHH3
E
Vasa Tj pHH3
E'
Vasa
E''
Tj
E'''
pHH3
F
Vasa Spec
F'
Vasa
F''
Spec
F'''
Dapi
G
Vasa Spec
G'
Vasa
G''
Spec
G'''
Dapi

Figure 2.12: Expression of Su(H)VP16 in the somatic lineage is sufficient to prevent cyst cell differentiation.

(A-F'') Testes labeled with markers for cyst cells. Scale bars = 20 μm. Staining as indicated. Asterisks denotes the apical tip of testes.

(A) Wildtype testis with expression of CySC- and cyst cell-specific markers.

(A, A') Zfh-1, a CySC marker is localized at the apical tip of the testes.

(A, A") Tj is also localized at the tip and in early-stage cyst cells.

(A, A'") Expression of Eya in late-stage cyst cells co-localizes with some Tj-positive cells (arrow).

(B, B') Su(H)VP16- expressing testes were filled with cells expressing CySC and early-stage cyst cell markers.

(B") Expression of Tj co-localizes with Zfh-1 expression suggesting cells maintain early-stage identity with activation of N in soma.

(B'") Eya expression is not observed in testes with activated N in cyst cells expressing both Zfh-1 and Tj.

(C, C') Wildtype testis with expression of cyst cell markers and pHH3, a marker for M-phase.

(C", C'") In wildtype testes, very few Tj-positive cyst cells are also positive for pHH3.

(D, D') N-activated testes with expression of cyst cell markers and pHH3.

(D") Cells positive for pHH3 were detected basal from the tip.

(D'") These pHH3-positive cells were also positive for Tj (arrows).

(E-F'') Testes labeled with markers for cyst cells and NICD. Scale bars = 20 μm. Staining as indicated. Asterisks denotes the apical tip of testes.

(E-E’’) Wildtype testes express high levels of NICD and Tj at the apical tip.

(F-F’’’) Su(H)-overactivated testes have expanded regions of expression of NICD and Tj far from the apical tip, supporting more early-stage cyst cells that also have high level of NICD expression.

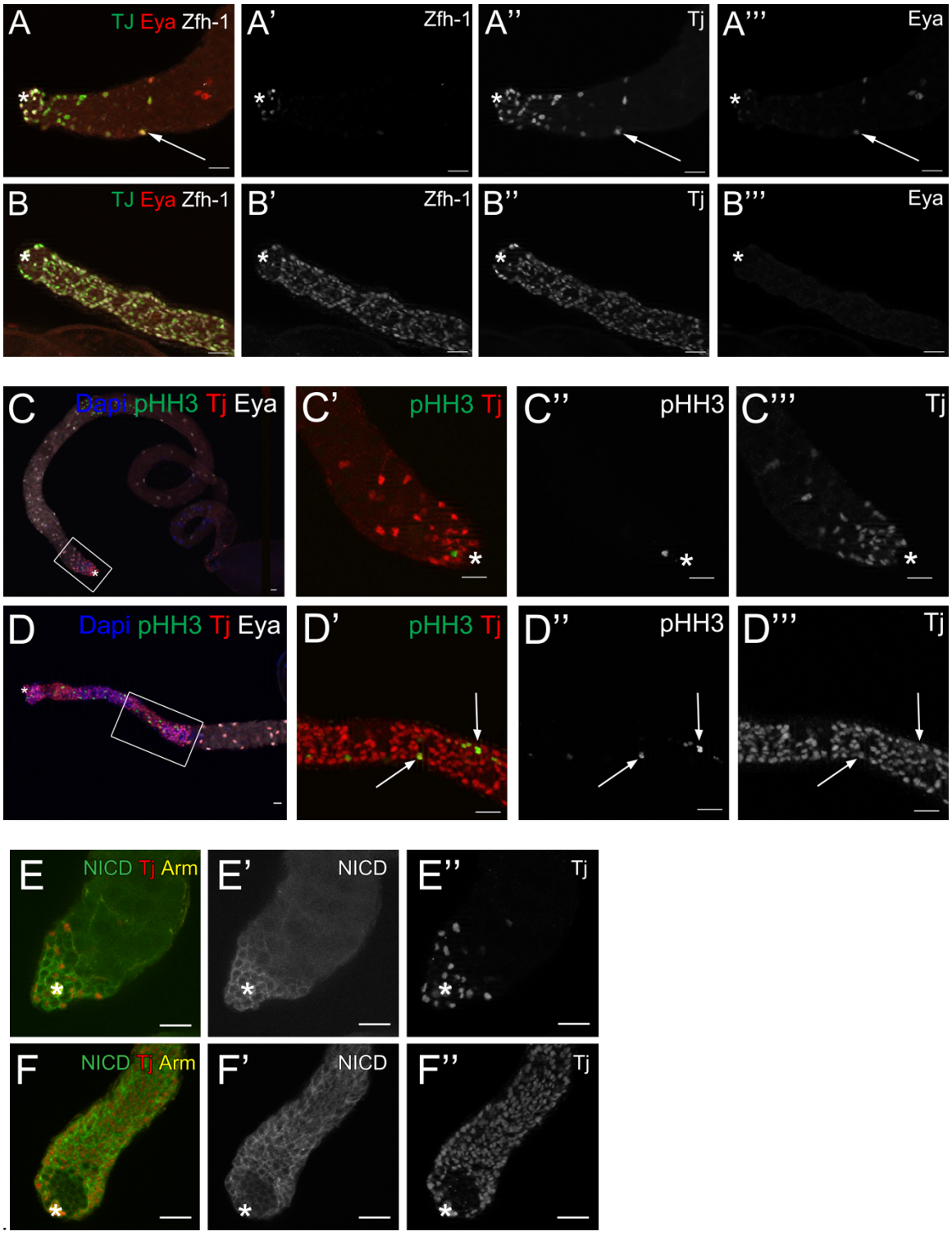

A
TJ Eya Zfh-1
A'
Zfh-1
A''
Tj
A'''
Eya
B
TJ Eya Zfh-1
B'
Zfh-1
B''
Tj
B'''
Eya
C
Dapi pHH3 Tj Eya
C'
pHH3 Tj
C''
pHH3
C'''
Tj
D
Dapi pHH3 Tj Eya
D'
pHH3 Tj
D''
pHH3
D'''
Tj
E
NICD Tj Arm
E'
NICD
E''
Tj
F
NICD Tj Arm
F'
NICD
F''
Tj

Figure 2.13: Activation of N in the somatic lineage is sufficient to prevent somatic and germ cell differentiation.

(A-D’’’) Testes labeled with somatic and germline markers. Scale bars = 20 μm. Staining as indicated. Asterisks denotes the apical tip of testes.

(A-A’’’) Wildtype testis with Tj-positive cyst cells (arrowhead) localized at the apical tip and Eya-positive cyst cells localized more basally.

(B-B’”) Over-activation of Notch in the soma caused an accumulation of Tj- and Eya-positive cells. Cyst cells further basal down the testes have persistent Tj-positive staining. (arrowhead).

(C, C’) Wildtype testis with spermatogonia (arrowhead) at apical tip and sperm bundles (arrow) at the base.

(C”, C”’) Magnified view of the testes tip in (C) showing narrow branched fusomes (arrowhead) of spermatogonia apical to the wider branched fusomes (arrow) of the spermatocytes.

(D, D’) N-activated testes were filled with early-stage germline cells (arrowhead). Testes lack sperm bundles.

(D”, D”’) Magnified view of the testis tip in (B) showing many spherical fusomes (arrow) indicative of GSCs and GBs and narrow branched fusomes (arrowhead).

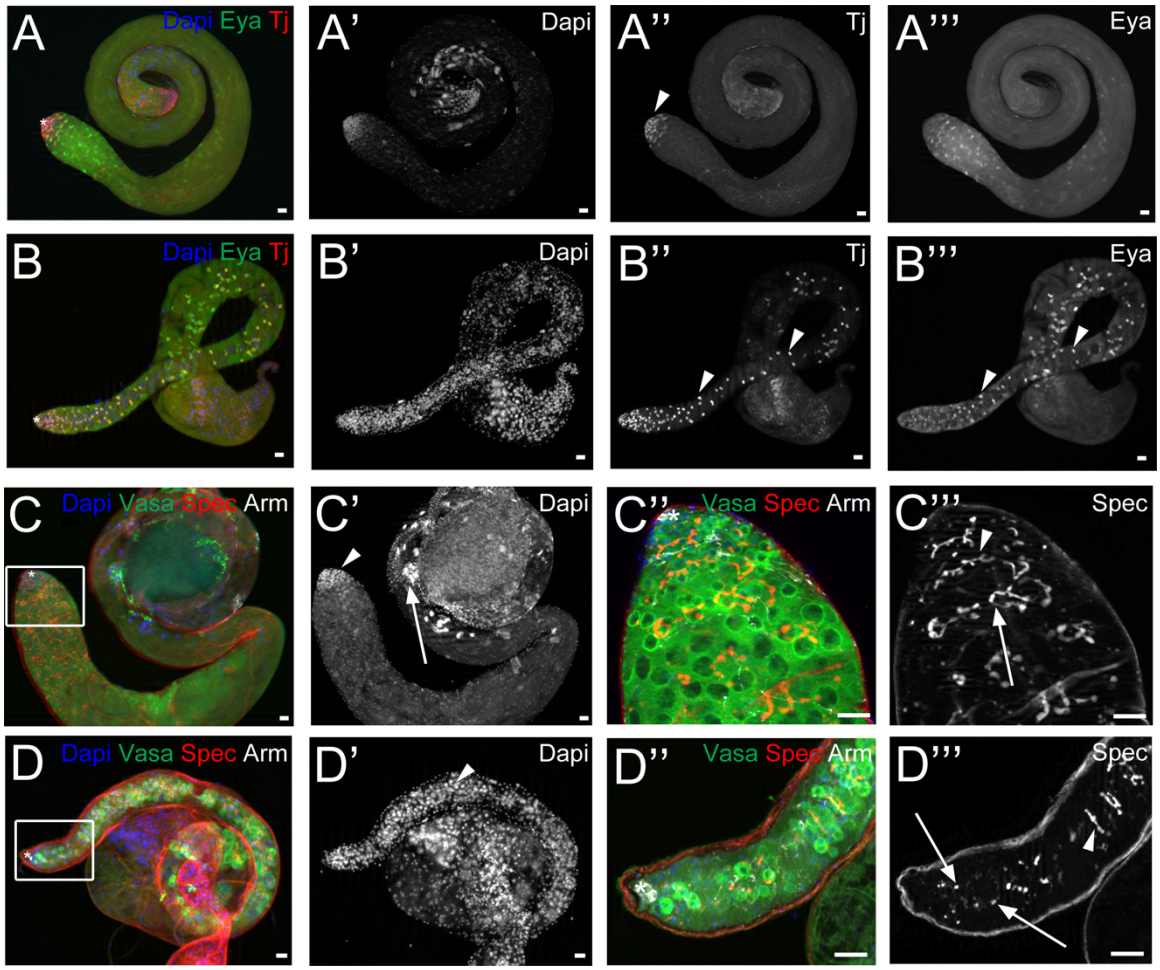

A
Dapi Eya Tj
A'
Dapi
A''
Tj
A'''
Eya
B
Dapi Eya Tj
B'
Dapi
B''
Tj
B'''
Eya
C
Dapi Vasa Spec Arm
C'
Dapi
C''
Vasa Spec Arm
C'''
Spec
D
Dapi Vasa Spec Arm
D'
Dapi
D''
Vasa Spec Arm
D'''
Spec

Figure 2.14: N target Hnt expression colocalizes with NRE-EGFP reporter.

