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The role of the *headcase* gene in the *Drosophila melanogaster* testis and intestinal stem cell niches

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The role of the *headcase* gene in the *Drosophila melanogaster* testis and intestinal stem cell niches

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Preface

The dissertation author, Luís Pedro Fernandes Resende, is a member of the 11th edition of the GABBA PhD Program (University of Porto) and started his PhD in January 2008. In September 2008 he joined Dr Leanne Jones research laboratory at the Salk Institute for Biological Studies, San Diego, California. The work presented in this document was performed between September 2008 and December 2012. Based on the findings here reported, 2 scientific original data articles are currently under revision, and one is being prepared for submission. Also, Luís Pedro Fernandes Resende published a first author review article that corresponds to the Appendix of this thesis. Citations of the mentioned papers are as follow:

1. Resende L.P.F, Boyle M., Tran D., Jones D.L. **Headcase promotes cell survival and niche maintenance in the *Drosophila* testis (submitted)**

2. Voog J., Sandall S., Hime G., Resende L.P.F., Loza-Coll M., Fuller M.T., and Jones D.L.. **Escargot regulates niche cell-stem cell conversion in the *Drosophila* testis (submitted)**

3. Resende L.P.F., Jones D.L. ***headcase* gene is required for maintenance of instestinal stem and enteroblast cells in the *Drosophila* intestine (in preparation)**

4. Resende L.P.F., Jones D.L. **Local signaling within stem cell niches: insights from *Drosophila***. *Curr. Opin. Cell Biol.* 2012, **24**:225–231.

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Author's motivation

Watching videos of vertebrate embryonic development I became fascinated with the power of **stem cells**. The primordial architects capable of transforming a single cell into a multicellular organism (embryonic stem cells), and latter the engineers behind the maintenance and regeneration of different tissues (adult stem cells).

The work here represented reflects my personal interest on studying stem cell biology *in vivo*, on the context of their natural microenvironment and never simplifying the complex and dynamic network of relationships between all the components of the **stem cell niche**.

While focusing on the role of specific gene (***headcase***) in two particular *Drosophila* tissues, I have always aimed to design experiments that could contribute to a better understanding of the basic principles behind the dynamic crosstalk between stem cells and their niche.

Acknowledgements

"Triste de quem vive em casa,
Contente com o seu lar,
Sem que um sonho, no erguer de asa,
Faça até mais rubra a brasa
Da lareira a abandonar!"

[Sad is the one who stays at home,
Contented with his homestead,
Without a dream, that in the lifting of wing,
Would turn redder the embers,
In the fireplace left behind!]

in Mensagem, Fernando Pessoa

I owe my deep gratitude to the GABBA program coordinators, for their visionary idea of creating such a unique program for PhD studies, for keeping the program alive in such a challenging time, and for accepting me as a member of the GABBA family.

I thank Dr. Leonor David for her wise professional guidance that started a few years before I began my PhD and that I was lucky to keep during this next important step of my scientific career. She always found the best way to help me to see things from the best perspective and to focus on the essential.

Joining Dr. Leanne Jones laboratory was undoubtedly the best professional decision I have ever made. She made me fell welcome in her lab since my very first day at the Salk and she perfected the role of a mentor. I thank her for the constant understanding, for the long scientific discussions and for always knowing when and how to push me towards excellence.

I am privileged to have worked with many talented scientists actual and former members of the "Jones lab". Many of them contributed in a very important way to the work here presented and from all of them I learned a lot.

A big "thank you" to all my family and friends - due to natural space limitation I can only mention a few of them. To Filipe Laranjeira for the encouragement to pursue a PhD and for being such an important friend for such a long time. To Nuno, Filipe, Bibiana, Zé Maria and Rui, together they were my "san diegan family".

To Ana, Luis and Rita, for being so important (and present) in my life. With them I started dreaming about living in California, with them I shared this dream during their several visits.

To my parents, the two persons I most admire. This work, and truly anything I achieve in life is a direct result of their continuous education and love. This thesis is dedicated to them.

Resumo

As células estaminais adultas residem em ambientes celulares especializados (nichos), onde desempenham um papel fundamental na manutenção dos diferentes órgãos e tecidos, e na resposta a danos. Os nichos de células estaminais são reguladores importantes de muitos aspectos da biologia das células estaminais como, por exemplo, a sua renovação, sobrevivência e diferenciação. Portanto, é essencial perceber o modo como as células estaminais respondem a alterações no seu nicho, num contexto de homeostasia do tecido, assim como em condições patológicas.

O testículo de *Drosophila* é um dos mais bem caracterizados e poderosos sistemas para o estudo dinâmica do nicho das células estaminais. Na extremidade apical do testículo de *Drosophila* na fase adulta, as *germline stem cells* (GSCs) e as *cyst somatic stem cells* (CySCs) rodeiam e contactam directamente um grupo de aproximadamente 10 células somáticas que formam um componente integral do nicho das células estaminais, denominado *hub.* Diversos estudos contribuiram nos últimos anos para o actual sólido conhecimento do potencial estrutural e de sinalização do *hub* mas é ainda desconhecido de que modo variações no número de células do *hub* influenciam o número de células estaminais. No âmbito de um procura de factores que desempenhassem funções reguladoras do tamanho do hub e da sua formação foi identificado o gene *headcase* (*hdc*), cuja função molecular é ainda desconhecida.

O primeiro capítulo desta tese corresponde às descobertas do autor relacionadas com as consequências fenotípicas autónomas e não-autónomas da perda de função do gene *headcase* no *hub*. Os resultados mostram que a função *de headcase* é requerida para evitar que as células do *hub* entrem em morte cellular programada. Isto representa a primeira associação conclusiva entre o gene *headcase* e o fenómeno de apoptose. A redução progressiva no número de células do *hub* em consequência da perda de função de *headcase* foi usada como paradigma para investigar questões abertas e interessantes relacionadas com a dinâmica dos nichos de células estaminais. Em particular, e igualmente parte integrante do primeiro capítulo, estão incluidos resultados sobre como as células estaminais respondem a alterações do tamanho do seu nicho *in vivo*, assim como no potencial regenerativo do nicho.

Após estas descobertas iniciais foi decidido investigar um eventual papel conservado para o gene *headcase* noutros nichos de células estaminais, como por exemplo no ovário e no intestino. O particular padrão de expressão no intestino juntamente com a experiência do laboratório neste tecido foram razões que levaram a

que fosse investigada uma eventual função de *headcase* no intestino. Resultados sobre como a função de *headcase* nas células estaminais e *enteroblast* é essencial para a manutenção de um epitélio functional estão descritos no segundo capítulo desta tese. O conjunto de dados incluídos no segundo capítulo representam um projecto numa fase mais preliminar, contudo, representam evidência complementar para a associação de *headcase* com o processo de morte cellular programada, reportado no primeiro capítulo. Sugerem que o fenótipo não é restricto às células do hub do testículo em particular, e pode ser observado noutros tipos celulares e/ou tecidos.

Experiências a decorrer e futuras visam expandir o conhecimento sobre o papel de *headcase* em diferentes tipos celulares, assim como sobre a sua função molecular e como esta está ligada com o processo o início de morte cellular programada. O gene humano homólogo de *headcase* (HECA) encontra-se desregulado em diferentes tipos de tumors. Assim sendo os resultados expostos nesta tese deverão provar-se informativos para perceber o papel da proteína codificada por este gene no contexto de tumorigenese, e ajudar a clarificar se a modulação da função de *headcase* poderá ser considerada para a elaboração de novas terapias para o cancro.

Abstract

Adult stem cells reside in specialized microenvironments (niches) where they play a crucial role in the maintenance of different organs and tissues, and in the response to injury. Stem cell niches are important regulators of many aspects of stem cell biology like self-renewal, survivor and differentiation. Therefore, it is essential to understand how stem cells respond to alterations in their niche in the context of tissue homeostasis and in pathologic conditions.

The *Drosophila* testis is one of the best-understood and powerful systems for the study of stem cell niche dynamics. At the apical tip of the adult *Drosophila* testis, germline stem cells (GSCs) and cyst somatic stem cells (CySCs) surround and directly contact a cluster of approximately 10 somatic cells that form an integral component of the stem cell niche, the hub. Several studies have contributed to a clear understanding of the structural and signaling potentials of the hub, but it is not known the degree to which the overall size of the hub influences the stem cell pool. In the course of a screen to identify factors involved in regulating hub size and maintenance, *headcase* (*hdc*) was identified, a gene whose molecular function is still unknown.

The first chapter of this thesis corresponds to the author's findings on the autonomous and non-autonomous phenotypic consequences of *headcase* loss-of-function in the hub. Results show that *hdc* gene function is required in the hub to prevent hub cells from entering programmed cell death. This represents the first direct association between *headcase* and apoptosis initiation. The progressive reduction in hub cell numbers after *headcase* loss-of-function was used as paradigm to investigate interesting and open questions about stem cell niche dynamics. Particularly, and also reported in first chapter, there are results on how stem cells respond to alterations in niche size *in vivo* and on the niche regeneration potentials.

After these findings in the testis, a putative conserved role for *headcase* in other cell types/tissues was investigated. Headcase expression was detected in other stem cell niches, like the ovary and the intestine. The particular interesting expression pattern in the intestinal midgut combined with laboratory expertise, were reasons that lead to the investigation of *headcase* function in the intestine. Results on how *headcase* function is required in intestinal stem and enteroblast cells to maintain a functional epithelium are described in the second chapter of this thesis. While still in a more preliminary stage, the dataset included in this second chapter provides further evidence on *headcase* association with the process of programmed cell death reported in the first chapter. It

suggests that this phenotype is not restricted to hub cells in the testis in particular, and can be observed in other cell types/tissue.

Ongoing and future experiments aim to expand the knowledge about *headcase* role in different cell types, but also it's molecular function and how that is linked to the process of apoptosis initiation. As HECA (Drosophila human homolog) is found misexpressed in different types of tumor, results included in this thesis should prove informative to understand the role of this protein in the context of tumor development and help to clarify if modulating *headcase* function should be considered for the design of new cancer therapies.

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List of Abbreviations

aa - aminoacid
AB – Antibody
ALS - Amyotrophic lateral sclerosis
Arm – Armadillo
BM – Basal membrane
BMP - Bone morphogenetic protein
Byn - Brachyenteron
CyCK – Cyclin K
CySC – Cyst stem cell
CB-SCs – Cord blood stem cells
CBCs- crypt base columnar cells
DE-Cad – Drosophila E-Cadherin
DN-Cad - Drosophila N-Cadherin
DIAP – *Drosophila* inhibitor of apoptosis
EE – Enteroendocrine
EB - Enteroblast
EC - Enterocyte
Esg – Escargot
FasIII – Fasciclin III
Flp-frt – Flippase recombination enzyme – flippase recognition target
Fig - Figure
$GAL80^{ts}$ – temperature sensitive GAL80
GFP – Green fluorescent protein
GSC – Germline stem cell
Hdc – Headcase
Hid – Head involution defective
HIV - Human immunodeficiency virus
ISC – Intestinal stem cell
JAK-STAT - Janus kinase Signal transducer and activator of transcription
nt - nucleotide
Tj – traffic-jam
Prosp – Prospero
RFP – Red fluorescent protein
Rpr – reaper
Wt – Wild type
Unk – Unkempt
Upd - Unpaired

Introduction

Stem cell niche, the concept

Stem cells are characterized by their ability to divide and generate both new stem cells (self-renew) as well as different specialized cell types (differentiation). Adult stem cells reside in specialized microenvironments within our body - “stem cell niches” - where they play a crucial role both maintaining tissue homeostasis as well as in response to injury. Inspired by findings on hematopoietic stem cells and the influence of stromal cells in their behavior, R. Schofield postulated the “the stem cell niche hypothesis” in the late 70’s, to explain the dependence of stem cells upon their microenvironment [1][2][3]. Studies in the model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* provided the initial identification and characterization of stem cell niches *in vivo [4]*[5][6][7][8]. From these studies, a diversity of components of stem cell niches were revealed and included: the stem cell themselves, progenitor and neighboring cells, soluble factors, extracellular matrix, neural inputs, cell adhesion components, and others [9] (Figure 1). In different organs or tissues, stem cells rely on a specific network of inputs from some of these components. Therefore, stem cell niches represent a basic unit of tissue physiology that serve different anatomic functions (structural and trophic support, topographical information and physiological cues), and ultimately are responsible for the regulation of stem cell behavior [10].

During the past few years, a remarkable success was witnessed in the identification and characterization of stem cell niches in different mammalian tissues including the skin [11][12], intestine [13][14], blood [15][16], and testes [17][18]. Current knowledge shows that stem cells invariably need niches, although the composition and mechanisms underlying niche function are extremely varied. Examples of reasons for niche existence include stem cells need for a special support for viability, niche action as an agent of feedback control, or niche role as an instrument of coordination among tissue compartments [19].

Stem cell niche, implications for regenerative medicine

The characterization of stem cell niches in mammals in particular, represented a breakthrough for regenerative medicine. The growing body of knowledge on how stem cell behavior is influenced by their *in vivo* microenvironment

represents important insight to be incorporated in different aspects or steps of stem cell based therapies (Figure 2) [20]. Most stem cell therapies involve the isolation of stem cells from a patient specific source (autologous) or a donner (heterologous) and further expansion *ex vivo*. But, stem cells often loose their hability for continued self-renewal when removed from their niche. Therefore, incorporation of niche cells or key signaling molecules normally secreted by these, should prove beneficial for this *ex vivo* steps. Also, incorporation of identified niche cells and/or signals in the explant during latter transplantation stages might help in the crucial step of homing the transferred cells to the correct tissue location in the patient [21]. Identification of niche intrinsic processes that go awry with age could explain cases of unsuccessful regeneration in older patients, and should also be an important factor to contemplate for age related diseases or when designing therapies for aged individuals (Figure 2C) [22]. On the specific context of cancer and the cancer stem cell model, targeting the niche also emerges an attractive approach to block tumor development [23].

Knowledge on the influence of the microenvironment in stem cell behavior have already been proved successful in treatments for a variety of human diseases, including macular degeneration [24], Amyotrophic lateral sclerosis (ALS) [25], Huntington's disease [26], HIV [27], and others. While the focus is often on the influence of the niche on stem cell behavior, it is important to note this relationship is bi-directional and stem cells can also influence the behavior of niche cells. Perhaps an interesting example of this situation comes from an approved clinical therapy for diabetes called “Stem cell Educator” [28]. In this therapy, there’s an isolation of lymphocytes from patients with type 1 diabetes followed by co-culture with immobilized multipotent cord blood stem cells (CB-SCs) in an hydrophobic chamber. This “re-educates” lymphocytes via immune modulation and proves to be an efficient to reverse type1 diabetes.

Success in the clinic will require a holistic view of stem cell regulation where the complexity of cellular players and molecular signals are all considered. Therefore, understanding the fundamentals of stem cell niche dynamics reveals essential for the future of regenerative medicine.

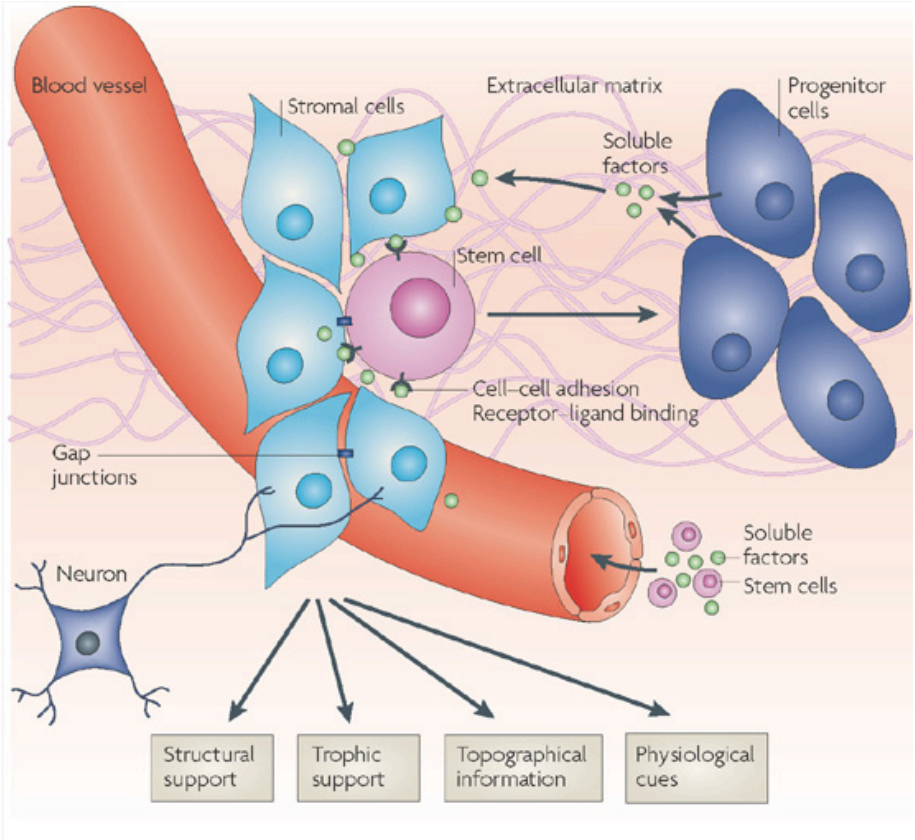


Figure 1. Components and Function of Stem cell Niches

Stem cell niches are composed of multiple cellular and acellular components capable of influencing stem cell behavior. Image from Jones D.L. *et al*, 2008

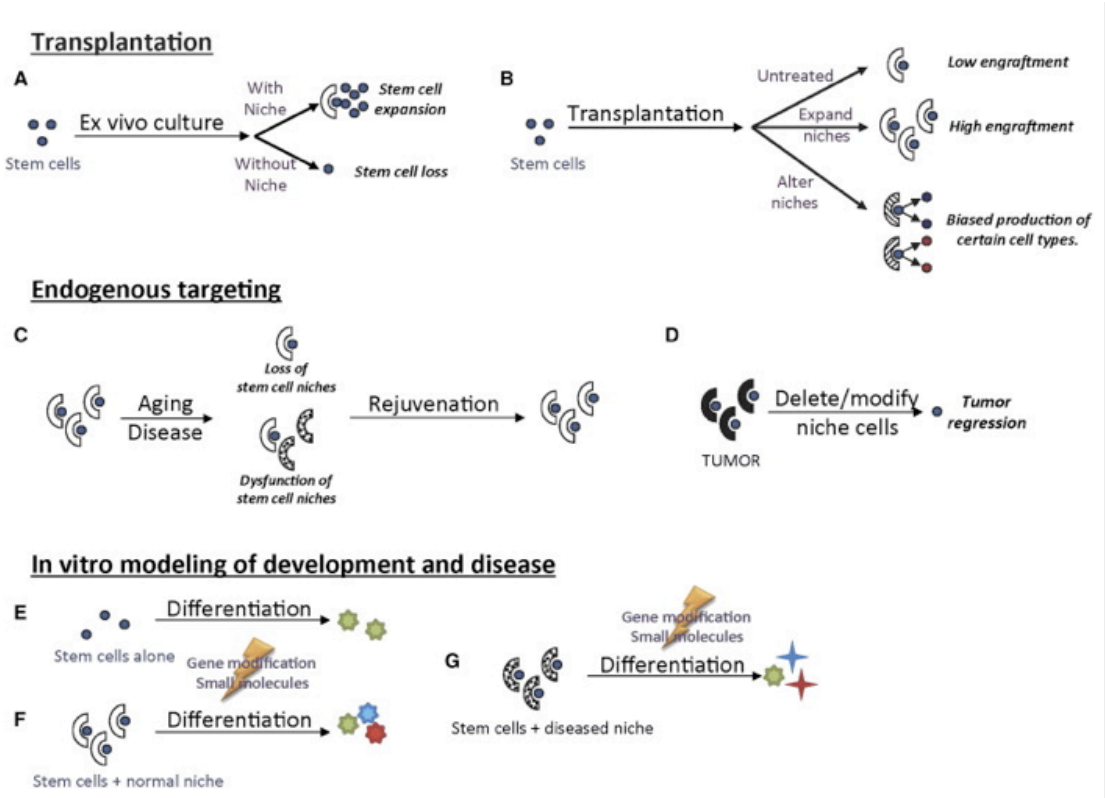


Figure 2. Potential entry points for stem cell niche-related therapies

Different possibilities for the therapeutic approach of tissue pathologies based on knowledge on the stem cell niche. Image from Wagers, 2012

Drosophila melanogaster testis stem cell niche

The process of spermatogenesis in *Drosophila* provides a robust, genetically tractable system for analyzing the relationship between stem cells and the niche. Germline stem cells (GSCs) and somatic cyst stem cells (CySCs) surround and are in direct contact with hub cells, a cluster of approximately 10 post-mitotic somatic cells (Figure 3). Hub cells can easily be identified immunohistochemically and/or by reporter genes that mark the expression of the cell adhesion molecules like Fasciclin III, DE-Cadherin, and DN-Cadherin; the signaling ligands Unpaired and Hedgehog; and the serine-threonine kinase Center-divider [29]. Attached to the hub by adherens junctions, GSCs divide asymmetrically by orienting their mitotic spindle perpendicular to the hub. In this way, one daughter cell will continue in close contact with the hub and maintain stem cell identity, while the other daughter is displaced away from the hub and initiates differentiation as a gonialblast (Figure 3B) [30][31]. Gonialblasts undergo four rounds of mitotic amplification divisions with incomplete cytokinesis to produce a cyst of 16 interconnected spermatogonia (germ cell cysts) (Figure 3B) [32]. The cytoplasmatic structure that interconnects the individual germ cell cysts is called fusome and can be visualized with antibody stain proteins like α-spectrin [33]. Like GSCs, CySCs divide mostly in asymmetric fashion [34]. Pairs of cyst cells will encapsulate germ cell cysts and provide essential signals for their proper differentiation [35][36][37]. A noteworthy characteristic of CySC behavior is the fact they seem capable of contributing to the hub [38]. Although CySC contribution to the hub might be rare during steady state, this fate change seems enhanced when CySCs carry mutations in the *lines* gene [39]. Interestingly, stem cell contribution to their own niche have been observed in other stem cell systems, like the mammalian intestinal crypt [13], and might represent a conserved mechanism to regulate tissue homeostasis and/or response to injury [40]. In addition to findings on CySC potential to contribute to the hub, lineage-tracing experiments show that hub cells and CySCs originate from a common precursor pool during embryogenesis [39], and data from our lab further suggest that convertion of hub cells to a CySC fate can happen in certain genetic backgrounds (Voog et al, submitted). Together, these studies point to a close relationship between the CySCs and hub, and to a remarkable cell plasticity of the soma in the testis stem cell niche.

At the molecular level, the testis stem cell niche function and architecture relies on a restricted production of the ligand Unpaired (Upd) by hub cells (Figure 3B, and also see detailed description on the role of JAK-STAT in the testis stem cell

niche in Appendix) [7][8] and reviewed in [41]. Upd is produced by and secreted from hub cells and activates JAK–STAT signaling in a non-autonomous manner. However, as shown nicely by biochemical studies, the protein is glycosylated and sticks tightly to the extracellular matrix, limiting its diffusion, and consequently only cells that are in close contact with the hub become activated for JAK-STAT (Figure 3C) [42][43].

Recent studies have shown that activation of the JAK–STAT pathway in CySCs was sufficient to drive proliferation of both CySCs and GSCs, suggesting the presence of another, secreted signal from somatic cells that regulates the behavior of GSCs [36][44]. Two genes, *Zfh-1* and *chinmo*, were recently identified as important downstream targets of Stat92E in cyst cells [36][45]. Clonal analysis revealed that both genes are required for CySC self-renewal, and overexpression of either in somatic cells resulted in expansion of both soma and germ line [36][45]. These studies indicate that GSC self-renewal is not solely dependent upon the hub and that factors from neighboring CySCs, likely downstream targets of STAT92E, play a critical role in regulating GSC behavior. In addition, the BMP pathway has been proposed to be involved in this cross-talk; however, it is not yet clear whether the signals are derived from the cyst lineage, hub cells or both [44][46][47][48].

