Genetic ablation of stem cells leads to transdifferentiation of quiescent niche cells and formation of ectopic niches in the *Drosophila* testis

and genetic ablation of somatic stem cells in the *Drosophila* ovary stem cell niche

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1. Abstract

Adult stem cells can self-renew and differentiate to maintain tissues and to regenerate them. These stem cells are thought to be maintained in special microenvironments called “niches” where they receive signals that control stem cell fate.

Two of the most studied niches reside in the *Drosophila melanogaster* testis and ovary where germline stem cells and somatic stem cells reside sustaining gametogenesis. Recent work on both niches showed what happens after germline stem cells are ablated. In the testis, differentiating germ cells start to dedifferentiate and to replace the lost germline stem cells. The same is true for the ovary stem cell niche. But not much was known about what happens when the soma is genetically ablated in these niches.

Former lab members found that induction of apoptosis in the cyst stem cell lineage can successfully ablate the soma. During recovery, the normally quiescent hub cells are capable of transdifferentiation into cyst stem cells to repopulate the niche. However, this process does not always occur without mistakes. We observed ectopic niche formation during the recovery of some of the ablated testes.

The first part of this thesis is aimed to further characterize these ectopic niches by using different conditions of ablation and recovery.

The results show that the number of testes having multiple niches increases with longer recovery time but decreases when ablation is induced at lower temperatures than 31°C. These results are indicative for the hypothesis that higher stress levels lead to formation of ectopic niches.

The second part of the thesis is intended to apply this method to genetically ablate somatic stem cells in the *Drosophila* ovary niche other than the testis. We invented an assay for escort cell ablation in the ovary stem cell niche. The initial results of recovery point towards a process that leads to germ cell tumor formation, loss of synchrony in replication and cycling escort cells. This indicates that the testis and the ovary niche do not use the same mechanism of somatic stem cell repopulation in the niche.

2. Introduction

Stem cells are undifferentiated cells that are able to proliferate, self-renew and produce a large number of differentiated functional progeny. These functions are very important for regeneration of tissues after injury (Loeffler, Roeder; 2002).
In metazoans (multicellular animals) tissue maintenance and regeneration depend on adult stem cells, which respond to the needs of the tissue they reside in (de Cuevas, Matunis; 2011).

2.1 Stem cell types

There are two distinct types of stem cells in mammals: embryonic stem cells and adult stem cells. Embryonic stem cells are derived in vitro from the inner cell mass of blastocysts whereas adult stem cells can be found in various tissues in the body. For example, hematopoietic stem cells can form all the types of blood cells and are found in the spleen; bone marrow stromal stem cells (mesenchymal stem cells) in the bone marrow generate bone, cartilage, fat, blood formation supporting cells and cells that form fibrous connective tissues (Spradling, Drummond-Barbosa, Kai, 2001).
Not only are the occurrences of the stem cell types different, but also their tasks. While embryonic stem cells can differentiate into specific cell types within the embryo, adult stem cells mostly have a repair and maintenance function in the tissue where they reside (Lander, Kimble, Clevers et al. 2012).
This requires the adult stem cell to have the ability of self-renewal – the ability to go through many cell cycles while staying in an undifferentiated state; and of potency – the ability to differentiate into a specialized cell type (Lu et al. 2012).
When an adult stem cell divides, one of the daughter cells can remain a stem cell (also known as self-renewal) while the other daughter differentiates into a specialized cell type (Spradling, Drummond-Barbosa, Kai, 2001). This process is also called “asymmetric division”.

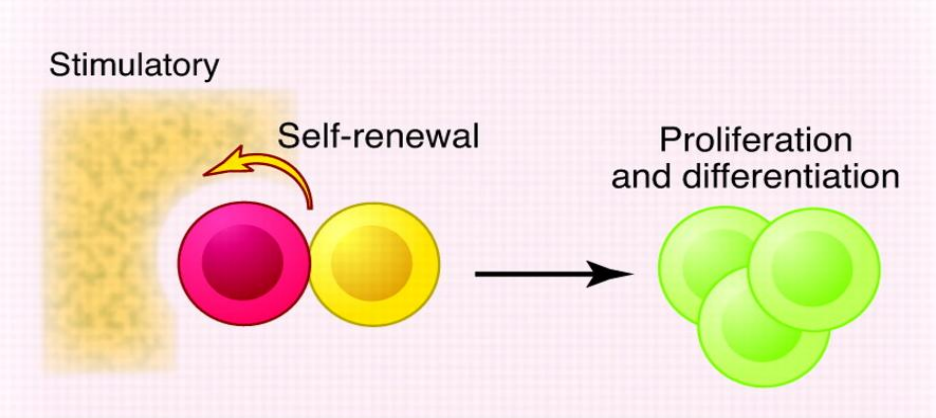


Figure 1: Asymmetric division of adults stem cell;
Li, Clevers Science 2010;327:542-545

2.2 Stem cell niches

Stem cells usually reside in specialized microenvironments, or niches.

Niches are a subset of tissue cells or extracellular substrates that accommodate one or more stem cells types and manage their self-renewal and differentiation *in vivo* (Spradling, Drummond-Barbosa, Kai; 2001). Stem cell niches are found in all creatures, including mammals and intervertebrates. They send out local signals to their surrounding stem cells to maintain the stem cell fate. A lot of work has been done to identify stem cell niches in different organisms using lineage tracing (permanent marking of cells and following their lineages).

This helps to further characterize niches and also helps to investigate stem cells in their natural environment.

The most well-characterized niches in mammals are the hematopoietic stem cell niche in the bone marrow, the germline niches in the reproductive tracts, epithelial niches in the skin and the hair follicles and endodermal niches in the crypts of the guts (Spradling, Drummond-Barbosa, Kai; 2001).

But why do stem cells need these special microenvironments to function properly?

One answer for this question is that stem cells have special requirements for viability. Another answer is that these niches act as feedback controls so that stem cell populations are maintained at a constant size.

This mechanism can be clearly seen when looking at the asymmetric division of germline stem cells in *Drosophila*.

One daughter cell always stays in contact with the niche and in turn continuously receives the maintenance signals from the niche and that it needs to stay a stem cell whereas its sister cell moves away from the niche and loses its contact to the niche.

It starts to differentiate because of a lack of stem cell fate maintenance signals (Tran, Lim, Xie, Chen, 2012).

The third answer to the question is that niches act as coordination instruments between different tissue compartments meaning that they help different tissues to interact as it is seen in the hair follicle stem cell niche. Here, the stem cells and their progenitor cells are competent of maintaining the epidermis, pigmentation, hair and connective adipose tissue by close interaction (Lander, Kimple, Clevers et al.; 2012).

Niches also play a role in cancer. During cancer progression and metastasis, cancer cells enter the blood stream or the lymph circulation system and travel to distant organs where they start to form new tumors. To be able to do so, cancer cells rely on the right microenvironment (niche) to maintain cell activity and fitness and not only on cell-intrinsic events. These metastatic niches evolve within a short period of time, usually in hypoxic regions, invasive fronts or perivascular sides of the tissue (Lander, Kimble, Clevers et al.; 2012).

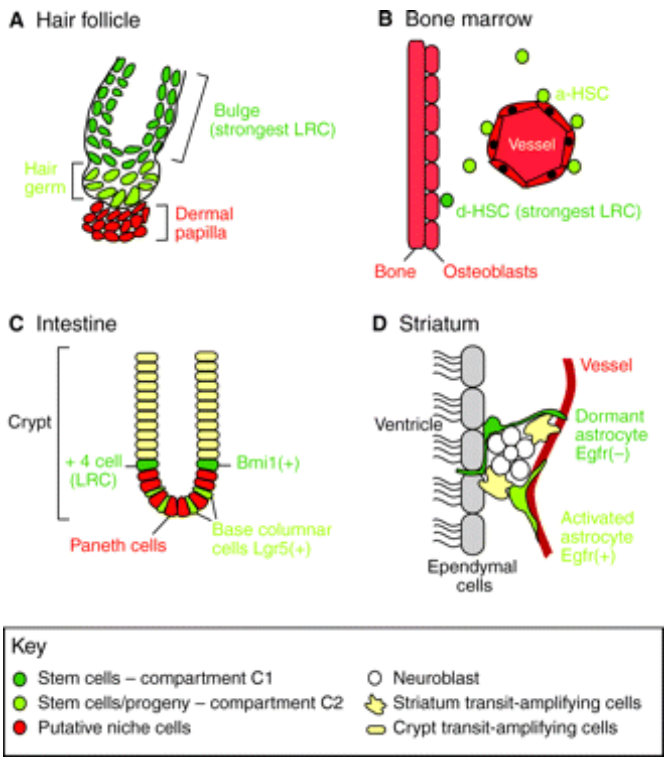


Figure 2: overview of mammalian stem cell niches
(A) hair follicle niche; (B) hematopoietic niche in the bone marrow; (C) intestinal crypt niche; (D) subventricula zone of striatum
Development 2010
http://dev.biologists.org/content/137/10/1586/F2.expansion.htmL

The best characterized and therefore most often used niches to study function and regulation of stem cells are found in the reproductive systems of the model organism *Drosophila melanogaster*. Both male and female flies have stem cell niches that house germline stem cells (GSCs) that give rise to sperm or eggs as well as somatic stem cells that help maintain the GSCs (de Cuevas, Matunis; 2011).

2.3 Stem cell niches in the Drosophila gonads – the male fly

Male adult *Drosophila melanogaster* contain a pair of testes that are each coiled around seminal vesicles. These testes are long tubes and the stem cell niche is located at the apical tip of the testis.

The microenvironment consists of a cluster of ten to twelve quiescent somatic cells called the “hub” that is usually in contact with the basement membrane.

Anchored to the hub are approximately ten germline stem cells. This number varies with the genotypes of the flies (Sheng et al. 2009; de Cuevas, Matunis; 2011; DiNardo et al. 2011).

Each of these GSCs is surrounded by about 2 somatic stem cells called cyst stem cells (CySCs) that contact the hub with thin cytoplasmic extensions (Hardy et al., 1979; Sheng et al., 2009; Cheng, 2011).

In the niche, GSCs divide asymmetrically to generate one daughter cell that remains a stem cell and another daughter cell that differentiates into first a gonialblast, then spermatogonia, spermatocytes and finally mature sperm. The sperm are then released into the seminal vesicle where they are stored until needed. CySCs divide asymmetrically as well to give rise to one stem cell and one further differentiated cyst cell. Cyst cells, unlike gonialblasts, do not divide anymore but grow to enclose the differentiating germ cells.

The *Drosophila* testis therefore contains all of the different stages of sperm development, beginning from GSCs and ending with mature sperms (Tran et al. 2012).Spermatogenesis is a well known and studied process that is characterized by a continuous production of highly differentiated sperms throughout the reproductive life from only a small number of GSCs. These stem cells are unipotent, meaning that they can only give rise to one specific cell type (Sheng, Brawley, Matunis, 2009; Kitadate 2007).