(A) Hindsight is detected in testes by Western blot around 250 kDa.

(B-C’’’) Immunofluorescence labeling of testes using anti-Hindsight antibody. Scale bars = 20 μm. Staining as indicated. Asterisks denotes the apical tip of testes.

(B-B”) Hindsight expression in NRE-EGFP reporter testes localizes to somatic lineage and also in the germline. Colocalization of Hindsight and GFP observed in somatic cells in the transition zone.

(C-C’’’) Hnt expression in wildtype testes colocalizes with Eya as Eya expression strongly increases in the transition zone (arrowheads).

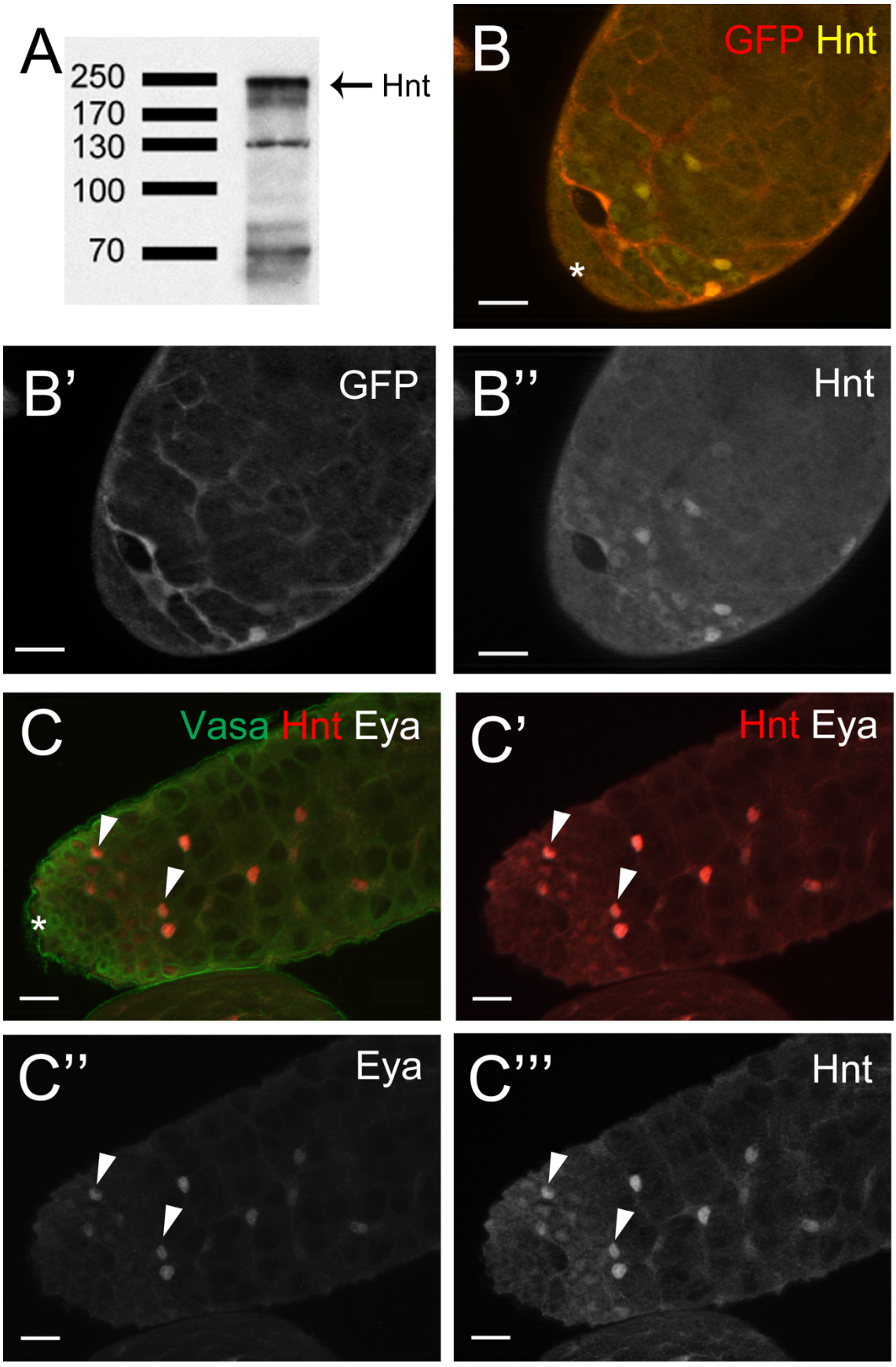

A
250
170
130
100
70
Hnt
B
GFP Hnt
*
B'
GFP
B''
Hnt
C
Vasa Hnt Eya
*
C'
Hnt Eya
C''
Eya
C'''
Hnt

Figure 2.15: Hnt expression expands in testes with overexpression of Dl in germline.

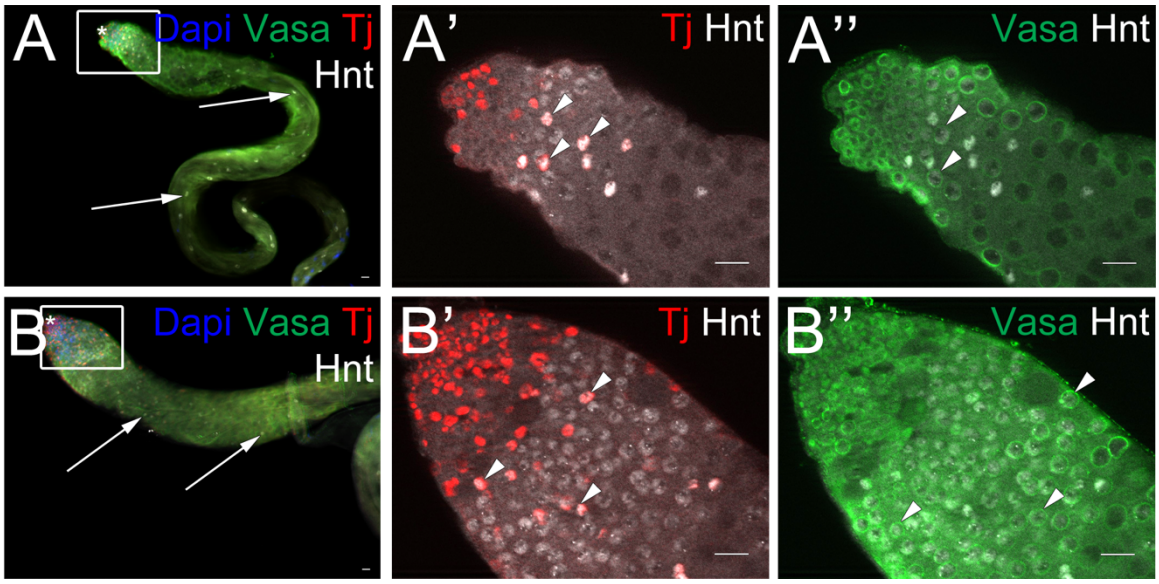
(A-B'') Hnt antibody staining in soma and germline. Scale bars = 20 μm. Staining as indicated. Asterisks denotes the apical tip of testes.

(A) In wildtype testes Hnt expression is present in the transition zone but also further basally along the length of testes (arrows).

(A') Tj-positive cyst cells colocalize with Hnt in the transition zone (arrowheads).

(A'') Hnt also colocalizes with Vasa in the germline in the transition zone (arrowheads).

(B-B'') In testes with Dl overexpression in germline, Hnt expression expands along with the increased number of Tj-positive cyst cells.


A
Dapi Vasa Tj
Hnt
A'
Tj Hnt
A''
Vasa Hnt
B
Dapi Vasa Tj
Hnt
B'
Tj Hnt
B''
Vasa Hnt

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CHAPTER 3

CSN MAINTAINS THE GERMLINE CELLULAR MICROENVIRONMENT AND CONTROLS THE LEVEL OF STEM CELL GENES VIA DISTINCT CRLS IN TESTES OF DROSOPHILA MELANOGASTER[1]

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Abstract

Stem cells and their daughters are often associated with and depend on cues from their cellular microenvironment. In Drosophila testes, each Germline Stem Cell (GSC) contacts apical hub cells and is enclosed by cytoplasmic extensions from two Cyst Stem Cells (CySCs). Each GSC daughter becomes enclosed by cytoplasmic extensions from two CySC daughters, called cyst cells. CySC fate depends on an Unpaired (Upd) signal from the hub cells, which activates the Janus Kinase and Signal Transducer and Activator of Transcription (Jak/STAT) pathway in the stem cells. Germline enclosure depends on Epidermal Growth Factor (EGF) signals from the germline to the somatic support cells. Expression of RNA-hairpins against subunits of the COnstitutively Photomorphogenic-9- (COP9-) signalosome (CSN) in somatic support cells disrupted germline enclosure. Furthermore, CSN-depleted somatic support cells in the CySC position next to the hub had reduced levels of the Jak/STAT effectors Zinc finger homeotic-1 (Zfh-1) and Chronologically inappropriate morphogenesis (Chinmo). Knockdown of CSN in the somatic support cells does not disrupt EGF and Upd signal transduction as downstream signal transducers, phosphorylated STAT (pSTAT) and phosphorylated Mitogen Activated Protein Kinase (pMAPK), were still localized to the somatic support cell nuclei. The CSN modifies fully formed Cullin RING ubiquitin ligase (CRL) complexes to regulate selective proteolysis. Reducing cullin2 (cul2) from the somatic support cells disrupted germline enclosure, while reducing cullin1 (cul1) from the somatic support cells led to a low level of Chinmo. We propose that different CRLs enable the responses of somatic support cells to Upd and EGF.

Introduction

The continuous production of specialized cells in animal tissue relies on the abilities of stem cells to self-renew and to produce differentiating daughter cells. Mechanistically, these traits are mostly regulated by extrinsic signals from the cellular microenvironment, or niche (Fuchs et al., 2004; Walker et al., 2009; Watt and Hogan, 2000). For example, in the bone marrow, quiescent Hematopoietic Stem Cells (HSCs) reside close to the bone within the osteoblastic niche that is composed of mesenchymal osteoblasts, osteoclasts, and CXC-chemokine ligand (CXCL) 12-Abundant Reticular (CAR) cells (Suarez-Alvarez et al., 2012). Signals from the osteoblastic niche regulate stem cell activity. Angiopoietin-1 from the osteoblasts directly binds to the Tie-1 receptor on the HSCs to regulate their quiescence and adhesion to the niche (Arai et al., 2004). The actively dividing HSCs are believed to reside more centrally within the bone marrow, in the vascular niche that lacks osteoblasts and osteoclasts. The vascular niche contains endothelial and CAR cells. The release of CXCL 12 from the CAR cells regulates their self-renewal, mobilization, and homing (Ara et al., 2003; Ceradini et al., 2004).