While CySCs indisputably play an instructing role on GSCs differentiation, it's importance on GSC self-renewal is not as clear, with different lines of evidence found in the literature [37][44]. Part of the controversy might arise from the variety of stem cell hallmarks used to identify GSCs in this system, like the activation of JAK-STAT signaling, the close association with the hub, mitotic activation, cell division orientation with respect to the hub, and others.

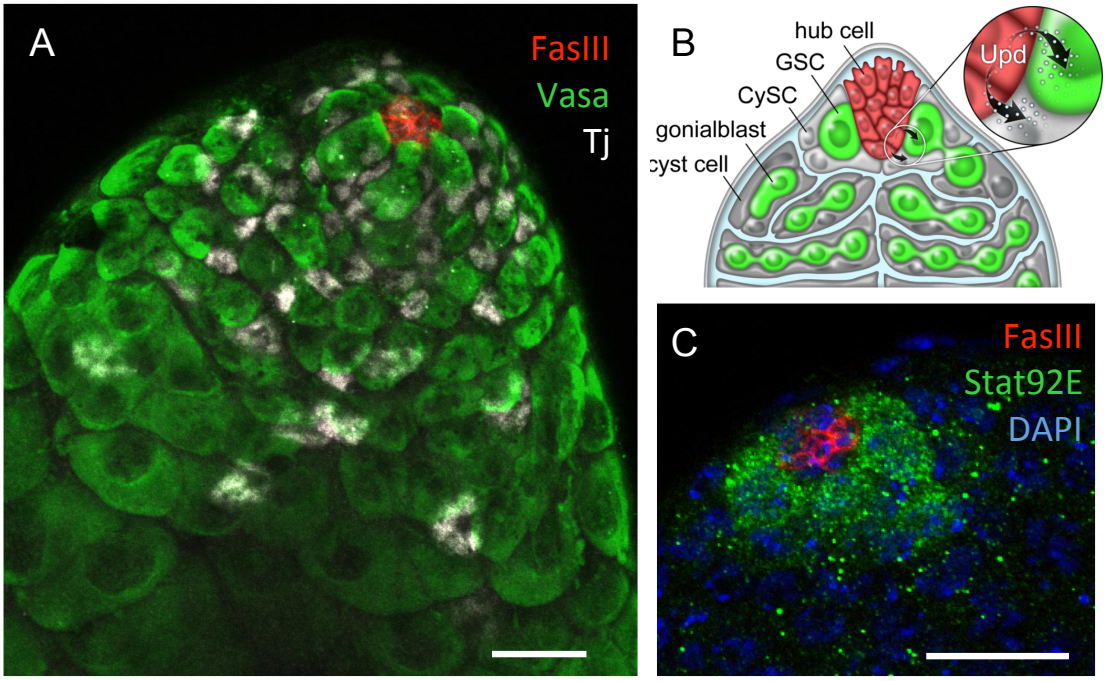


Figure 3. The *Drosophila* testis stem cell niche

A) Immunofluorescence image of the apical tip of the testis, where the stem cell niche is located. Vasa (green) marks the germ cells, and Tj (White) marks the cyst cells. GSCs and CySCs are in close association with a cluster of approximately 10 post-mitotic cells that form the hub (FasIII, red). **B)** Schematic representation of the testis stem cell niche shown in A). Upd is produced and secreted exclusively by hub cells, activating JAK-STAT signaling only in cells immediately adjacent to hub, as evident with Stat92E stain shown in **C)**

Drosophila melanogaster intestinal stem cell niche

Derived from endodermal lineages, the *Drosophila* posterior midgut shares many evolutionary conserved regulatory signals with other endodermal-derived organs in vertebrates [49]. In the adult, the *Drosophila* midgut is composed by a simple, monolayer epithelium, enveloped by two layers of visceral muscle (one circular and one longitudinal). Similar to the mammalian intestine [50], the *Drosophila* midgut also undergoes a dynamic process of self-renewal [51][52], and shares key regulatory signals like Wnt, EGFs, and Hippo [53]. Intestinal stem cells (ISCs) were recently identified and characterized in the posterior midgut providing an additional versatile model for the study of adult somatic stem cell behavior [51][52] and reviewed in [54]. Derived from adult midgut progenitors (AMPs) [55][56] and reviewed in [57], ISCs are located basally, immediately adjacent to the basement membrane, in close proximity to the visceral muscle (Figure 4).

These multipotent ISCs are capable of symmetric division (self-renewal) as well as asymmetric division to give origin to a type of commited progenitor cells called enteroblasts (EBs) [58] (Figure 4). Contrary to transit amplifying cells (TAs) in the mammalian intestinal crypts, EBs rarely, if ever, divide. Enteroblasts differentiate into either a secretory enteroendocrine cell (EE) that expresses the transcription factor Prospero (Pros) or an absorptive enterocyte (EC), which undergoes endoreplication to become polyploid (Figure 4). The choice between an EC or EE cell fate outcome depends on Notch signaling between the ISC and the enteroblast (EB), shortly after division. An ISC that expresses high levels of the Notch ligand Delta (presumably leading to high levels of Notch activation in EB) will signal strongly and induce EC differentiation, whereas ISCs with low Delta expression will trigger EB differentiation toward an EE fate [59][60][61]. Cell adhesion molecules like DE-Cad have also been show to mediate this cell fate process, by prolonging the interaction between the ISC and EB, ensuring a correct Notch signaling [62]. In intestines from young flies, the ratio of enteroendocrine cells to enterocytes is roughly 1:9 [51][59]. In addition to Notch signaling, a number of signaling pathways (Hippo, JNK, JAK-STAT, EGFR) have been implicated in the regulation of ISC behavior and EB differentiation under both homeostatic conditions and in response to aging, bacterial infection, and other environmental changes [54]. The *Drosophila* posterior midgut is a very attractive model system to study regeneration. Several studies have shown that injured enterocytes that are about to die secrete signals that act to stimulate ISC division and differentiation, so that the lost cells are replenished. Therefore, feedback from differentiated cells in the midgut is a key feature for maintenance of tissue homeostasis.

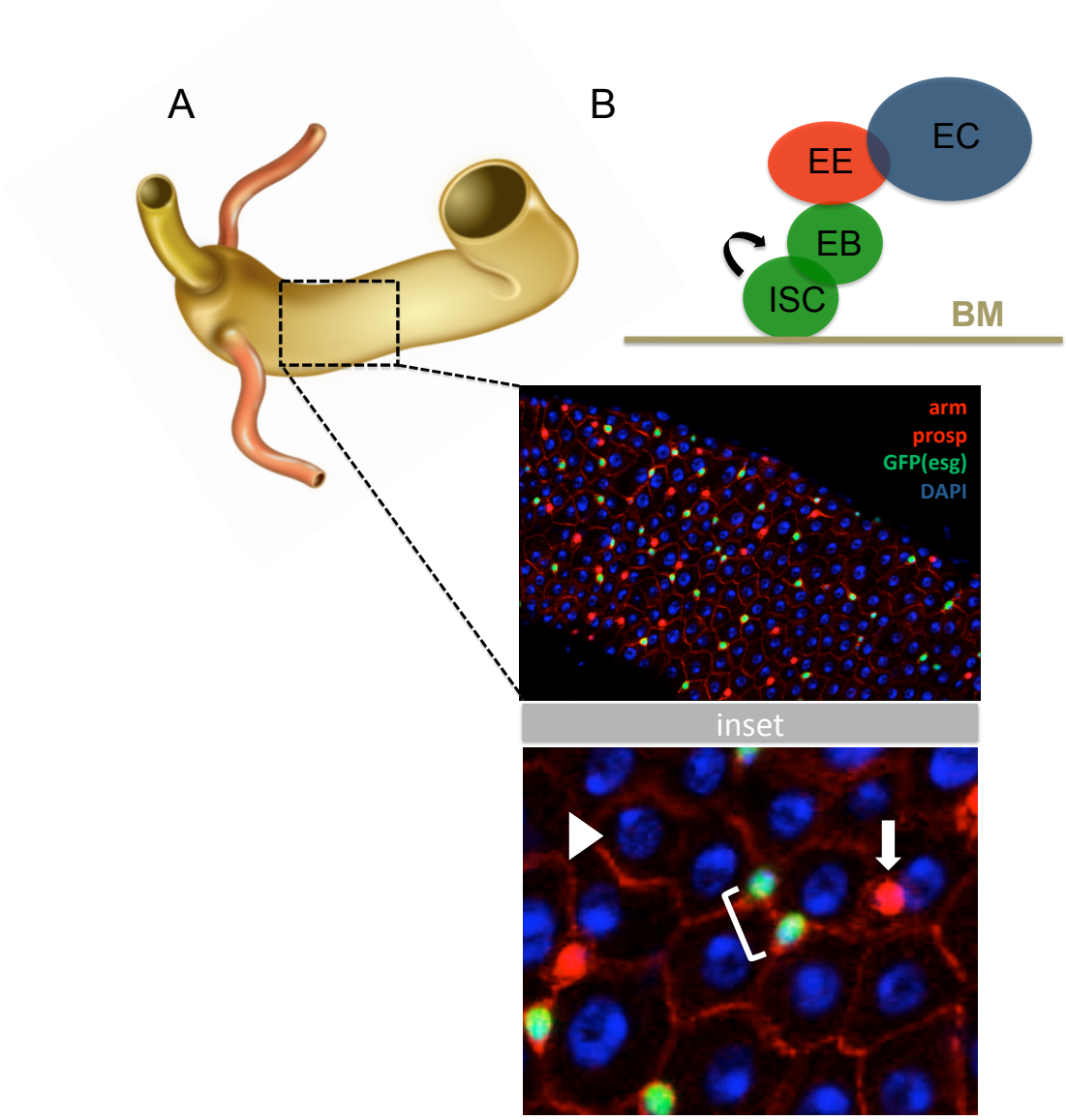


Figure 4. The Drosophila intestinal stem cell niche

A) Intestinal stem cells (ISCs) reside in the posterior midgut, right after the pylorous and the malpighian tubules. In the midgut epithelium, ISCs and EBs are found in ISC/EB nests (white bracket, inset, Esg^{pos}/GFP^{pos}), and are in close association with the basement membrane and the intestinal musculature. Enteroendocrine (EEs, $prospero^{pos}$, nuclear red, white arrow) and Enterocytes (large poliploid cells, white arrowhead) are the differentiated cell types found in the midgut. **B)** Schematic representation of the different cell types found in the posterior midgut

Chapter I

The role of *headcase* in the *Drosophila* testis stem cell niche

Results and discussion

headcase function is required for maintenance of the apical hub

Studies from our lab have demonstrated that failure to maintain the hub during development or conditional ablation in adults leads to loss of GSCs and CySCs [Voog et al, submitted]. In addition, intrinsic changes in hub cells occur during aging, including decreased expression of *upd* and E-cadherin, which appear to contribute to stem cell loss over time [63]. However, it remains unclear to what degree a reduction in hub cell number leads to stem cell depletion. To address this question it was decided to perform a candidate screen for regulators of hub maintenance.

For the identification of factors involved in hub maintenance a GAL4-UAS system was used in combination with RNAi-mediated knock-down to manipulate gene expression specifically in hub cells, and hub cell number was quantified upon eclosion (1d) and after 10 days (10d).

Hub cell number varies with genetic background, even within controls; therefore, the average number of hub cells was measured in a variety of genetic backgrounds at both time points. All genotypes tested had an average hub cell number ranging between 9 and 11 (Fig5E and Table1), which is consistent with what has been described previously for wild-type testes. In addition, these data indicate that hub cell number does not change with 10 days after eclosion [63].

Of the candidates tested, reduction of *headcase* (*hdc*), a previously described downstream target of the JAK-STAT pathway, lead to the strongest phenotype of all the candidate genes analysed (Table1) [64]. Interestingly, during tracheal development, *hdc* expression appears to be regulated non-autonomously by the transcriptor factor escargot (esg) [65], a gene whose function is required to maintain hub cells [Voog *et al*, submitted]. In wild-type hub cells, antibody staining with a Hdc antibody revealed cytoplasmatic expression in all cells at the tip of the testis, with enrichment in 8-16 spermatogonial cysts (Fig5C and Fig7). This expression pattern was confirmed using a *hdc* nuclear reporter line (*hdc* enhancer trap from the Carnegie protein trap library [66], Figure 7 C to C’’’) A function for Hdc has not been described previously; however, the mammalian homolog of Hdc, HECA, is misexpressed in diferent types of cancer [67][68][69].

The effects of *hdc* reduction in hub cells was verified using 3 RNAi constructs (see Materials and Methods), and all RNAi lines reavealed efficient to knock-down of *hdc* expression (Fig5D). A majority of testes where *hdc* expression was knocked

down in the hub using the strongest RNAi line showed a complete loss of these cells upon eclosion (67%, N=30, Fig5B and E and Table1), while the remaining testes had a residual hub composed of 1 to 3 cells (Fig5B and E, Fig6B and Table1). Experiments using RNAi contructs targeting other, distinct sequences revealed a normal number of hub cells on day 1, but loss of hub cells was observed after 10 days (Fig5E and Table1), providing further evidence that *headcase* function is required to prevent hub cell loss. Hub cell loss after RNAi mediated knock down could also be achieved by using a different hub cell specific promoter (*FasIII*Gal4) and could also be observed using multiple hub cell markers (Fig6A and B).

To exclude the possibility that hub cell loss was consequence of a disruption in hub formation during development, expression of the UAS-*hdc*RNAi[1] line was suppressed during development using a temperature sensitive allele of the Gal80 repressor. No hub cell loss or niche degeneration was observed when the flies were maintained at 18°C to suppress Gal4 with Gal80ts, during development (N=35). In contrast, when the flies were shifted to 29°C to initiate transgene expression, total hub loss was observed in 35% of the testes examined (N=27) after 10 days; remaining testes had hubs composed of 1-3 cells (Fig6C). Similar results were obtained with two other *hdc*RNAi lines, which showed normal hub cell number on day 1 and significant hub cell loss after 10 days at 25°C (Fig6C). Altogether, these experiments indicate that *hdc* function is required for hub cell maintenance.

Table 1. Results of candidate gene screen for regulators of hub size

genotype	1d	10do
UpdGal4;+ **(control)**	**10.06 +/- 2.31 (N=31)**	**10.94 +/- 2.45 (N=31)**
UpdGal4;UASLacZ **(control)**	**10.18 +/- 2.46 (N=33)**	**10.93 +/- 2.07 (N=30)**
UAShdcRNAi^{1}/+ (control)	**8.53 +/- 1.48 (N=30)**	**10.19 +/- 2.83 (N= 16)**
UpdGal4;UASZfh1RNAi	**11.79 +/- 1.88 (N=28)**	**11.07 +/- 2.40 (N=29)**
UpdGal4;UASHdcRNAi^{1}	**0.43 +/- 0.73 (N=30)**	**ND**
UpdGal4;UASHdcRNAi^{2}	**10.22 +/- 1.73 (N=27)**	**4.20 +/- 1.44 (N=25)**
UpdGal4;UASHdcRNAi^{3}	**10.30 +/- 2.37 (N=30)**	**5.17 +/- 2.27 (N=35)**
UpdGal4;UASHdcRNAi^{1};Gal80ts	**7.04 +/- 2.12 (N=27)**	**1.6 +/- 1.36 (N=26)**
UpdGal4;UASHdcRNAi^{2};Gal80ts	**12.78 +/- 2.73 (N=18)**	**8.34 +/- 2.74 (N=26)**
UpdGal4;UASHdcRNAi^{3};Gal80ts	**11.81 +/- 2.76 (N=26)**	**9.04 +/- 1.80 (N=23)**
UpdGal4;UAShdc$^{RA/C}$;Gal80ts	**ND**	**9.28 +/- 3.21 (N=29)**
UpdGal4;UAShdc^{Gsd404};Gal80ts	**ND**	**8.79 +/- 2.15 (N=28)**
UpdGal4;;UASUnkRNAi	**9.69 +/- 1.99 (N=16)**	**8.71 +/- 2.91 (N=17)**
UpdGal4;UASCycKRNAi	**11.08 +/- 1.73 (N=12)**	**6.24 +/- 2.94 (N=33)**
UpGal;UASreaper	**Embrionic lethal**	**Embrionic lethal**
UpdGal4;UASreaper;Gal80ts	**8.96 +/- 2.40 (N=23)**	**9.07 +/- 2.08 (N=29)**
UpdGal4;;UAShid,UAS-reaper	**Embrionic lethal**	**Embronic lethal**
UpdGal4;;UAShid,UAS-reaper/Gal80ts	**9.08 +/- 1.56 (N=23)**	**5.15 +/- 1.49 (N=26)**
UpdGal4;UASDIAP2RNAi	**3.80 +/- 2.20 (N=36)**	**1.40 +/- 1.82 (N=20)**

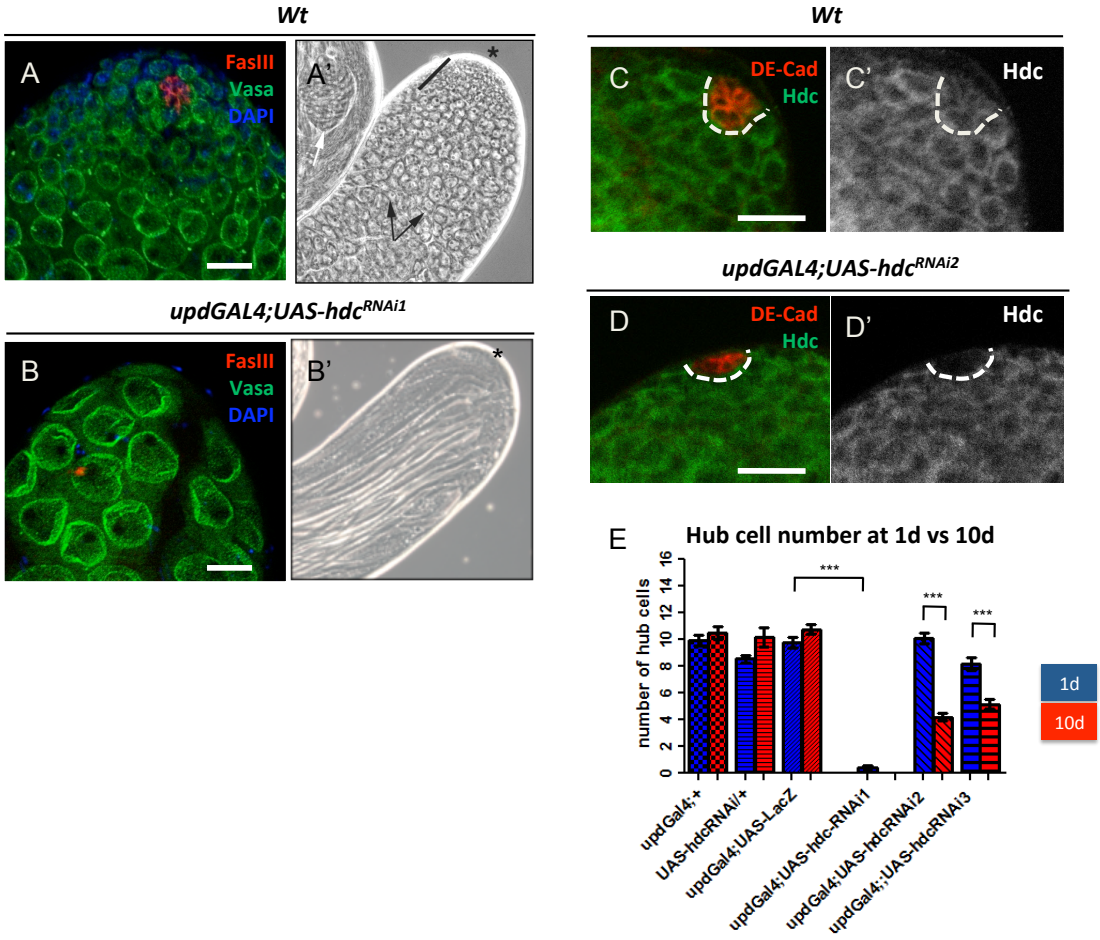


Figure 5. *Headcase* is required for maintenance of hub cells

(A) Immunofluorescence image of *wild-type* (*wt*) testis; FasIII (hub, red); Vasa (germ line, green); DAPI (DNA, blue). **(A')** Phase-contrast image of a *wt* testis; Asterisk denotes apical tip; transit-amplifying spermatogonia (black bar); spermatocytes (arrows); **(B and B')** Reduction of *hdc* in hub cells leads to loss of hub cells and niche degeneration; Note absence of FasIII^{+} hub cells (red) and presence of large spermatocytes **(B)** or mature sperm **(B')** at the apical tip; **(C and C')** Testis from *wt* male showing Hdc expression in all cells at the apical tip; **(D and D')** RNAi-mediated knock-down of *hdc* in hub cells results in loss of Hdc protein. Similar results were obtained for all RNAi lines tested; **(E)** Hub cell quantification at 1d (blue) and 10d(red) in controls and upon reduction of *hdc*. N=30 for each genotype/time point; Mean and SEM are shown; ***P<0.0001 (one-way ANOVA (Kruskal-Wallis test, followed by a Dunn's multiple comparison test); Scale bars, 20μm; See also Table S1 and Figure S1.

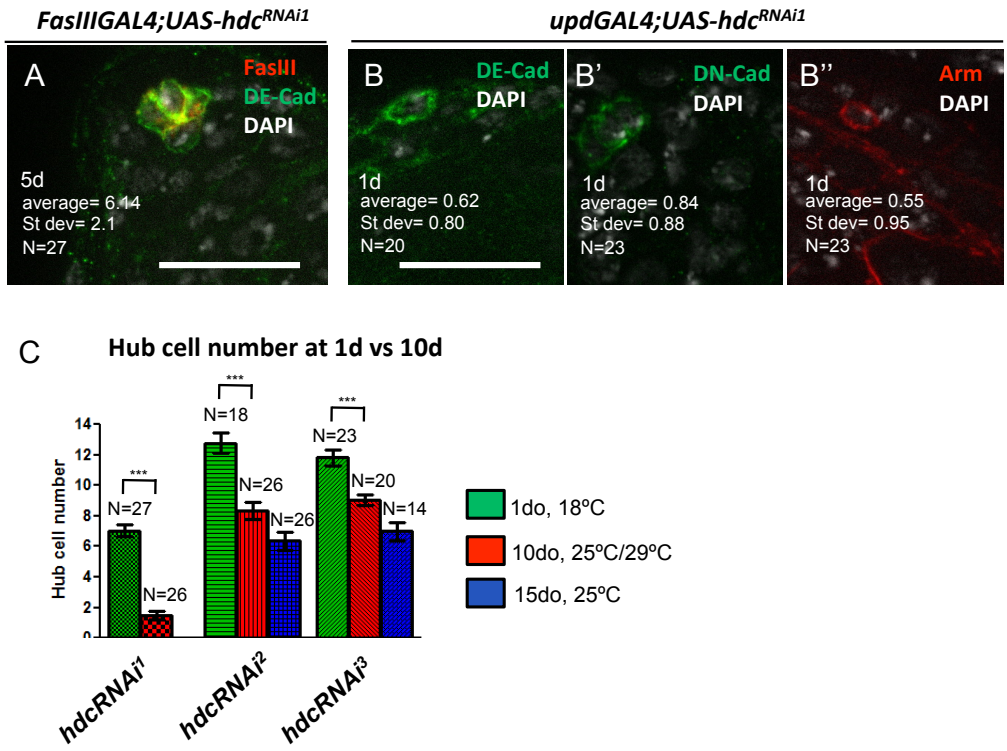


Figure 6. Hub cell loss is evident using multiple paradigms and is not due to developmental defects.