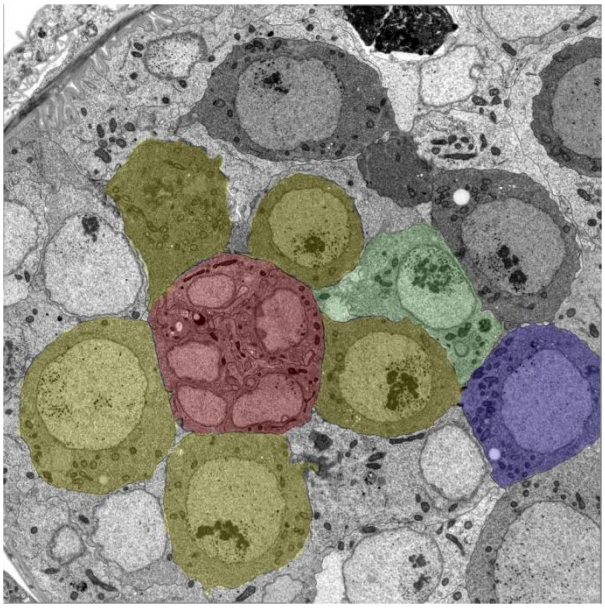


Figure 3: EM image of Drosophila testis niche
Cells are pseudocolored, red – hub cells, yellow – GSCs, green – CySC and blue – differentiating GSC daughter
Matunis laboratory

2.4 Stem cell niches in the Drosophila gonads – the female fly

Female *Drosophila* have two ovaries that are composed of 16 to 21 tube-like structures, also called ovarioles. The ovarioles consist of six to eight egg chambers that differ in their development stage. The stem cell niche is located at the tip of the ovarioles called the germarium (Bolivar, Pearson et al. 2006).

The ovary stem cell niche also consists of a cluster of somatic quiescent cells called cap cells (CCs). Five to seven of these CCs are attached to eight to ten terminal filament cells (TFC) that are also non-mitotic (Eliazer et al., 2011).

Two to three GSCs are located adjacent to the niche and have direct contact to it.

As in the testis, GSCs also undergo asymmetric divisions producing one daughter cell that differentiates and one that remains a stem cell. The differentiating daughter cell first undergoes incomplete divisions to form a cyst and later functional oocytes.

The germarium also contains somatic cells that inhabit the stem cell niche. Escort cells (ECs), also called inner germarium sheath cells, line the anterior region of the germarium and have long cytoplasmic extensions to wrap around cyst cells (Eliazer et al., 2011; Morris and Spradling 2011).

There are about 25 ECs in the germarium, their number decreases slightly with age. Escort stem cells are non-mitotic cells (quiescent cells) that usually do not undergo apoptosis or necrosis (Pritchett, Tanner, McCall, 2009). New evidence from in vitro studies suggests that these cells do not cycle even though they are capable of division and stay attached to the basement membrane. They are thought to divide only when the ratio of ECs to GCs or cysts increases (usually a ~2:1 ratio). They have smaller nuclei than GCs, are flat and curved in shape. Usually, ECs stay in the anterior germarium and pass on cysts from one cell to the next by using dynamic membrane activity (Morris and Spradling 2011). Also somatic epithelial follicle cells (FCs) can be found in the posterior region of the *Drosophila* germarium (Bolivar et al., 2006; Margolis, Spradling, 1995).

Oogenesis is the female counterpart to spermatogenesis and is also a well studied field in stem cell research. As it is the case in the testes, the ovary continuously produces oocytes from a small number of germline stem cells.

The same asymmetric division takes place in the ovary where one daughter cell stays in the niche and maintains the germline stem cell population whereas the other daughter cell differentiates to become an oocyte. Also here the niche and its cells regulate the divisions by providing essential signals to the germline stem cells (Morris, Spradling, 2011).

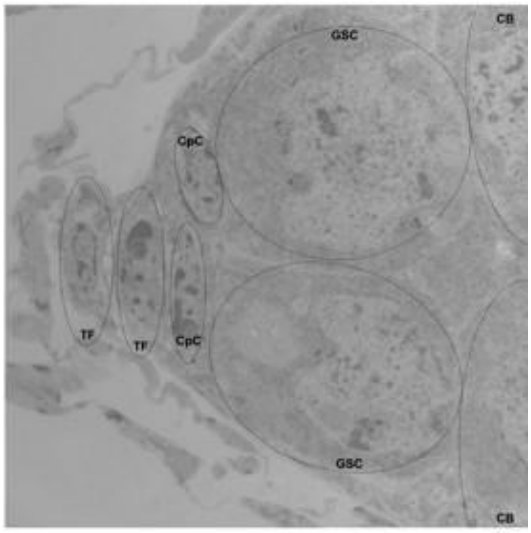


Figure 4: EM image of Drosophila ovary niche
TFCs (terminal filament cells) and CCs (cap cells) form the niche; 2 GSCs (germline stem cells) and differentiated daughters (CB – cystoblast)
Dr. Gerd Hause, Biocenter, Martin Luther Univ. Halle-Wittenberg, http://www.spp1356.de/project-area-a/reuter/

2.5 Niches as the source of signaling – the male niche

The hub in the *Drosophila* testis is the source of the self-renewal ligand Unpaired (Upd) that activates the Janus Kinase/Signal transducer and activator of transcription pathway (Jak/STAT pathway) in surrounding cells (GSCs and CySCs) by binding to its receptor. Upon this binding, STAT is phosphorylated and translocated into the nucleus where it regulates the transcription of its responsive genes (de Cuevas and Matunis, 2011; Hombria et al., 2002).

The Upd ligand only has a very short acting range, so only cells that are in close proximity to the hub receive the maintenance signal. Daughter cells that are displaced further away from the niche are out of the signal's range and start to differentiate (Leatherman et al., 2008; Yamashita et al., 2003). Another task of the Jak/STAT pathway is to regulate adhesion of the GSCs and CySCs to the hub (Leatherman and DiNardo, 2008).

Another pathway that regulates self-renewal in the *Drosophila* testis stem cell niche is the Bone morphogenetic protein signaling pathway (BMP pathway).

When BMP binds to its receptor, Mothers against dpp (Mad) is phosphorylated and translocates into the nucleus, where it affects transcription of its target genes (Affolter and Basler, 2007). The two most important BMP ligands that are expressed by the hub are Glass bottom boat (Gbb) and Decapentaplegic (Dpp). These have an effect on bag of marbles (bam) that, when up-regulated, leads to differentiation of GSCs and CySCs (Song et al., 2004; Okegebe et al. 2011). Another important ligand that is secreted by the hub cells is Hedgehog (Hh) that is required for the cell-autonomous regulation of the CySC self-renewal and maintenance (Amoyel et al. 2012; Michel et al. 2012; Hatini et al. 2005).

2.6 Niches as the source of signaling – the female niche

Self-renewal signals are expressed by CCs and TFCs, most important are Hedgehog (Hh) and Wingless (Wg) that control somatic stem cell self renewal.

The attachment of GSCs to their niche is regulated via cadherin-mediated adhesion that is controlled by the Jak-STAT pathway and BMP signals. These signals are also short range signals that affect only cells in close proximity (Kirilly and Xie, 2007; Michel et al. 2012).

GCs that are wrapped by ECs communicate using Notch signals, EGFR ligands but also Jak-STAT and BMP signals are involved so that the ratio between these cell populations can be maintained (Morris and Spradling, 2011).

2.7 The *UAS-Gal4* system

To study gene function in Drosophila, ectopic expression of genes is often used. The altered gene expression either leads to an induced cell fate change, changes of fates in neighbouring cells or it alters the physiology of the cell. By using ectopic expression of genes it can be found out if a certain gene is sufficient for cell identity and if its mode of action is autonomous or not.

The technique is also used to find out whether certain genes play a role in signalling pathways or the response of a given cell or tissue. The most important system for ectopic gene expression in Drosophila is the *UAS-Gal4* system.

It uses two components; first the transcriptional activator *Gal4*, isolated from yeast, and second a transgene under the control of the *UAS* (upstream activation sequence) that gets bound by *Gal4*.

To bring the two components together, a genetic cross is needed. In the progeny, the transgene is then only transcribed in the cells or tissues that express the Gal4 protein.

To temporary control the *Gal4* system, another yeast protein (*Gal80*) that binds to the trans-activation domain of the Gal4 protein to prevent it from activating transcription is used. To be more flexible in the expression of trans-genes, a temperature sensitive $Gal80^{ts}$ protein was genetically engineered. Here the *Gal4* is alleviated by shifting the flies to a higher temperature (Elliott and Brand).

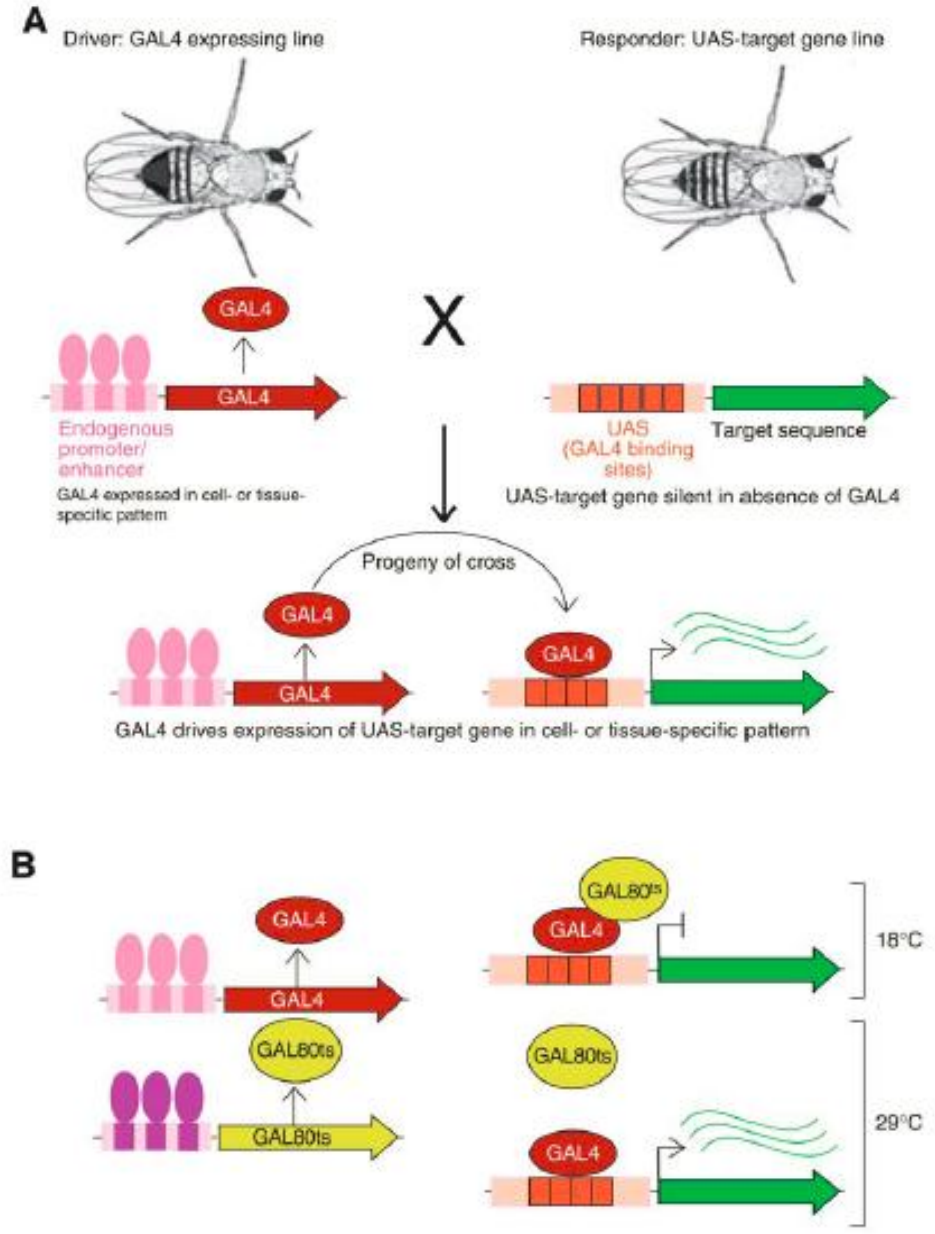


Figure 5: UAS-Gal4 system in Drosophila melanogaster. (A) Scheme of the UAS-Gal4 system. (B) Gal4 activity can be antagonized by GAL80 or by the use of a temperature sensitive GAL80 (GAL80ts) that allows greater temporal control over onset of gene expression. (Elliott and Brand)

2.8 Apoptosis

Apoptosis is a mechanism of “self-destruction” in mammalian cells that is often referred to as programmed cell death. If the regulation of apoptotic pathways does not work properly, tissues are not maintained in an appropriate way and cancers can occur.