Similarly, germline cells occupy different microenvironments dependent on their developmental stage. In mammalian testes, all germline cells are enclosed by somatic Sertoli cells. The Spermatogonial Stem Cells (SSCs) and their mitotic daughters are found along the basal membrane of the seminiferous tubules but the distinct morphology of the SSC microenvironment is yet to be distinguished (de Rooij, 2009). According to the current understanding, the SSCs are concentrated in those areas of the seminiferous tubules that are above interstitial cells and bloods vessels (Chiarini-Garcia et al., 2001;

Yoshida et al., 2007). SSC self-renewal is regulated by several extrinsic factors, such as Glial cell line-Derived Neurotrophic Factor (GDNF) and Fibroblast Growth Factor 2 (FGF2) from the Sertoli cells, and Colony Stimulating Factor 1 (CSF1) from the interstitial Leydig cells (Goriely et al., 2005; Kokkinaki et al., 2009; Meng et al., 2000). Differentiating germline cells are displaced away from the basal membrane and receive instructive signals for their differentiation from the Sertoli cells. These signals include Steel factor, Activin A and Bone Morphogenic Protein 4 (Filipponi et al., 2007; Griswold, 1998; Nagano et al., 2003).

Despite the importance of the cellular microenvironment for tissue homeostasis, comparably little is known about how microenvironment cells are regulated. In the gonad of Drosophila melanogaster, two different microenvironments were identified, one for GSCs and one for developing GSC daughters. A plethora of genetic manipulations and molecular tools have been established that allow for the study of these microenvironment cells. In testes, GSCs and CySCs contact an apical stem cell organizing center, called the hub. Two CySCs have cytoplasmic extensions around one GSC and into the hub (Fig. 3.1A) (Hardy et al., 1979). The hub cells and the CySCs provide regulatory signals to the GSCs and are considered the GSC cellular microenvironment (de Cuevas and Matunis, 2011). Specifically, hub cells signal via the ligand Upd to activate the Jak/STAT pathway in the contacting germline and somatic support cells (Fig. 3.1B). Upon phosphorylation, pSTAT localizes into the nuclei of cells to regulate the transcription of target genes. In the CySCs, two effectors of Jak/STAT signaling have been identified, Zfh-1 and Chinmo. Loss of any of the components of the pathway leads to loss of CySCs, while hyperstimulation of the pathway, or overexpression of Zfh-1 or Chinmo induces CySC

fate in the somatic support cells in positions away from the hub (Flaherty et al., 2010; Kiger et al., 2001; Leatherman and Dinardo, 2008, 2010; Tulina and Matunis, 2001).

GSCs and CySCs self-renew and produce daughters that differentiate as gonialblasts and cyst cells, respectively. The gonialblast recruits two cyst cells to grow cytoplasmic extensions around it. The growth of cytoplasmic extensions from the somatic support cells (CySCs and cyst cells) and the differentiation of the cyst cells depend on signaling from the germline cells to the somatic support cells via EGF. Stimulation of the EGF-receptor (EGFR) activates downstream signaling cascades that act through the small monomeric GTPases, Rac1, and MAPK, respectively (Fig. 3.1B) (Hudson et al., 2013; Sarkar et al., 2007; Schulz et al., 2002). Once phosphorylated, pMAPK translocates into the nucleus to regulate the expression of target genes (Schlessinger, 2004). However, the transcriptional targets of EGF in testes remain unknown.

Once a gonialblast is enclosed by two cyst cells the three cells develop as a unit, called a cyst. The enclosed gonialblast enters mitotic transit amplifying divisions to generate spermatogonia. Cytokinesis is incomplete in germline cells. Thus, each group of cells derived from a GSC remains interconnected by cytoplasmic bridges that facilitate their synchronous development and gives them the appearance of clusters of cells. After spermatogonia exit transit amplifying divisions the clusters of cells develop into spermatocytes. Spermatocytes grow in size, divide by meiosis, and differentiate into spermatids (Fig. 3.1A) (Fuller, 1993; Hardy et al., 1979). The cyst cells continue to enclose and co-differentiate with the germline, as evident by their increase in size and the expression of stage-specific nuclear markers. CySCs express high levels of Zfh-1, CySCs and early-stage cyst cells (cyst cells associated with spermatogonia) express high levels

of Traffic jam (Tj), and late-stage cyst cells (cyst cells associated with spermatocytes) express high levels of Eyes absent (Eya, Fig. 3.1A-A') (Fabrizio et al., 2003; Leatherman and Dinardo, 2008; Li et al., 2003). Signals from the cyst cells regulate germline differentiation (Zoller and Schulz, 2012). Signaling via Transforming Growth Factor-beta (TGF-β), for example, restricts the number of mitotic amplification divisions in spermatogonia (Bunt and Hime, 2004; Matunis et al., 1997; Schulz et al., 2004).

Here, we address a requirement for the CSN and distinct downstream Cullins in the somatic support cells. The CSN complex is composed of eight subunits of varying sizes, termed CSN1 to CSN8. CSN was originally identified due to its role in light responses in Arabidopsis thaliana (Stratmann and Gusmaroli, 2012; Wei et al., 1994). Subsequently, other members of the complex were identified in numerous species (Freilich et al., 1999; Mundt et al., 1999; Seeger et al., 1998; Wei and Deng, 1998; Wei et al., 1998). Subunits of the CSN are nuclear enriched and have been implicated in important processes, such as cell proliferation, lipid metabolism, and cancer (Licursi et al., 2014; Richardson and Zundel, 2005; Yan et al., 2003). In Drosophila, the CSN has been associated with many developmental processes, including muscle development, development of the immune system, and the temporal regulation of gene expression during the transitions between larval stages (Bech-Otschir et al., 2002; Goubeaud et al., 1996; Harari-Steinberg et al., 2007; Oron et al., 2007).

The CSN complex modulates the activity of CRL complexes. A CRL complex consists of a catalytic region containing the E3 RING Ubiquitin ligase, a Cullin scaffold, and a substrate-binding region that connects to Cullin, often via an adaptor protein. The combination of different Cullins and substrate receptors provides the complexes with

specificity in targeting different substrates for poly-ubiquitination by the proteasome (Bosu and Kipreos, 2008; Sarikas et al., 2011). The activity of CRLs is regulated by the presence or absence of a covalently linked Ubiquitin-like molecule, termed Nedd8, to the Cullin scaffold (Pan et al., 2004). CSN, specifically, removes Nedd8 from the Cullin scaffold. This modification prevents auto-ubiquitination and promotes disassembly of the CRL (Bosu and Kipreos, 2008; Cope and Deshaies, 2006; Cope et al., 2002; Lyapina et al., 2001).

Here, we show that knockdown of CSN-subunits and distinct cullins disrupted the function of the somatic support cells in Drosophila testes. Expression of an RNA-hairpin directed against the subunit CSN2, also known as Drosophila alien, or other subunits of the CSN complex in the somatic support cells resulted in two distinct responses: a failure of the somatic support cells to grow and maintain cytoplasmic extensions around the germline cells, and a reduction in the levels of the CySC-specific transcription factors Zfh-1 and Chinmo in the somatic support cell nuclei. The upstream signaling pathways regulating germline enclosure and CySC fate, EGF and Jak/STAT, respectively, appeared to be stimulated normally, based on the nuclear localization of the signal transducers, pMAPK and pSTAT. This places CSN function downstream of, or parallel to these signaling events. Finally, we show that Cul2 is required for germline enclosure, while Cul1 is required for maintaining a high level of Chinmo in the CySCs. We propose that CSN acts via different CRLs to enable the somatic support cell's response to the external EGF and Upd signals.

Material and methods

Fly husbandry

All fly lines in this study were raised and maintained on standard cornmeal molasses agar diet at room temperature, unless otherwise stated. The following flies carrying RNAi and overexpression constructs were obtained from the Bloomington Stock Center: *UAS-alien-RNAi*$^{TRiP.HM05119}$ [BL#28908]; *UAS-lin19-RNAi*$^{TRiP.HM05197}$ [BL#29520]; *UAS-cul-2-RNAi*$^{TRiP.HM05237}$ [BL#30494]; *UAS-cul-3-RNAi*$^{TRiP.HMS01572}$ [BL#36684]; *UAS-CSN1b-RNAi*$^{TRiP.JF02612}$ [BL#27303]; *UAS-CSN3-RNAi*$^{TRiP.HMS00242}$ [BL#33369]; *UAS-CSN4-RNAi*$^{TRiP.GL01169}$ [BL#42798]; *UAS-CSN5-RNAi*$^{TRiP.JF03159}$ [BL#28732]; *UAS-CSN6-RNAi*$^{TRiP\ HMS02392}$ [BL# 41991]; *UAS-CSN7-RNAi*$^{TRiP.HMS00073}$ [BL#33663]; *C784-Gal4* [BL#6985]; *tubulin-Gal80*^{ts} [BL#7018]. *UAS-cul-4-RNAi*$^{P\{GD14006\}}$ [v44829] was obtained from the Vienna Drosophila Research Center and the *tj-Gal4* stock from the Kyoto stock center [#104-055]. The *eya-Gal4* stock was a gift from Steve DiNardo.

UAS/Gal4 expression studies

Animals carrying the Gal4-transactivators and the temperature sensitive tubulin- Gal80 were crossed to animals carrying the UAS-RNAi-constructs. Flies were placed on apple juice plates in an 18°C incubator with humidity control and day/night cycle. Apple juice plates were transferred into food bottles and the progeny were raised at 18°C. After enclosure, adult animals carrying all three constructs were either kept at 18°C as non-shifted controls or shifted to 29°C to induce high activity of the UAS/Gal4-system (Duffy, 2002). Animals carrying only the Gal4- and Gal80-constructs (Gal4/Gal80), and wild-type animals were raised and kept under the same conditions and served as controls.

Unless otherwise stated, testes from experimental animals were dissected seven days after the temperature shift.

Immunofluorescence experiments and microscopy

Immunofluorescence experiments were performed as previously described (Flaherty et al., 2010; Schulz et al., 2002). The following hybridoma/monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242: mouse anti-α-Spectrin (3A9) (1:10), mouse anti-Eya (1:10), mouse anti-Fasciclin III (Fas III, 1:10), rat anti-ECad (1:5), and rat anti-NCad (1:5). Goat anti-Vasa (1:150) and rabbit anti-Arm (1:500) were obtained from Santa Cruz Biotechnology. Rabbit anti-pMAPK (P-p44/42 MAPK, T202/Y204, 1:200) was obtained from Cell Signaling and rabbit anti-pHH3 (1:800) was obtained from Millipore. Guinea-pig anti-Tj (1:5000) was a gift from Dorothea Godt.
Rabbit anti-pSTAT (1:1000) and rabbit anti-Chinmo (1:1000) were gifts from Erika Bach. Rabbit anti-Zfh-1 (1:100) was a gift from Ruth Lehman. Alexa-488-, Cy3, and Cy5-conjugated secondary antibodies (Invitrogen) were used at 1:1000. Testes were embedded in SlowFade Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) and were observed using a Zeiss Axiophot microscope. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software, using the same exposure time within each set of control and experimental genotypes. Pixel counts were performed using the Axiovision Rel Software. The TUNEL assay was performed following the manufacturer's (Roche) instructions.