(A) Loss of hub cells is observed using an alternative hub driver (*FasIIIGal4*); Testis from *FasIIIGal4; UAS-hdcRNAi*^{1} male at 5 days (compare to Fig. 5F); **(B, B' and B'')** Strong hub cell loss marked by staining for FasIII (see Figs. 5C and F) was confirmed with other hub cell markers [DE-Cadherin (DE-Cad), DN-Cadherin (DN-cad) and Armadillo (Arm)]; **(C)** Hub cell quantification in flies where *hdcRNAi* lines expression by *updGal4* was suppressed at 18ºC during development, and activated at 25ºC (without Gal80^{ts}) or 29ºC (With Gal80^{ts}) for 1, 10, and 15 days; Means and SEM are shown; ***P<0.001 (one-way ANOVA (Kruskal-Wallis test, followed by a Dunn's multiple comparison test)**;** Scale bars 20μm.

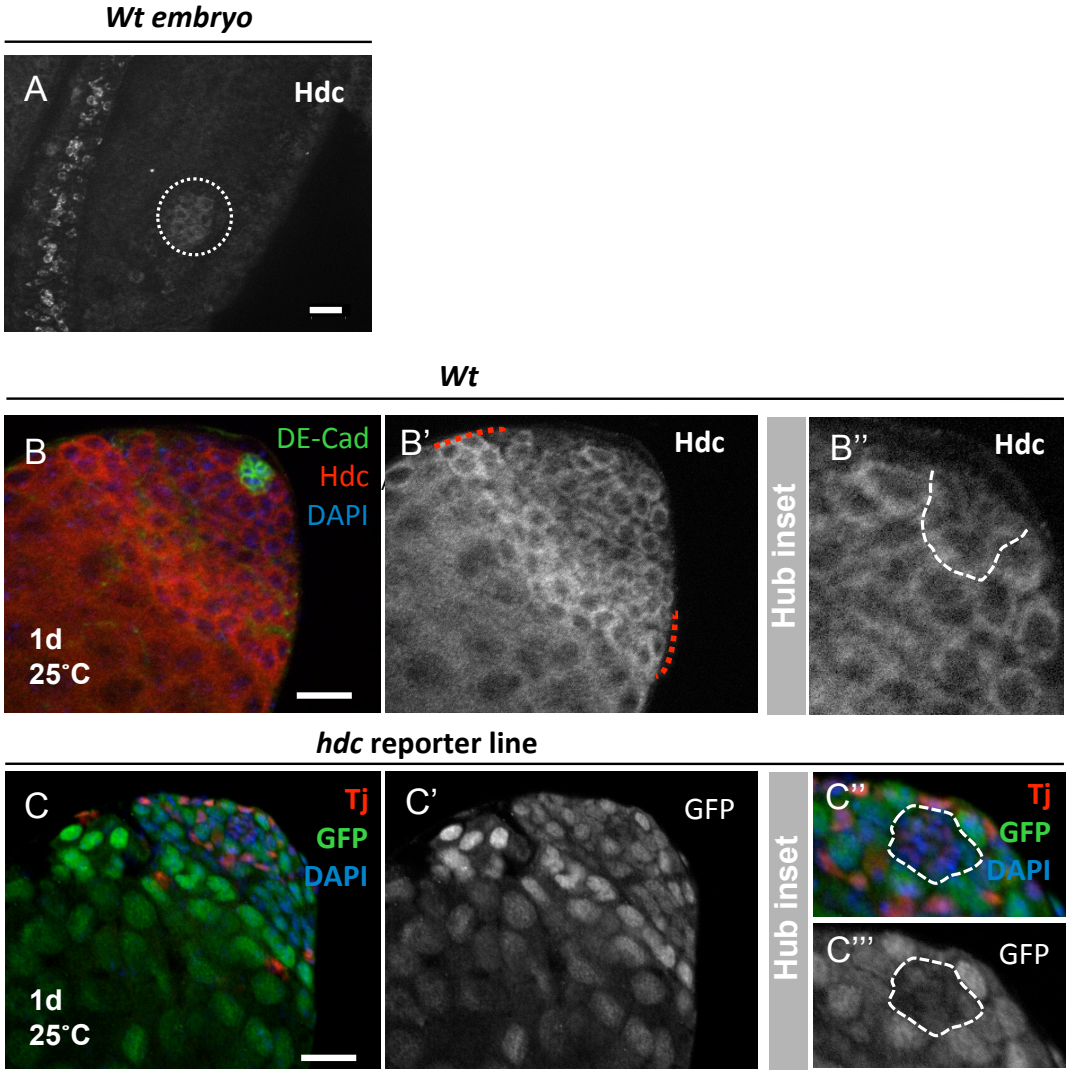


Figure 7. *headcase* expression pattern in the testis stem cell niche

(A) *headcase* expression was detected in early stages of testis stem cell niche formation, here showing expression in the embryonic gonad outlined by white dotted circle **(B)** *headcase* expression pattern in wt testis, DE-Cad (green marks the hub); Inset in A'' shows Headcase protein in the cytoplasm of hub cells outlined by white dotted line; *headcase* is expressed in all cell types that compose the testis stem cell niche but highest levels of expression are found within the germline in the transit-amplifying region – red dotted lines; **(C)** *headcase* nuclear reporter line shows a very similar expression pattern as observed with the AB stain. Note that in C'' and C''' expression can be detected in CySCs (marked by Tj in red); Scale bars 20μm.

headcase over-expression does not afect hub cell number

Headcase over-expression in the hub was tested using the *upd*Gal4;;GAL80ts driver to express both a UAS-*hdc*$^{RA/C}$ UAS-hdc^{GSD404} (see material and methods).

Flies were kept at 18°C throughout development and shifted to 29°C on eclosion day (1d). Altought, both constructs revealed efficient to increase Headcase levels in the hub as measured by antibody stain (Fig8A and B), no effect on hub cells numbers was dectected at after 10 days of induction (Fig8C). Therefore, it was concluded that increasing *headcase* expression in the hub does not impact hub cell fate.

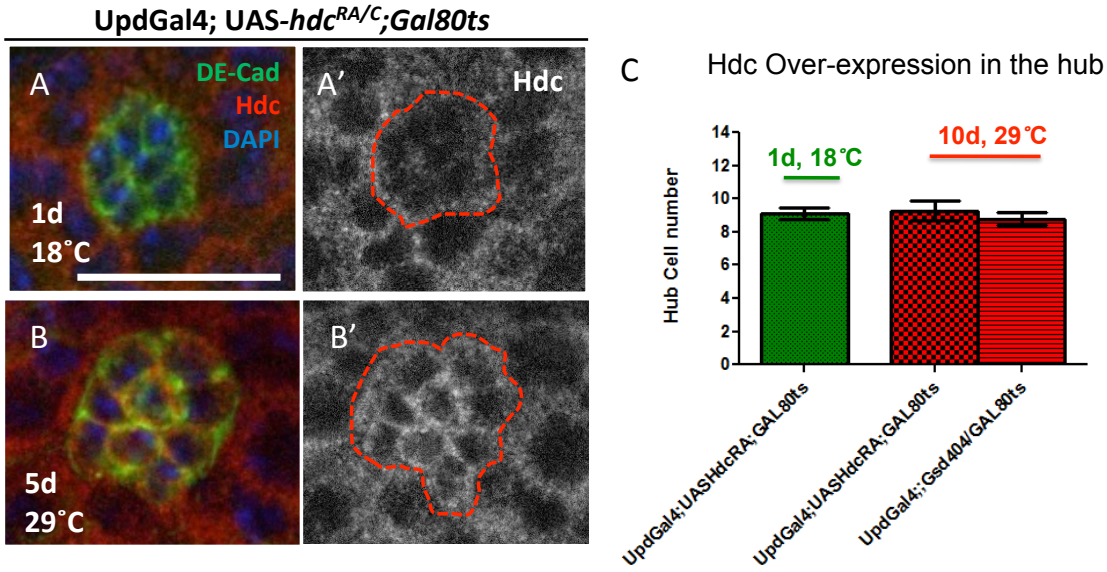


Figure 8. *headcase* over-expression in the hub

(A) *headcase* expression in the hub of UpdGal4;UAS-hdcRA;Gal80ts when the construct is OFF by raising the flies at 18C; **(B)** When the GAL4-UAS system is activated, *headcase* high levels of expression are detected in the hub; **(C)** quantification of hub cell numbers in controls and in two independent constructs to express *headcase* (after 10 days of expression); Means and SEM are shown no statistical significance between different datasets (one-way ANOVA (Kruskal-Wallis test, followed by a Dunn's multiple comparison test); Scale bars 20µm.

Hub cell loss in response to reduced *hdc* function occurs due to apoptosis

Several scenarios could explain the loss of hub cells in response to loss of *hdc*. Cells could be lost due to programmed cell death or hub cells could be losing hub cell characteristics and converting to the cyst cell lineage [Voog *et al*, submitted]. To determine the fate of lost hub cells, we first assayed for apoptotic hub cells, upon induction of *hdc* RNAi. Consistent with previous studies, apoptotic hub cells were rarely observed in *wild-type* testes (1/113 testes analysed) (Fig9C) [70]. In contrast, apoptotic hub cells were detected in testes where *hdc* levels were reduced (Fig9A, B

and C). Two categories of apoptotic cells were observed: one corresponded to apoptotic cells that were clearly within the hub domain (N=1/113 in controls, and N=5/131 in testes from *hdc*^{RNAi1} males) (Fig9A), while the other corresponded to putative hub cells in later stages of apoptotis (N=0/113 in controls and N=7/131 in testes from *hdc*^{RNAi1} males) (Fig9B). Loss of hub cells was suppressed by co-expressing *hdc*^{RNAi} constructs and the anti-apoptotic baculovirus protein, p35, which has been shown to supress cell death efficiently in *Drosophila* (Fig9E and F) [71].

Based on these results, we concluded that apoptosis was a likely cause for loss of hub cells in respose to reduced levels of *hdc*. This conclusion can be supported by the identification of *hdc* in screen for specific regulators of apoptosis in the eye, where *hdc* mutant phenotype resembled that of *crumbs,* a gene required to prevent programmed cell death in ommatidia [72][73]. Therefore, a putative role in preventing apoptosis may be conserved amongst tissues. Interestingly, loss of *hdc* suppressed the overgrowth phenotype induced by ectopic activation of the JAK-STAT pathway in the *Drosophila* eye [64]. As some of the mammalian STAT family members, STAT92E has been shown to have an antiapoptotic role [74], it is tempting to speculate that mutations in the *hdc* gene might suppress tumor progression in the eye by leading cells to enter apoptosis.

This represents the first description of apoptosis in the hub and suggests that hub cell loss could be achieved by induction of the apoptotic pathway.

Given that the number of apoptotic cells was relatively low, we wanted to test whether hub cells could also be lost due to conversion to the cyst cell lineage. To answer this question we took advantage of a lineage tracing using G-TRACE, which allows both a real-time readout of cell-type specific GAL4 activity by expression of DsRed while the fate of DsRed positive cells can be followed by expression of GFP [75] (Fig10A). Using this tool with *updGal4* driver we expected to see DsRed and GFP expression in hub cells and any GFP positive cells would be representative of an event of hub cell convertion to the cyst lineage (Fig10B). Interestingly, in our initial experimental paradigm, where G-trace cassete was driven with the *updGal4* driver throughout development, only 64% (N=50) of the control testis had hub restricted expression of GFP, while the remaining 36% showed GFP^{pos} cells outside of the hub at 1d (Fig10C). This is consistent with previous data demonstrating that hub cells and CySCs are derived from a common progenitor pool during development [39], and our observation that larval testes contain, on average, approximately twice as many hub cells as adults [76]. Therefore, the conversion of hub cells to the cyst lineage may be a normal, programmed event during development of the male germ line. So, in order to test for hub cell conversion to the cyst lineage in the adult stages, we changed our

experimental paradigm to include the GAL80ts and flies were kept at 18ºC until eclosion day. No statistical difference in the number of GFP positive cells outside the hub was observed between control flies (7%, N=44, genotype: *updGal4; G-TRACE; Gal80^{ts}*) and *hdc* loss-of-function in the hub (15%, N=20; genotype: *updGal4; UAShdcRNAi^{1}; G-TRACE/Gal80^{ts};* and 15%, N=20, genotype: *updGal4; G-TRACE; UAShdcRNAi^{3}/Gal80^{ts}*) (Fig11,B and C). This indicates that reduced levels of *hdc* do not influence the ability of hub cells to maintain their identity.

Altogether these data indicate that hub cell loss upon reduction of *hdc* is due to apoptotic cell death. These data represent the first conclusive association of this gene with programmed cell death and provides insight into the role of the human homolog, HECA, a gene that is been found misexpressed in a diversity of tumors.

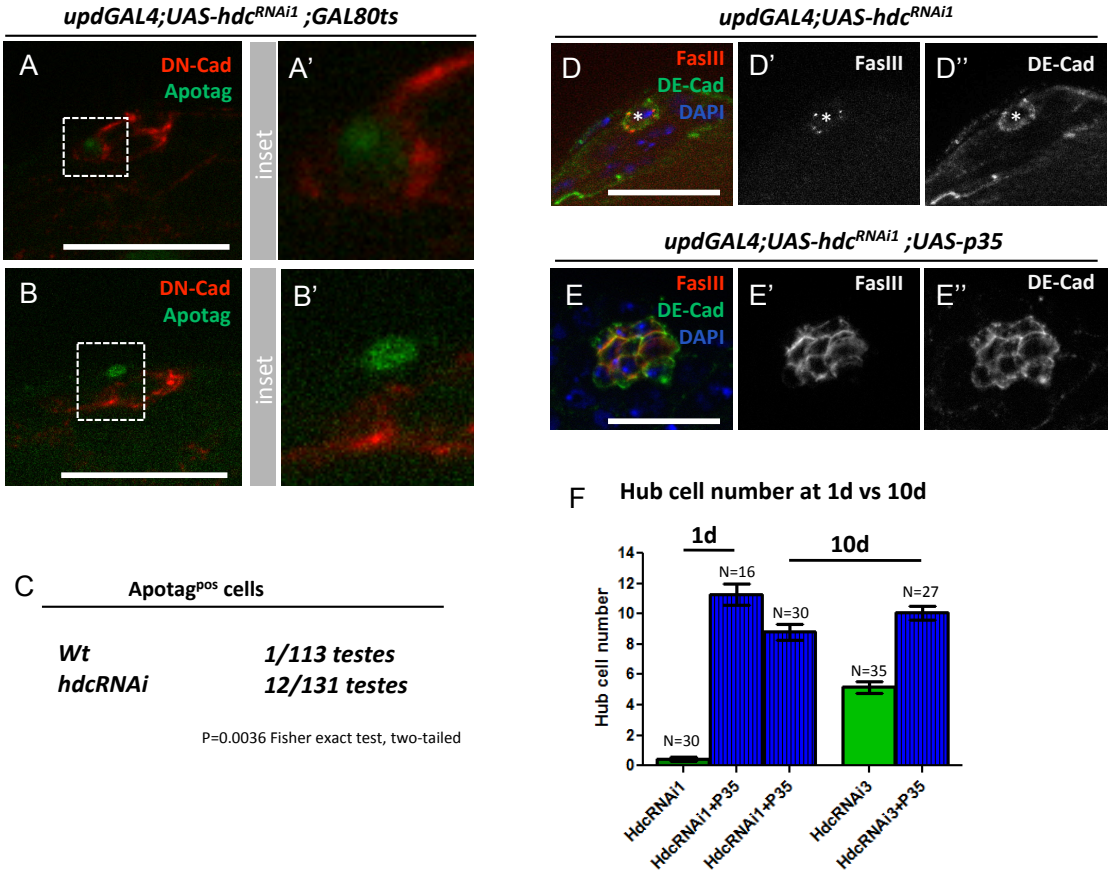


Figure 9. Loss of hub cells upon *hdc* reduction occurs via apoptosis

(A and B) Two types of apoptotic hub cells found after RNAi-mediated reduction in *hdc*; DN-cadherin (red), Apotag (green); **(A,A')** An apoptotic hub cell (Apotag^{+}/DN-Cad^{+}) and **(B, B')** Putative late-stage apoptotic hub cell (Apotag^{+}/DN-Cad^{low}) - Genotype: *updGal4;UAS-hdcRNAi^{1};Gal80^{ts}*. **(C)** Summary of apoptotic cells found in testes from *wt* and *hdc*RNAi males. **(D, E)** Co-expression of p35 rescues the strong phenotype observed with *HdcRNAi^{1}*; Hub stained with both FasIII (red) and DE-Cadherin (green); **(D)** Example of testis containing 1 hub cell (*); **(E)** Expression of p35 efficiently suppresses hub cell loss. **(F)** Quantification of hub cell number in the presence and absence of p35. Mean and SEM are shown; Scale bars 20μm; See also Figure 10.

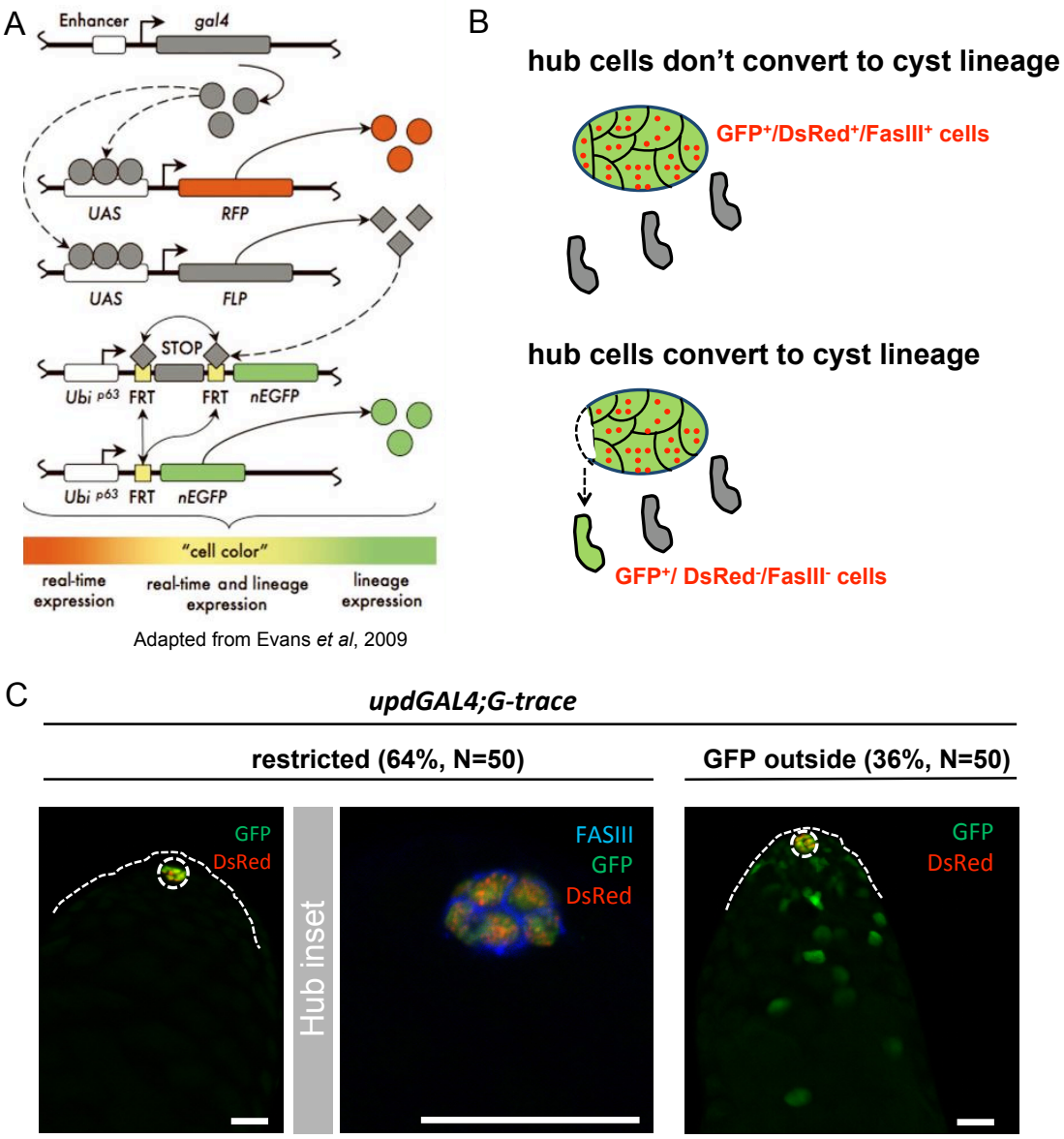
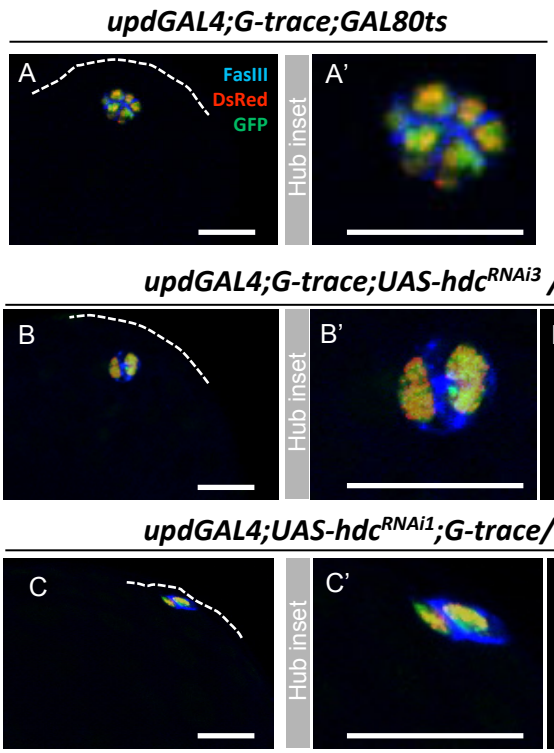


Figure 10. Hub cell convertion to a cyst lineage during development

(A) G-trace, a techique for lineage tracing using the GAL4-UAS system. Real-time expression of the driver of interested can be visualized by DsRed, while expression of a Flp-FRT leads to a permanent expression of GFP. **(B)** Model for the two possible scenarios when the G-trace cassette is expressed in the hub with the UpdGal4 driver. **(C)** Real data from testes where G-trace was active throughout developmental stages**.** Two examples and corresponding percentages of testis from the two phenotypic classes: hub restricted GFP expression vs non-resctricted. Scale bars 20μm.



D

genotype	GFP restricted to hub	GFP outside the hub
updGAL4;G-trace;GAL80ts	93%	7% (N=44)
updGAL4;G-trace;UAShdcRNAi^{3}/GAL80ts GAL80ts	88%	12% (N=85)
updGAL4;UAShdcRNAi^{1};G-trace/GAL80ts	85%	15% (N=20)

Figure 11. Hub cell conversion to the cyst lineage does not happen after *hdc* loss-of-function in the hub

Lineage-tracing analysis using G-TRACE in **(A, A'**) controls (genotype: *updGal4;G-TRACE;Gal80^{ts}*) or **(B-C'')** upon loss of *hdc* function (genotype: *updGal4;G-TRACE;UAS-hdcRNAi^{3}/Gal80^{ts}* and *updGal4;UAS-hdcRNAi^{1};G-TRACE/Gal80^{ts}*) shows restricted expression of dsRed and GFP in hub cells; **(B'' and C'')** No change in the levels of *upd* promoter activity (DsRed) was observed in the different categories of hub cell loss**; (D)** Summary of lineage-tracing experiments; Control and *UAShdcRNAi^{3} G-TRACE* flies were all kept at 18°C during development and shifted to 29°C on eclosion day; Males flies were dissected at 5, 10, and 15 days; *UAShdcRNAi^{1}* G-TRACE flies were analyzed at 5 and 10 days, as total hub cell loss was observed in most testes after this time point; No difference was observed between controls and the two *hdc* loss-of-function conditions (P=0.68 Chi-square test); Scale bars, 20µm.

Hub cell: stem cell ratio is decreased upon hub cell loss

Altough it is clear that the hub plays an important signaling and structural role [4] [7][8], it is not known the degree to which the overall size of the hub influences the stem cell pool. In the course of the experiments described above, hubs consisting of single cells appeared capable of sustaining multiple GSCs, corresponding to a clear alteration in the normal hub cell: GSC ratio (Fig12A and Video S1). In addition to being attached to the hub, these germ cells maintained additional hallmarks of GSCs, including spherical spectrosomes, positive staining for Stat92E, and markers of cell cycle progression (Fig12B and C). Similarly, mitotically active somatic cyst cells positive for the early cyst cell marker Zfh1 [36], were found in close proximity to the hub (Fig12D).