To ensure proper function, caspases are needed. There are different classes of caspases according to the length of their prodomains. Executioner caspases have short domains whereas initiator caspases have long domains.

These domains contain protein-protein interaction motifs to be able to bind to adaptor molecules. Different stimuli like DNA-damage, infections, low levels of oxygen (hypoxia) as well as presence of reactive oxygen species lead to the activation of caspases through death inducer genes.

Once activated, caspases form the apoptosome with the help of cytochromeC and Apaf-1 (adaptor protein) and the cell dies. This pathway is further regulated by IAPs (inhibitors of apoptosis proteins) that bind to caspases and inhibit their function.

Recent work in the model organisms *Drosophila melanogaster*, *Xenopus* and *Hydra* showed that apoptotic caspases play a crucial role in the production of mitogenic signals to stimulate proliferation of stem cells and their progenitors and to aid in tissue regeneration (Bergmann and Steller, 2010).

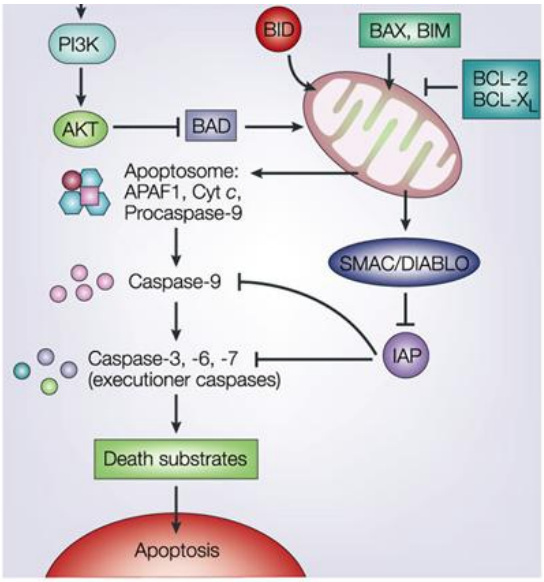


Figure 6: Apoptotic pathway; stress signals can initiate apoptosis. Pro-apoptotic BCL2 family proteins (BAX, BID, BAD, BIM) are important mediators of these signals. Activation of mitochondria leads to the release of cytochrome c (Cyt c) into the cytosol, where it binds apoptotic protease activating factor 1 (APAF1) to form the apoptosome that activates initiator caspase-9. Apoptosis can be inhibited by inhibitors of apoptosis proteins (IAPs), which are regulated by SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI). http://www.nature.com/nrc/journal/v2/n4/fig_tab/nrc776_F4.html; Frederik H. Igney & Peter H. Krammer, 2002

2.9 Ectopic niches in the Drosophila melanogaster testes

Former work showed that if GSCs get lost through damage or aging, they can be replaced by spermatogonia (GSC progeny in later stages of their development) through dedifferentiation (Brawley, 2004; Chen, 2008; Sheng, 2009; Kai et al. 2004). However, not a lot of work has been done to understand if CySCs get replaced when they are lost through damage.

To investigate this question, *c587-GAL4* (Kai and Spradling, 2003; Manseau et al., 1997), a CySC lineage specific *GAL4* driver was used to express a pro-apoptotic gene *Grim* within the CySCs and early cyst cells under specified conditions.

The flies were raised at the permissive temperature of 18°C when *GAL4* activity is being inhibited. At this temperature, no difference in the niche can be found when compared to wild type flies. But when the flies get shifted to the restrictive temperature of 31°C for two days when *GAL4* is fully active, the apoptotic gene *Grim* is expressed in the CySC lineage and induces apoptosis in these cells (Hétié et al. submitted).

Immediately after CySC ablation, all the CySCs are gone. Most of the GSCs are also lost due to loss of support from CySCs. Only hub cells that do not express the *c587-GAL4* driver remain for some time in the testes (Hétié et al, submitted; Lim and Fuller, 2012).

When shifting the flies back to the permissive temperature of 18°C and letting them recover for two weeks, most testes were able to regain functional CySCs and early cyst cells and looked very similar to wild type testes.

After ablation, hub cells and later cyst cells (confirmed by antibody staining) are the only somatic population left in the testes, these two populations of cells can be the source of the new CySCs. Both cell types are non-mitotic in the wild-type conditions, meaning they do not normally actively divide. However, when we did EdU experiments on the ablated testes during recovery we found that a small amount of testes show that some hub cells are positively marked with EdU, meaning that these hub cells indeed re-enter the cell cycle and start to divide. No EdU positive late cyst cells were found, so the hub cells are considered as the primary source for the repopulation of testes after CySC ablation.

Another important finding was that after two weeks of recovery, about 50% of testes that had regained CySCs had a hub at the apex and additional ectopic hubs located nearby. These ectopic niches seem to be completely functional and are surrounded by their normal populations of stem cells.
It is not clear where these ectopic niches come from but they might arise from transdifferentiating hub cells that are not able to reprogram completely into CySCs and therefore go back to hub cell fate to build new niches by recruiting GSCs and CySCs (Hétié et al. submitted).

2.10 Germline cell ablation in the Drosophila ovary stem cell niche

In 2003 work was published that showed what happens to the female stem cell niche when its germline stem cells are genetically ablated. Under these conditions, cap cells stay active for several weeks and escort cells enter the empty niche but fail to divide. Consequently, follicle cell progenitors and somatic stem cells (escort stem cells and follicle stem cells) enter the niche. Follicle stem cells but not escort stem cells start to proliferate due to the signals sent out by the remaining cap cells.
These results show that the female *Drosophila* niche is a small but stable structure that is a critical factor for the extent of proliferation of the cells (Kai and Spradling, 2003). It is also published that germline stem cells need escort cells to be able to proliferate. Escort cells interact physically with GSCs and send out signals. However it is still unclear whether the differentiation status of GSCs has an effect on escort cells and how these interactions between the cell populations is regulated (Kirilly et al.2011).

3. Materials and Methods

Experimental procedures

3.1 Fly stocks and culture

The flies were raised on standard yeast and molasses medium at 25°C. To build the crosses the following stocks were used:

- *c587-Gal4; +* (Zhu and Xie, 2003; gift from A. Spradling)
- *tub-Gal80ts* (Bloomington #7018)
- *+/Y; UASGrim/Sm6B* (Chen et al., 1996; gift from J. Abrams)
- *Hh-lacZ/Tm6B* (Forbes, 1996)
- *FasIII-Mi-GFP/Sm6B* (Bloomington #30688)
- *UAS-GFP::nls* (Bruce Edgar)

To get the correct genotype needed for the following experiments, crosses where set up using the stocks mentioned above and raised at the permissive temperature of 18°C:

- *c587-Gal4; **FasIII-Mi-GFP**/Sm6B* (W/W) *X* *UASGrim; Gal80ts* (W/Y)
- *c587-Gal4; +; Gal80ts* (W/W) *X* *+/Y; UASGrim/Sm6B; **Hh-lacZ**/Tm6B* (W/Y)
- *c587-Gal4; Gal80ts* (W/W) *X* ***UAS-GFP*** (W/Y)

To induce gene expression that is under the control of the UAS Gal4 system, the flies were shifted to higher temperatures for the experiments.

3.2 X-Gal staining

Testes and ovaries are fixed for 15 minutes using a fixative containing 1% Glutaraldehyde and 50mM Na-Cacodylate. After rinsing the samples with 1X staining buffer these are incubated in the 1X staining buffer for 30 minutes at room temperature.

Then the staining buffer containing 1X staining buffer, 50mM K-ferri, 50mM K-ferro and 20% X-Gal is added and incubated at in a 37°C water bath for several hours.

To make sure that the solution works, the samples are examined for the presence of a blue precipitate after 15 minutes, 1 hour and 2 hours. The reaction is stopped by rinsing the testes using PBS-1mM EDTA, mounted in the glycerol based mount and samples are stored at 4°C.

The samples are observed under the phase contrast microscope or a bright field microscope. (Dr. Erika Matunis laboratory, Johns Hopkins Medical School, Baltimore, Maryland, USA).

3.3 Immunostaining

Flies are dissected in 1XRingers solution and then fixed for 30 minutes at room temperature with fixative (one part Formaldehyde and three parts of 1X PBX (1XPBS with 0.1%Trition-100)). After fixation, the testes or ovaries are washed two times briefly and two times ten minutes in 1XPBX.

The washing step is followed by one of the most important steps – the blocking step to prevent unspecific binding of the primary antibodies. The block is performed over night at 4°C using 2% normal goat serum (NGS) in PBX-3%BSA-0.02% Sodium Azide.

After blocking, the sample gets incubated at 4°C over night in primary antibody diluted in PBX-3%BSA-0.02% Sodium Azide to its final concentration.

Again a washing step is performed using PBX, two times briefly and two times ten minutes. Secondary antibodies are incubated either for one hour at room temperature or at 4°C over night. The sample stays in the dark (covered with aluminum foil) after addition of secondary antibody. The sample has to be washed again using PBX twice (briefly) and two times ten minutes and then ten minutes using PBS.

The stained testes are stored in Vectashield at -20°C or mounted onto a glass slide. (Dr. Erika Matunis laboratory, Johns Hopkins Medical School, Baltimore, Maryland, USA).

The primary antibodies used to perform the stains where: rabbit anti Vasa (#103, NYU Medical Center) dilution 1:200; mouse anti β-galactosidase (catalog # Z3781, System Lot # 217305, Promega) dilution 1:500; rabbit anti GFP (catalog # TP401, Lot # 071519, Torre Pines Biolabs Inc.) dilution 1:1000; rabbit anti β-galactosidase (Abcam Limited, 31 Cambridge Science Park, Cambridge, CB40FX UK) dilution 1:500; mouse anti Armadillo (Eric Wieschaus, Department of Molecular Biology, Princeton University) dilution 1:50; mouse anti 1B1 (Howard D. Lipshitz, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, CANADA) dilution 1:50;

mouse anti laminC (Fisher P.A., School of Medicine, Dept. of Pharmacological Sciences, State University of New York at Stony Brook, NY 11794-8651) dilution 1:20 and rabbit anti PH3 (upstate cell signaling solutions, Lake Placid, NY 12946) dilution 1:200

Secondary antibodies used included: goat anti mouse (invitrogen, Molecular probes Inc.) 568nm (Alexa Fluor 568 goat anti mouse IgG (H+L), catalog # A11004, Lot# 43497A) and 488nm (Alexa Fluor 488 goat anti mouse IgG (H+L), catalog # A11001, Lot# 1110070); goat anti rabbit (invitrogen, molecular probes Inc.) 568nm (Alexa Fluor goat anti rabbit IgG (H+L), catalog # A11011, Lot# 1126593) and 488nm (Alexa Fluor goat anti rabbit IgG (H+L), catalog # A11008, Lot# 913909) as well as DAPI (4',6-diamidino-2phenylindole, dihydrochloride; Nucleic Acid Stain, D-1306, Molecular probes Inc.).