Results

Expression of an RNA-hairpin against alien in the somatic support cells resulted in an accumulation of early-stage germline cells

To investigate a role for CSN in the adult male gonad an RNA-hairpin construct directed against alien (*UAS-alienRNAi*) was expressed in the testes using the tissue- specific UAS/Gal4 expression system (Brand and Perrimon, 1993; Phelps and Brand, 1998). Staining with the molecular marker, anti-Vasa, distinguishes the early-stage germline cells (GSCs, gonialblasts, and spermatogonia) from the spermatocytes. While the early-stage germline cells are small (Fig. 3.1C, arrowhead) and found in the most apical region of a control testis, the spermatocytes are large (Fig. 3.1C, small arrow) and found more basally. The long spermatids can be seen in the lumen and at the base of the testis (Fig. 3.1C, large arrow, n>100). Expression of *UAS-alienRNAi* in the somatic support cells of otherwise wild-type testes (cyst cell-*alienRNAi*-testes) using three different somatic Gal4-transactivators (tj-Gal4, eya-Gal4, or C784-Gal4) produced a drastic phenotype in 100% of the testes. When shifted to 29°C for one week, the apical region of a testis contained large areas of small, Vasa-positive cells (Fig. 3.1D, small arrowheads), indicative of early-stage germline cells (n>100). The remainder of the testis was filled with cell debris indicating that expression of *UAS-alienRNAi* induced cell death in the developing germline-soma cysts (Fig. 3.1D, large arrowhead). We never observed such a phenotype in non-shifted animals kept at 18°C for up to two weeks (n>50), or in testes from shifted control *UAS-alienRNAi* or control Gal4/Gal80 flies (n>50, data not shown). We conclude that the observed germline phenotype was due to the expression of *UAS-alienRNAi* in the somatic support cells. We did not observe a mutant phenotype upon expression of UAS-

alienRNAi in the germline cells (n>30, data not shown), suggesting that alien may be required specifically in somatic support cells.

The germline cells at various stages of differentiation contain subcellular structures of different shape and size, the fusomes (Lin et al., 1994). These can be visualized with antibodies against α-spectrin. GSCs and gonialblasts are single cells that each contain a single round fusome (Fig. 3.2A-A', arrowheads). Developing germline cells within one cyst are interconnected by cytoplasmic bridges. In these cells, the fusome grows and reaches through the intercellular bridges, making it appear progressively more branched as the number of interconnected germline cells increases (Fig. 3.2A-A', small arrows). The fusomes in the spermatocytes are wider (Fig. 3.2A-A', large arrows) than the fusomes in spermatogonia due to the differences in cell sizes. The germline cells in all of the cyst cell-alienRNAi-testes contained round fusomes (Fig. 3.2B-B', arrowheads) and thin, slightly branched fusomes (Fig. 3.2B-B', small arrows) normally seen in early-stage germline cells but lacked fusomes characteristic of spermatocytes (n>50).

Another excellent criterion for determining the developmental stage of the germline cells is their cell division pattern revealed by the expression of cell cycle-specific proteins (Gonczy and DiNardo, 1996). The single GSCs and gonialblasts divide independently of the divisions of other cells and when labeled with Vasa and a mitosis marker, anti-phosphorylated Histone-H3 (pHH3), are detectable as single Vasa-positive, pHH3-positive cells located close to the hub (Fig. 3.2C, arrowhead). The clusters of spermatogonia within one cyst, on the other hand, divide in synchrony and are detectable in groups of two, four (Fig. 3.2C, arrow), or eight Vasa-positive, pHH3-positive cells located away from the hub. In cyst cell-*alienRNAi*-testes, single dividing, Vasa-positive

germline cells (Fig 2D, arrowheads, inset) and groups of two or four dividing cells (data not shown) were scattered throughout the apical region (100%, n>50). This finding confirms that the accumulating germline cells in cyst cell-*alienRNAi*-testes were groups of interconnected spermatogonia and single cells that could have been GSCs, gonialblasts, or both.

To examine if the single germline cells away from the hub were GSCs or gonialblasts, we used antibodies targeted against pSTAT. In control (Fig. 3.2E, arrow, n>30) and cyst cell-*alienRNAi*-testes (Fig. 3.2F, arrow, n>30), we always detected germline cells with nuclear pSTAT located in the stem cell position next to the hub, but germline cells with nuclear pSTAT away from the hub were never observed. Together, our data show that the accumulating germline cells in cyst cell-*alienRNAi*-testes were gonialblasts and spermatogonia.

Somatic support cells in cyst cell-alienRNAi-testes lacked cytoplasmic extensions

Differentiation of the germline cells is dependent on their enclosure by cytoplasmic extensions from the somatic support cells (Schulz et al., 2002). The cytoplasmic extensions can be visualized using antibodies against cell surface markers. For example, the Vasa-positive germline cells are enclosed in cytoplasmic extensions that are positive for the adherens junction protein Armadillo (Arm). The cytoplasmic extensions are seen as a net-like pattern that surrounds the clusters of germline cells (Fig.3.3A-A', small arrows). We never detected this net-like pattern of cytoplasmic extensions around the germline upon expression of UAS-*alienRNAi* in the somatic support cells. In cyst cell-*alienRNAi*-testes, Vasa-positive germline cells were not enclosed by Arm- positive

cytoplasmic extensions (Fig. 3.3B-B'), even though Arm was expressed in the apical hub and some cytoplasmic extensions were seen near the testis sheath (Fig. 3.3B', large arrow, n>50). Despite the lack of cytoplasmic extensions around the germline, the early-stage somatic support cells were readily detectable in cyst cell-*alienRNAi*-testes based on nuclear Tj-staining (compare Fig. 3.3D to Fig. 3.3C, arrowheads, n>100). Other cell surface markers, such as the cell adhesion molecules E-Cadherin (ECad) and N- Cadherin (NCad) also detected cytoplasmic extensions in a net-like pattern in control testes (Fig. 3.3C-C' and 3E-E', respectively, small arrows, n>50). Only very few and short cytoplasmic extensions (Fig. 3.3D-D' and 3. 3F-F', respectively, large arrows, n>50) but no net-like pattern of cytoplasmic extensions were seen in cyst cell-*alienRNAi*- testes. We conclude that the knockdown of alien disrupted the cellular microenvironment of the germline cells.

A timeline experiment revealed that the cytoplasmic extensions were quickly lost in response to the knockdown of alien. Testes from cyst cell-*alienRNAi* and control flies were dissected prior to (day 0) and 1, 2, or 3 days after the shift from 18°C to 29°C. All testes from non-shifted cyst cell-*alienRNAi*-animals (Fig. 3.4A-A') and from all other control animals appeared normal when co-labeled for Tj and Arm. However, by day 1 after the shift to the restrictive temperature, holes in the net-like pattern of cytoplasmic extensions were observed in 50% of the cyst cell-*alienRNAi*-testes (Fig. 3.4B-B', small arrowheads, n>50). By day 2, large areas that did not contain Arm-positive cytoplasmic extensions were observed in 70% of the cyst cell-*alienRNAi*-testes (Fig. 3.4C-C', large arrowhead, n>50), and by day 3, the cytoplasmic extensions had almost vanished from the majority (95%) of the cyst cell-*alienRNAi*-testes (Fig. 3.4D-D', n>50). The

cytoplasmic extensions from the somatic support cells are rapidly growing to accommodate the dividing, enclosed germline cells. A simple explanation for the quick response to reduction in alien is a requirement for alien either in the growth or in the stability of the cytoplasmic extensions. None of the testes from control animals displayed disruptions in the cytoplasmic extensions (data not shown, n>50). No signs of cell death were observed in the Tj-positive somatic support cells in the apical region of cyst cell-*alienRNAi*-testes based on the Terminal deoxynucleotidyl transferase dUtp Nick End Labeling (TUNEL) cell death assay (data not shown, n>50). This suggests that knockdown of alien did not impair viability of these somatic support cells.

A possible explanation for the failure of the somatic support cells in cyst cell- *alienRNAi*-testes to maintain cytoplasmic extensions could be a lack of EGF signaling. In control testes, pMAPK was detected in the nuclei of all Tj-positive somatic support cells (Fig. 3.4E-E', large arrows). We also detected pMAPK in the cytoplasm of all somatic support cells (Fig. 3.4E-E', small arrows), resembling the net-like pattern of the cytoplasmic extensions seen with the surface markers shown above (n>50). In 100% of cyst cell-*alienRNAi*-testes, pMAPK always co-localized with Tj in the somatic cell nuclei (Fig. 3.4F-F', large arrows, n>50). This finding shows that somatic support cells in cyst cell-*alienRNAi*-testes did receive and transduce the EGF signal, and excludes the possibility that Alien regulates MAPK phosphorylation or nuclear translocation. Due to the lack of cytoplasmic extensions, we did not detect a net-like pattern of pMAPK- staining in cyst cell-*alienRNAi*-testes (Fig. 3.4F-F').

The levels of transcription factors that act downstream of Jak/STAT signaling were reduced in cyst cell-alienRNAi-testes

Expression of UAS-*alienRNAi* in the somatic support cells led to a severe reduction in the levels of CySC stage-specific transcription factors. STAT activation in CySCs appeared normal in control and cyst cell-*alienRNAi*-testes, as all Tj-positive cell nuclei next to the hub were positive for the pSTAT antibody (compare Fig. 3.5B-B' to Fig. 3.5A-A', arrows). In response to JAK/STAT signaling, CySCs express high levels of the transcription factors Zfh1 and Chinmo. In control testes, we always detected Zfh1 at a high level in CySC nuclei (Fig. 3.5C-C', arrow) and at lower levels in the cyst cell nuclei (n>50). Surprisingly, Tj-positive nuclei in the CySC position next to the hub did not express a high level of Zfh1 in any of the cyst cell-*alienRNAi*-testes (Fig. 3.5D-D', arrow, n>50). For unknown reasons, a high level of Zfh-1 was detected in random Tj-positive nuclei positioned away the hub (Fig. 3.5D-D', large arrowhead) and in nuclei of the testis sheath (Fig. 3.5D-D', small arrowhead).

In control testes, Chinmo was expressed at high levels in CySC nuclei (Fig. 3.5E-E', arrows) and to some degree in the cyst cell nuclei. In cyst cell-*alienRNAi*-testes, Chinmo was hardly detectable in Tj-positive nuclei next to the hub (Fig. 3.5F-F', arrows). Pixel counts that quantify the level of Chinmo confirmed that Chinmo was significantly reduced in the Tj-positive cells next to the hub in cyst cell-*alienRNAi*-testes compared to controls (Fig. 3.6A, Table 1). Together, this suggests that knockdown of alien either reduces the expression or leads to degradation of the transcription factors Zfh-1 and Chinmo in the CySCs. Notably, the expression levels of Tj did not appear to be affected

in cyst cell-*alienRNAi*-testes (Fig. 3.5B, 3.5D, and 3.5F), suggesting that the observed reduction in Chimno and Zfh-1 was due to a selective process.