To obtain a better view of how hub cell and stem cell loss is correlated, hub cell and stem cell numbers were quantified at additional time points between 1 and 10 days. Samples were divided into classes according to hub cell number (1-2;3-4;5-6;7-8), and for each of these classes the number of GSCs and CySCS was counted. GSCs were scored as germ cells adjacent to the hub and positive for Stat92E (Fig13A and A'), while presumptive CySCs were scored as $Zfh1^{+}$ cells within a 15μm distance from the center of the hub (Fig13B and B'). Both GSCs and CySCs were sensitive to alterations in hub cell number, as a decrease in the number of both stem cell populations was observed as hub cells numbers drop (Fig13 D). However, loss was not as dramatic as would have been predicted, given the important structural and signaling role of the hub in regulating stem cell activity. Hubs composed of only one or two cells maintained an average of approximately half of the original number of both GSCs and CyScS (Fig13 A', B' and D).

In addition, total stem cell loss and loss of early stages of spermatogenesis was only observed after a complete loss of the hub and never in testes that had at least one hub cell remaining (N>30).

Because proximity to the hub is essential for both GSCs and CySCs to maintain stem cell identity, we hypothesized that stem cell loss might correlate more closely with reduction of hub area, rather than total hub cell number. Indeed, the average hub area in previously defined categories revealed that a 5 fold reduction in hub cell number (7.5 vs 1.5 hub cells) corresponded to only a 2.8 fold reduction in the hub cell area (112.98 to 40.12μm^2) (Fig13C,C' and E). The importance of the area defined by hub cells and its effect on the stem cell pool can be supported by findings in other systems where stem cells are regulated by signals from the support cells that

act in a short-range fashion. For example, in the *Drosophila* ovary, cap cells play an analogous role to hub cells. An expansion of cap cells leads to an increase in the number of GSCs, and the area defined by cap cells seems to be the limiting step for a bigger increase in the number of GSCs [77][78]. Additional parallels can be found in mammalian stem cell niches, such as the small intestine. In this system, genetic ablation of Paneth cells in vivo leads to a loss of $Lgr5^{+}$ crypt base columnar (CBC) cells (intestinal stem cells); however, in crypts where a single Paneth cell is remaining, multiple CBCs can be found cluster around it (similar to what we observe in testis composed of only one hub cell) [14]. Strong *upd* promoter activity was detected in hub cells in both controls and during progressive hub cell loss in response to reduction of *hdc* (Fig11 B'' and C''). In addition, no obvious differences were noted in the levels of JAK-STAT pathway activation in GSCs upon hub cell loss, as determined by Stat92E staining in adjacent GSCs (Fig13A and A'), indicating that remaining hub cells appear functional. These data are consistent with previous observations that over-expression of Upd in hub cells does not result in an increase in the number of GSCs and suggest that distance from the source of Upd is more important in this system than variations in signal intensity [63][41].

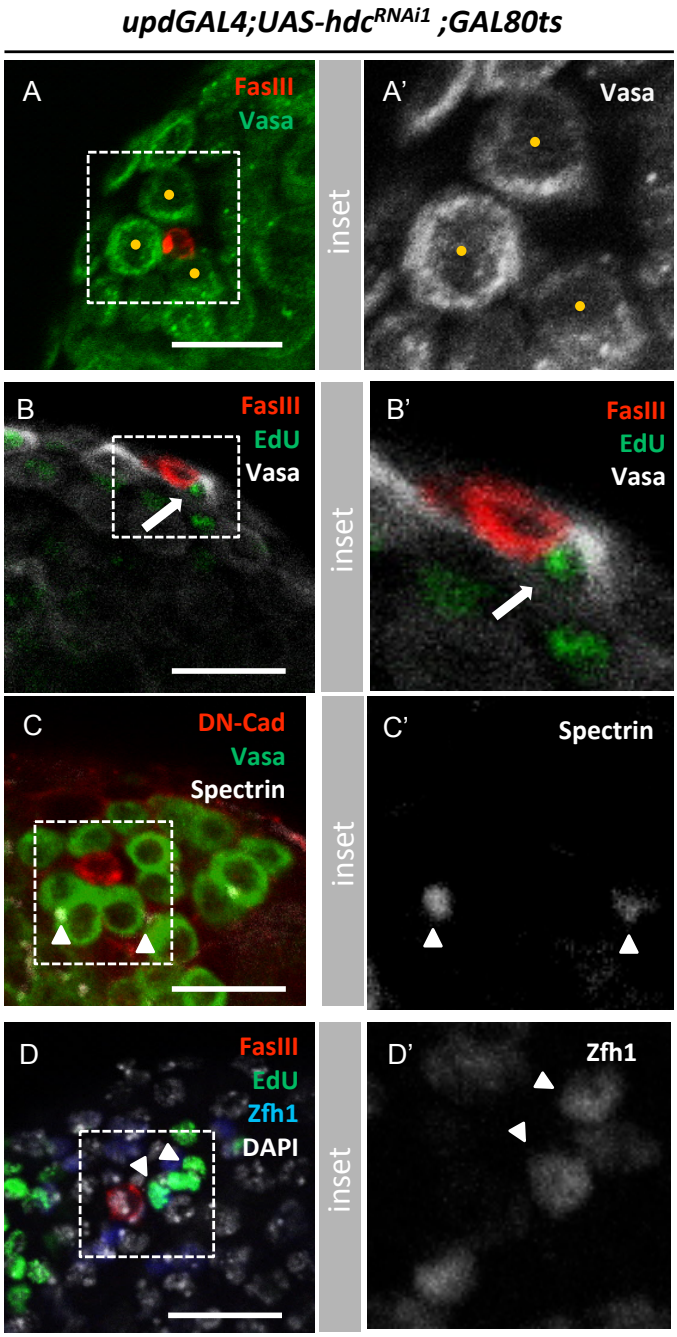


Figure 12. Smaller hubs are functional and capable of maintaining stem cells.

(A and A') Three Vasa^{pos} germ cells attached to a single cell hub (yellow dots); FasIII (hub, red) and Vasa (germ line, green); **(B, B')** Dividing germ cell (white arrow) attached to a 2-cell hub; FasIII (hub, red), Vasa (germ line, white) and EdU (green); **(C, C')** Germ cells attached to a 1-cell hub with spherical fusomes (white arrowheads); DN-Cadherin (hub, red), Vasa (germ line, green), and α-spectrin (fusome, white); **(D, D')** Two dividing Zfh1+ cyst cells (presumptive CySCs, white arrowheads) adjacent to a 2-cell hub; FasIII (hub, red), Zfh-1 (cyst cells, blue), Edu (green), DAPI (DNA, blue); In all cases, testes are from *updGal4;UAShdcRNAi^{1};Gal80^{ts}* males 10 days after shifting to 29°C to induce RNAi expression; Scale bars, 20μm. See also Video S1 and S2.

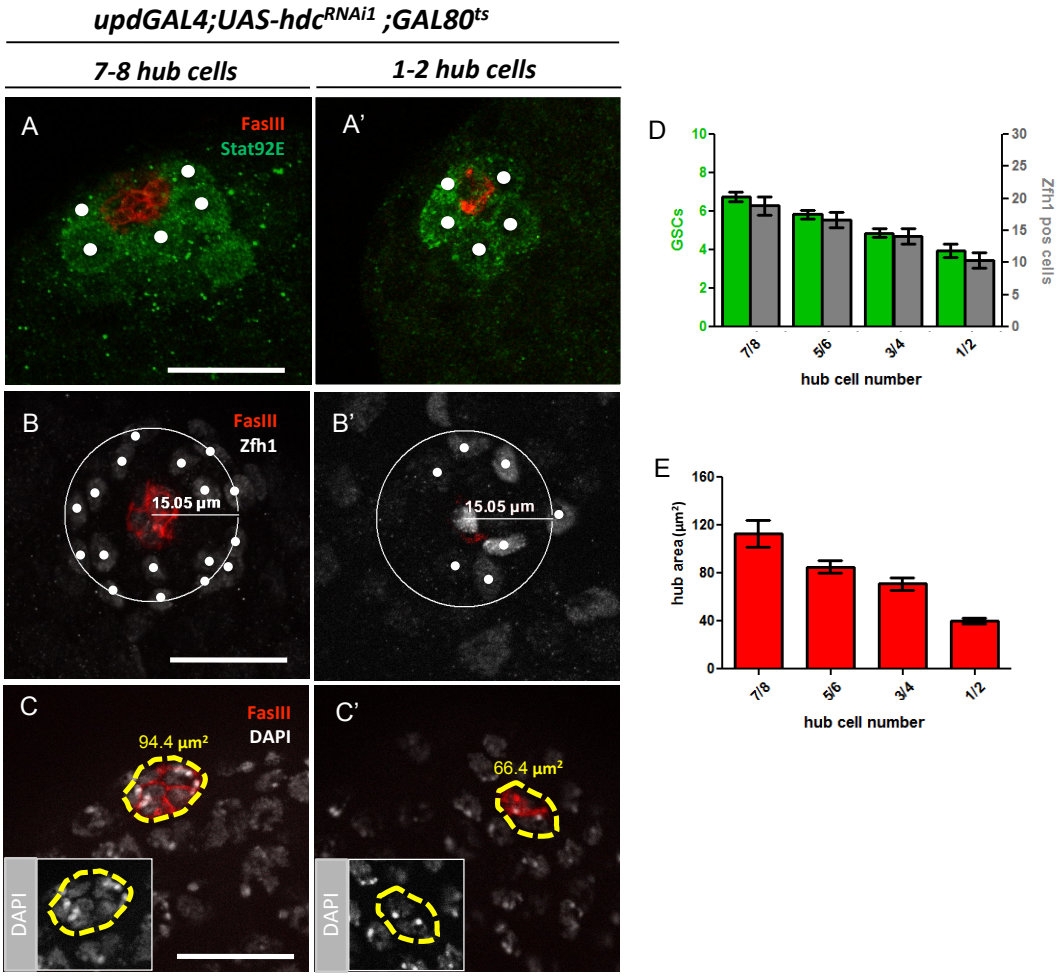


Figure 13. Alterations in GSCs, CySCs, and hub area during progressive hub cell loss.

(A to C, left panels) Testes with 7-8 hub cells (FasIII, red) from 1-day old *updGal4;UAS-hdcRNAi*^{1}*;Gal80*^{ts} males raised at 18ºC; **(A' to C', right panels)** Testes with 1-2 hub cells from *updGal4;UAS-hdcRNAi*^{1}*;Gal80*^{ts} males after 7-9 days at 29ºC to induce transgene expression; **(A, A')** GSCs were counted as Stat92E^{+} germ cells (green) contacting the hub **(B, B')** CySCs were counted as Zfh1^{+} cells (white) within a 15µm radius from the center of the hub; **(C, C')** Hub area was measured based on FasIII^{+}/DAPI^{+} cells; **(D)** Graph representing hub cell:GSC:CySC ratio during progressive hub cell loss. **(E)** Graph of hub area during hub cell loss; N>/=20 testes for each genotype/timepoint; Means and SEM are shown. Scale bars, 20µm.

Headcase knock-down in the hub leads to Zfh1 up-regulation and cell-cycle re-entry

In the course of our investigation on how the number of CySCs was affected by hub cell loss after *hdc* loss-of-function, it was noticed that individual hub cells often expressed very high levels of Zfh1 (Fig13B and B'). Expression of this transcription factor is typically high in CySCs and early cyst cells and homogeneous low levels are found within hub cells (Fig 14A) [36]. In order to confirm, in quantifiable manner, the difference in Zfh1 levels of expression between hub cells and cyst cells, pixel intensity corresponding to the Zfh1 AB stain was measured within individual hub cells and compared to levels in cyst cells. In wild-type and control testes it was never observed the case where a testis had a hub cell expressing Zfh1 higher than any cyst cell (Fig14A, D and F). On the contrary, when *hdc* was knocked-down, 19% (N=32) of testes were found where the highest Zfh1 expressing cell was a hub cell, not a cyst cell (Fig14B, C and F). Based on the fact that, at the testis tip, Zfh1 high levels are restricted to somatic cells capable of proliferation, it was speculated that hub cells where Zfh1 was upregulated might be re-entering the cell-cycle. Indeed, EdU stain revealed hub cells re-entering the cell-cycle. Preliminary experiments suggest that these cells were the highest Zfh1 expressing cells within those testes (Figure 15). These two results together open the interesting hypothesis that *headcase* loss-of-function in the hub might be leading to these cells to adopt a different cell fate and re-enter the cell cycle, but putative failure to complete this conversion might lead to apoptosis. An alternative scenario is also being explored in which these proliferating hub cells might be consequence of a process called "compensatory proliferation" [79]. In this process, cells undergoing apoptosis send proliferative signals to their neighbor cells as serve as a strategy for tissue regeneration [80][81]. Ongoing and future experiments from our lab should help to understand better the mechanisms behind apoptosis initiation on the context of *headcase* loss-of-function. Also, they will aim to clarify if apoptotic cells send mitogenic signals to their neighboring cells, to induce compensatory proliferation.

Headcase expression is not affected in the testis of escargot mutant flies

Previously, in a study developed in the *Drosophila* trachea, it was reported that *headcase* expression was regulated by the transcriptor factor *escargot [65]*. In this system, *hdc* is normally expressed in a subset of cells that extend fusion sprouts to

interconnect the tracheal network, but it is not expressed in fusion cells of *esg* mutant flies. Conversely, *hdc* is ectopically expressed when *esg* is over-expressed. Since *escargot* plays a very important role in the regulation of somatic cell fate in the testis, and because its function is required to prevent hub cell loss (Voog *et al*, submmited), it was decided to test if *esg* expression was also required of *hdc* expression in the testis. When *escargot* mutant gonads (esg^{shof}), were stained for *hdc*, normal levels of *hdc* expression were found both in hub cells and cyst cells (Figure). Note that gonads were used because hub cells are quickly lost during development of these *esg* mutants and the majority of testis have a complete lost of hub cells at eclosion day, that progressively also leads to loss of stem cells and all early stages of spermatogenesis. When adult flies were stained, *hdc* expression was still detected in somatic cells that were still found at the testis tip, confirming results observed in gonads.

Based on these results we concluded that the results previously reported in the tracheal development on the role of *escargot* in the regulation of *hdc* expression are likely to be tissue/cell type specific. Moreover, as regulation of *hdc* expression by *esg* in the trachea happens in a non-autonomous fashion, it is likely to be consequence of a cell fate change induced by alteration of a development program of the specific group of cells acting together to form tracheal branches.

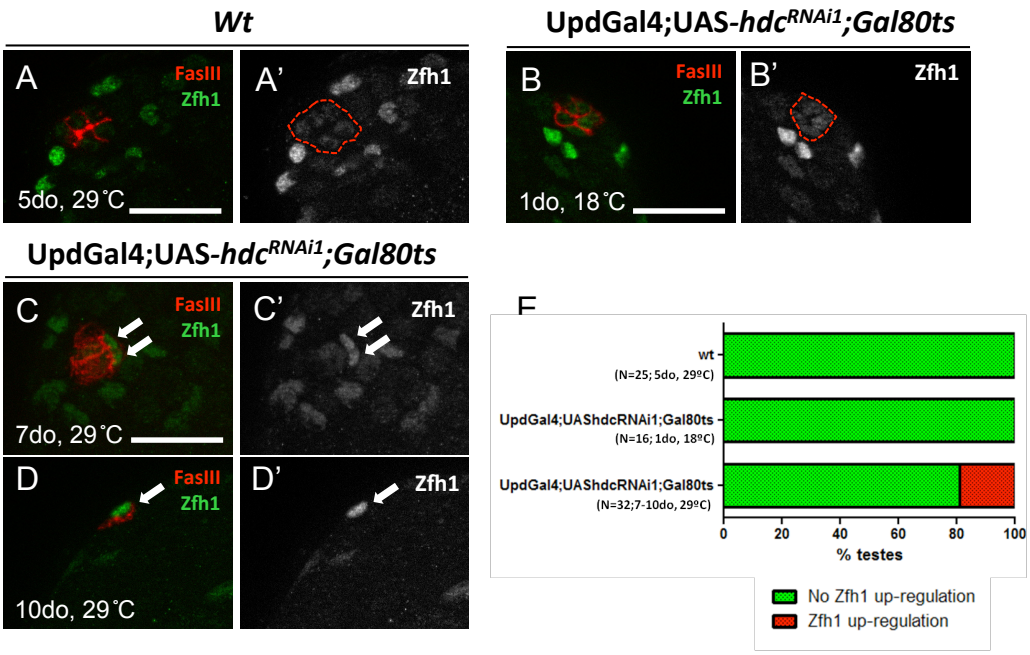


Figure 14. Zfh1 is upregulated in the hub after *hdc* loss-of-function

Both in wt **(A)** and control situations **(B)** Zfh1 expression was found as described in the literature [36], with high levels of expression within CySCs and early cyst cells, and low levels of expression in the hub; hub is marked with FasIII (red) and outlined in **A'** and **B'** by red dotted line; **(C and D)** In the course of our experiments knocking-down in the hub, several examples were found where Zfh1 expression within individual hub cells seemed higher than other hub cells from the same testis (see **C** and **C'**) and were the highest Zfh1 expressing cells from that testis (more than any CySCs or early cyst cell); **(F)** Quantification of Zfh1 upregulation in the hub of wild-type, control and *hdc* knock-down situation; Upregulation was measured using pixel intensity and by scoring only the testis where the highest Zfh1 expressing cell was a hub cell, and not a cyst cell; N>30 for each genotype; Scale bars, 20µm

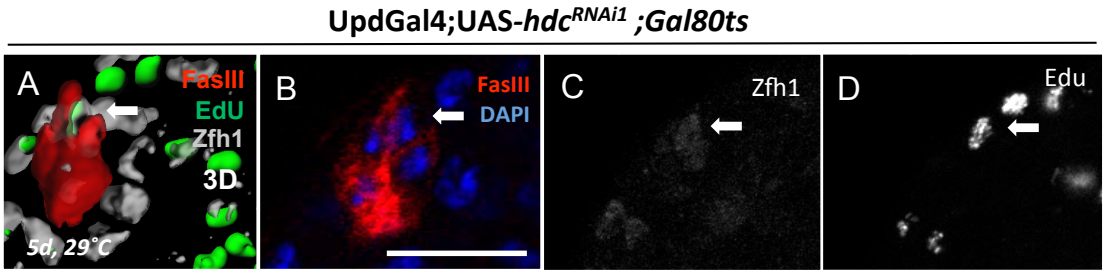


Figure 15. Zfh1 upregulation and cell-cycle re-entry in the hub after *hdc* loss-of-function

(A to D) Example of a UpdGal4;UAS-*hdc*RNAi[1];Gal80ts fly testis where you can see an EdU positive hub cell, which is the cell expressing the highest level of Zfh1 within that testis (higher than any cyst cell, measured by pixel intensity). Please note that Zfh1 signal intensity in the image is very weak as compared to other Zfh1 stains included in this thesis (for example in Figure 14). The reason for this is of technical nature, and is related to the secondary AB used for this confocal image (Alexa 633) which as always correspond to a weaker signal compared to other secondaries used; Scale bars, 20μm

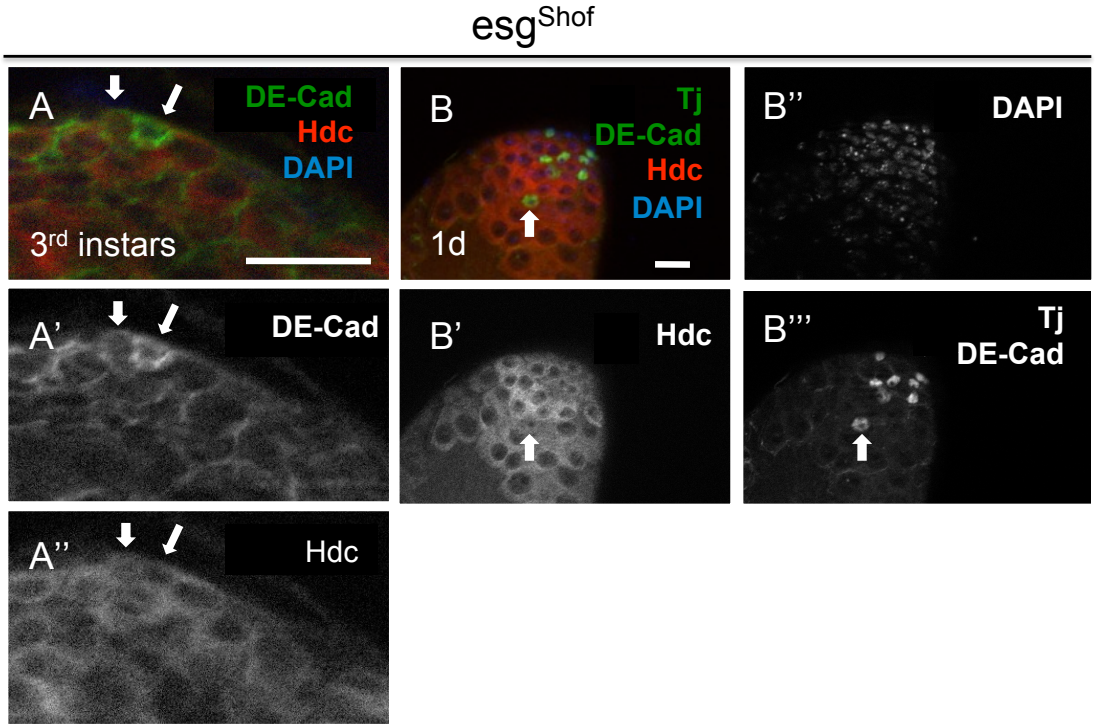


Figure 16. *headcase* expression in esg[shof] mutant flies

(A to A'') esgshof 3rd instars larvae, high DE-Cad staining (Green) highlights remaining hub cells; white arrows point to two hub cells showing normal *hdc* expression; **(B to B''')** adult 1day-old esg[shof] flies; white arrow points to a cyst cell (Tj[pos], nuclear green), where normal levels of *hdc* are found; N=10 for 3rd instars, N=15 for 1d; Scale bars, 20μm

Hub cell death can be achieved by genetic manipulation of the apoptotic pathway

The results here presented on hub cell loss after *headcase* loss-of-function represented the first description of apoptosis in the hub. This raised interest in the study of the pathway in the hub. Initial experiments to force apoptosis in the hub included hub specific over-expression of the gene *reaper*, or a combination of *reaper* (*rpr*) and *head involution defective* (*hid*) (genotypes: *updGAL4;UAS-reaper* or *updGAL4;;UAS-reaper,UAS-hid*). Both of these experiments resulted in embryonic lethality, and could not be used to test for a phenotype in the hub (Table1). Lethality was efficiently rescued by repression of the GAL4-UAS system by co-expression $GAL80^{ts}$, and by keeping flies at 18°C during development *(*genotypes: *updGAL4;UAS-reaper;GAL80^{ts}* and *updGAL4;;UAS-reaper,UAS-hid/GAL80^{ts}*) (Table1 and Fig16D). Activation of the GAL4-UAS system and consequent induction of the *UAS-reaper* transgene for the first 10 days post-eclosion did not result in hub cell loss (Table1 and Fig16D). On the contrary, when both *UAS-reaper* and *UAS-hid* were co-expressed they lead to a significant hub cell loss after 10 days of induction (Table 1, Fig16A,B and D). Interestingly, in the central nervous system, *hid* and *reaper* have been show to act cooperatively to induce cell death [82]. In addition to over-expression of the genes *reaper* and *hid*, hub cell loss was also observed when the DIAP2 (*Drosophila* inhibitor of apoptosis 2) gene was knocked-down (Table 1 and Fig16B and D). The Inhibitors of Apoptosis (IAP) are a family of functionally and structurally related proteins, which serve as endogenous inhibitors of programmed cell death [83]. In Drosophila only two DIAPs have been identified so far. DIAP1 has been shown to be required to prevent cells from entering apoptosis in different cell type/tissue contexts [84][85][86]. On the other hand, DIAP2 putative role preventing apoptosis is still unclear [87][88]. These results prove that induction of apoptotis using classical genes from the pathway can lead to a progressive adult hub cell loss, similarly to what happens when *hdc* expression is reduced in these cells. Altogether these data endorces the hub and the testis stem cell niche as a valid system for the study of apoptosis.