3.4 EdU labeling (thymidine analog ethynyl deoxyuridine)

The Click-it EdU imaging Kit (Invitrogen) is used to identify cells in S-phase of the cell cycle. Flies are fed 6 to 24 hours with a mixture of 85µL apple juice, 10µL EdU and 5µL green food dye that is pipetted onto a piece of filter paper. The flies are kept at the permissive temperature during feeding process. After feeding, the flies are dissected in 1X Ringers solution and fixed the same way as with immunostaining. The samples are washed two times ten minutes using 1XPBX and then incubated for 30 minutes at room temperature in the dark in a mix of 2.5µL EdU buffer additive, 212.5µL distilled water, 25µL 1XEdU reaction buffer and 0.625µL Alexa Fluor Azide. After incubation in the reaction cocktail the samples are washed three times five minutes, then blocked and stained as usual (see immunostaining; Dr. Erika Matunis laboratory, Johns Hopkins Medical School, Baltimore, Maryland, USA).

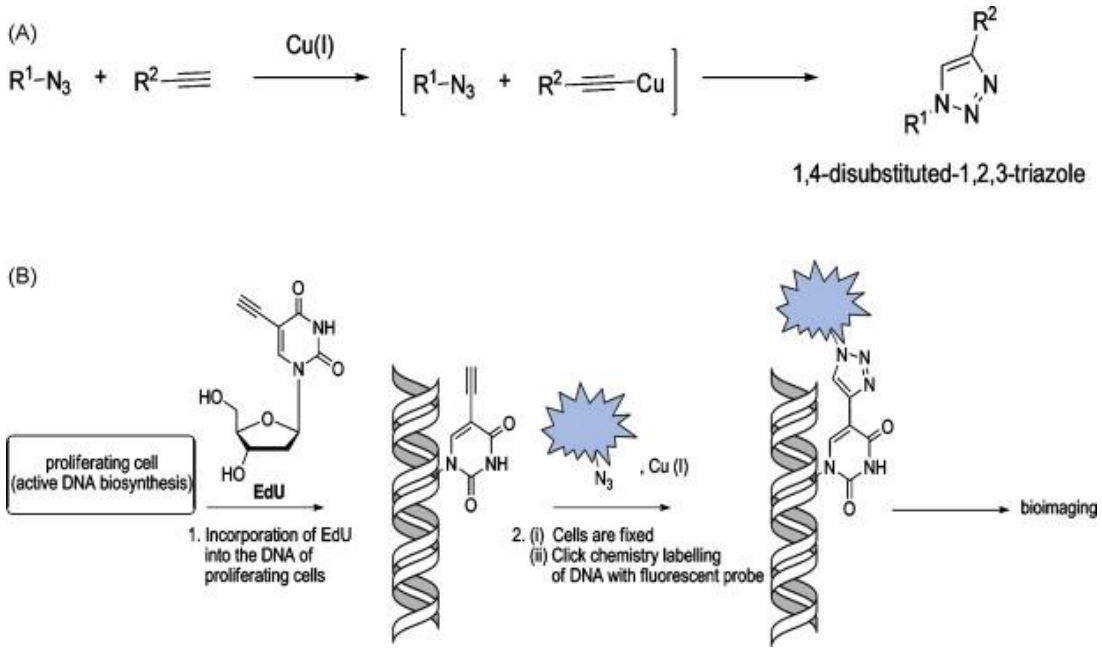


Figure 7: Click" chemistry. (A) The Cu(I) catalysed 1,3-dipolar cycloaddition reaction of organic azides (R1-N3) with terminal acetylenes (R2-). This "click" chemistry reaction affords exclusively the 1,4-disubstituted 1,2,3-triazole regioisomer. (B) Click-iT™ assay by Invitrogen™.

4. Results

4.1 Determination of number of testes having multiple niches under previously established conditions

To be able to start with the testes project, I tested how often I see ectopic hubs. It was previously demonstrated that following a two day ablation at 31°C and subsequent two week recovery at 18°C, 50% of testes acquire multiple hubs.

To repeat this experiment, I dissected, fixed and immunostained *FasIII-Mi-GFP* and *Hh-lacZ* flies (2-3 days old) before ablation, after ablation and after recovery. The testes before ablation all looked wild type with only one hub, surrounded by ~10 GSCs that are flanked by CySCs.

After ablation for two days, 70.5% (n=12/17) testes are fully ablated, the others are only partially ablated or not ablated at all.

After recovery, 26.7% (n=27/101) of all testes (*FasIII-Mi-GFP* and *Hh-lacZ*) have multiple hubs. 67/102 testes only had one hub where 11/101 had a GFP signal that did not co localize with DAPI stain so it might be only background signal (Panel 1, B-G). This difference (26.7% compared to previous 50%) might be due to variations in the fly batches as we used different starting stocks to build the cross.

I also looked at the percentages of testes with multiple hubs within each genotype. When looking only at *Fas-III-Mi-GFP* testes, 25/97 (15.5%) acquire multiple hubs after 2 weeks of recovery. *Hh-lacZ* testes show in 50% of the testes multiple hubs after two weeks of recovery. This number cannot be used for further conclusions as the “n” was very low (n=2/4).

I also did the experiment with 13 day old flies. Here 68.4% of testes (n=7/19) developed ectopic niches. This is an increase of 41.7%. Unfortunately only 19 testes were observed, therefore this experiment has to be repeated to increase the “n” and to be able to draw any conclusions. Hub tumors (big sheaths of cells that are positive for hub specific markers, Panel 1, G) are seen in both genotypes (*FasIII-Mi-GFP* and *Hh-lacZ*). They occur at about the same rate when comparing two weeks recovery to three and four weeks of recovery but the number of testes having tumors increases about two-fold when looking at testes from males that were 13 days old when the experiment started.

This relation to age (increase in testes having multiple hubs and tumors in older males) needs to be further investigated to increase the number of testes that were looked at. Other ages of flies also need to be considered.

I further investigated ectopic hub formation phenomenon using different methods and different conditions and compared the results to the ones obtained after two weeks recovery (26.7%).

4.2 X-Gal staining can also be used to investigate multiple hubs but is not as reliable

Knowing the observed frequency of the appearance of ectopic hubs in ablated and recovered testes, I wanted to invent an assay that is faster than immunostaining to determine the number of hubs. I chose to do X-Gal staining, a chemical staining method that is not only faster than immunostaining but also cheaper. This method detects the expression of *Hh-lacZ*, which is normally restricted to the hub and therefore is a good marker to find newly formed niches.

Again I looked at testes before CySC ablation, after CySC ablation and after recovery from ablation and compared X-Gal results to immunostaining results.

Before ablation, I could only see a single hub each of the testis. After ablation, all the CySCs were gone.

After two weeks recovery, 18.8% (n=9/48) of testes are likely to have ectopic hubs. 31.3% (n=15/48) of testes have multiple blue dots that might indicate ectopic niches but also might only be dying cells that start to express the β-gal protein. The rest of the testes (50%) show only one blue dot, indicating the original hub (Panel 1, H-K).

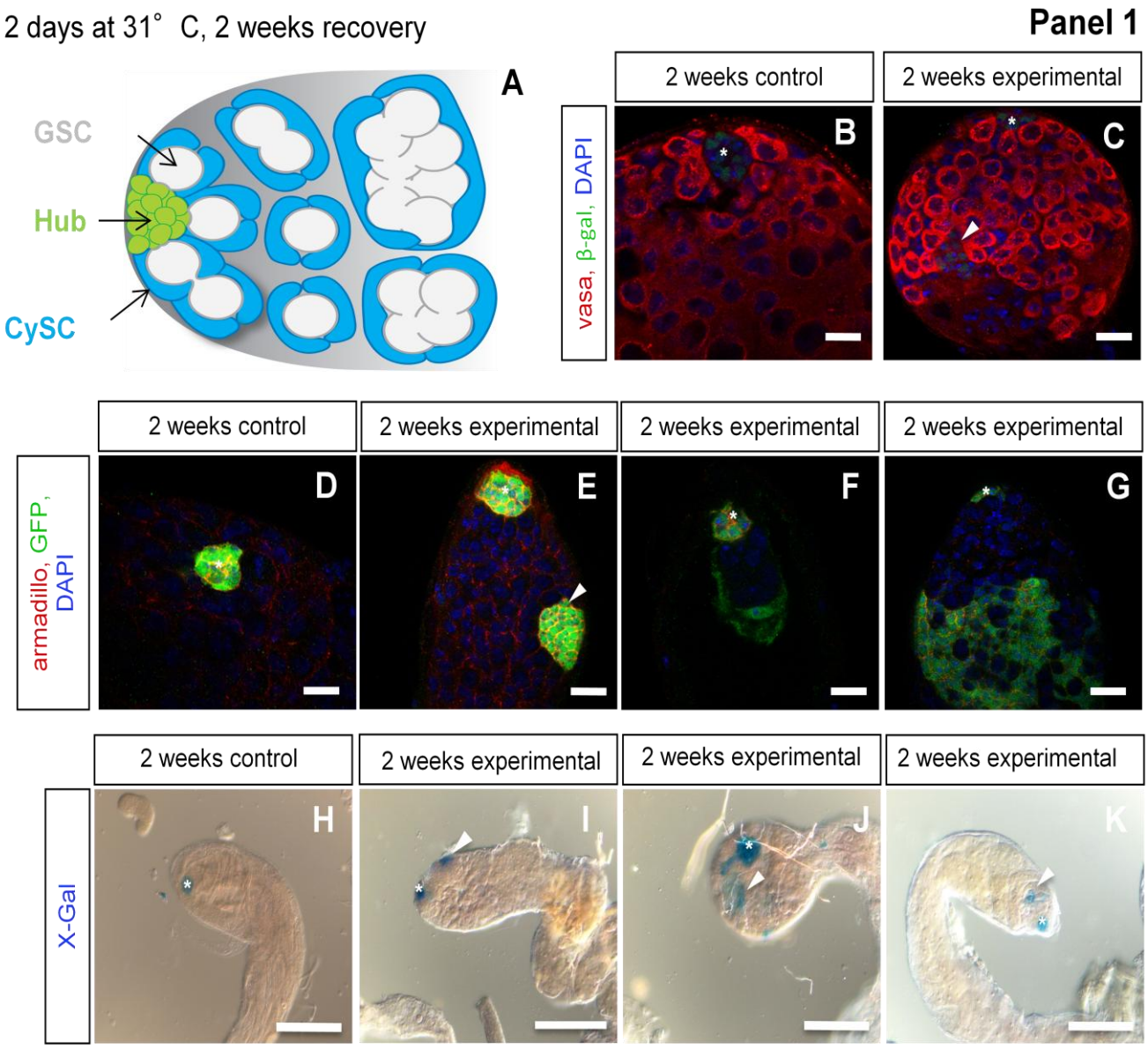


Figure 8: Multiple hubs can be detected using X-Gal staining but the method is not very specific
asterisks indicate potential original hubs, arrowheads show ectopic niches (or background signal),
(A) cartoon of the testis stem cell niche showing the hub in green, germline (stem) cells in white and cyst (stem) cells in blue; (B) hh-lacZ control, 2 days of ablation and 2 weeks of recovery; (C) hh-lacZ experimental testis showing 2 niches (original niche asterisk, ectopic niche arrowhead) after 2 days of ablation and 2 weeks of recovery; (D) FasIII-Mi-GFP control (same conditions as in B and C); (E) FasIII-Mi-GFP experimental showing two hubs (asterisk showing original niche, ectopic niche shown by arrowhead); (F) FasIII-Mi-GFP experimental testis having 1 hub and some GSCs left but also showing a GFP signal spreading out (does not co localized with DAPI stain); (G) FasIII-Mi-GFP experimental showing one original hub (asterisk) and a “hub tumor” (arrowhead); (H)-(K) X-Gal stains of testes after 2 days of ablation and 2 weeks of recovery; (H) hh-lacZ control having only 1 hub; (I) hh-lacZ experimental testis having 2 dark blue dots indicating hubs (asterisk original hub, arrowhead shows ectopic hub); (J) hh-lacZ experimental testis showing multiple blue dots, asterisk shows original hub that spreads out, arrowhead indicates light blue dots that might only be dying cells but not an ectopic niche; (K) hh-lacZ experimental testis showing the original hub (darker blue dot, asterisk) and a light blue dot that might be an ectopic niche but might also be only a bunch of dying cells

Comparing to immunostaining, I found X-Gal is less reliable than immunostaining. Thus I decided to perform my subsequent experiments using immunostaining. Moreover, both *Hh-lacZ* and *FasIII-Mi-GFP* can be detected via immunostaining. Therefore both types of testes can be used to analyze the ectopic niches formation.