Despite the reduction in the levels of Zfh-1 and Chinmo, knockdown of alien did not disrupt the ability of the somatic support cells to divide by mitosis. In control testes, Tj-positive, pHH3-positive cells were mostly found close to the hub (Fig. 3.6B, arrowheads). In cyst cell-*alienRNAi*-testes as well, we detected Tj-positive, pHH3-positive somatic support cells close to the hub (Fig. 3.6C, arrowheads) indicating that the somatic support cells in the stem cell position responded to proliferative signals.

Finally, we investigated if the somatic support cells in cyst cell-*alienRNAi*-testes had the ability to differentiate into late stage. The transcription factor Eya is normally only expressed at low levels in early-stage cyst cells but at high levels in late-stage cyst cells (Fig. 3.6D-D', arrows). In cyst cell-*alienRNAi*-testes, Eya was barely detectable (Fig. 3.6E-E', arrows), strongly implicating that the somatic support cells remained at early stage. As the upstream signaling pathway activating high levels of Eya is not known, we were not able to examine whether or not the pathway was activated upon knockdown of alien from somatic support cells.

Alien acts through its role in the CSN and via distinct Cullins to regulate germline enclosure and the level of Chinmo

Cyst cell-RNAi against several other members of CSN (CSN1b, CSN3, and CSN6) caused identical phenotypes to cyst cell-*alienRNAi*. For example, expression of a RNA-hairpin directed against CSN1b (cyst cell-CSN1bRNAi-testes) always resulted in cells that expressed Tj but lacked Arm-positive cytoplasmic extensions (compare Fig. 3.7B to

Fig. 3.7A, n>50). Furthermore, somatic support cells in cyst cell-*CSN1bRNAi*- testes and cyst cell-*CSN3RNAi*-testes had a low level of Chinmo in the Tj-positive nuclei next to the hub (Fig. 3.6A, Table 1). Cyst cell-RNAi against other subunits of the CSN complex (CSN4, CSN5, CSN7) produced similar but weaker defects in germline enclosure and differentiation (data not shown). We conclude that Alien acts though its role in the CSN in the somatic support cells of the testes.

Finally, we asked if a loss or reduction of any of the *cullins* produced a similar phenotype to knockdown of *alien*. For this, we expressed available hairpins against *Drosophila cul1, cul2, cullin3 (cul3), or cullin4 (cul4)* in the somatic support cells. We discovered that the expression of an RNA-hairpin against *cul2* led to strong defects in germline enclosure in 100% of the testes (Fig. 3.7C-C'). These defects were identical to the defects seen upon expression of the RNA-hairpin against *alien* (compare to Fig. 3.3B). Expression of RNA-hairpins against *cul1, cul3, or cul4* did not disrupt germline enclosure, as cytoplasmic extensions were clearly detected in a net-like pattern around the germline in 100% of the cyst cell-cul1RNAi-testes (Fig. 3.7D-D', small arrows, n>30), cyst cell-*cul3RNAi*-testes (data not shown, n>30), and cyst cell-*cul4RNAi*-testes (data not shown, n>30). We cannot exclude the possibility that the RNA-hairpins did not fully knockdown cul1, cul3, and/or cul4 in our experiments. However, the RNA-hairpin against cul2 produced a different phenotype than the RNA-hairpins against *cul1, cul3, or cul4*, strongly suggesting that they play distinct roles.

While a reduction in *cul2* disrupted germline enclosure it did not affect the level of Chinmo. In cyst cell-*cul2RNAi*-testes, normal levels of Chinmo were easily detectable in the somatic support cells next to the hub (Fig. 3.7E-E', arrows, Fig. 3.6A, Table 1). In

contrast, Chinmo was hardly detectable in the somatic support cells next to the hub in cyst cell-*cul1RNAi*-testes (Fig. 3.7F-F', arrows, Fig. 3.6A, Table 1). We conclude that CSN and Cul2 maintain the germline cellular microenvironments while CSN and Cul1 maintain a high level of Chinmo in the CySCs.

Discussion

Here we show that knockdown of alien from somatic support cells disrupted the cellular microenvironment of the germline cells. Interestingly, Alien activity was associated with the regulation of nuclear hormone signaling. When transformed into Hela cells, Alien interacts with the thyroid hormone receptor in a ligand-dependent manner. A similar role for Alien as a co-regulator for the Ecdysone receptor (EcR) has been suggested (Dressel et al., 1999). However, Alien does not appear to act as a co-regulator of the EcR in somatic support cells as depletion of EcR signaling molecules from these cells did not produce a mutant phenotype (Qian et al., 2014). Instead, Alien acts though its role in the CSN. As with the expression of the RNA-hairpin directed against alien in the somatic support cells, the expression of RNA-hairpins against other subunits of the CSN complex in these cells disrupted germline enclosure. The CSN has not been associated with the maintenance or function of cellular microenvironments in any species. Given the importance of cellular microenvironments for tissues homeostasis and the conservation of the CSN complex among plant and animal species, it seems highly likely that a similar role for CSN may be discovered in other tissue maintained by stem cells.

The knockdown of alien from the somatic support cells also caused an accumulation of early-stage germline cells within the mutant testes. A similar germline

phenotype was observed in other mutants that affect the structure of the microenvironment cells, such as those in Tj, Dynein Light Chain, or those in components of EGF signaling (Joti et al., 2011; Li et al., 2003; Schulz et al., 2002). We therefore propose that the observed failure of germline cells to differentiate in cyst cell-alienRNAi-testes was an indirect effect due to the loss of the cellular microenvironment.

In addition to the defects in germline enclosure, cyst cell-alienRNAi-testes had reduced levels of Zfh-1 and Chinmo in the somatic support cells next to the hub, suggesting that these cells were not CySCs. Yet, these cells occupied the CySC position, had pSTAT and nuclear pMAPK in their nuclei, and divided by mitosis, indicating that they had CySC characteristics. These latter observations suggest that high levels of Zfh-1 and Chinmo may be dismissible for CySC fate or function. A low level of Zfh-1 and Chinmo could be sufficient for CySC fate as long as the cells remain in contact with the hub. Possibly, pSTAT or other signal transducers activate additional downstream effectors for CySC fate or function.

The CSN complex has been associated with stem cell maintenance in human embryonic stem cells and GSCs in the *Drosophila* ovary (Chia et al., 2010; Pan et al., 2014; Yan et al., 2003). In the *Drosophila* ovary, germline cells were depleted of CSN function using mosaic analysis. In this technique, the homozygous mutant GSCs were quickly lost from the stem cell position while the heterozygous GSCs were maintained (Pan et al., 2014). Recently, competition for the position next to the hub has been shown to be one of the mechanisms to assure that a healthy population of stem cells is maintained in the gonad (Issigonis et al., 2009; Sheng et al., 2009). In the context of mosaic analysis, a CSN-depleted CySC may be disadvantaged and lose the competition

for the position next to the hub. In our experiments, we depleted the CSN from all CySCs. CySCs, in turn, are instructive for GSC fate (Leatherman and Dinardo, 2010). Thus, we can assume that we created a non-competitive environment that allowed CSN-depleted CySCs to be maintained next to the hub.

In the ovary, CSN4 was reported to specifically interact with the cytoplasmic differentiation factor Bag of marbles (Bam) and this interaction was proposed to regulate the balance between GSC self-renewal and differentiation (Pan et al., 2014). fmc is not expressed in somatic support cells of the testes and thus is not likely to regulate CSN function in the CySC lineage. We did not observe a loss of germline cells in the position next to the hub upon expressing the RNA-hairpins against subunits of CSN in all germline cells (data not shown). This could be due to a lack of competition between the CSN-depleted GSCs, or because we did not sufficiently reduce the CSN subunits from the germline.

We showed that different Cullins, which are known to function downstream of the CSN complex, mediate different cellular responses of the somatic support cells. Cyst cell-*cul1RNAi*-testes had reduced levels of Chinmo. We propose that Cul1 promotes CySC fate via controlling the levels of Zfh-1 and Chinmo downstream of Jak/STAT activation. Cyst cell-*cul2RNAi*-testes showed defects in germline enclosure. We propose that Cul2 regulates germline enclosure in the CySCs and the cyst cells downstream of MAPK activation, possibly by regulating EGF-targets. Different roles for the Cullin scaffolds downstream of the CSN have been described in dendrite morphogenesis. In this aspect of development, the CSN acts through Cul1 to stimulate dendritic branching and through Cul3 to inhibit it (Djagaeva and Doronkin, 2009a, b). It would be interesting to address if

and how selected protein degradation acts in mammalian tissues maintained by stem cells. Specifically, studying the role of different Cullins may provide useful information for furthering our understanding of tissue homeostasis.

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Table 3.1: The levels of Chinmo in the Tj-positive cells next to the hub.

The levels of Chinmo in the Tj-positive cells next to the hub. Genotypes, number of testes, and number of somatic support cell next to the hub analyzed (cells) are as indicated. A reduction of Chinmo (measured as pixels per inch) was observed upon knockdown of subunits of the CSN and upon knockdown of Cul2, s.d.= standard deviation.

Genotype	**#testes**	**#cells**	**Chinmo**	**s.d.**
UAS-alienRNAi/+	20	51	222.12	58.76
Cyst cell-*alienRNAi*	21	40	96.05	36.87
UAS-CSN1bRNAi/+	27	65	125.12	45.42
Cyst cell-*CSN1bRNAi*	18	53	52.65	15.18
UAS-CSN3RNAi/+	25	61	193.72	56.89
Cyst cell-*CSN3RNAi*	23	56	114.46	41.20
UAS-cul2RNAi/+	24	59	230.77	94.50
Cyst cell-*cul1RNAi*	30	69	122.60	50.47
Cyst cell-*cul2RNAi*	18	33	208.26	64.12
Cyst cell-*cul3RNAi*	25	67	200.99	87.52

Figure 3.1: Expression of the RNA-hairpin against *alien* in somatic support cells produced a strong phenotype.

(A) Illustration of the arrangement and development of germline and somatic support cells. The apical hub is shown in yellow, germline cells in light blue, and cyst cells are color-coded according to their developmental stage; CySCs in pink, early-stage cyst cells in purple, and late-stage cyst cells in green.