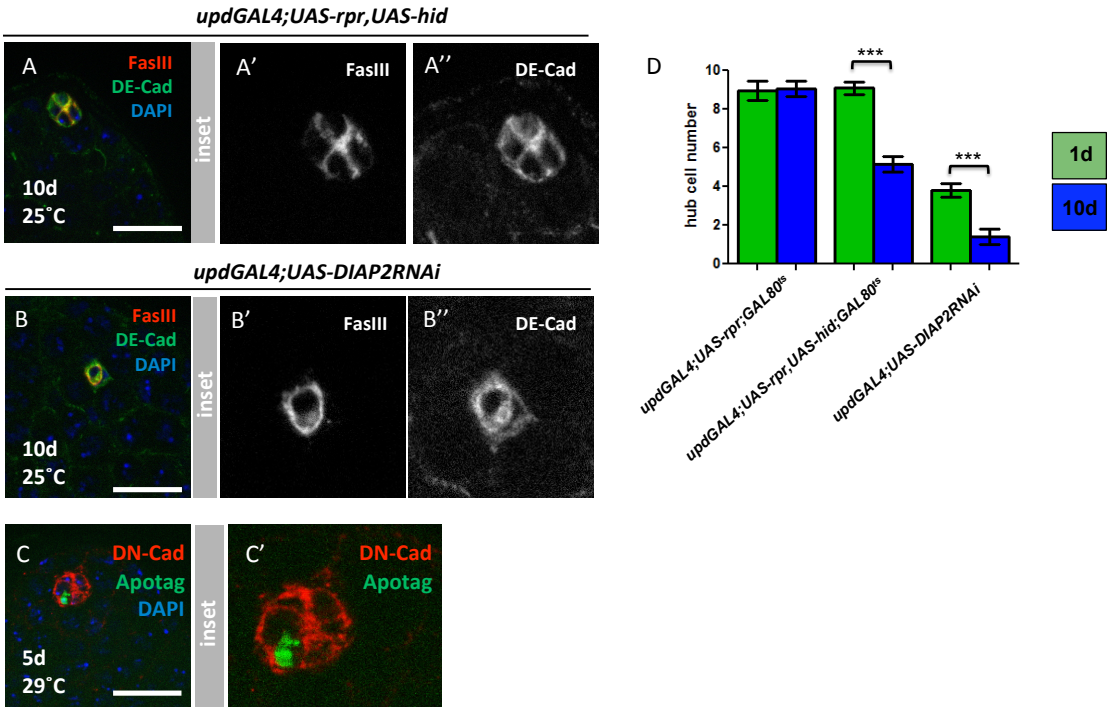


Figure 17. Hub cell loss by activation of the apoptotic pathway

Hub cell loss was observed when the genes *rpr* and *hid* were co-expressed **(A to A'')** in the hub, or when *DIAP2RNAi* was expressed **(B to B'')**; **C)** Apotag positive hub cell in a *updGal;UAS-DIAP2RNAi testis* (N=3/39 testes), D) Hub cell quantification at 1d and 10d of flies with hub expression of *rpr*, *rpr* and *hid* together, and *DIAP2RNAi*; Scale bars, 20µm

Hub regeneration

Adult stem cells play an important role maintaining tissue homeostasis but also serve a crucial function in the response to tissue injury. Regeneration potential varies dramatically depending on the animal or tissue context, and also it can happen via different processes like dedifferentiation, stem cell activation or transdifferentiation [89]. Planarians, urodeles limb and mammalian skin represent outstanding examples of regeneration potential [90][91][92]. On the other hand, the adult human heart or brain are a good examples where regeneration seems much more limited [93][94].

No studies have been reported on hub regeneration in the testis stem cell niche. Hub cell loss after *hdc* loss-of-function together with the technical possibility to turn ON and OFF the RNAi via the $GAL80^{ts}$ provided us an interesting opportunity to investigate hub regeneration. To test for hub regeneration, *updGal4;UAS-hdcRNAi^{1};Gal80^{ts}* flies were raised at 18ºC, shifted to 29ºC at eclosion day for 10 days to induce hub cell loss, and then again shifted to 18ºC to allow for recovery (Fig17A). Hub cell numbers were measured at 10d, after hub cell loss induction, and then after the recovery period. No statistical significance difference was observed between the average hub cell number in the two experimental situations (Fig17B). Although, an interesting trend was observed in the distribution of hub cell numbers after damage with the percentage of testis with 1 to 3 cells was reduced after the recovery period. This might suggest that hub cell regeneration might only be trigged when hubs are severely compromised (reduced to only 3 cells?) and stop shortly after they recover only a few cells, not necessarily recover to the original size (hypothetical model for regeneration – Figure 18). A model with such characteristics requires very high numbers of sample analysis, which is currently being tested with the described paradigm. But also, it should be confirmed in other regeneration paradigms. This is also under ongoing investigation, more precisely, it is being tested using the paradigms where hub cell loss is being induced by activation of the apoptotic pathway via expression of *reaper* and *hid* simoultaneously, or $DIAP2^{RNAi}$ (Table 1, Fig16A,B).

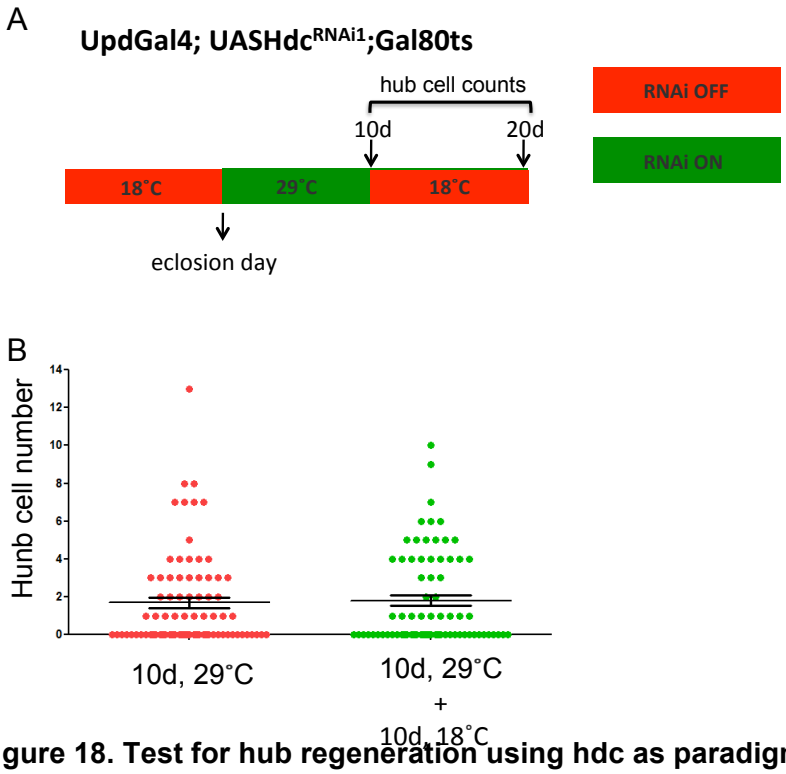


Figure 18. Test for hub regeneration using hdc as paradigm

A) Experimental paradigm to test for hub regeneration; Hub cell loss was achieved by expression of *hdc*-RNAi[1] in the hub during the first 10 days post-eclosion and then the GAL-UAS system was turned OFF by shifting the flies to 18ºC (10 days recovery period). **B)** Graph showing hub cell numbers after damage and recovery period; N>70 for each timepoint.

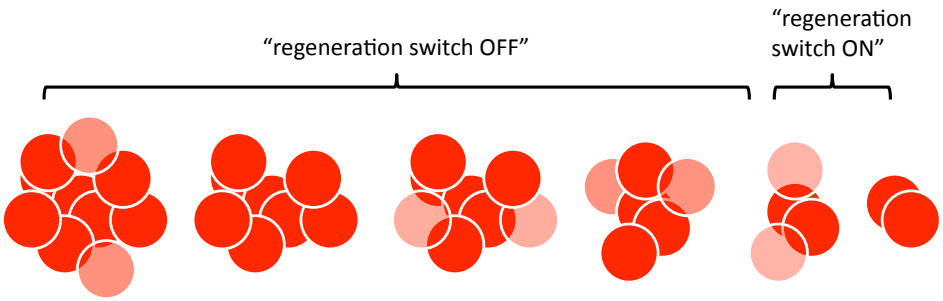


Figure 19. Hypothetical model for hub regeneration

As hub cells are lost, and if a mechanism for hub regeneration exists, a regeneration switch might be very sensitive and turned ON only one or two hub cells are lost. Alternatively, this switch can be activated only after a very significant hub cell loss occurs. While is still inconclusive if hub regeneration exists, data collected so far using paradigm described on Figure 16, points to the second scenario, where regeneration might happen only after severe damage and stop shortly after hub recover only a few cells.

headcase role the male germline

At the testis tip, *headcase* expression was not restricted to hub cells. In fact, higher levels of Headcase protein were found in germ cells, particularly those within the transit-amplifying region (Figure 7B). Although our initial interest in *hdc* was related to it's role in the hub, the particular high levels of expression of this protein within the germline were suggestive of a putative role for *headcase* in the germline. To test for a role for *hdc* in the germline, it was decided to use the *nanosGal4:VP16* driver and the *UAS-hdc*^{RNAi} lines described previously in hub experiments to knock-down *hdc* within these cells. Attempts to investigate for a *hdc* loss-of-function phenotype in the germline using this strategy failed as all three RNAi lines tested revealed inefficient to knock down *hdc* in this cell type, as revealed by AB staining. This is not surprising, as these RNAi constructs are well known to have very weak expression or not being expressed at all in the germline. Advances in the field have already addressed this issue and generated a new collection of UAS-RNAi lines designed specifically to perform well in the germline (pVALIUM20 vector) [95] but no line targeting *hdc* is available yet. Accordingly it was decided to investigate *hdc* loss-of-function using mosaic analysis [96]. Using this technique we are able to generate cells that are mutant for *hdc* within a heterozygous background animal (for detailed description see material and methods). Wild-type and *hdc* mutant clone induction was successful and happened at the same rate, and in both cases, GSCs clones were maintained throughout a period of 8 days, suggesting that *hdc* is not required for GSC maintenance in the Drosophila testis (Fig19). Complementary experiments to prove that *hdc* is not required for GSC maintenance should include the analysis of other *hdc* mutant alleles, and dissections at latter time points than 8 days post heat-shock.
Still with the aim of testing for a role for *headcase* in the germline, gain-of-function experiments were also performed. The *nanosGal4:VP16* driver was used to express both the *UAS-hdc*$^{RA/C}$ and *UAS-hdc*^{GSD404} . Over-expression of *hdc* in the germline resulted in a elongated testis tip, most likely resulting from a delay in the initiation of differentiation, as visible with markers that distinguish early germ cell stages from more differentiated stages (Fig21). Further characterization of this phenotype is ongoing.

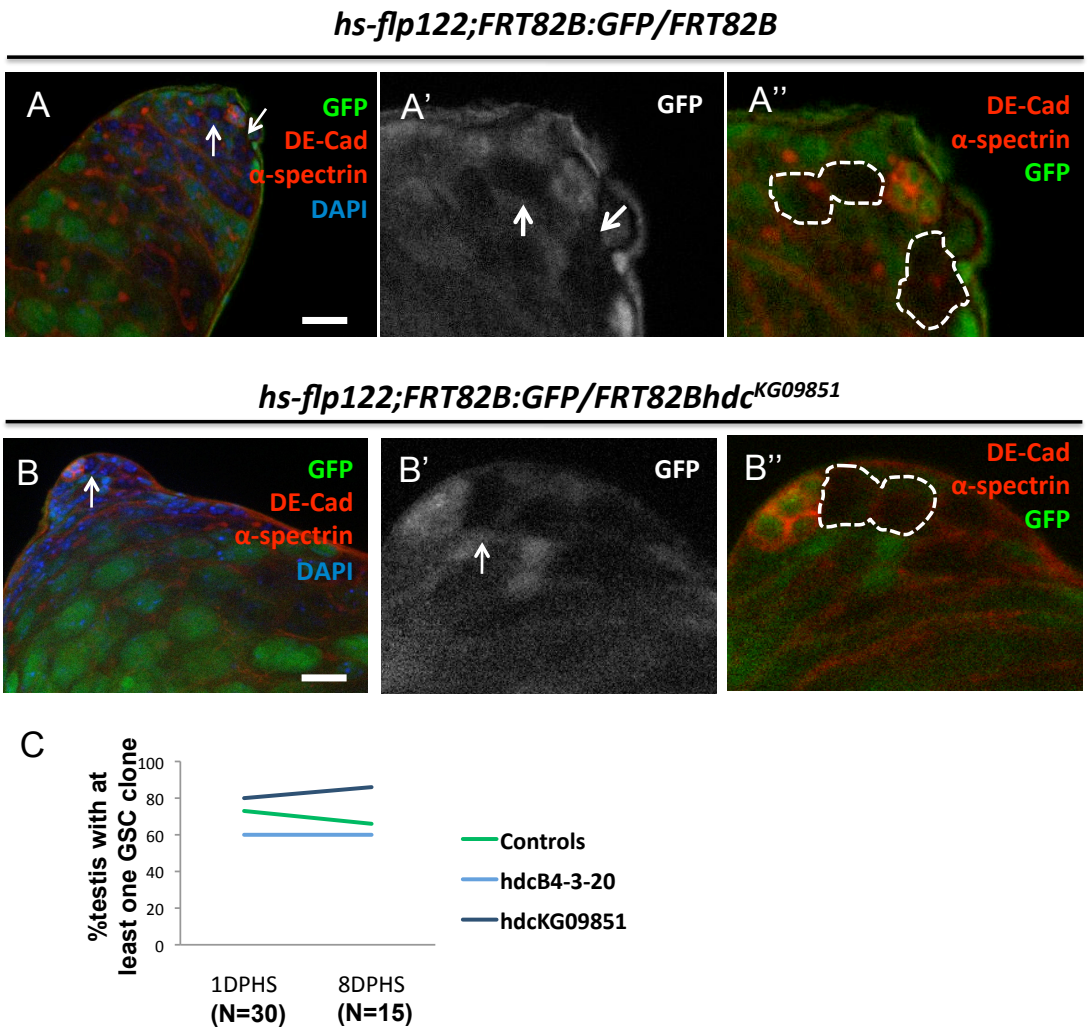


Figure 20. h*eadcase* is not required for male GSC maintenance

A) Example of a testis with two negatively marked control GSC clones (see absence of GFP in cells pointed by arrows in A'; GSC control clones are outlined by dotted white lines in A'' where it is evident a dotted fusome separating the GSC from it's daughter cell, the gonialblast); **B)** Example of a negatively marked *hdc* mutant clone, pointed by white arrow in B', and outlined by white dotted line in B'; scale bars, 20μm.

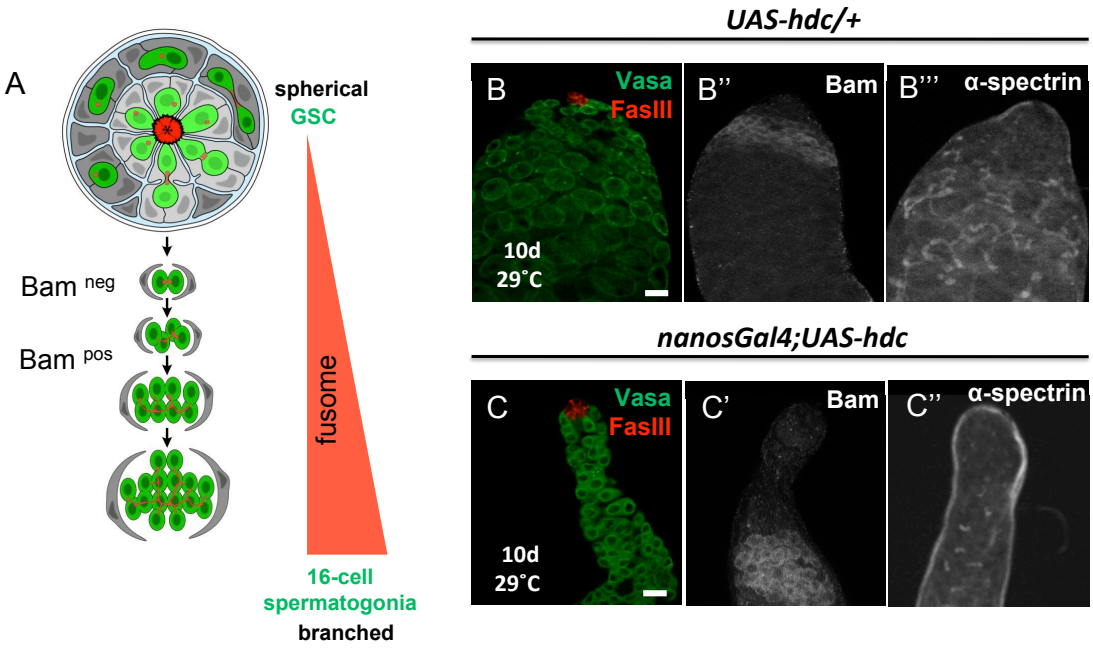


Figure 21. Headcase over-expression in the germline

A) Schematic diagram of the testis stem cell niche at the tip of the testis and early stages of spermatogenesis; The fusome goes from a spherical shape in GSCs to a branched shape in latter stages of germ cell development; the marker Bam is abscent from earlier stages of germ cell differentiation but it starts being expressed in 4 to 8 germ cell cysts; **B)** Genetic control for the Hdc over-expression experiment showing typical staining of a wild-type testis for Bam and α-spectrin; **C)** Hdc over-expression in the germline affects germ cell development; note the anatomic changes to the tip that is much thinner than control testis, and the accumulation of germ cells in early stages of differentiation (negative for Bam and with spherical fusomes); N>30 for each genotype; scale bars, 20μm.

headcase is ectopically expressed in *Drosophila* testis tumors models

The *Drosophila headcase* human homolog (HECA) is misexpressed in different types of cancers, although different lines of evidence exist for *hdc* acting as a putative oncogene or tumor suppressor, or simply a marker with no role on tumor development [67][68][69][97]. Over the past two decades *Drosophila melanogaster* has become an important model system for cancer studies. In conjunction with studies using other model organisms, flies have contributed greatly to our understanding of the mechanisms involved in cancer initiation and progression, revealing previously unknown molecular components and concepts [98]. The particular ability to conduct large-scale genetic screens studies, coupled with reduced redundancy in the fruit fly genome compared to humans, the power of genetic tools available to manipulate gene expression in tissue or cell type specific

fashion, has enabled its use to determine the molecular characterization of important developmental processes involved in many aspects of tumor development (cell division, survival, growth, metastasis, death, metabolism, etc) [99][100][101].

In the Drosophila testis, over-expression of certain genes lead to an uncontrolled proliferation of either germ cells or cyst cells, or both, leading to an accumulation of undifferentiated cells, with uncontrolled cell division, leading in some cases to a massive increase in organ size. Therefore, this phenotypically mimics general tissue alterations that occur in cancer, and can be used as models tumor development. One good example is the germline over-expression of the cytokine Upd (normally produced only in hub cells), that leads to an over-proliferation of both somatic and germ cells, and a dramatic increase on organ size evident immediately after a few days of over-expression (Fig21) [7][8]. It was decided to investigate if *hdc* expression was affected in this particular tumor model. Like suggested by studies on *headcase* human homolog (HECA), *hdc* was found ectopically expressed in this particular tumor models, both in over-proliferating germ and somatic cells (Fig22). To test if this result independent of the tumor model used, other tumor models were used. When over-expressed in cyst cells, the genes esg and Zfh1, lead to an uncontrolled proliferation of somatic cells corresponding to an alternative tumor model it could be used to investigate if *hdc* is misexpressed. Indeed, when Zfh1 or *esg* were over-expressed using the somatic specific driver c587Gal4, *hdc* was found ectopically expressed (Fig23). More importantly, it revealed to be a very good marker to follow the level of tumor progression as high levels of *hdc* expression were found to be coincident with the areas of over-proliferative cells in the initial stages of tumor development (Fig23A). Therefore, results show that *headcase* is ectopically expressed in different testis tumor models in Drosophila. These results, also suggest that HECA over-expression detected in humans, is likely to be consequent of a more abundant number of cells in the tissue in a proliferative state the ones where typically high levels of *hdc* are found.

Ongoing and future experiments aim to answer the question if blocking *hdc* expression affects tumor progression. Based on findings previously described in this chapter on *hdc* role specifically in hub cells, it is temped to speculate that high levels of *headcase* found in tumor cells might serve an anti-apoptotic role, essential for these cells to undergo continuous proliferation and accumulate in the tissue.

Data generated on hdc role during tumor development in the *Drosophila* testis should prove important to understand HECA potential as a novel cancer therapy target.

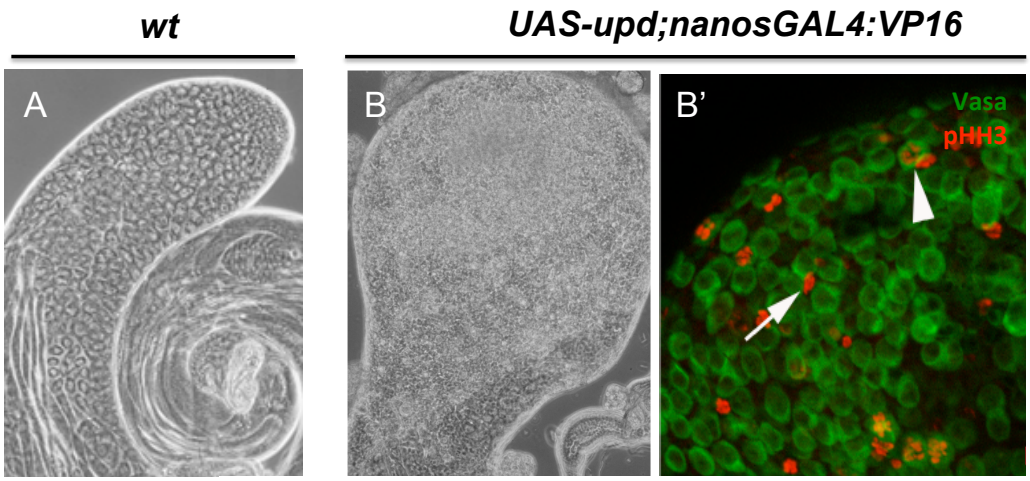


Figure 22. Generation of tumors in the Drosophila testis

A) Phase-contrast image of a wild-type testis; **B)** Phase-contrast image of a testis where *Upd* is being over-expressed in the germline. This leads to a dramatic over-proliferation at the testis tip and evident anatomical changes of the testis. **B')** At the tip of these testes there's an uncontrolled proliferation (pHH3, red) of both germline (Vasa positive, Green, white arrowhead) and soma (VASA negative cells, white arrow). IF image courtesy of Monica Boyle

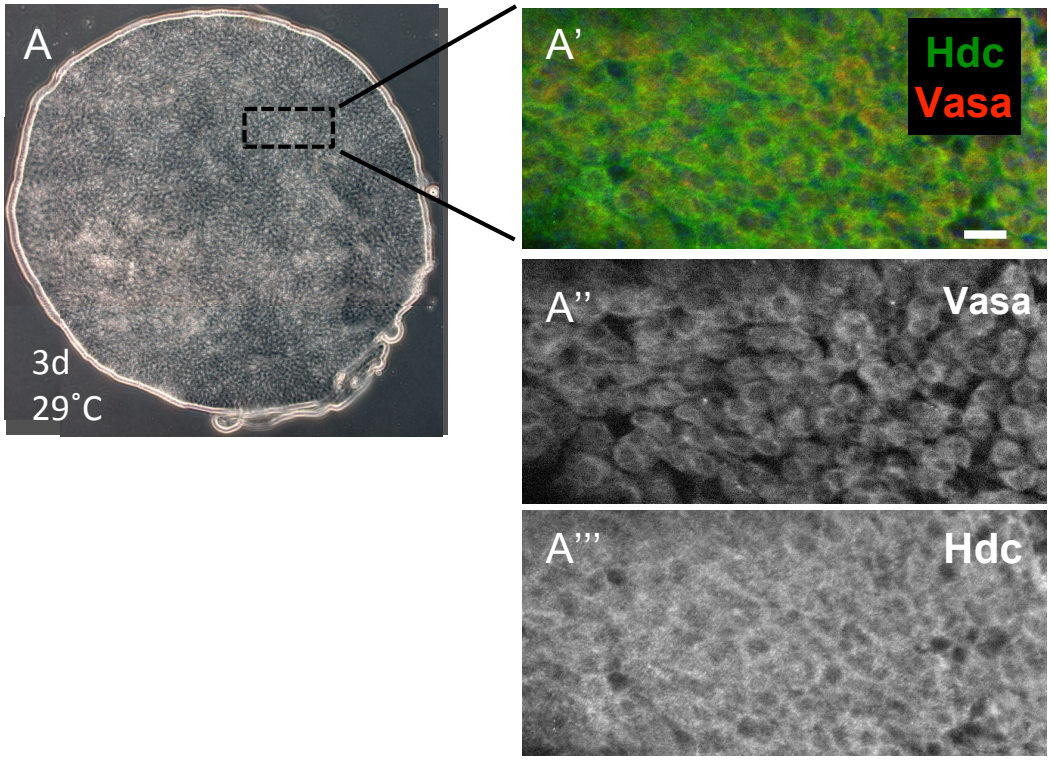


Figure 23. *headcase* ectopic expression in germline tumors in the testis

A) phase-contrast image of testis tumor caused by Upd over-expression in the germline (see also Figure 19); IF image shows that over-proliferation germ cells in the tumor ($Vasa^{pos}$ in A'') express high levels of *headcase* (A'''); scale bars, 20µm.