4.3 The number of testes with ectopic niches increases slightly after longer recovery

The first question I tried to answer was if the number of testes having multiple hubs increases with longer recovery time.

To answer this question, I ablated CySCs in *Hh-lacZ* and *FasIII-Mi-GFP* flies and examined testes after three and four weeks of recovery.

I already knew that after two weeks of recovery I can expect to see 26.7% of testes have multiple hubs. This number was used to compare the following results.

After three weeks of recovery, 28% of all testes *(FasIII-Mi-GFP* and *Hh-lacZ*, n=14/50) have multiple hubs, an increase of 1.3% when compared to two weeks of recovery (Panel 2, A-H).

When the two genotypes are separated, 30.8% of *Fas-III-Mi-GFP* flies have multiple hubs (4/13) and 27% of *Hh-lacZ* flies show the phenotype (10/37).

After four weeks of recovery, 35.1% of testes (n=13/37) have multiple hubs. This is an increase of 8.4% when compared to two weeks of recovery and an increase of 7.1% when compared to three weeks of recovery (Panel 2, I-O).

Again I looked at both genotypes separately, where *Fas-III-Mi-GFP* flies have ectopic niches in 66.7% (n=4/6) and *Hh-lacZ* flies in 29% (n=9/31).

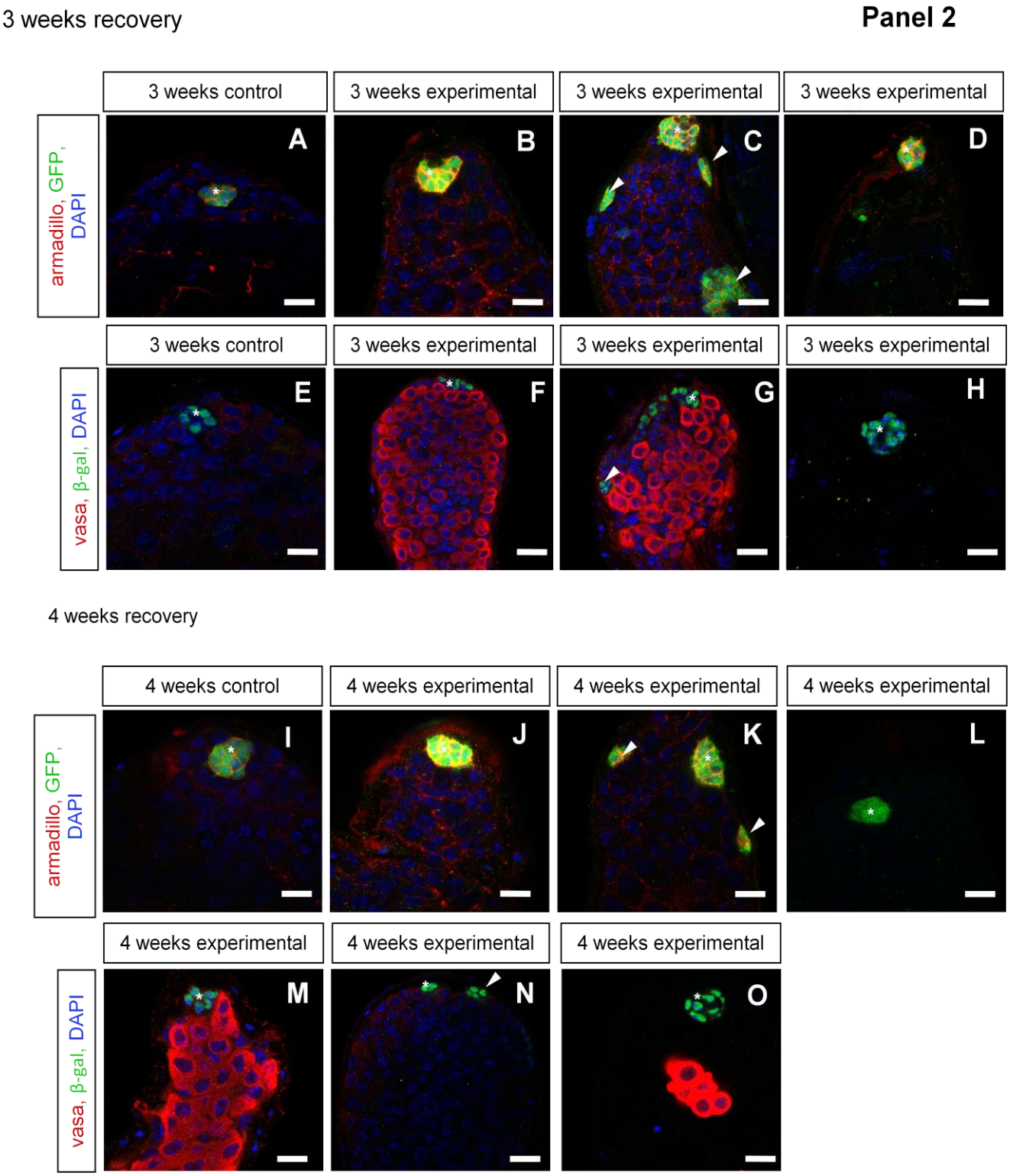


Figure 9: The number of testes with ectopic niches increases over time

(A)-(H) testes after 2 days at 31°C and 3 weeks of recovery at 18°C; (A) FasIII-Mi-GFP control; (B) FasIII-Mi-GFP experimental testis, completely recovered with only 1 hub; (C) FasIII-Mi-GFP experimental testis having 1 original hub (asterisk) and 3 ectopic niches (arrowheads); (D) FasIII-Mi-GFP experimental testis that did not recover. Only one hub and some GSCs are left; (E) hh-lacZ control; (F) hh-lacZ experimental testis that fully recovered having only one hub; (G) hh-lacZ experimental testis having one original hub (asterisk) and one ectopic niche (arrowhead), the original hub is stretched that might indicate that it is going to form a new niche; (H) hh-lacZ experimental testis not recovered (completely empty, just the hub remains);

(I)-(O) testes after 2 days at 31°C and 4 weeks of recovery at 18°C; (I) FasIII-Mi-GFP control; (J) FasIII-Mi-GFP experimental, completely recovered with only 1 hub; (K) FasIII-Mi-GFP experimental testis having 1 original hub (asterisk) and 2 ectopic niches (arrowheads); (L) FasIII-Mi-GFP experimental testis that did not recover, only one hub left; (M) hh-lacZ experimental testis that fully recovered having only one hub; (N) hh-lacZ experimental testis having one original hub (asterisk) and one ectopic niche (arrowhead); (O) hh-lacZ experimental testis not recovered, only some germ cells and the hub are left in the testis

4.4 Ablation is possible using lower temperatures than 31°C

To find out whether these ectopic niches only occur after ablation at 31°C, I tried to ablate testes by inducing *UAS-Grim* expression at 29°C and 25°C.

As we didn't know if total ablation works under these temperatures and how long it would take, I first had to figure that out.

I shifted flies of both genotypes to 29°C for different period of time. As I knew that CySCs are completely ablated after two days ablation at 31°C, I started with three days of *Grim* induction at 29°C. CySCs in 19% of testes (n=3/16) are completely ablated after that period.

After four days of ablation, 88.8% of testes (n=16/18) get completely ablated and after five days of ablation 94.1% of testes lack CySCs (n=16/17). As after five days of ablation, many testes are completely empty (GSCs are also gone). Therefore, I decided to ablate for four days to do the following recovery experiments (Panel 3, A-D).

The same procedure was used at 25°C to find out if and how long it takes to completely ablate testes. As it took at least three days at 29°C, I started with an incubation time of four days at 25°C. CySCs in 50% of testes (n=4/8 testes) get completely ablated under this condition. This is not sufficient for further recovery experiments; therefore I also tried five and seven days of ablation at 25°C.

After five days at 25°C, 55% of testes get completely ablated, this increases to 60% after seven days at 25°C (Panel 3, E-H).

Table 1: % of testes that are completely ablated after certain days of incubation

Days of incubation	29°C	25°C
3	19%	--------
4	88.8%	50%
5	94.1%	55%
7	--------	60%

These results that confirm that *Grim*-induced CySC ablation is possible even at lower temperatures.

To confirm that all CySCs and early daughters were ablated, the experiment needs to be repeated using additional antibodies (traffic jam, somatic cell specific antibody) for immunostaining.