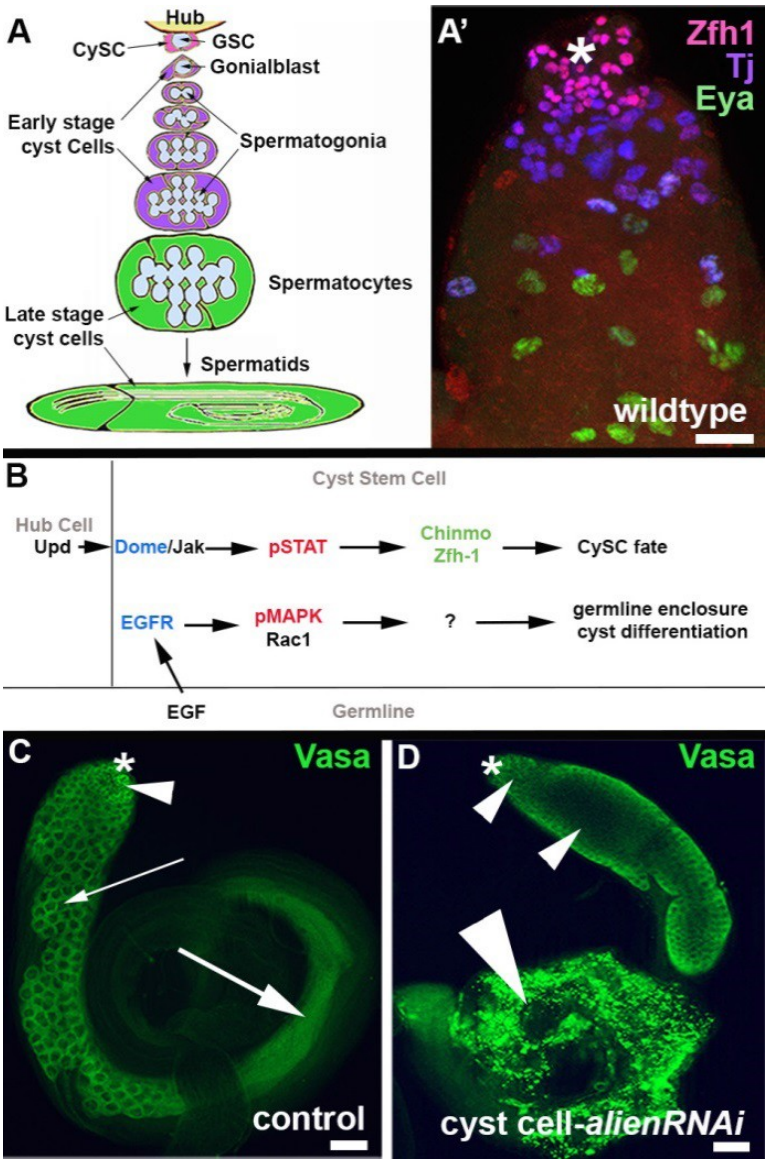
(A') The apical region of a wild-type testis labeled for stage-specific, nuclear markers for somatic support cells, as indicated.

(B)) Illustration of the key players in Jak/STAT and EGF signaling that regulate CySC fate, germline enclosure, and cyst differentiation. Cell types in light grey, receptors in blue, signal transducers that translocalize into nuclei in red, other signal transducers in black, transcription factors in green.

(C) A whole testis from a control animal showing small, Vasa-positive, early-stage germline cells (arrowhead), and large, Vasa-positive spermatocytes (small arrow). Sperm bundles are seen at the base (large arrow).

(D) A whole testis from a cyst cell-*alien*RNAi-animal with excess small, Vasa-positive, early-stage germline cells (small arrowheads) in the apical region but lacking spermatocytes and sperm bundles. Note that cell debris (large arrowhead) is filling the basal region of the testis, indicative of massive cell death.

Asterisks mark the hubs, scale bars: 30 μm.


A
Hub
CySC
GSC
Gonialblast
Early stage
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Spermatogonia
Spermatocytes
Late stage
cyst cells
Spermatids
A'
Zfh1
Tj
Eya
wildtype
B
Cyst Stem Cell
Hub Cell
Upd
Dome/Jak
pSTAT
Chinmo
Zfh-1
CySC fate
EGFR
pMAPK
Rac1
?
germline enclosure
cyst differentiation
EGF
Germline
C
Vasa
control
D
Vasa
cyst cell-alienRNAi

Figure 3.2: Germline differentiation was blocked in cyst cell-*alienRNAi*-testis.

(A)-(F) Immuno-labeling with molecular markers, as indicated.

(A) and (A') A control testis tip with a round fusome (arrowheads) in GSCs and gonialblasts, thin fusomes (small arrows) interconnecting spermatogonia, and thick fusomes (large arrows) interconnecting spermatocytes.

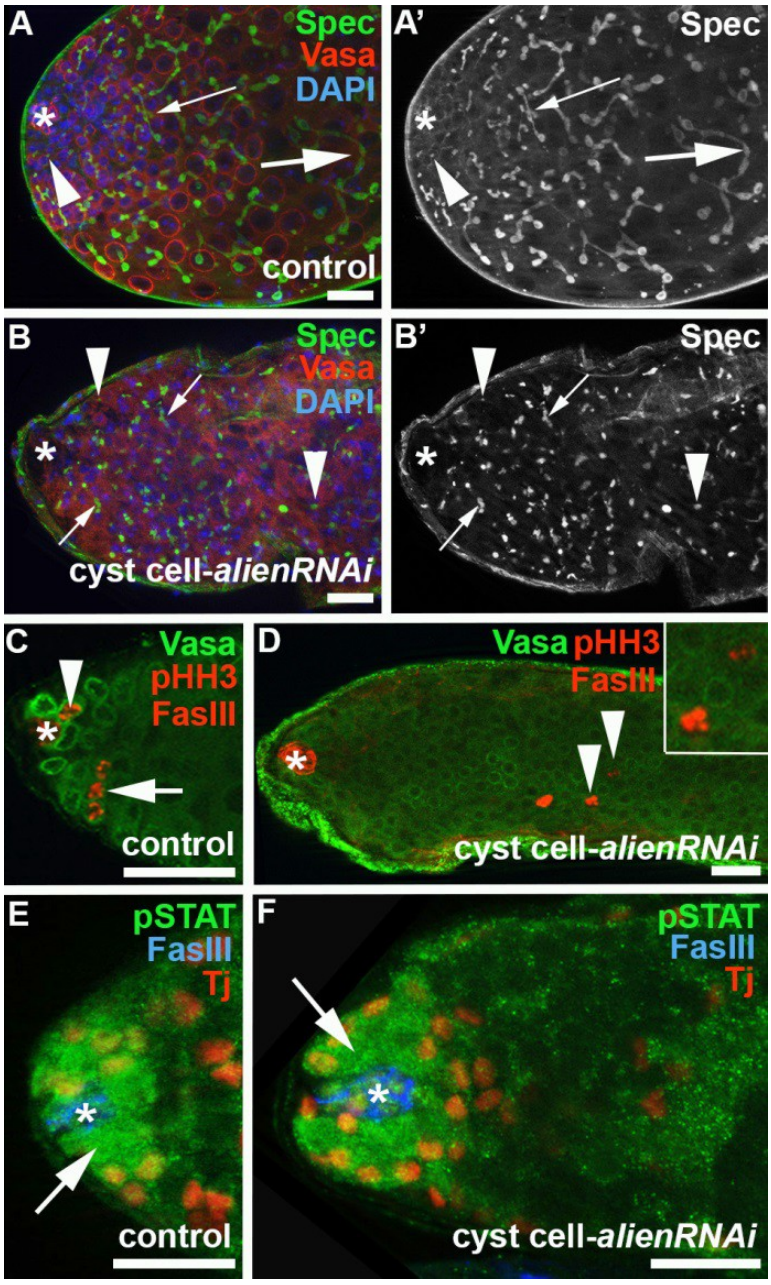
(B) and (B') Apical region of a cyst cell-*alienRNAi*-testis showing round fusomes (arrowheads) and short, thin fusomes (arrows).

(C) A control testis tip with a single dividing GSC (arrowhead) next to the hub and a group of four dividing spermatogonia (arrow) displaced away from the hub.

(D) Apical region of a cyst cell-*alienRNAi*-testis showing single dividing germline cells (arrowheads) in positions away from the hub. Inset shows two dividing Vasa-positive germline cells at a high magnification.

(E) and (F) Nuclear pSTAT (green) was only detected in the cells around the apical hub in (E) a control testis and (F) a cyst cell-*alienRNAi*-testis.

Asterisks mark the hubs, scale bars: 30 μm.


A
Spec
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cyst cell-alienRNAi
B'
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C
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pHH3
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FasIII
cyst cell-alienRNAi
E
pSTAT
FasIII
Tj
control
F
pSTAT
FasIII
Tj
cyst cell-alienRNAi

Figure 3.3: In cyst cell-*alienRNAi*-testis, the germline cells lacked their cellular microenvironment.

(A)-(F') Apical regions of testes immuno-labeled with molecular markers, as indicated. Small arrows point to cytoplasmic extensions surrounding the germline cells, large arrows point to cytoplasmic extensions in the mutant testes, and arrowheads point to somatic support cell nuclei.

(A) and (A') A control testis showing an Arm-positive, net-like pattern of cytoplasmic extensions around the germline.

(B) and (B') A cyst cell-*alienRNAi*-testis without an Arm-positive, net-like pattern of cytoplasmic extensions around the germline. Note that the hub is positive for Arm.

(C) and (C') A control testis showing Tj-positive cyst cell nuclei and an ECad-positive, net-like pattern of cytoplasmic extensions.

(D) and (D') A cyst cell-*alienRNAi*-testis contains Tj-positive cyst cell nuclei but lacks the ECad-positive, net-like pattern of cytoplasmic extensions.

(E) and (E') A control testis with Tj-positive cyst cell nuclei and an NCad-positive, net-like pattern of cytoplasmic extensions.

(F) and (F') A cyst cell-*alienRNAi*-testis contains Tj-positive cyst cell nuclei but lacks the NCad-positive, net-like pattern of cytoplasmic extensions.

Asterisks mark the hubs, scale bars: 30 μm.

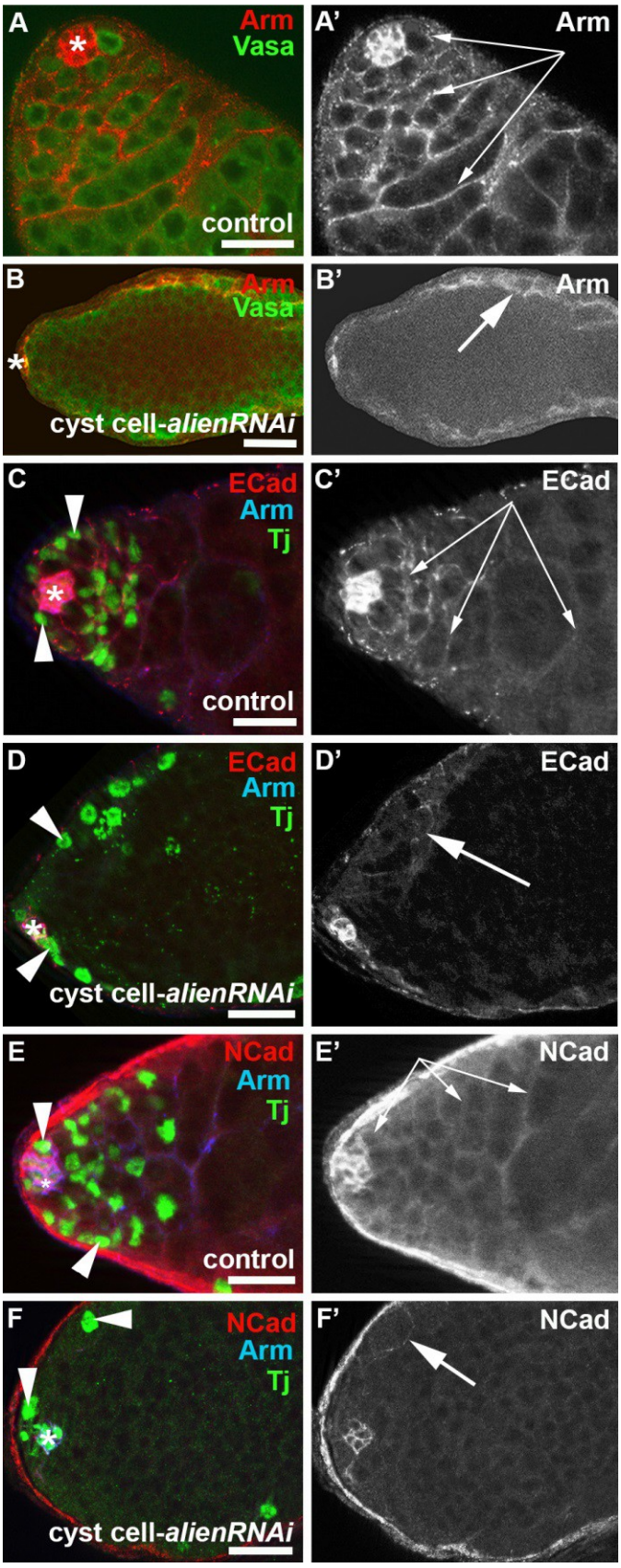

A
Arm
Vasa
control
A'
Arm
B
Arm
Vasa
cyst cell-alienRNAi
B'
Arm
C
ECad
Arm
Tj
control
C'
ECad
D
ECad
Arm
Tj
cyst cell-alienRNAi
D'
ECad
E
NCad
Arm
Tj
control
E'
NCad
F
NCad
Arm
Tj
cyst cell-alienRNAi
F'
NCad

Figure 3.4: Reduction of *alien* in somatic support cells led to a quick loss of cytoplasmic extensions.