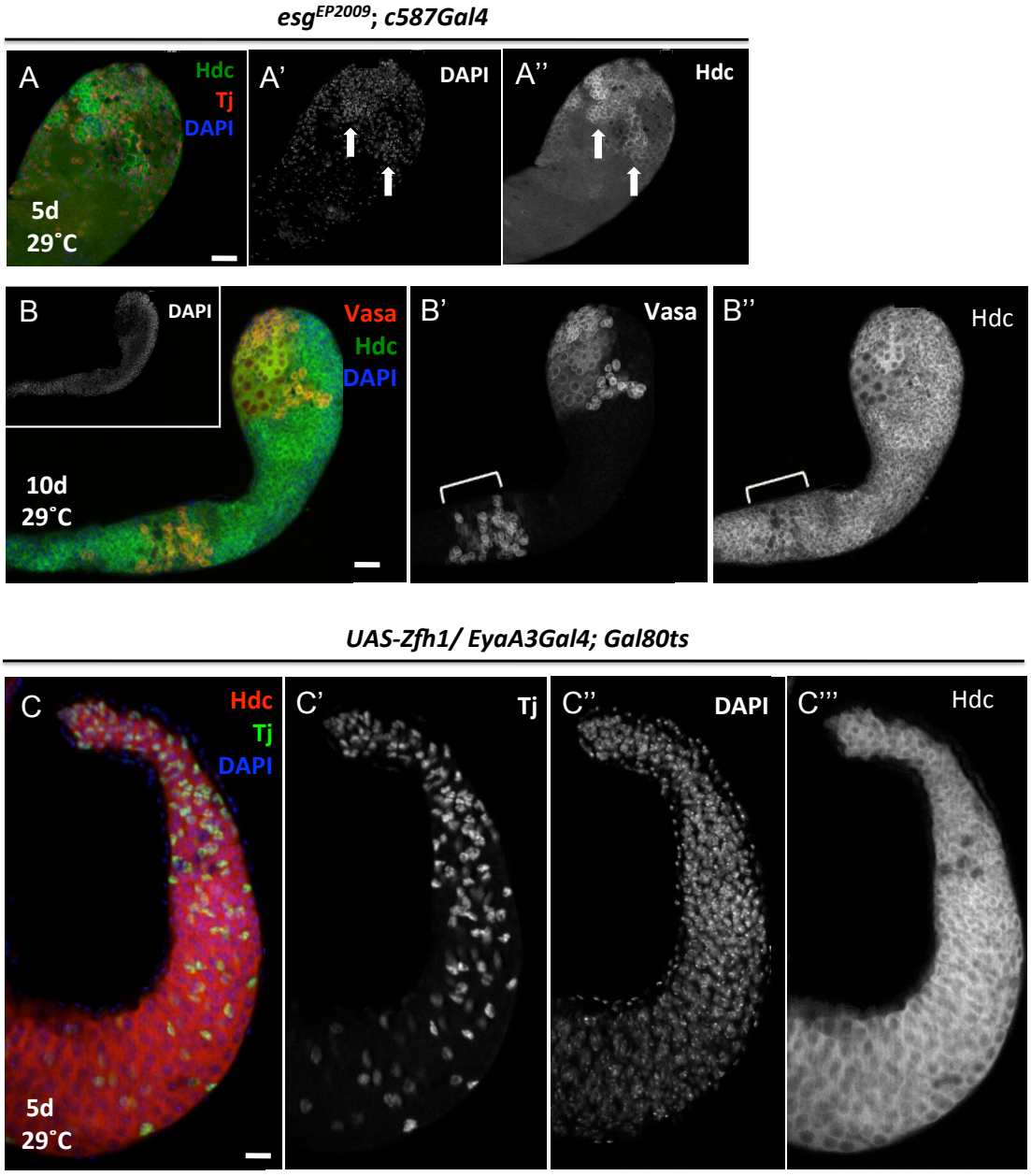


Figure 24. Hdc ectopic expression in somatic tumors in the testis

A) Initial stages (5d) of tumor development caused by Esg over-expression in the soma**;** Note the high levels of Headcase in the accumulating bright DAPI small cells pointed by white arrows in A' and A''; **B)** Same genotype and as in A) but now in a latter stage of tumor development (10d); The testis is now filled with bright DAPI cells and high levels of Hdc are found in over-proliferating cyst cells ($Vasa^{neg}$), and also in over-proliferating germ cells ($Vasa^{pos}$, white bracket in B' and B''); **C)** Hdc ectopic expression in a different model for somatic tumor, in this case caused by Zfh1 over-expression in cyst cells; Scale bars, 40μm in A and B, and 20μm in C.

Chapter conclusion

The transparency of the *Drosophila* testis, the particular spatial-temporal organization of the tissue, the power of genetic tools available for transgene expression, lineage tracing, mutant analysis, and immunohistochemistry to identify multiple cell types make it an ideal system to study the stem cell niche [102].

In this chapter it is reported that the *hdc* gene of *Drosophila* plays a role in maintaining survival of apical hub cells, which comprise a critical signaling center within the male germline niche [7][8]. Reduction in the levels of active *hdc* resulted in loss of hub cells, which could be blocked by expression of the anti-apoptotic protein p35. This corresponds to the first report of hub cell death, and provided an interesting paradigm to investigate how the number of niche cells (hub cells) affected the two stem cell populations of the testis, CySCs and GSCs. A comprehensive analysis of testis with hub cell numbers, revealed that stem cell are lost as hub cells are lost. This is was predictable due to the signaling and structural importance of the hub in this system [41]. More surprising was the observation that stem cell loss is not dramatic and even after severe damage induced to the hub. Hubs with one or two residual cells seem capable of maintaining multiple stem cells around them, corresponding to an interesting change in normal ratio of stem cells:niche cells and reveals a dynamic architecture of the stem cell niche (Fig24). Parallels with this observation are found in other stem cell niches, like the mammalian intestinal crypt, where in cases of genetic ablation of paneth cells, multiple $Lgr5^{+}$ crypt base columnar (CBC) cells can be found in close association with a individual paneth cell [14].

Importantly, we noted that total hub area is not compromised in a proportional manner to the loss of hub cells. It is proposed here that this is the explanation for the high functionality of compromised hubs. These remaining one or two cells still show high levels of *upd* activity, and as long as there is physical space for GSCs or CySCS to be in close contact to the cytokine source, the signal transfer seem to be efficient.

These findings provide insight into how in similar mammalian niches the stem cell/niche size ratio scales and, therefore, inform the design of 3D niches *in vitro* for the expansion and manipulation of stem cells in culture.

Ongoing studies aim to explain the mechanism behind apoptosis initiation in the hub after *headcase* loss-of-function, and place *headcase* into a developmental

pathway capable of controlling cell death. Future studies should provide a description of other important genes acting to prevent apoptosis in long-lived, post-mitotic hub cells. Preliminary data shows that hub cell apoptosis can be induced via classical genes of the apoptotic pathway, and that compromised hubs in these experiments recapitulate findings on the influence of the niche size on the stem cell pool observed after *hdc* loss-of-function. This highlights the importance of findings reported as they seem to be valid for different experimental paradigms and should reflect a general characteristic of the testis stem cell niche.

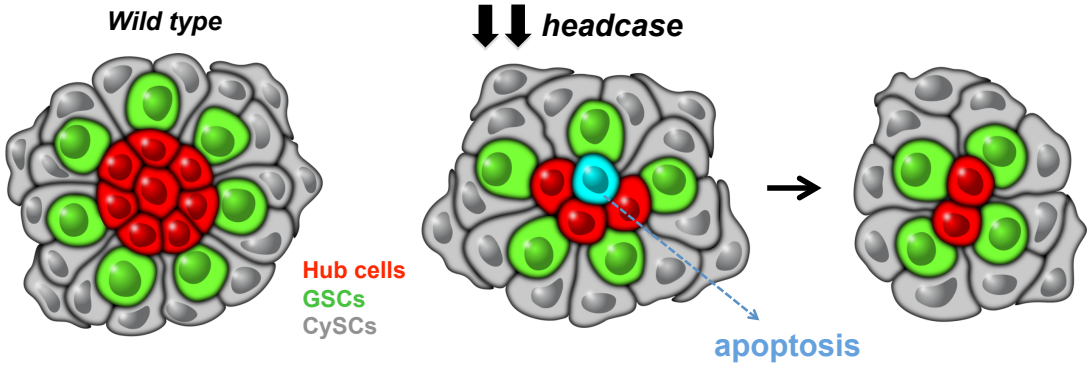


Figure 25. Model summary of findings on *headcase* role in hub cells of the Drosophila testis

Reduced headcase levels in hub cells results in loss of hub cells due to apoptosis. As hub cells are lost, stem cell niche architecture changes; however, severely compromised hubs composed of only 1 or 2 cells remain functional and capable of maintaining active stem cells around them.

Chapter II

The role of *headcase* in the *Drosophila* intestinal stem cell niche

Results and Discussion

headcase expression is expressed in ISCs and EBs in the *Drosophila* midgut

After findings on a role for *headcase* in the *Drosophila* testis, it was decided to investigate if this gene was expressed in other fly stem cell niches. We found it was expressed in the ovary, in stem cells and different cell types that compose the stem cell niche at the tip of the germarium (data not shown), and a very interesting expression pattern was detected in the intestinal midgut. The *hdc* monoclonal AB showed that *headcase* was expressed in ISCs and EBs (Fig25). Expression could also be detected in hindgut stem cells [103][104] and malpighian tubule stem cells [105][106] (Figure 25, A and A'). If *hdc* was expressed in ISCs and EBs in the posterior midgut, reporter lines for *esg* should reveal an identical expression pattern of GFP and the *hdc* AB. Indeed that was observed (Fig25 B to B'''), and therefore it was concluded that *hdc* is expressed in progenitor cells in the posterior midgut. A *hdc* reporter line (see Chapter I) confirmed expression of *hdc* in ISCs and EBs (Fig25 C to D'''). As ISCs and EBs are the cell types where the JAK-STAT pathway is active in the posterior midgut [107][108][109], this expression pattern might be seen as further evidence for a link between *hdc* and the JAK-STAT pathway, as proposed in a study in eye development [64]. Also, hyper-activation of the pathway in the testis, lead to *hdc* overexpression (see chapter I), representing one more evidence for this relationship. Although, high levels of expression in cell types where the JAK-STAT pathway is inactive, like hub cells or 4-8 germ cell cysts in the testis (Figure 7, chapter I), or malpighian tubule stem cells in the intestine (Fig 25), suggest that *hdc* expression can not be simply explained by level of activation of the JAK-STAT pathway.

headcase is required for maintenance of ISCs and EBs

After the characterization of *hdc* expression pattern in the posterior midgut, it was decided to test for a putative role for this gene in this tissue. Using the same strategy used in the first chapter where the role of *hdc* in the testis stem cell niche was investigated, experiments were designed to knock down *hdc* expression in a cell type specific manner using the GAL4-UAS system. The *esgGal4* driver was used to express different hdc^{RNAi} lines in ISCs and EBs. Expression of any of the three different RNAi lines for *hdc* (described in chapter I and in material and methods),

lead to embryonic/larval lethality. Again, similar to strategies used in chapter I to suppress RNAi expression during development, it was used the GAL80^{ts} allele as an attempt to suppress in a temporal fashion the GAL4-UAS system. Indeed, when kept at 18°C, lethality of *esgGAL4/UAS-hdc^{RNAi1};GAL80^{ts}* flies was successfully rescued. These flies were then shifted to 29°C at 1d to activate the RNAi expression, and test for a putative role for *hdc* in ISCs and EBS. Expression of *hdc^{RNAi1}* (the line corresponding to the stronger phenotype observed in the testis hub cell experiments) in ISCs and EBs lead to significant loss of these progenitor cells after 10d of RNAi induction (Figure 26 A to D). ISC/EB nests can be identified as *esg* positive cell doublets (see white brackets in Figure 25B, insets, and orange brackets, in Figure 26 A''), but in intestines where *hdc* was knocked down in these cells for 10days, GFP$^+$ were very rarely found in doublets (B''), and quantification of total number of GFP$^+$ cells in the the 1st field of view of the posterior midgut (see material and methods) showed that approximately 2/3 of ISCs/EBs cells were lost (Figure 26D). This phenotype was even more severe after 20days of RNAi induction, resulting in a dramatic loss of ISCs and EBs – only 5-15 found in the 1st field of view (Figure 26C) as opposed to approximately 100 typically found in young control flies).

ISC/EB loss was also observed when *hdc^{RNAi2}* was used, but surprisingly this line originated a much stronger phenotype, as opposed to what was observed in the testis where this line originated a weaker hub cell loss when expresseed in the hub, and when compared to *hdc^{RNAi2}*. Expression of *hdc^{RNAi2}* for 10days in progenitor cells in the posterior midgut, was enough to lead to a total loss of ISCs and EBs in the majority of flies analysed (N>20), and the remaining intestines where a total loss was not observed had very few (1 to 15) *esg* positive cells left (Figure 26E). Preliminary data suggests that flies can live at least for 3 weeks after the majority of their progenitor cells in the midgut were lost. It is now under investigation how the exact lifespan of these flies is affected and also if the intestinal functionality is affected.

ISC/EB loss after *hdc* loss-of-function can be prevented by blocking apoptosis

The observations on *hdc* role preventing hub cells from entering apoptosis described in chapter I, and the finding that *hdc* loss-of-function in the posterior midgut lead to a strong loss of the progenitor cells (ISCs and EBs) opened the hypothesis that *hdc* role in preventing cell death might be conserved in other cell types/tissues. To test if this is true in the *Drosophila* posterior midgut, *hdc^{RNAi}* was co-expressed with the apoptosis blocker baculovirus protein P35, to check for a putative phenotype rescue (same rationale of experiments performed in chapter I).

Indeed, and blocking apoptosis via expression of p35 successfully rescued the loss of ISCs and EBs in the context of *hdc* knocked down (Fig27). Flies where both the *hdc*^{RNAi1} and the p35 constructs were expressed in ISCs/EBs (*esgGAL4/UAS-hdc*^{RNAi1}*;UAS-p35/GAL80*^{ts} showed a normal epithelium at 10d, and even at 20d, time point when flies that carried the *hdc*^{RNAi1} construct alone (*esgGAL4/UAS-hdc*^{RNAi1}*;GAL80*^{ts}*)* had a very severe loss of progenitor cells (Fig27). This result expands the importance of findings reported on *hdc* biological role preventing hub cell death, to what probably represents a conserved role in more cell types/tissues.

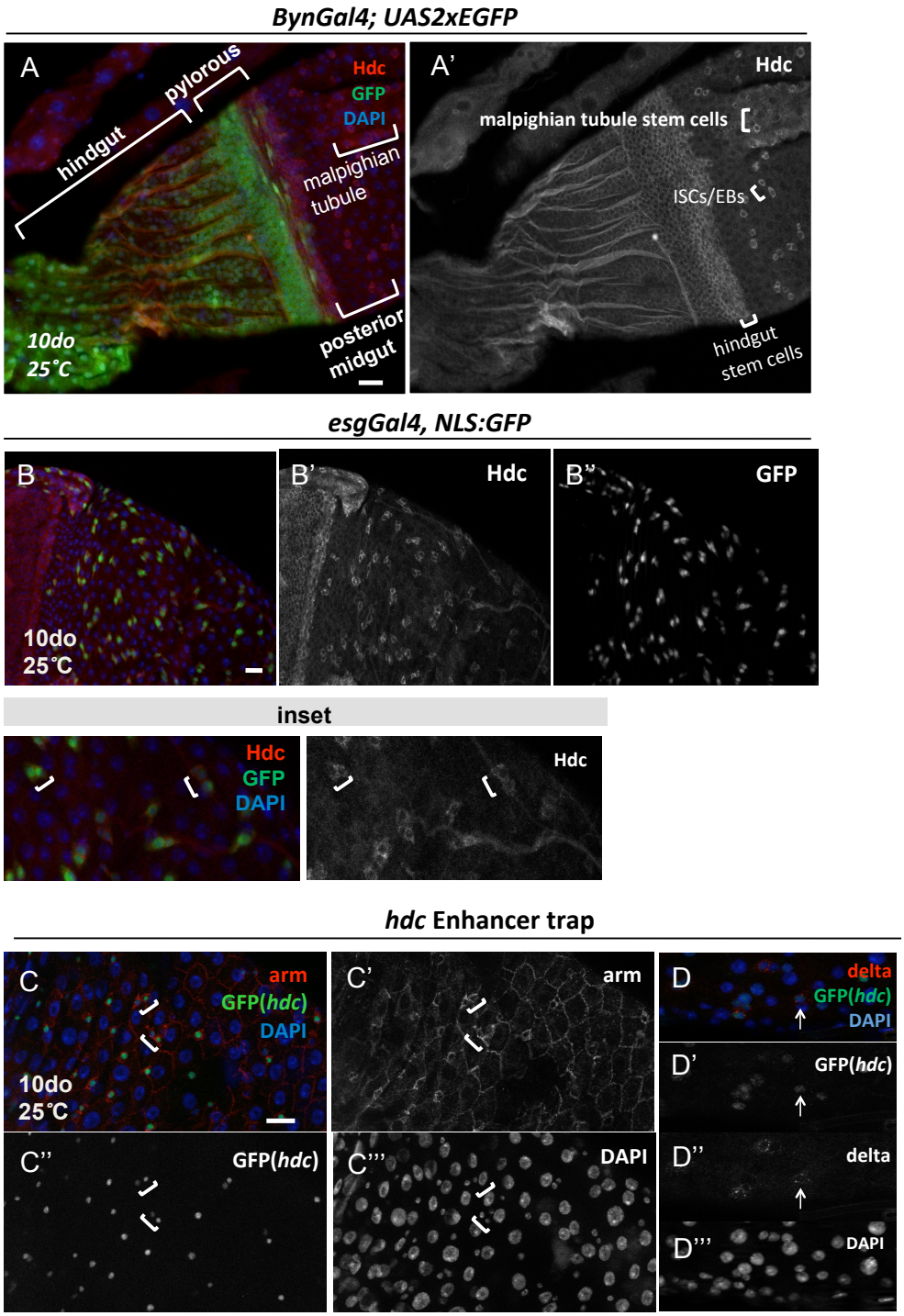


Figure 26. *Headcase* expression in the Drosophila intestine

A and A') Immunofluorescence image of part of the *Drosophila* intestine that includes the most anterior part of the hindgut, the pylorous, and the most posterior part of the midgut. Also visible are the malpighian tubules, which are connected to the midgut epithelium right close to the pylorous. BynGal4 (Green in A) is expressed in all cells from the hingut and in the most posterior cells of the pylorous. Hdc is expressed in ISC/EBs nests in the posterior midgut, in the pyolorous, including the hindgut stem cells, and in the malpighian tubule stem cells (Red in A, and white in A') **B)** *headcase* and *esg* share the same expression pattern in the posterior midgut; C) Hdc enhancer trap line revealing the same expression pattern observed with the hdc monoclonal AB; Scale bars, 20µm

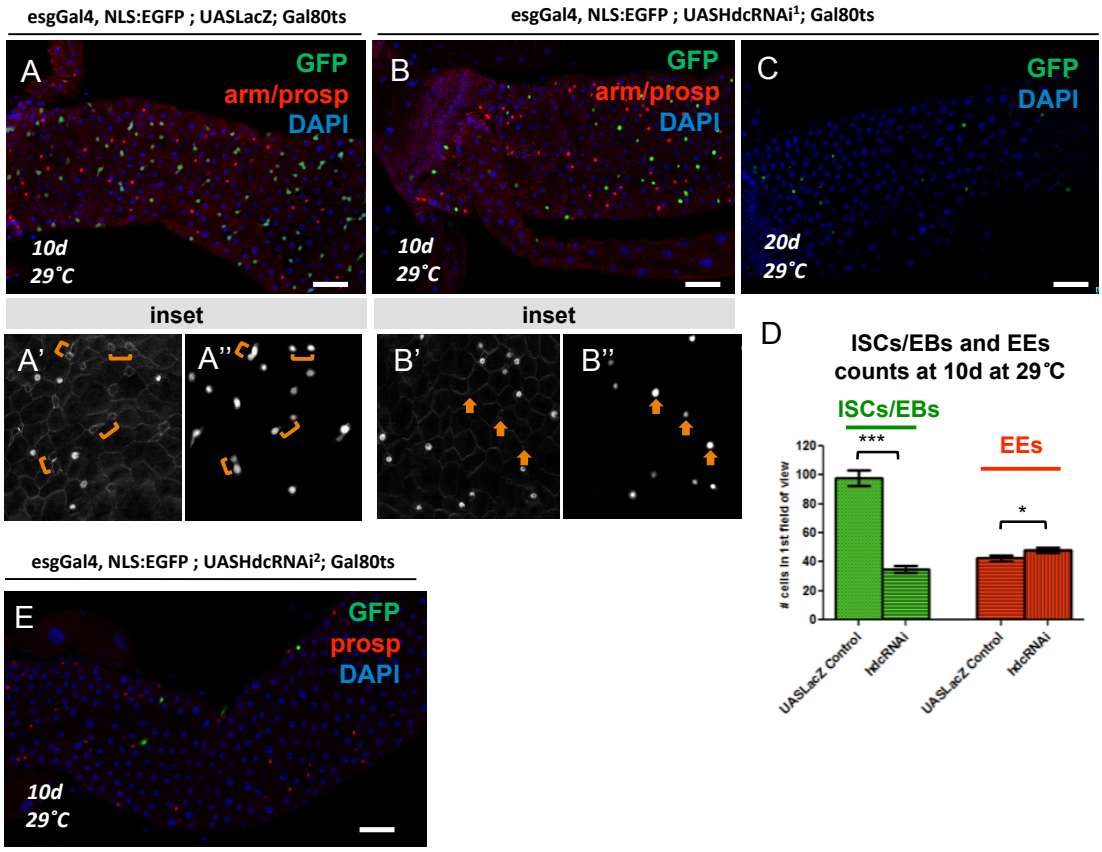


Figure 27. *headcase* is required for ISC/EB maintenance in the posterior midgut

Control **(A)** and *esgGal4:NLSEGFP/UAS-hdc^{RNAi1};Gal80ts* **(B)** 10day-old posterior midguts; *hdc* knock-down in ISCs/EBs leads to loss of these progenitor cells (esg^{+}, GFP); - compare GFP^{+} doublets of cells in (orange brackets in A', A'') to single GFP^{+} cells in (orange arrows in B'. B''); **C)** loss of ISCs/EBs is even more dramatic after 20days of RNAi induction; **D)** quantifications of the number of ISCs/EBs and EEs at 10d in control and hdc loss-of-function situations; **D)** *hdc*^{RNAi2} expression in ISCs/EBs leads to stronger ISC/EB loss at 10d; Scale bars, 40μm