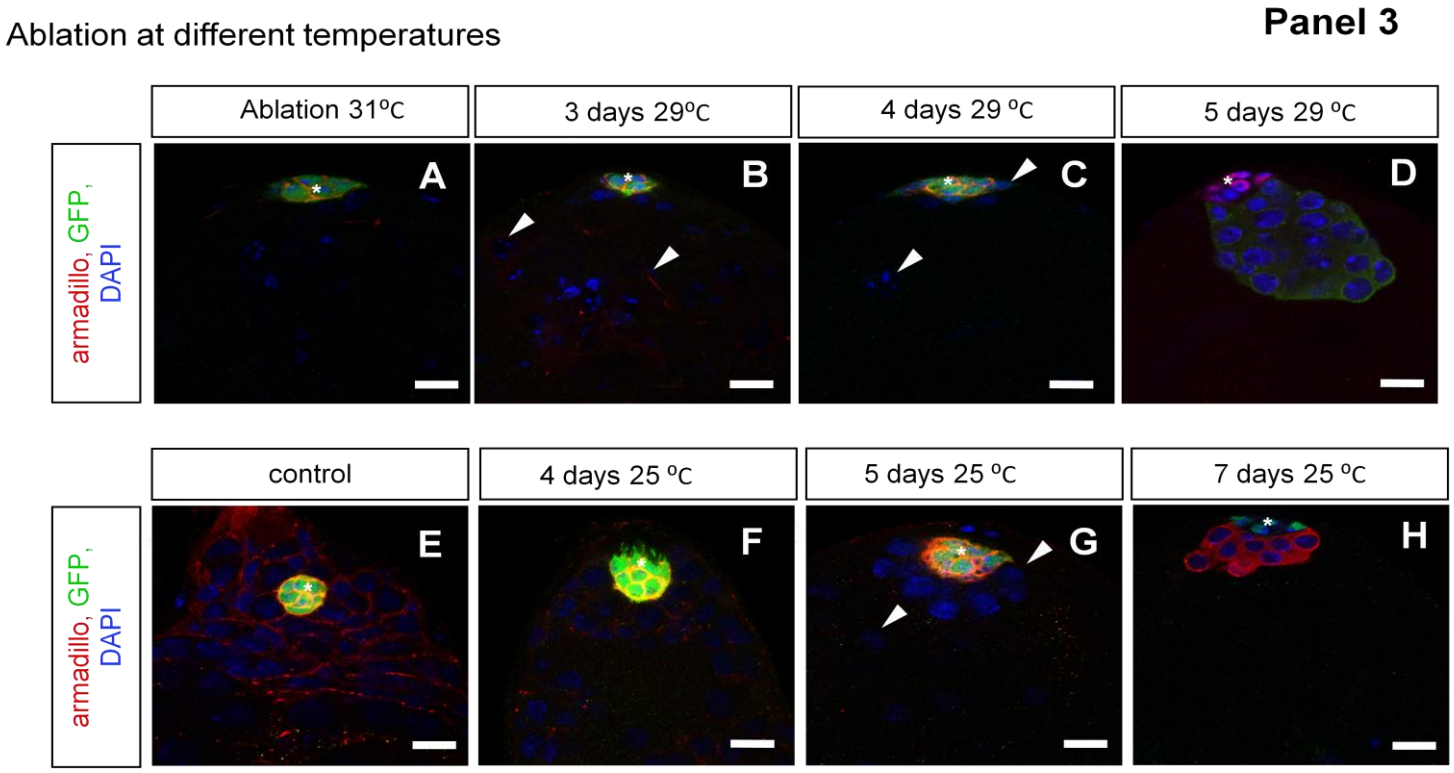


Figure 10: Ablation is possible using lower temperatures – but it takes more time
(A) fully ablated testis after 2 days at 31°C, used as a comparison/control for ablations at lower temperatures; (B)-(D) ablations at 29°C; (B) testis only partially ablated after 3 days at 29°C (19% fully ablated), arrowheads show remaining somatic cells indicated by armadillo stain; (C) completely ablated testis after 4 days at 29°C (88.8%), surrounding the hub are GSCs; (D) completely ablated testis after 5 days at 29°C (94.1%). Some germline cells are remained but most testes are completely empty and only the hub is left
(E)-(H) ablations at 25°C; (E) FasIII-Mi-GFP control; (F) FasIII-Mi-GFP experimental testis gets partially ablated after incubation for 4 days at 25°C; (G) 55% FasIII-Mi-GFP experimental testis fully ablated after 5 days at 25°C; (H) 60% FasIII-Mi-GFP experimental testis fully ablated after 7 days at 25°C

4.5 Multiple hubs occur even after ablation at lower temperatures – but at a lower rate

When doing ablation at 29°C for four days and letting theses flies recover for two weeks at 18°C, 16.3% of all testes (n=7/43) show multiple hubs. 14/43 of all testes were not recovered at all; the rest (22/43) were recovered but had only one hub (Panel 4, A-D and L). 3/20 (15%) *FasIII-Mi-GFP* testes had ectopic niches whereas 4/23 (17.4%) of *Hh-lacZ* acquired them under these conditions.

I performed the same experiment using the ablation conditions for 25°C (seven days at 25°C, two weeks of recovery at 18°C). Under this condition, only 7.8% of all testes formed multiple hubs (n=5/64), which is significantly lower than under harsher conditions of 31°C (Panel 4, E-K and L). In *FasIII-Mi-GFP* testes, 3.3% of testes had multiple hubs (n=1/30), this is a significant decrease in testes having ectopic niches when compared to two weeks of recovery after ablation at 31°C. In *Hh-lacZ* testes I could see 11.8% that had multiple niches (n=4/34) which is not a significant change.

This might indicate that ectopic niches are more likely to form when the tissue gets severely damaged.

Table 2: Comparison % testes having multiple hubs under different conditions

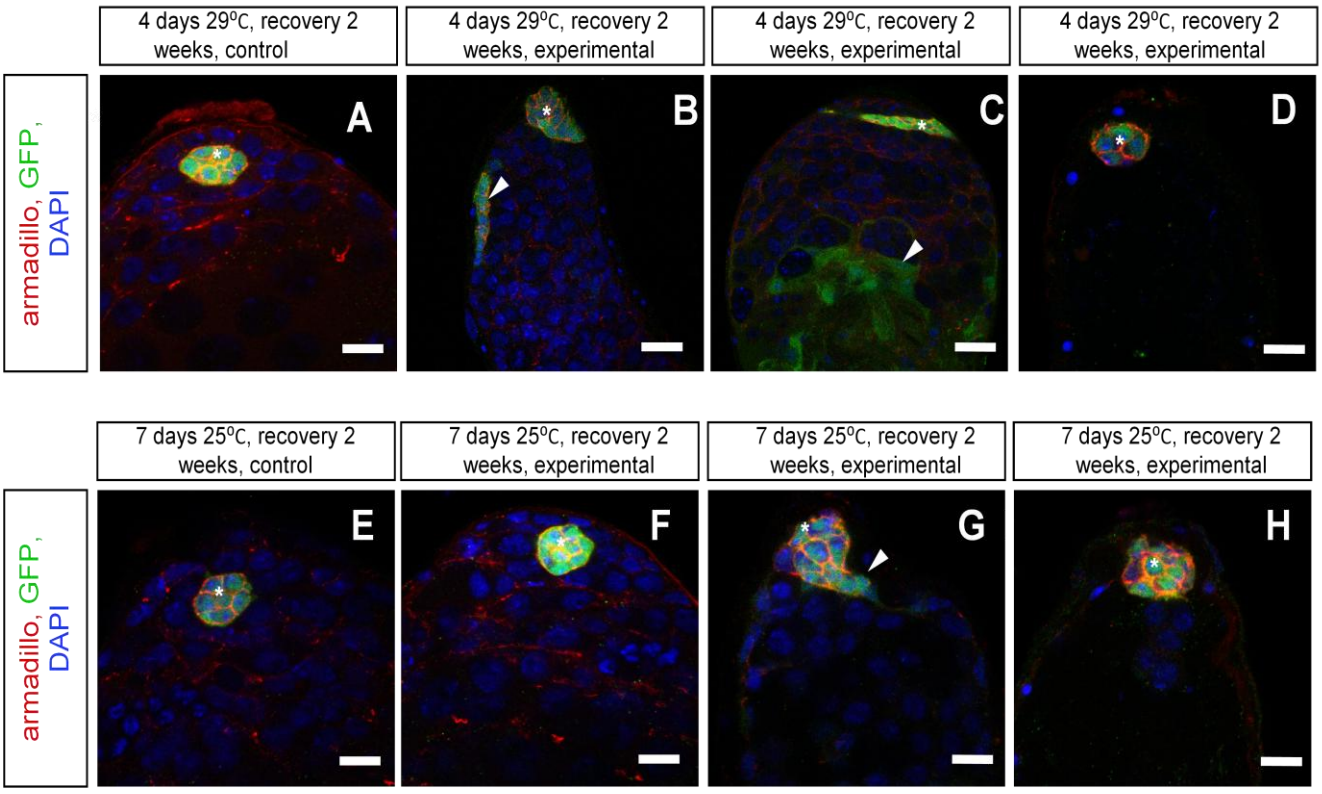
	2 weeks	3 weeks	4 weeks	29°C, 2 weeks	25°C, 2 weeks
FasIII-Mi-GFP	25/97 15.5%	4/13 30.8%	4/6 66.7% P=0.0513	3/20 15%	1/30 3.3% P=0.0046
Hh-lacZ	2/4 50%	10/37 27%	9/31 29%	4/23 17.4%	4/34 11.8%
Total	27/101 26.7%	14/50 28%	13/37 35.1%	7/43 16.3%	5/64 7.8% P=0.0024

The formation of “hub-cell-tumors” (Panel 1, G) can be seen in both genotypes, but is more clearly visible in *FasIII-Mi-GFP* testes. These sheaths of cells might consist of cells that are still in their transdifferentiating state. This is considered with the fact that these cells express a lower level of β-gal, suggesting that they haven’t decided yet whether to become a CySC or a hub cell.

There seems to be an increase in this hub-cell- tumor formation when older flies are used for ablation and recovery experiments but this has to be further determined.