(A)-(F') Apical tips of testes, immuno-labeling as indicated.

(A)-(D') Arm-positive cytoplasmic extensions in cyst cell-*alienRNAi*-testis

(A) and (A') appear normal prior to the shift to 29°C (day 0),

(B) and (B') show holes (small arrowheads) by day 1 after the shift to 29°C,

(C) and (C') have large disruption (large arrowhead) by day 2 after the shift to 29°C,

(D) and (D') are almost gone by day 3 after the shift to 29°C.

(E) In a control testis, Tj-positive somatic support cells had pMAPK in their nuclei (large arrows) and in their cytoplasm (small arrows).

(F) and (F') In a cyst cell-*alienRNAi*-testis, pMAPK co-localized with Tj in somatic support cell nuclei.

Asterisks mark the hubs, scale bars: 30 μm.

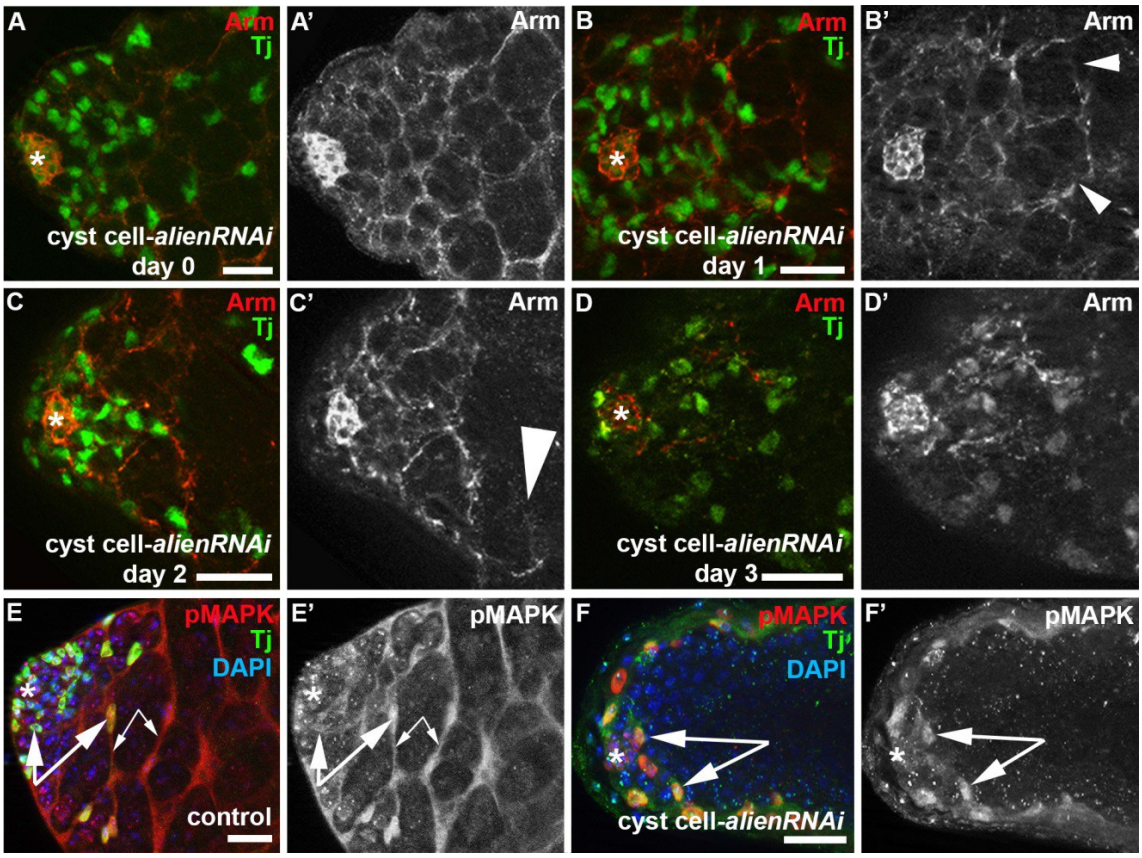

A
Arm
Tj
cyst cell-alienRNAi
day 0
A'
Arm
B
Arm
Tj
cyst cell-alienRNAi
day 1
B'
Arm
C
Arm
Tj
cyst cell-alienRNAi
day 2
C'
Arm
D
Arm
Tj
cyst cell-alienRNAi
day 3
D'
Arm
E
pMAPK
Tj
DAPI
control
E'
pMAPK
F
pMAPK
Tj
DAPI
cyst cell-alienRNAi
F'
pMAPK

Figure 3.5: *alien*-depleted somatic support cells have lower levels of the transcription factors Zfh-1 and Chinmo.

(A)-(F') Apical testes tips immuno-stained with molecular markers, as indicated.

(A)-(B') pSTAT in Tj-positive nuclei next to the hub in (A) and (A') a control testis and (B) and (B') a cyst cell-*alienRNAi*-testis.

(C) and (C') In control, Tj-positive nuclei next to the hub had a high levels of Zfh-1 (arrows) while the Tj-positive nuclei away from the hub had decreasing levels of Zfh-1.

(D) and (D') In a cyst cell-*alienRNAi*-testis, Zfh-1 was hardly detectable in Tj-positive nuclei next to the hub (arrows). Note that a high level of Zfh-1 was detected in nuclei of the testis sheath (small arrowhead) and random Tj-positive cells located away from the tip (large arrowhead).

(E) and (E') In a control testis, Tj-positive cell nuclei (large arrows) next to the hub showed a high level of Chinmo.

(F) and (F') In a cyst cell-*alienRNAi*-testis, a low level of Chinmo was detected in Tj-positive nuclei next to the hub (large arrows).

Asterisks mark the hubs, scale bars: 30 μm.

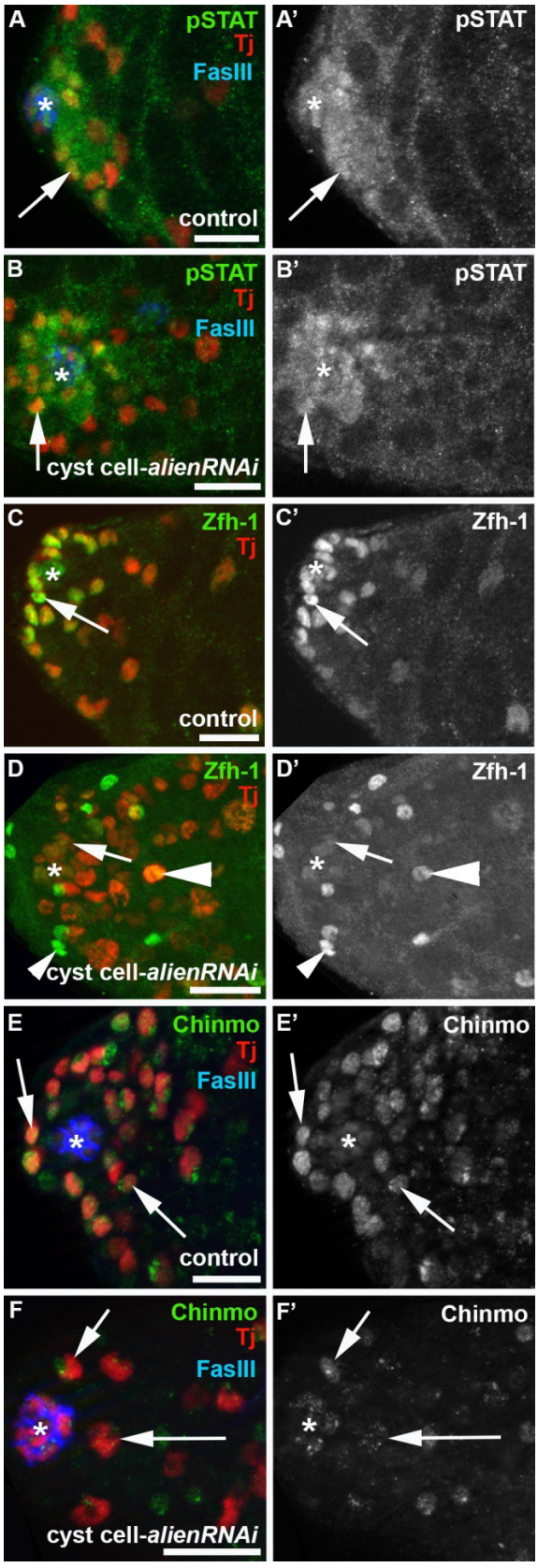

A
pSTAT
Tj
FasIII
control
A'
pSTAT
B
pSTAT
Tj
FasIII
cyst cell-alienRNAi
B'
pSTAT
C
Zfh-1
Tj
control
C'
Zfh-1
D
Zfh-1
Tj
cyst cell-alienRNAi
D'
Zfh-1
E
Chinmo
Tj
FasIII
control
E'
Chinmo
F
Chinmo
Tj
FasIII
cyst cell-alienRNAi
F'
Chinmo

Figure 3.6: *alien* is required for the levels of transcription factors but not for mitosis.

(A) Bar graph showing pixel counts for Chinmo in a variety of genotypes, as indicated. Pixel counts of control genotypes shown as light grey bars, and those of experimental genotypes as black bars. Control and experimental genotypes are grouped as they have been stained and analyzed the same day. P= statistical relevance of the difference in pixel count between experimental genotypes and the corresponding control genotype.

(B-E) Apical testes regions immuno-labeled with molecular markers, as indicated.

(B) and (C) Tj-positive cells next to the hub undergoing mitotic divisions (arrowheads) in (B) a control, and (C) a cyst cell-*alienRNAi*-testis.

(D) and (D') A control testis with Tj- and Eya-positive (arrows) cyst cell nuclei.