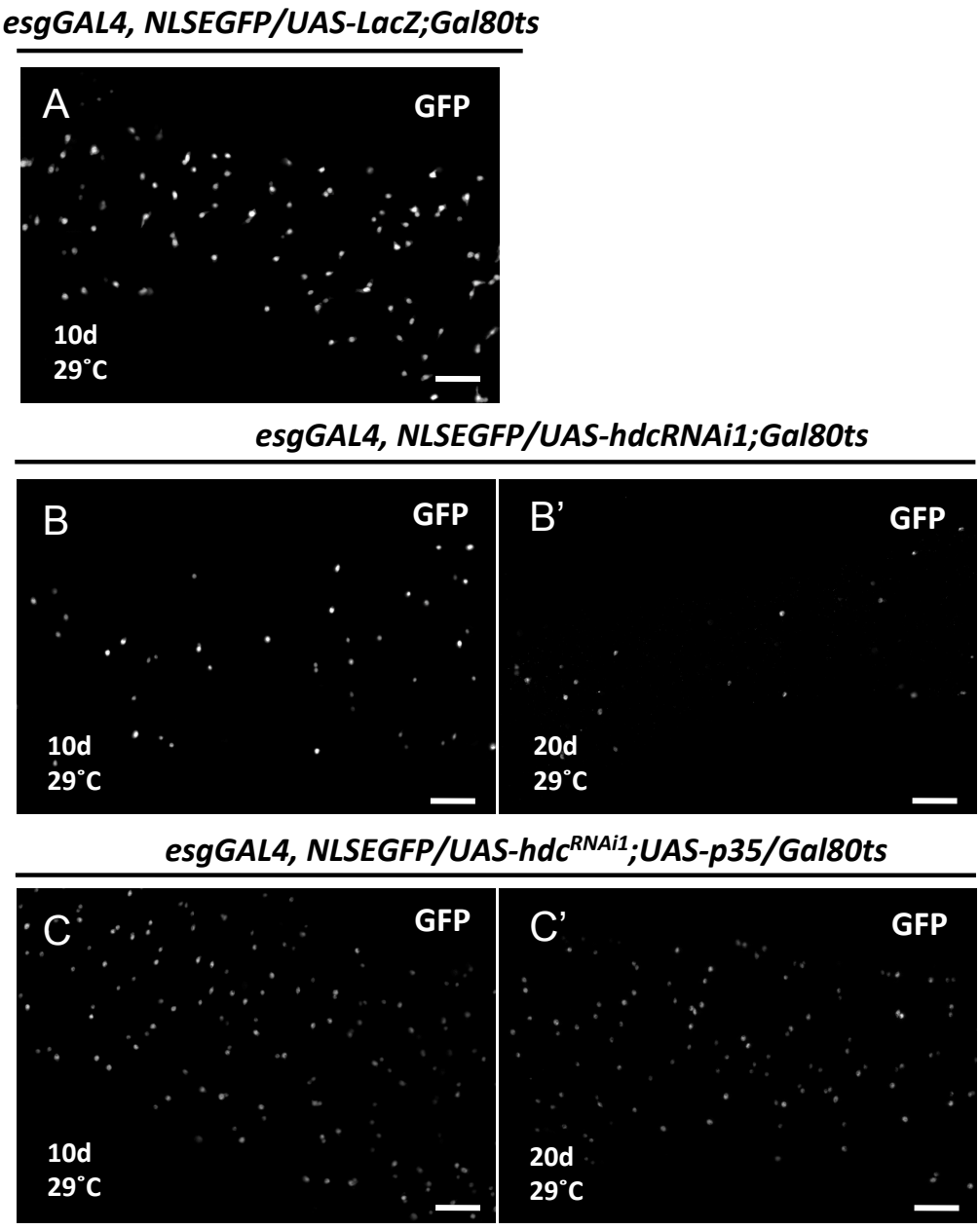


Figure 28. Apoptosis block rescues ISC/EB loss after headcase loss-of-function

(A) *esgGal4, NLS:EGFP/UAS-LacZ;Gal80ts* 10day-old controls flies **(B)** *esgGal4:NLSEGFP/UAS-hdc*^{RNAi1} 10day-old posterior midguts; *hdc* knock-down in ISCs/EBs leads to loss of these progenitor cells (esg^{+}, GFP, white); **(B')** ISC/EB loss after *hdc* loss-of-function is even more dramatic at 20d; **(C and C')** Apoptosis block with p35 rescues ISC/EB loss after *hdc* knock-down. (compare to B and B'); Scale bars, 40μm

Chapter Conclusion

The discovery and characterization of *Drosophila* intestinal stem cells provided an excellent system for the study of stem cell behavior. We observed an interesting expression pattern of *headcase* in the posterior midgut, restricted to intestinal stem cells and enteroblasts - the progenitor cell types in the intestine, and absent from the differentiated specialized cell types - the secretory enteroendocrine cells, and the absorptive enterocytes.

Loss-of-function experiments revealed that, like hub cells in the testis, *headcase* function is required in ISCs and EBs to prevent them from entering programmed cell death. Also, similar to experiments in the testis this phenotype was successfully rescued when the apoptosis inhibitor p35 was expressed. Some of these experimental paradigms lead to a total loss of progenitor cells in of the posterior midgut, transforming dramatically the epithelium into a simple composition of two differentiated cell types: EEs and ECs. Surprisingly, these flies seem to live at least up to 3 weeks after total loss of progenitor cells, suggesting that these are not absolutely required for intestinal function in “unchallenged” flies. Ongoing experiments aim to understand how the longevity of these flies where ISCs and EBs are lost is affected. Current experiments are also trying to characterize the intestinal functional consequences of this dramatic ISC/EB loss. It is tempted to hypothesize that challenges induced in a posterior midgut where progenitor cells were ablated would be lethal to those flies. Also, it would be very interesting to know if these flies are particularly sensitive to other types of challenges (tissue injury), induced in the intestine, as the cells normally acting during regeneration are absent. As it is still not very well understood how the *Drosophila* midgut epithelium turnover happens, findings on this particular situation on which the progenitor cells area ablated should reveal important data to access how tissue functionality is affected over a long period of time.

The *hdc gene* was also found to be expressed in hindgut stem cells and malpighian tubule stem cells it would be interesting to test for a role for this gene in these alternative tissues. The identification of stem cells in these two tissues only happened recently, therefore much is still unknown about the characteristics of stem cell niches within the pylorus and the malpighian tubules. Therefore, *hdc* could serve as an interesting tool to explore phenotypic consequences of gain and loss-of-function experiments in the different cell types that compose these tissues, and

hopefully shed some light on the dynamic processes acting to regulate hindgut and malpighian tubule stem cells.

Material and Methods

Drosophila life-cycle and experimental design

The majority of experiments mentioned in this thesis were performed in the adult stages of *Drosophila melanogaster* (as a fly). After egg deposition, when kept at 25ºC, development lasts approximately 10 days and culminates with the emergence of the adult fly (hatching) (Fig28). Temperature has a large impact on the rate of *Drosophila* development. Temperatures (29ºC for instance) accelerate development while lower temperatures slow-down development (18ºC for instance). When describing an experiment, there is a reference to "eclosion" or "hatching" this refers to the moment when the insect splits the pupal case and emerges as an adult fly (Fig27). When it is mentioned that a process occurred "during development" or "during developmental stages", it refers to life-cycle stages comprised between egg and eclosion, and therefore, includes embryonic, larval and pupae stages (Fig27).

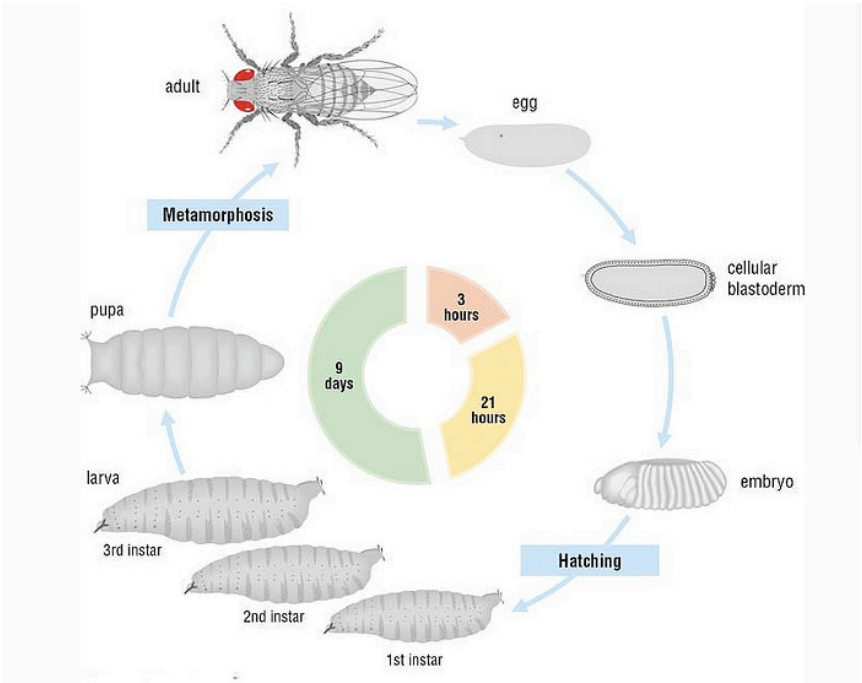


Figure 29. Drosophila melanogaster life-cycle

Drosophila melanogaster development is approximately 10 days and includes an embryonic stage, followed by three larval stages (1st, 2nd and 3rd instar), pupa and finally the adult fly. and Image obtained from *FlyMove* (flymove.uni-muenster.de/)

Fly husbandry and stocks

Flies were raised on standard cornmeal-molasses-agar medium. Male progeny from experimental crosses were collected and maintained with less than 30 flies per vial. Flies were turned onto fresh food every two days.

The following stocks were used; more information on them can be found in Flybase (http://flybase.bio.indiana.edu): *updGal4*(X); *FasIIIGal4*; *UAS-p35* (Bloomington stock center #5072 and #5073), *UAShdc*^{GSD404} (gift from Dr. Don Van Meyel lab), *hdc* enhancer trap (Carnegie protein trap library), *UAS-reaper, UAS-reaper, UAS-hid* (gift from Dr. Eric Rulifson laboratory), *UAS-lacZ*^{NLS} (Bloomington stock center #3956); *UAS-DsRed*,*UAS-flp*, *ubi>stop>GFP*/Cyo;MKRS/TM6b and Cyo/Sco; *UAS-DsRed,UAS-flp, ubi>stop>GFP*/TM6b (G-TRACE cassettes on II and III) (gift from U. Banerjee) [Evans et al, 2009] ; *hdc*^{RNAi} lines used were from Vienna *Drosophila* stock center and labeled as *UAS-hdc*^{RNAi1}, *UAS-hdc*^{RNAi2} and *UAS-hdc*^{RNAi3}, *UAS-unk*^{RNAi}, *UAS-cycK*^{RNAi}, *UAS-DIAP2*^{RNAi}, corresponding to VDRC#45069, VDRC#104322 and VDRC#39877, VDRC#4267, VDRC#110774 and #2973 respectively. All three RNAi lines target distinct *hdc* sequences that map across the gene: UAS-*hdc*^{RNAi2} (mild phenotype) targets all three isoforms while UAS-*hdc*^{RNAi1} (strong phenotype) and UAS-*hdc*^{RNAi3} (mild phenotype) only target two of the 3 isoforms (Fig29). Note that hub cell loss could not be tested using *hdc* mutants, as all alleles tested (*hdc*^{50}, *hdc*$^{Fus-6}$, *hdc*^{43}, *hdc*$^{B4-3-20}$ and *hdc*^{KG09851}) exhibited embryonic/larval lethality.

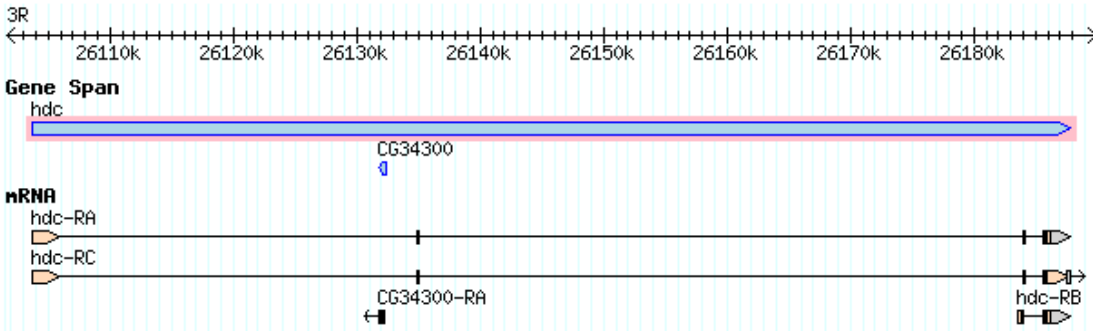


Figure 30. Headcase gene model and products

Image obtained from flybase: www.flybase.org; *headcase* gene isoforms A, B and C (A – 4592 nt / 650 aa C – 4592 nt / 1080 aa B – 2437 nt / 201 aa)

Candidate screen for regulators of hub maintenance and temporal expression of RNAi transgenes

Male flies carrying diferent *UAS-RNAi* lines corresponding to different target genes were crossed to virgin females flies carrying the *updGal4* driver. Crosses were set at 25°C and male flies were dissected at eclosion day (1d) or collected and kept at 25°C before dissection at 10 days (10d). Statistical significance between hub cell numbers within different genotypes/timepoints was determined using a one-way ANOVA (Kruskal-Wallis test) followed by a Dunn's multiple comparison test.

Suppression of expression of the UAS-*rpr,* UAS-*hdcRNAi*^{1} *and G-TRACE* constructs during development was achieved using a temperature sensitive allele of Gal80 (*Gal80*^{ts}). Flies were raised at 18°C and shifted to 29°C upon eclosion (day 1) to activate transgene expression for 10 days, or as otherwise noted. Expression of *UAS-hdcRNAi*^{2} and *UAS-hdcRNAi*^{3} was suppressed during development by raising flies at 18°C (no Gal80^{ts} used).

Generation of UAS-*hdc* flies

Sequences corresponding to *headcase* gene longer isoforms A/C or the shorter isoform B (Fig29) were cloned into a pUAST vector (Fig30) [110]. Transgenic flies were generated by injection of these constructs using Genetic Services Inc company (http://www.geneticservices.com/). In the case of the isoform B a myc and GFP tag were also incorporated on the construct cloned into the vector and sent to injection.

Antibodies

Testes were stained with: rabbit anti-Vasa (1:2,000) (gift from P. Lasko); mouse anti-Hdc (1:5) (gift from R. White); guinea pig anti-Zfh-1 (1:3,000) (gift from C. Doe); rabbit anti-Stat92E (1:800) (gift from D. Montell); mouse anti-Fasciclin III (7G10) (1:10); rat anti-DE-cadherin (1:20), rat anti-DN-cadherin (1:20), mouse anti-αspectrin (1:10), mouse anti-armadillo (1:15) and mouse anti-Prospero (1:50) (Developmental Studies Hybridoma Bank (developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa,

Department of Biological Sciences); rabbit anti-GFP(1:5000) (Molecular Probes). Secondary antibodies were diluted 1:500 (Molecular Probes).

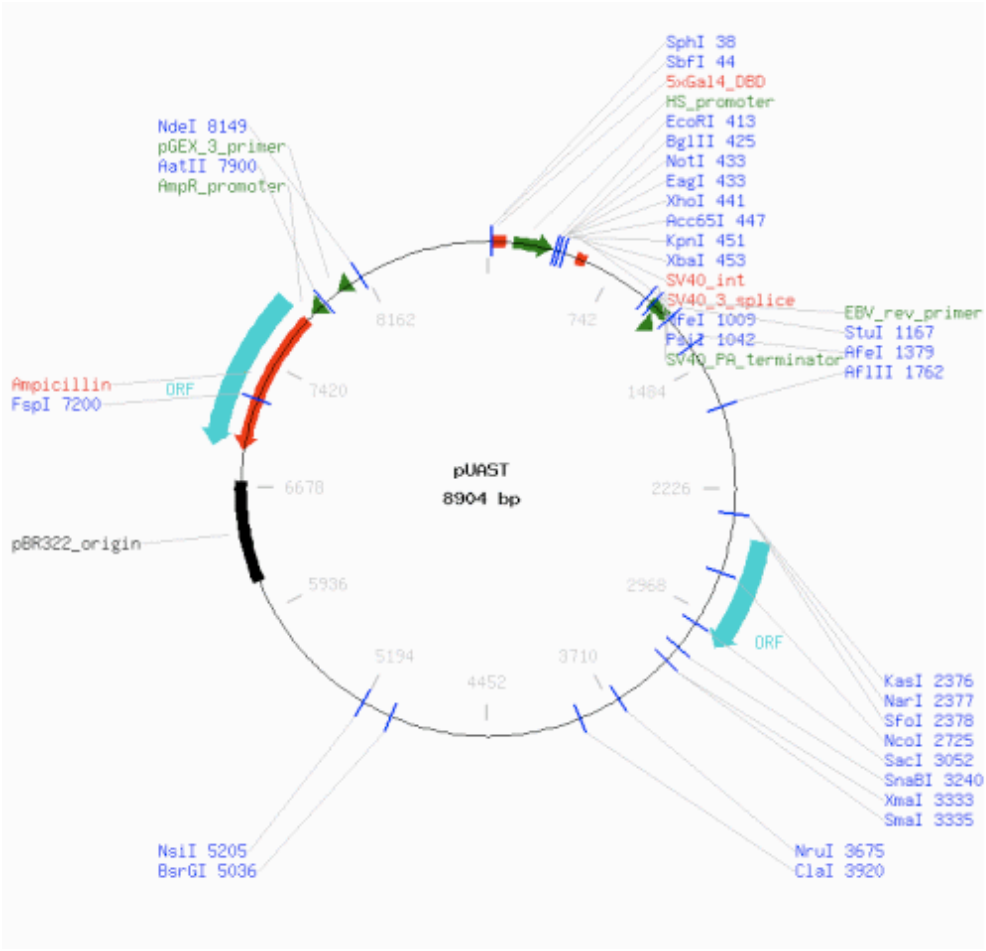


Figure 31. pUAST vector map and features

Image obtained from lablife (www.lablife.org)

Immunofluorescence and microscopy

Phase contrast images of squashed testes were obtained using a Leica DM5000 microscope equipped with a DC500 camera, using Firecam imaging software (version 1.7.1; Leica Microsystems). Immunofluorescence (IF) microscopy was performed on whole-mount testes dissected into PBS and fixed in 2% PFA as previously described (Boyle et al., 2007). Images were obtained using either a Zeiss LSM 710 Laser Scanning confocal microscope or Zeiss Axiovert 200 microscope equipped with Apotome. Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Pictures were analysed in AxioVision (version 4.8; Carl Zeiss) and Adobe Photoshop software (Mountain View, CA).

Cell counts and hub area measurements

All experiments involving cell counts (hub cells, GSCs or CySCs) were performed using at least 10 sections (Z-stacks). Hub cells were counted as $DAPI^+$ nuclei that were $FasIII^+$, DE-cad^+ or DN-Cad^+, using a 63X objective. GSCs were counted as STAT92E positive germ cells contacting the hub. Presumptive CySCs were scored as $Zfh1^+$ found in a 15μm radial distance from the center of the hub. Hub area was measured using the AxioVision (version 4.8; Carl Zeiss) software to calculate the area defined by $FasIII^+$ cells.

Statistical analysis

For the purpose of comparing hub cell averages between multiple genotypes/timepoints found on Figure 5 and Figure 6 the Kruskal–Wallis one-way analysis of variance test was used); On Figure 8, 9, 16 and 17 statistical significance of the different hub cell averages was tested between different pairs of columns (genotypes), and a Welch's *t*-test was used. For both cases, p-values were represented in graphs using the following asterisk code: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Statistical significance of the different frequencies of apoptotic hub cells of different experimental conditions was tested with the Fisher's exact test. Analysis of the G-trace data and the frequency of testis with hub resctricted GFP expression vs non restricted was done with a chi-square test.

Apoptosis Assay

Testes were fixed and processed using the Chemicon ApoTag Fluorescien Kit, according to manufacturer's instructions (detailed description at http://labs.genetics.ucla.edu/lusis/protocols/Apoptosis%20Protocol.pdf), followed by immunofluorescence as described above.

Ex vivo EdU incorporation

EdU incorporation was performed and analyzed using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen - www.invitrogen.com), with the following modifications. All procedures were performed at room temperature with minimal exposure to light. Crude dissection of testes was performed in 1X Ringer's buffer and then transferred

immediately to 1X Ringer's buffer in a glass embryo dish for no more than 10 minutes. Testes were subsequently transferred to 30mM EdU diluted in 1X Ringer's buffer for 30'. After incorporation, testes were fixed for 20' in 4% paraformaldehyde diluted in 1X PBS, followed by two washes with 1X PBST (0.5% Triton-X100) and blocked with 3% BSA in 1X PBS. Testes were bathed in the Click-iT reaction cocktail for 30 minutes. IF was performed as indicated above.

Clonal analysis

For the generation of GSC clones and analysis in the testis (Figure 18, Chapter I) the classical negatively marked mosaic analysis were used [111][112]. A detailed schematic of the principles behind generation of negatively marked clones with this technique are shown in Figure 31.

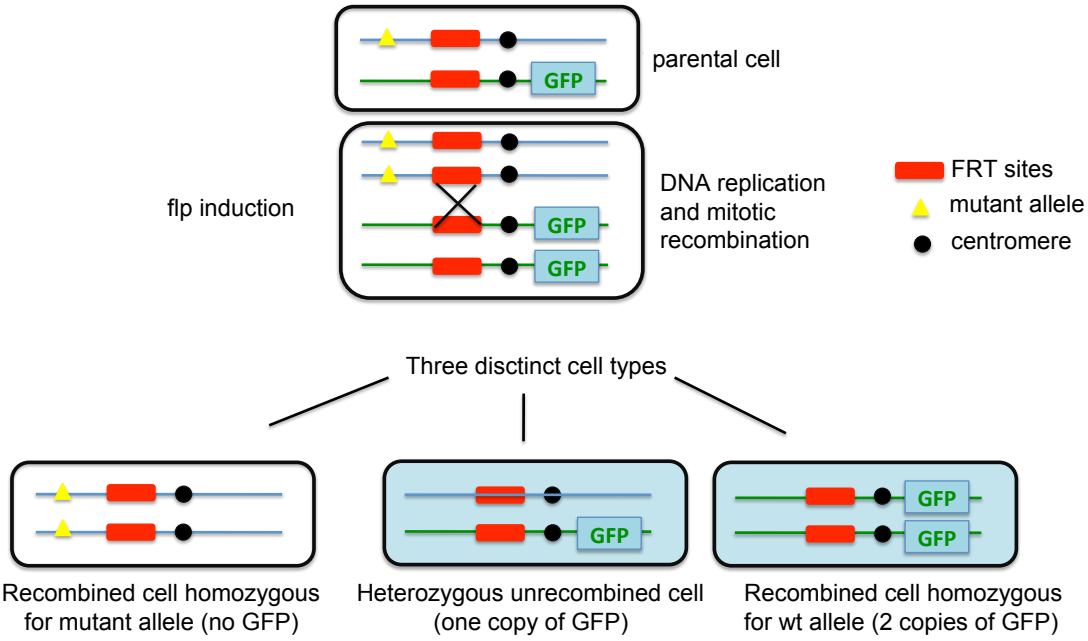


Figure 32. Mosaic analysis with a repressive cell marker

FLP/FRT-based mitotic recombination system for generating negatively labelled clones in a positively labelled background; Induction of FLP-recombinase gene, under the control of an hsp70 promoter, recognizes and promotes recombination specifically at the level of the FRT sequences, during mitosis. This can generate three distinct genotypes within cells from the same animal - unrecombined heterozygous (GFP pos), recombined homozygous for mutant allele (GFP negative), or recombined homozygous for wt allele (GFP positive).