Recovery after abaltion at 29˚ C and 25˚ C

Panel 4



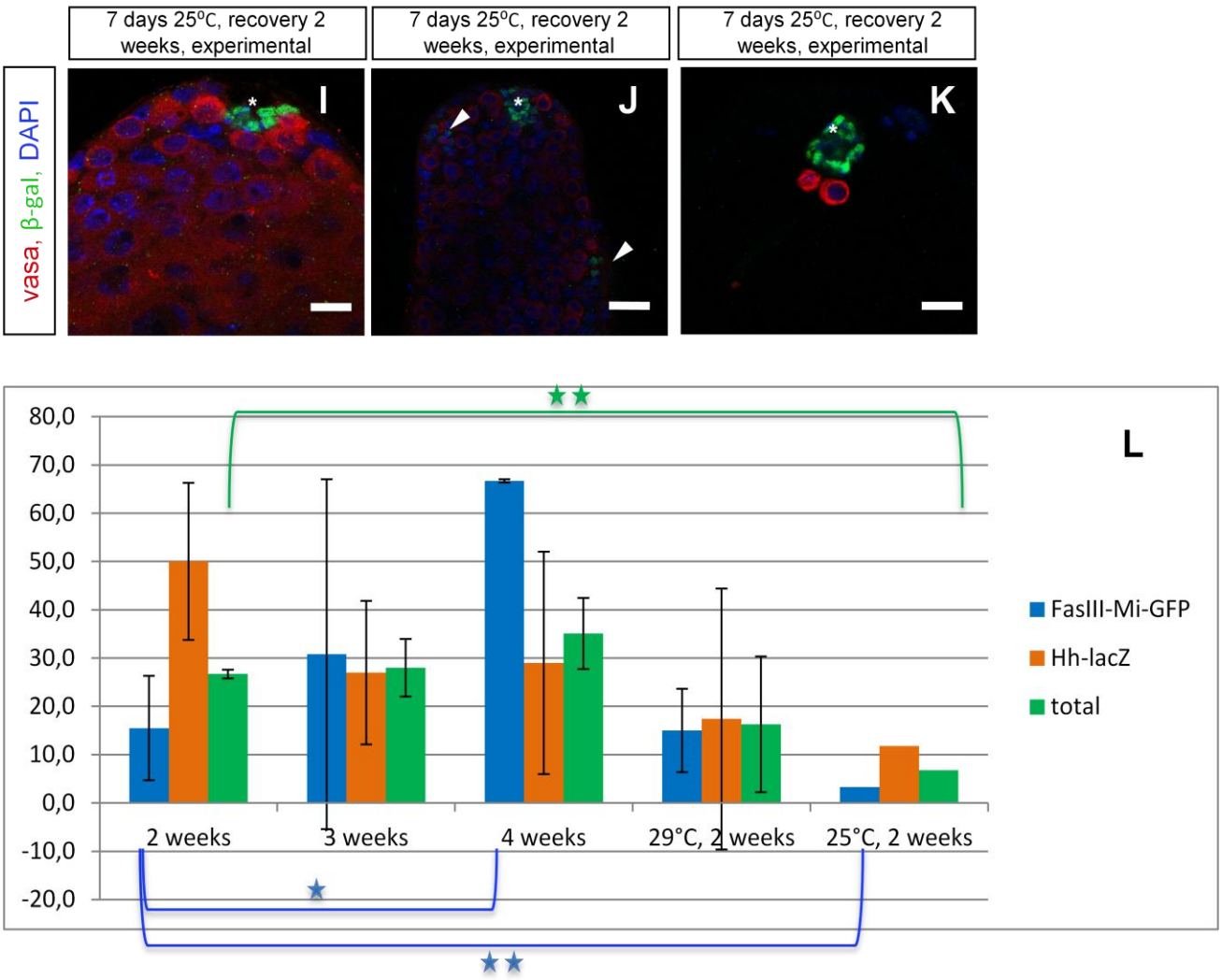


Figure 11: Ectopic niches occur to a lower extent when using lower temperatures for ablation
(A)-(D) FasIII-Mi-GFP flies after 4 days ablation at 29°C and 2 weeks recovery; (A) FasIII-Mi-GFP control; (B) FasIII-Mi-GFP experimental testis that fully recovered and has 2 hubs; (C) FasIII-Mi-GFP experimental testis having one hub (asterisk) and a weird GFP signal; (D) FasIII-Mi-GFP experimental testis that did not recover; (E)-(K) FasIII-Mi-GFP and hh-lacZ flies after 7 days of ablation at 25°C and 2 weeks of recovery; (E) FasIII-Mi-GFP control; (F) FasIII-Mi-GFP experimental testis that fully recovered; (D) FasIII-Mi-GFP experimental testis that did fully recover having a hub that seems to start forming a new niche; (H) FasIII-Mi-GFP experimental testis that did not recover but still having some GSCs left surrounding the hub; (I) hh-lacZ experimental testis that fully recovered; (J) hh-lacZ experimental testis having one original hub and 2 ectopic ones (arrowheads); (K) hh-lacZ experimental testis that did not recover with 2 GSCs left around the hub; (L) graph comparing % of testes having multiple hubs under different recovery and ablation conditions, blue – FasIII-Mi-GFP testes having multiple hubs, orange – Hh-lacZ testes, green – total (FasIII-Mi-GFP and Hh-lacZ), significant values are indicated with a star (FasIII-Mi-GFP 2 weeks recovery compared to 4 weeks of recovery; FasIII-Mi-GFP 2 weeks recovery compared to ablation at 25°C and recovery and total 2 weeks recovery compared to ablation at 25°C and recovery are significant values)

4.6 Somatic cells in the ovary need longer for ablation than in the testes

As we already knew that testes stem cell niches recover over time in most cases when all the somatic stem cells and their early daughters are ablated, I was curious if the same thing happens in the *Drosophila* ovary niche.

To answer this question, I first determined if I can induce apoptosis in the somatic cells in the ovary. I used the same driver (*c587-GAL4*) as in the testis. This driver is expected to be expressed in escort cells and early follicle cells in the ovary (Kirilly, Wang, Xie, 2011). To confirm that, I immunostained ovaries expressing *c587-GFP*.

The GFP-signal was strong in escort cells and early follicle cells, which makes it possible to use the same driver as in the testes to study ovary ablation (Panel 5, A)

I used X-Gal staining to determine if ECs were ablated after induction of *UAS-Grim* expression. Normally, *Hh-lacZ* is expressed in terminal filament cells, cap cells and slightly in escort cells and early follicle cells. After two days of ablation at 31°C the weak signal indicative of ECs disappeared in most cases (31/32 germaria), suggesting that the ablation might already work under these conditions (Panel 5, B-C). As the ECs are stained very weakly using X-Gal staining I decided to perform all the other experiments using immunostaining.

The same ablation conditions used in the testes were used in the ovary (two days at 31°C). ECs express the *Hh-lacZ* signal therefore they can be stained using the mouse α β-gal antibody that was used in the testes as well. The signal is very weak therefore I doubled the amount of primary antibody used in the testes. After two days at 31°C, 66.7% of germaria (18/27) still have some ECs and FCs left, the highest amount of cells was 6 ECs per germarium where usually about ten to 25 ECs can be found (Morris and Spradling, 2011). This result is not satisfying to perform the following recovery experiments. Therefore I wanted to determine how long it really takes to ablate all of the ECs and FCs in the female ovary stem cell niche.

I tried three, four, five and seven days of ablation at 31°C.

After three days at 31°C, 26.7% (n= 4/15) germaria still had β-gal positive cells (excluding cap and terminal filament cells). These remaining cells are mostly detached from the membrane, indicating that they may die soon.

When the flies were incubated at 31°C for four and five days, 90% of germaria lack hh-positive ECs, 1/10 (4 days) and 1/11 (five days) still have one or two ECs left.
After seven day ablation at 31°C, no β-gal positive cells away from the niche can be observed (0/11). This indicates that all ECs got ablated under this condition (Panel 5, D-I)

Table 3: % of germaria having ECs left

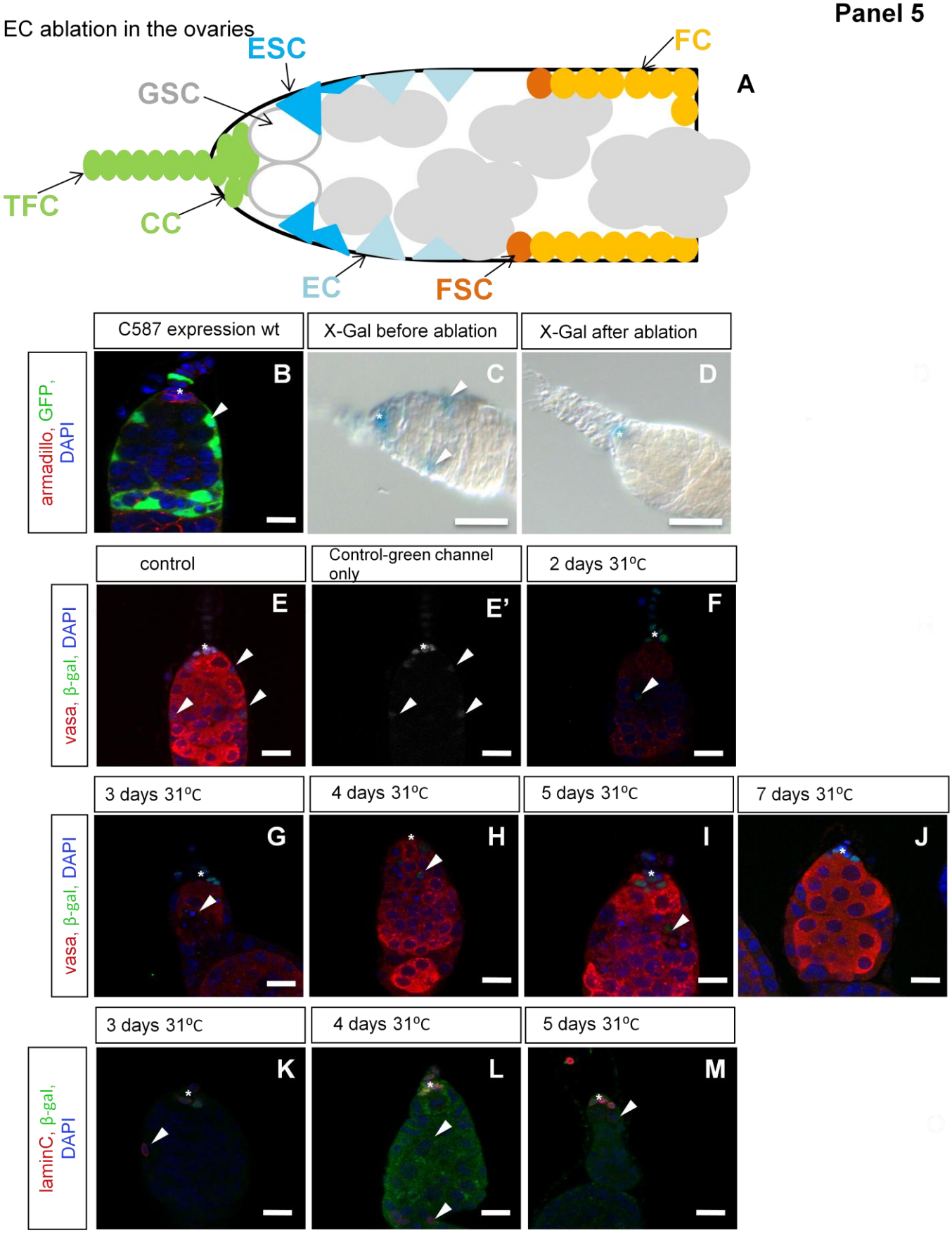
2 d. 31°C	3 d. 31°C	4 d. 31°C	5 d. 31°C	7 d. 31°C
66.7%	26.7%	10%	9.1%	0%
18/27	4/15	1/10	1/11	0/11

The experiment above was repeated using anti- laminC antibody to stain CCs and terminal filament cells and make them better distinguishable from the ECs that are located next to the niche. When incubating the flies for three, four or five days at 31°C, the laminC antibody does not only stain CCs and TFCs but is also slightly expressed from other cells in the germarium. Especially at the stalk where the egg chambers are connected many cells acquire this marker. GCs in the germarium and somatic cells near the basement membrane also start to express this marker after 3 days at 31°C (Panel 5, J-L).

4.7 Germ cell tumors are formed after two week recover after partial ablation of ECs and FCs

After EC ablation, the germaria seem to be very skinny. I wanted to know what happens if I let them recover for two weeks as we did with the testes.
I used flies that were shifted to 31°C for two and three days (these germaria lack part, but not all of their ECs). After two weeks of recovery at 18°C, these germaria seem to acquire more germ cells or even germ cell tumors. Also, the recovered germaria appear fatter than wild type ones (Panel 5, M-O).
The state of these germ cells is still unclear, but it could be determined using the 1B1 antibody which stains the fusome in germ cells.
A dot fusome indicates GSC fate whereas a branched fusome is typical for later stages of germ cell development (Lin, Yue, Spradling, 1994).

Panel 5


EC ablation in the ovaries
ESC
FC
GSC
A
TFC
CC
EC
FSC
C587 expression wt
X-Gal before ablation
X-Gal after ablation
armadillo, GFP, DAPI
B
C
D
control
Control-green channel only
2 days 31ºC
vasa, β-gal, DAPI
E
E'
F
3 days 31ºC
4 days 31ºC
5 days 31ºC
7 days 31ºC
vasa, β-gal, DAPI
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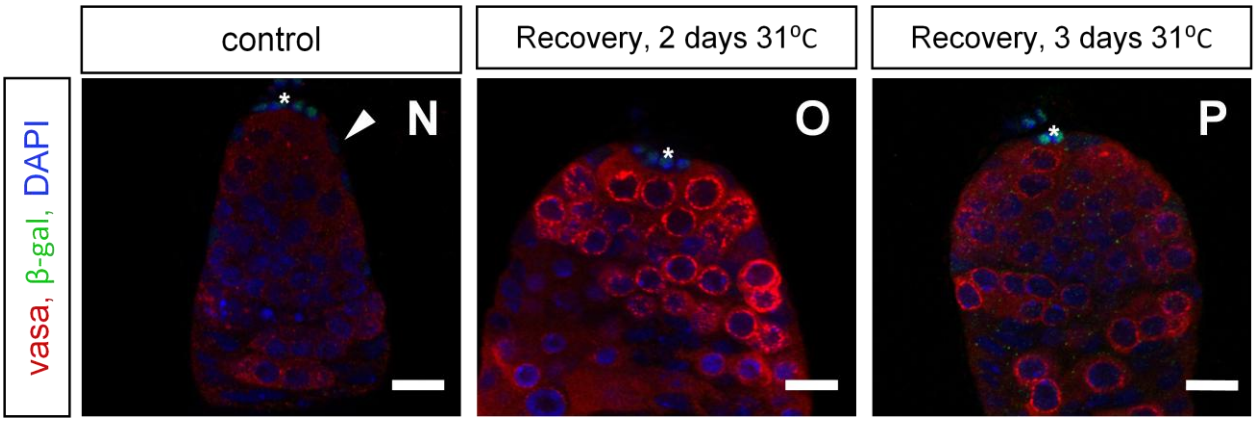