(E) and (E') In a cyst cell-*alienRNAi*-testis, many nuclei were positive for Tj, but Eya was hardly detectable (arrows).

Asterisks mark the hubs, scale bars: 30 μm.

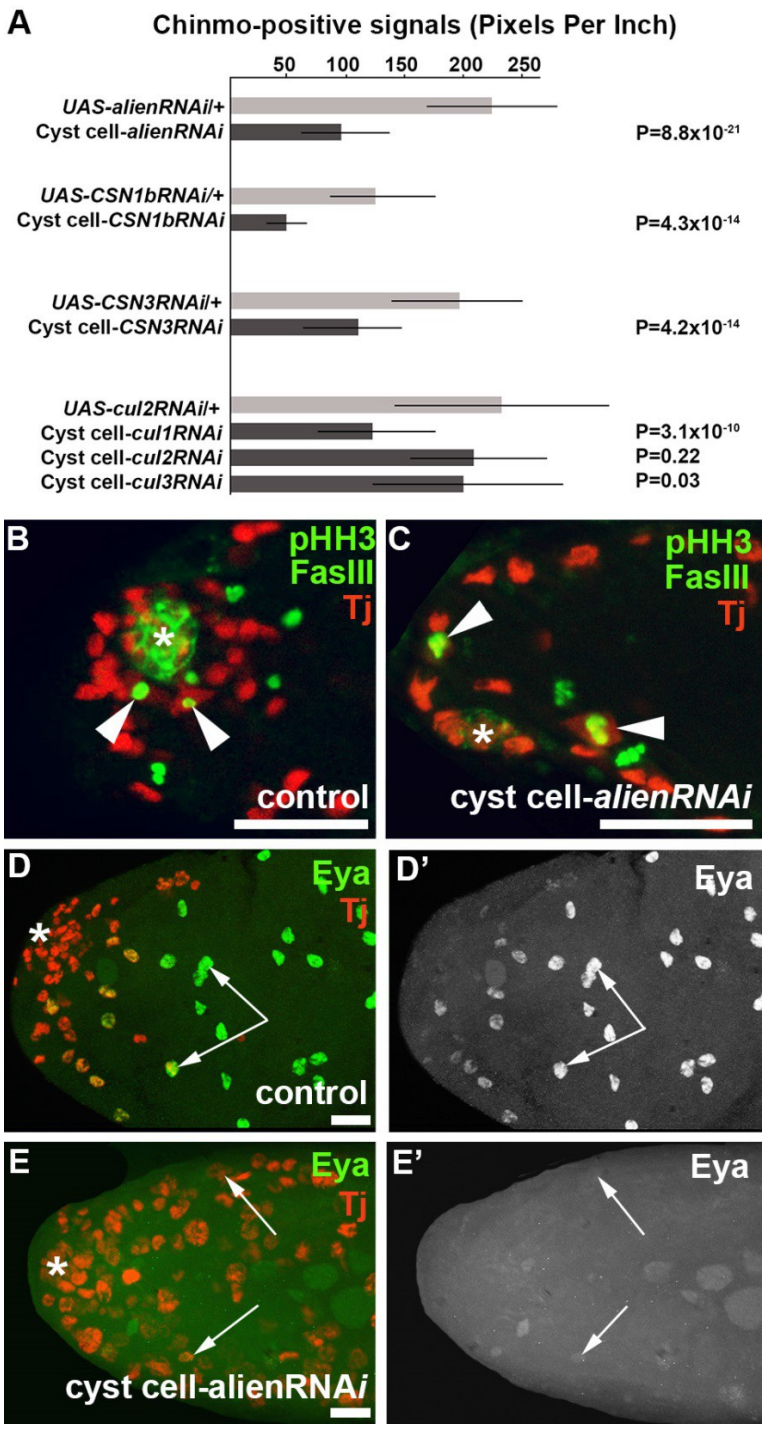

A
Chinmo-positive signals (Pixels Per Inch)
50
100
150
200
250
UAS-alienRNAi/+
Cyst cell-alienRNAi
P=8.8x10-21
UAS-CSN1bRNAi/+
Cyst cell-CSN1bRNAi
P=4.3x10-14
UAS-CSN3RNAi/+
Cyst cell-CSN3RNAi
P=4.2x10-14
UAS-cul2RNAi/+
Cyst cell-cul1RNAi
Cyst cell-cul2RNAi
Cyst cell-cul3RNAi
P=3.1x10-10
P=0.22
P=0.03
B
pHH3
FasIII
Tj
control
C
pHH3
FasIII
Tj
cyst cell-alienRNAi
D
Eya
Tj
control
D'
Eya
E
Eya
Tj
cyst cell-alienRNAi
E'
Eya

Figure 3.7: Alien acts through the CSN and Cullins.

(A-G') Apical testes tips immuno-labeled with molecular markers, as indicated.

(A) A control testis with Tj-positive nuclei (arrowhead) and an Arm-positive, net-like pattern of cyst cell cytoplasmic extensions (red).

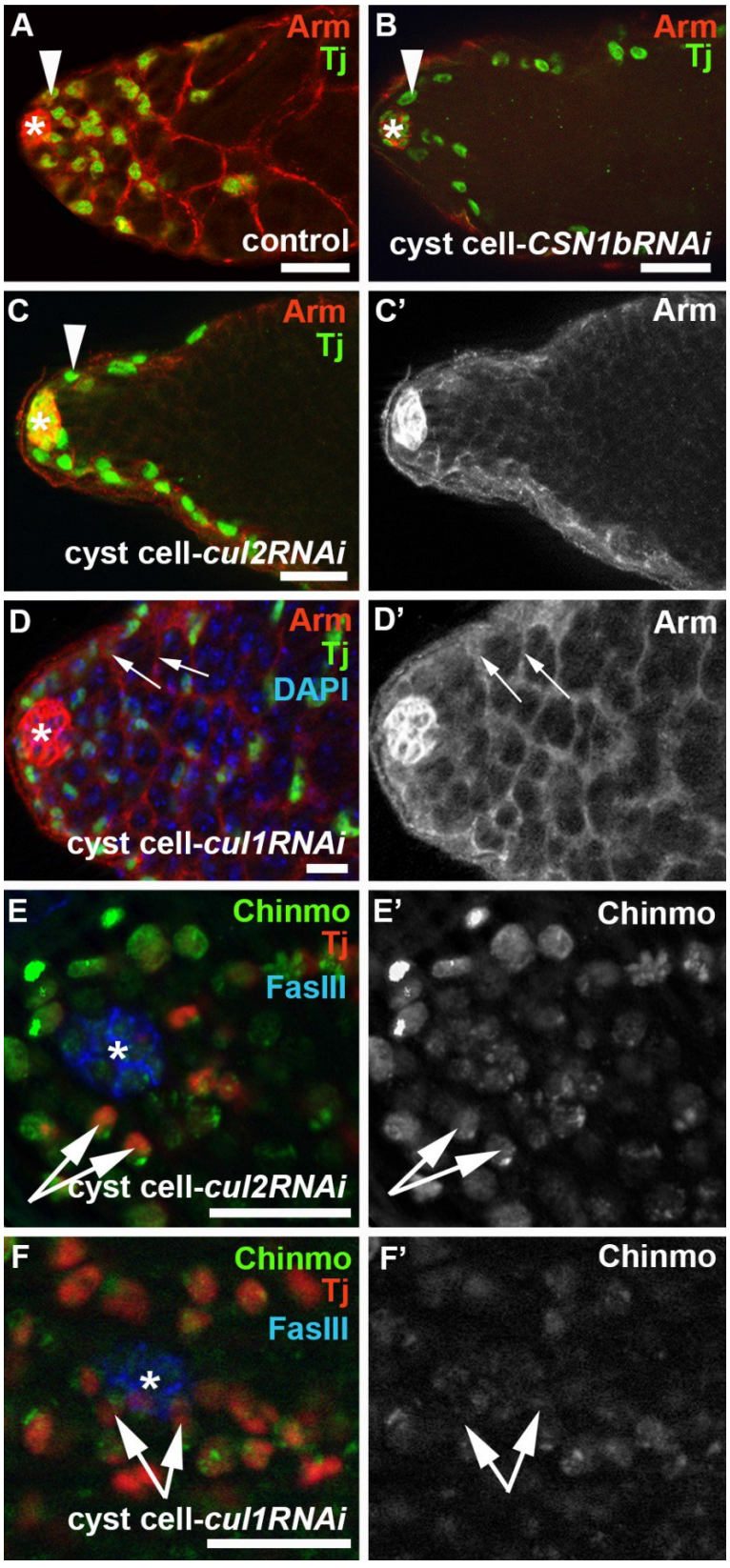
(B) A cyst cell-*CSN1b*RNAi-testis with Tj-positive nuclei (arrowhead) but lacking cytoplasmic extensions.

(C) and (C') A cyst cell-*cul2RNAi*-testis with Tj-positive nuclei (arrowhead) but lacking cytoplasmic extensions.

(D) and (D') A cyst cell-*cul1RNAi*-testis with cytoplasmic extensions around the germline (small arrows).

(E) and (F') Tj-positive nuclei (arrows) next to the hub had (E) and (E') a high level of Chinmo in a cyst cell-*cul2RNAi*-testis, but (F) and (F') a low level of Chinmo in a cyst cell-*cul1RNAi*-testis.

Asterisks mark the hubs, scale bars: 30 μm.


A
Arm
Tj
control
B
Arm
Tj
cyst cell-CSN1bRNAi
C
Arm
Tj
cyst cell-cul2RNAi
C'
Arm
D
Arm
Tj
DAPI
cyst cell-cul1RNAi
D'
Arm
E
Chinmo
Tj
FasIII
cyst cell-cul2RNAi
E'
Chinmo
F
Chinmo
Tj
FasIII
cyst cell-cul1RNAi
F'
Chinmo

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CHAPTER 4

CONCLUSION

In this dissertation, I have discussed the important interactions between germline and somatic cells during spermatogenesis of *Drosophila melanogaster*. In Chapter 2, I reported the localization of N-specific components to testes and the role of N in maintaining germline-somatic cysts. In Chapter 3, I showed that CSN acts through CRLs to target CySC proteins for degradation. The signaling pathways in these studies are likely critical to mediate cell-to-cell communication between germline and soma. The *Drosophila* gonad serves as an important tool with its genetic tractability and relative simplicity compared to other model systems to explore this intimate relationship. While the exact mechanisms involved between these two cell populations to regulate spermatogenesis are complex and context-dependent, there remain important questions to be answered: (1) how do multiple signaling factors interact to coordinate the spatial-temporal organization of the gonads; (2) subsequent to signaling from the germline, how do somatic cells respond to the germline to coordinate cell differentiation; (3) how closely conserved are the signaling pathways regulating *Drosophila* spermatogenesis in higher-order organisms such as humans and mouse? In order to try and address these questions, we will need a more comprehensive approach involving the use of current or developing molecular, biochemical, and genomic tools to specifically target and label cell signaling events.

APPENDIX

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Title: CSN maintains the germline cellular microenvironment and controls the level of stem cell genes via distinct CRLs in testes of Drosophila melanogaster

Author: Yue Qian,Chun L. Ng,Cordula Schulz

Publication: Developmental Biology

Publisher: Elsevier

Date: 1 February 2015

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