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Appendix

Signalling within stem cell niches: insights from *Drosophila*

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Abstract

Tissue stem cells are found in specialized microenvironments (niches) where they are exposed to diverse systemic and local signals that are integrated with cell intrinsic factors to regulate stem cell behavior. In general, systemic signals are utilized to coordinate the response of tissues to acute or long-term changes that affect the whole organism, such as variations in nutrient availability or aging. In contrast, local signaling regulates tissue maintenance by balancing stem cell self-renewal with differentiation under homeostatic conditions and in response to local damage. In this review, we highlight the role of the JAK–STAT pathway in two *Drosophila* stem cell systems, the testis and intestine, and compare and contrast how activation of this pathway leads to tissue maintenance under both homeostatic conditions and in response to stress or injury.

Introduction

Adult stem cells reside in highly organized and specialized microenvironments, known as niches, within the tissues they sustain. The stem cell niche represents a complex system composed of the stem cells themselves, as well as diverse cellular and acellular components that provide inputs to regulate stem cell behavior [1]. Stem cell maintenance, survival, self-renewal and the initiation of differentiation all depend on the intimate relationship between stem cells and their niche. Therefore, local signaling must be tightly controlled to balance stem cell behavior with the demands upon the tissue.

A number of stem cell niches have been characterized in *Drosophila*, including the ovary, testis, and intestine, which have served as paradigms for the identification of stem cell niches in more complex mammalian systems (reviewed in [2]). Within these local microenvironments, multiple signal transduction pathways have been shown to regulate stem cell behavior, as well as the size and activity of the niche. Here we review how signaling via the JAK–STAT pathway in *Drosophila* is utilized in the testis and intestine to regulate stem cell behavior under homeostatic conditions and in response to damage or stress.

The JAK-STAT pathway in *Drosophila*

The Janus kinase (JAK)–Signal transducer and activator of transcription (STAT) pathway is an evolutionary conserved signal transduction pathway which is utilized in an array of important biological processes, such as embryonic patterning, cellular proliferation, sex determination, immunity, and regulation of stem cell behavior [3]. The study of the JAK–STAT pathway in vertebrates is complicated by the fact that it functions downstream of numerous cytokine and growth factor receptors and functional redundancy of multiple JAK and Stat homologs. In *Drosophila*, core components of the pathway include three ligands [Unpaired (Upd, also known as Outstretched/Os), Upd2 and Upd3], a transmembrane receptor, Domeless (Dome); one JAK, Hopscotch (Hop); and one transcription factor, STAT92E (Figure 1; [3]). Molecular and functional data indicate a high level of conservation between the structural components of the insect and mammalian pathways, which is exemplified best by the fact that hyperactivating mutations in mammalian JAKs, which are associated with leukemias and/or myleoproliferative disorders, cause similar blood cell neoplasias in flies [4] and reviewed in [5].

JAK–STAT signaling is initiated in flies when an extracellular ligand, such as Upd, binds to Dome, causing a conformational change that results in phosphorylation and

activation of the associated JAKs. JAK phosphorylation creates a docking site for cytoplasmatic STATs. Once bound to the receptor/JAK complex, STATs become phosphorylated, allowing the formation of STAT dimers, which translocate to the nucleus to activate transcription of downstream targets (Figure 1). Conserved negative regulators of the pathway also exist, such as Suppressors of cytokine signaling (SOCS) and Protein inhibitors of activated Stats (PIAS) [3].

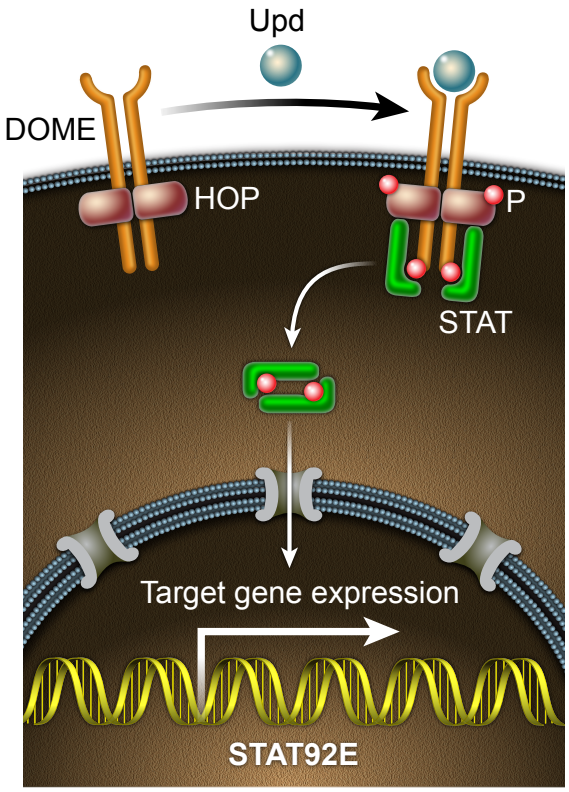


Figure 1. The canonical JAK-STAT pathway in *Drosophila*
Binding of the ligand Upd to the transmembrane receptor Dome leads to cross phosphorylation and activation of the receptor-associated Hop kinase. Activation of Hop leads to phosphorylation of the receptor, providing a docking site for cytoplasmatic STAT. Once bound to the Dome/Hop complex, STAT also becomes phosporylated, resulting in the formation of stable dimers that translocate to the nucleus to activate gene expression.

The *Drosophila* testis

The process of spermatogenesis in *Drosophila* provides an excellent system to study the role of local signaling in the regulation of stem cell behavior, as two populations of stem cells are located within the same niche at the apical tip of the *Drosophila* testis, providing a means to compare and contrast how different stem cells respond to the same local signals [6] and [7]. Germline stem cells (GSCs) arise from primordial germ cells, which form at the posterior end of the developing embryo and

follow a programmed migration to coalesce with the somatic component of the gonad [8], while cyst stem cells (CySCs) are derived from a cluster of somatic gonadal precursors present in the embryonic gonad [9]. In the adult, both stem cell populations surround and are in direct contact with a cluster of approximately 10 post-mitotic somatic cells called the hub (Figure 2). Whereas GSCs sustain spermatogenesis, CySCs produce cyst cells that encapsulate the maturing germ cells and ensure differentiation [[10], [11] and [12]]. In addition, clonal analysis has demonstrated that CySCs have the potential to generate cells that contribute to the hub, which is a critical component of the stem cell niche in the testis [13], [14] and [15]].

Jak-Stat signaling in the testis

Early studies revealed that hub cells specifically produce and secrete Upd, which activates the JAK–STAT pathway in adjacent stem cells to regulate stem cell behavior. Loss of function mutations in *hop* or clonal analysis with null alleles of *STAT92E* resulted in loss of both stem cell populations (GSCs and CySCs), whereas ectopic activation of the pathway led to an expanded number of cells that resemble GSCs and CySCs [13] and [14]. Upd is produced by and secreted from hub cells and can activate JAK–STAT signaling in a non-autonomous manner; however, biochemical studies indicated that the protein is glycosylated and sticks tightly to the extracellular matrix, potentially limiting its diffusion [16] and [17•]. Interestingly, whereas ectopic expression of *upd* in germ cells leads to overproliferation of both GSCs and CySCs, forced expression of *upd* in hub cells does not result in stem cell overproliferation [18], suggesting that hub cells may possess factors that are responsible for modifying Upd in such a way as to limit diffusion. These data also highlight the importance of extracellular matrix as component of stem cell niches.

Therefore, the biochemical properties of the secreted ligand, in combination with restricted expression to a small subset of cells, creates a limited signaling environment localized strategically at the tip of the testis. Accordingly, JAK–STAT activation is apparent only in cells in close proximity to the hub [18] and [19]. Both CySCs and GSCs possess mechanisms to orient mitotic spindles perpendicular to hub cells to facilitate an asymmetric outcome to stem cell divisions: upon stem cell division, one daughter cell remains adjacent to the hub and close to the source of Upd, while the other daughter cell is displaced away from the hub and initiates differentiation (Figure 2) [20] and [21].

Elegant studies subsequently demonstrated that activation of the JAK–STAT pathway in CySCs was sufficient to drive proliferation of both CySCs and GSCs, suggesting the presence of another, secreted signal from somatic cells that regulates the behavior of GSCs [22] and [23••]. Two genes, *Zfh-1* and *chinmo*, were recently identified as important downstream targets of STAT92E in somatic cells [22] and [24]. Clonal analysis revealed that both genes are required for CySC self-renewal, and overexpression of either in somatic cells resulted in expansion of both soma and germ line [22] and [24]. These studies indicate that GSC self-renewal is not solely dependent upon the hub and that factors from neighboring CySCs, likely downstream targets of STAT92E, play a critical role in regulating GSC behavior. In addition, the BMP pathway has been proposed to be involved in this cross-talk; however, it is not yet clear whether the signals are derived from the cyst lineage, hub cells or both [23••] and [25]. Thus, although data indicate that both stem cell populations depend upon the JAK–STAT pathway for maintenance and are competent to respond to Upd, current models suggest that the primary role of the JAK–STAT pathway is to regulate the self-renewal of CySCs directly, which then regulate the proliferation of GSCs in a non-autonomous manner [22] and [23••]. In addition, activation of JAK–STAT signaling in GSCs directly may regulate adhesion to hub cells [23••]. Modulation of the strength of JAK–STAT signaling throughout the niche, accomplished via negative regulators such as Socs36E, also influences the balance of signaling between soma and germ line [26••].

Future studies should determine how Stat function is regulated differentially in somatic and germ line cells, resulting in the activation of distinct targets unique to each cell type. Such specificity may be achieved by the presence of cell type-specific co-factors or epigenetic modifications that restrict access to promoter regions. Furthermore, it will be interesting to identify factors that are involved in regulating the expression and diffusion of Upd from hub cells, as well as the potential role of other Upd family members, which appear to vary in intensity and duration of JAK–STAT activation [17•]. Lastly, future studies should also reveal the mechanisms by which additional signals, such as the BMP/TGF-β pathway, are integrated with JAK–STAT signaling to regulate tissue homeostasis in the testis [10], [23••], [27] and [28].

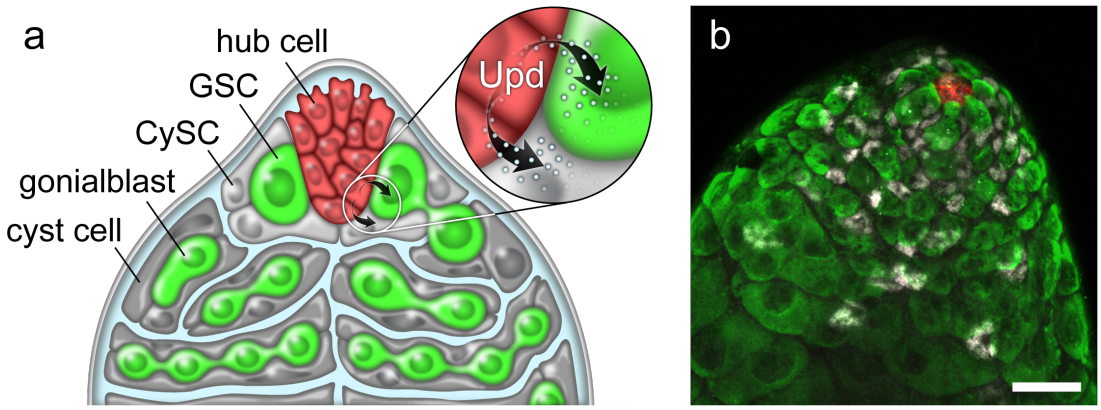


Figure 2. JAK-STAT signaling in the male germ line
(A) Schematic diagram of the apical tip of the testis. Hub cells (red) Germline stem cells (GSC, green) and Cyst stem cells (CySCs, light grey) are in direct contact with hub cells. Inset highlights the hub as a source of Upd.
(B) Immunoflurescence image of the testis apex. Hub cells are marked by expression of the cell surface marker Fasciclin III (FasIII;red). Germ cells are visualized by the germ cell specific marker Vasa (green), and CySCs and cyst cells are apparent based on expression of the transcription factor Traffic-Jam (Tj; white) [48]; Scale bar 20μm

The *Drosophila* intestine

The characterization of intestinal stem cells (ISCs) in the *Drosophila* posterior midgut has provided an additional, versatile model for the study of mechanisms regulating somatic stem cell behavior [29] and [30] reviewed in [31]. ISCs are located basally, immediately adjacent to the basement membrane, in close proximity to visceral muscle (Figure 3). In contrast to the testis, ISCs do not reside within a stromal niche adjacent to support cells such as the apical hub [32]. These multipotent ISCs can divide and give rise to a new ISC (self-renewal) and to a population of progenitor cells called enteroblasts (EBs), and recent studies have revealed both symmetric and asymmetric modes of ISC division in response to growth stimuli [33]. Enteroblasts differentiate into either a secretory enteroendocrine cell (EE) that expresses the transcription factor Prospero (Pros) or an absorptive enterocyte (EC), which undergoes endoreplication to become polyploid (Figure 3). In intestines from young flies, the ratio of enteroendocrine cells to enterocytes is roughly 1:9 [30] and [34].

Signaling in the *Drosophila* midgut

A number of signaling pathways have been implicated in the regulation of ISC behavior and EB differentiation under both homeostatic conditions and in response to aging, bacterial infection, and other environmental changes. For example, ISCs express the Notch ligand Delta (Dl), which activates Notch signaling in the EB

daughter to induce differentiation. Accordingly, loss of Notch signaling in the EB causes a hyperplastic phenotype due to ISC-like cells that fail to differentiate and continue proliferating [[30] and [34]]. Moreover, it has been shown that the level of N pathway activation in EBs determines their fate (EC vs. EE): ISCs with low Dl expression are typically associated with prospective EE cells that express the Pros+, whereas ISCs with abundant Dl expression are associated with Pros^{-}, presumptive ECs [[34] and [35]].

Numerous other factors, in addition to Notch, have been demonstrated to influence the behavior of ISCs and regulate differentiation of progenitor cells. For example, Wingless, which is secreted from the underlying visceral muscle, has been shown to regulate self-renewal of adjacent ISCs [36], [37] and [38], and signaling via the EGF receptor pathway has been implicated in influencing ISC behavior under homeostatic conditions and in response to stress [38], [39], [40] and [41]. In addition to local signaling, systemic signals also contribute to the regulation of ISC behavior and gut homeostasis, as signaling via the *Drosophila* Insulin receptor (*dInR*) has been shown to regulate ISC proliferation and maintenance [33], [42] and [43].

JAK-STAT signaling in the intestine

In addition to the signaling pathways described above, the JAK–STAT pathway also plays an important role in the regulation of ISC proliferation and tissue homeostasis in the intestine. In young, healthy flies, expression of the Upd ligand has been detected in visceral muscle cells, as revealed by staining with antibodies and promoter reporters (*upd-lacZ*) [44]. Accordingly, activation of the JAK–STAT pathway is evident in ISCs and EBs, but not EEs and ECs [44], [45••], [46••], [47] and [48]. These data suggest that Upd diffusion from the muscle could signal to ISCs and, thus, act as a local, secreted signal to influence stem cell behavior, similar to its role in the male germ line.

However, JAK–STAT signaling also plays a prominent role in regulating ISC proliferation in response to damage or bacterial infection in the intestine, in concert with the stress-sensing Jun N-terminal Kinase (JNK) pathway. Jiang *et al*. elegantly demonstrated that ISC proliferation is stimulated as a consequence of signaling by the damaged ECs that line the lumen of the intestine [45••]. In this way, ISC proliferation is perfectly balanced with the need for replacement of terminally differentiated cell types. Interestingly, reporter lines reveal that Upd expression, which can normally be detected in visceral muscle cells, is now observed in enterocytes, and data indicate that damage leads to increased expression of all three *upd* family members (*upd, upd2*, and *upd3*) in the intestine, as measured by

quantitative PCR [[45••], [46••] and [49], with *upd3* being the most highly upregulated of the three [45••] and [49]. Notably, the Hippo (Hpo) pathway, an evolutionarily conserved pathway implicated in organ size control and tumorigenesis, has been shown to induce expression of Upd and EGFR ligands in response to damage, both of which would stimulate ISC proliferation [50•], [51•] and [52•]. This transient increase in secreted ligands that stimulate ISC division serves as a remarkably elegant strategy to replenish injured cells in a timely fashion to mediate tissue homeostasis.

In summary, low level signaling, mediated by secretion from the visceral muscle, contributes to homeostatic turnover of the tissue, whereas upregulation of ligand expression, in response to damage or bacterial infection, leads to rapid tissue regeneration and maintenance of integrity of the intestinal epithelium. This dual role of JAK–STAT signaling *Drosophila* midgut underscores the plasticity and dynamic nature of stem cell niches and provides a paradigm for how normal homeostatic signaling can be modulated to accommodate tissue replacement and repair.

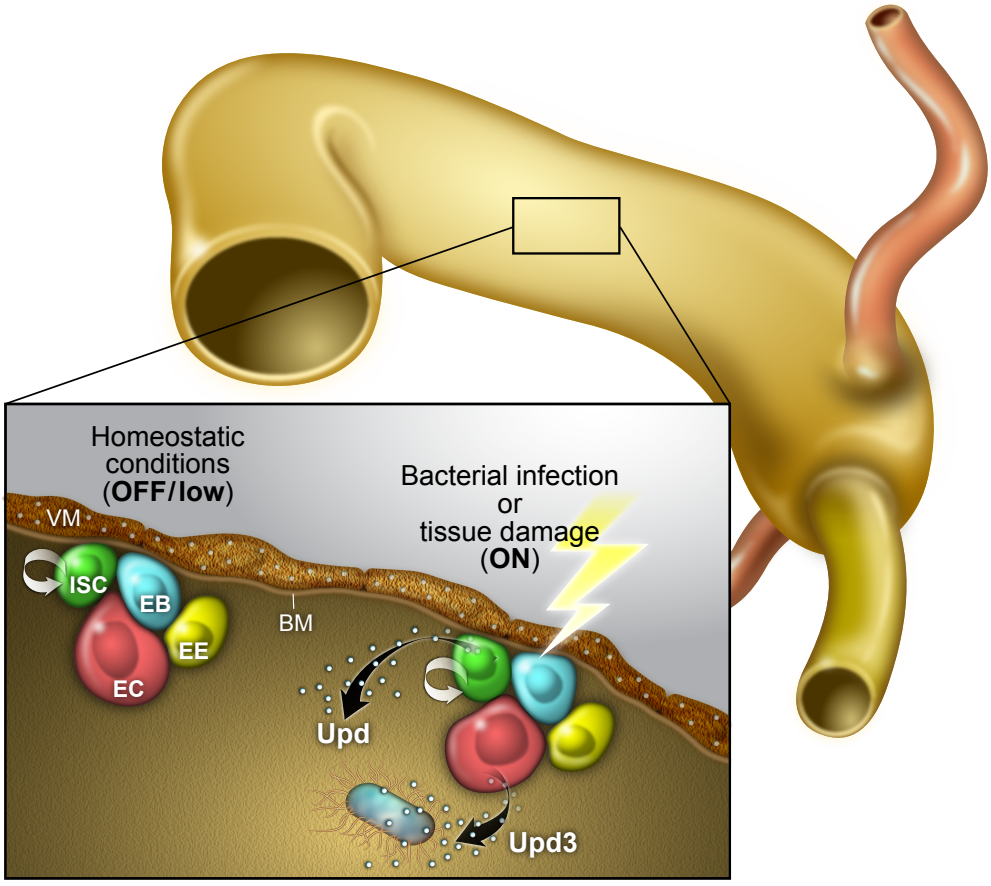


Figure 3. JAK-STAT signaling in the posterior midgut
(A) Schematic illustration of the midgut epithelium: Intestinal stem cells (ISCs, green), enteroblasts (EBs, blue), enteroendocrine cells (EEs, yellow) and enterocytes (ECs, red). Under homeostatic conditions, Upd is expressed in the adjacent visceral musculature and activates JAK-STAT signaling in ISCs and EBs. After tissue injury Upd cytokines are upregulated: Upd becomes expressed in progenitor cells (ISCs and EBs), while Upd3 is secreted by enterocytes.

Conclusions and perspectives

Using Drosophila as model system to study local signaling within stem cell niches has provided unique insights into how the JAK–STAT pathway can be used to regulate stem cell behavior in multiple tissues both under homeostatic conditions and in response to injury or stress. Moreover, these studies have provided fundamental insight into how local signaling pathways are used to coordinate stem cell behavior with the demand for replacement of differentiated cells.

In both the testis and intestine, stem cells rely on production of Upd by support cells within the niche (hub cells in the testis and visceral muscle in the posterior midgut)

for a basal level of pathway activation and a normal homeostatic turnover of the tissue. However, whereas stem cells within the testis appear to be exposed to a single, localized source of Upd, multiple cells within the intestinal niche can signal to ISCs via Upd and Upd3. It will be interesting to learn whether common targets of STAT92E, in addition to Socs36E and Dome, are expressed in GSCs, CySCs, and ISCs or whether cell type-specific strategies are utilized to elicit different transcriptional outputs. Future studies should also focus on elucidating the roles of other Upd family members in the male germ line and comparing and contrasting post-translation modification of the Upd proteins that could affect association with the extracellular matrix and ultimately diffusion throughout the niche.

Decreased signaling or pathway inactivation could result in progressive stem cell loss and tissue degeneration, such as what is observed in the testis of aging males [18]. Conversely, an increase in the intensity and/or duration of pathway activation stimulates an increase in stem cell activation in response to damage or infection, to replace damaged enterocytes. Interestingly, in aged flies, precocious activation of ISC proliferation coupled with a block in terminal differentiation of EBs leads to disruption of epithelial integrity (reviewed in [53]), which closely resembles a chronic inflammatory response. Thus, the mechanism by which pathway activation returns to baseline, once damaged cells have been replaced, is of utmost importance. Similar to what occurs in the testis, upregulation of negative regulators of the pathway, such as SOCS36E, likely suppresses signaling to attenuate the response to injury or infection [26••] and [45••].

As outlined above, the JAK–STAT pathway does not act in isolation to regulate stem cell behavior within the niche. Therefore, one challenge will be to understand how local signals from multiple pathways, sometimes providing antagonistic instructional cues, are perceived by stem cells and integrated with systemic and cell autonomous factors resulting in cell fate decisions. Future studies will continue to elucidate how crosstalk between all of the pathways that regulate stem cell proliferation and maintenance via local signaling ultimately converge to regulate tissue homeostasis and regeneration.

Stem cell niches in invertebrate and vertebrate models share many common features, including architectural organization, cellular and acellular components, and key signaling molecules, such as the JAK–STAT pathway (reviewed in [2]). For example, stem cells in the murine small intestine, the trachea, muscle satellite cells, and long-term hematopoietic stem cells all increase their turnover rate in response to injury. Basic mechanisms that regulate stem cell behavior to accommodate varying

tissue demands, such as those discussed above, provide important insights into common strategies also used by mammalian systems (reviewed in [31]). Therefore, knowledge from studies in *Drosophila* will continue to enhance our understanding of how stem cell deregulation could contribute to disease initiation and progression, and ultimately, result in better therapeutic strategies for regenerative medicine.

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