Figure 12: Ablation of escort cells in the ovary niche takes longer than ablation of somatic cells in the testis

(A) ovary stem cell niche cartoon showing terminal filament (TF) and cap cells (CCs) in green, germline stem cells (GSCs) in white and their progeny in grey (CB, GB) escort (stem) cells (ESCs and ECs) in blue and follicle (stem) cells (FSCs and FCs) in orange; (B) C587 GFP expression in the ovary, strong GFP signal in ECs and FCs; (C)-(D) X-Gal images of germarium; (C) before ablation, cap cells stained very good, escort cells only faintly (arrowheads); (D) after ablation (2 days at 31°C) only the stained CCs are left indicating that ECs really can be ablated; (E)-(J) hh-lacZ ovaries after different time points of incubation at 31°C to determine how long it takes to ablated ECs completely; (E) hh-lacZ control; (E') hh-lacZ control showing only the green channel (β-gal signal), signal is strong in CCs and TFCs but only very weak in ECs; (F) hh-lacZ experimental germarium after 2 days at 31°C, arrowhead shows EC that rounded up and detached from the memabrane (%); (G) hh-lacZ experimental germarium after 3 days at 31°C, again the arrowhead points out a β-gal positive cell (EC); (H) hh-lacZ experimental germarium after 4 days at 31°C (same as in F and G); (I) hh-lacZ experimental germarium after 5 days at 31°C (same as in F, G and H); (J) hh-lacZ experimental germarium after 7 days at 31°C, no β-gal positive cells (ECs) can be found in these germaria

(K)-(M) laminC stain to point out cap cells and terminal filament cells; hh-lacZ experimental germarium after 3, 4 and 5 days at 31°C, other cells than cap cells and terminal filament cells start to express laminC (arrowhead) (N)-(P) germaria after 2 weeks of recovery; (M) hh-lacZ control; (N)-(O) hh-lacZ experimental germarium after 2 and 3 days at 31°C and 2 weeks recovery at 18°C, the germarium appears to be fatter than in the control and seems to have many more GCs

4.8 Cap cells do not enter mitosis after partial/complete EC ablation

To be able to draw a connection between the testes stem cell niche and the ovary stem cell niche, I wanted to find out whether quiescent cap cells enter mitosis after somatic cell ablation as it is the case in the testes.

I shifted female *Hh-lacZ* flies to 31°C for 7 days and let them recover for 14, 12, 9, 7, 5 and 3 days. I stained ovaries with EdU, a marker for S-Phase during mitosis. No EdU positive cap cells could be found in all (n=59) germaria observed. Moreover, very few other cells in the germarium were stained with EdU. The location of the EdU positive cells shown in Panel 6 C suggests that these cells are escort cells.

Normally these cells are quiescent but start to cycle when the EC to GC ratio is increased (Morris and Spradling, 2011). This seems to be the case in recovered germaria as they have lots more germline cells than wild type germaria.

I also stained recovered ovaries (14 days of recovery) with PH3, an M-Phase marker. With this marker, more cells per germaria are marked indicating that they are dividing, but again no PH3 positive cap cells were detected (Panel 6, A-C'). The PH3 antibody stain shows two dividing cells in a four cell cyst in some cases. This division pattern is usually not seen in wild type conditions and was first described by Snapp et al. in 2004 and Malmanche et al in 2004.

These papers describe the phenomenon as a loss of synchrony or "semi-sterility" that leads to ovaries that are able to form a smaller number of functional eggs but produce a larger number of nurse cells. If this pattern occurs.

Also in ablated and recovered germaria could be determined by looking at later stages of egg development and counting of oocytes and nurse cells.

Compared to the recovery process in the testes, dividing hub cells are only found in the first few days (two to four days) of recovery, so it would be interesting to look at PH3 staining on ovaries that are at an earlier time point during recovery.

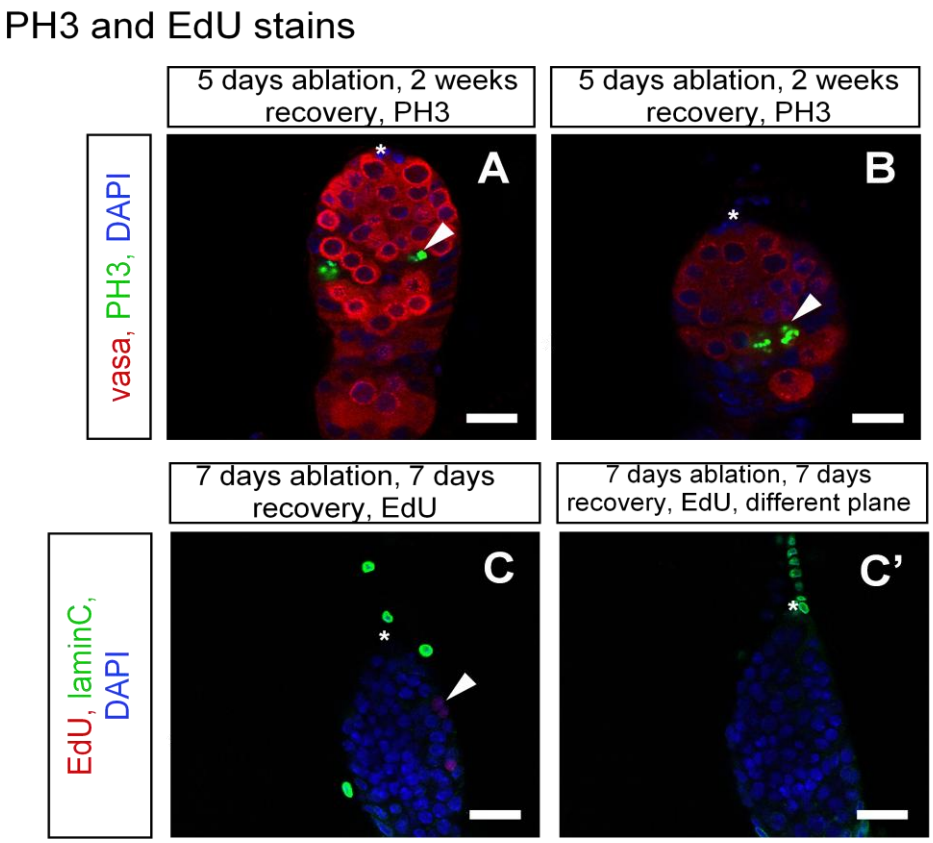

Figure 13: Cap cells and Terminal filament cells seem not to re-enter mitosis after full/partial ablation of ECs and recovery

(A)-(B) PH3 stain of hh-lacZ germaria after 5 days of ablation and 2 weeks of recovery showing some cells that are in M-Phase (PH3 positive cells) but they are far away from the niche, (B) PH3 stain located in only 2 cells of a 4 cell cyst, this indicates that the GCs lose their synchrony in division

(C) EdU stain of hh-lacZ germarium after 7 days of ablation and 7 days of recovery showing some cells (arrowhead) that are in S-Phase (EdU positive cells), these cells seem to be somatic ECs. Again no evidence that cap cells enter mitosis; (C') different plane of same image to show location of stem cell niche (cap cells, asterisk)

5. Discussion

Previous work showed that GSCs that are lost through damage or aging can be replaced by spermatogonia that dedifferentiate to produce new GSCs (Brawley, 2004; Chen, 2008; Sheng, 2009). This mechanism is valuable for both, the male and female stem cell niches.

Now we also know that hub cells forming a stem cell niche in the *Drosophila* testis are not permanently withdrawn from the cell cycle. They re-enter the cell cycle when all CySCs and their early daughters are genetically ablated.

These testes, when recovered for some time at their permissive temperature, start to form ectopic niches that recruit stem cells and continue to expand in an uncontrolled manner (Hétié et al. submitted).

This is the first known example where a damaged stem cell niche is repaired through the transdifferentiation of formerly quiescent cells which can then go on to form additional ectopic niches.

However, similar processes occur in human cancers of the esophagus and cervix where chronic damage of tissue promotes tumorigenesis by activating cells that are normally quiescent to produce progenies and grow uncontrollably. The mechanisms are still poorly understood in mammals; therefore it is of great importance to characterize these ectopic niches in the *Drosophila melanogaster* testis further (Hétié et al. submitted).

X-Gal staining was shown to work beautifully when used on clones expressing high levels of β-galactosidase (Margolis and Spradling, 1995). The clones can be easily distinguished from dying cells in the tissue because of the darker stain due to higher β-galactosidase expression.

Only the hub cells get stained in *Hh-lacZ* testes but due to its low β-galactosidase expression, the X-Gal stain is very strong.

Ectopic niches seem to express a lower level of β-galactosidase than wild type hubs as they get stained weaker. Therefore, it is always hard to distinguish between ectopic hub cells and clusters of dying cells because both types of cells are stained weakly using this method.

The same is true for X-Gal staining in the ovary stem cell niche. Cap cells are stained stronger because of their stronger β-galactosidase expression than escort cells.
These results lead to the conclusion that X-Gal staining is very helpful in some cases but might not a suitable method to investigate ectopic niches in the testis or EC ablation in the ovary due to its lack of specificity.

My finding that a longer recovery time leads to a higher number of testes forming multiple niches suggests that the occurrence of ectopic niches is related to the recovery time or the age of flies at the time of CySC ablation.
The percent increase is not significant when the results from three and four weeks are compared to that from two weeks recovery. The fact that there is only a slight increase in testes having multiple hubs when comparing three weeks of recovery to four weeks of recovery might indicate that if testes recover for too long (maybe five weeks), ectopic niches start to disappear again. This could be due to the fact that these ectopic niches expand uncontrollably but as soon as two niches contact each other, cellular signaling might be confused that then leads to the breaking up of the ectopic niches. This hypothesis could be proven by letting flies recover after ablation for five weeks and comparing the number of testes with multiple hubs to two, three and four weeks of recovery.
It is also interesting that flies that were older before shifting yield a higher percentage of testes having multiple hubs then when using young flies for the experiment. These are just preliminary results as the “n” of the old flies was very low. Therefore, the experiment should be repeated with old flies to see whether this has an influence on ectopic niche formation or not.

The “hub tumors” were first thought to be only visible when using *FasIII-Mi-GFP* flies. Now it is known that they also occur in *Hh-lacZ* testes but they seem to have a weaker β-galactosidase expression. Therefore they could be intermediate stages of ectopic niche formation and that these cells do not know whether to become a hub cell or differentiate back into a CySC. The number of testes having tumors seems to increase with the age of the flies. When shifting young flies (2-3 days old) to 31°C for two days and then to 18°C for two weeks, very few testes show this phenomenon (~3 in a batch of ~30 testis).

But when old flies are used for the experiment (13 day old flies) it occurs about twice as much (n=6/19). Also here the “n” of old flies was too low to draw any further conclusions and the experiment has to be repeated.
It would also be interesting to see what happens when CySCs are ablated in even older flies. One possibility is that even more testes with ectopic niches will be created and that also the number of “hub-cell-tumors” increases. Another possibility is that nothing happens because the flies are too old and die during the experiment.

The *c587-Gal4* driver was first used in 2003 whereas the *UAS-Grim* construct was first introduced in 1996 (Kai and Spradling, 2003; Chen et al. 1996). These two tools were not used in combination before. The Matunis lab then used *c587-GAL4* driver to induce *Grim* expression in the CySC lineage and successfully ablated all the CySCs and their early progeny (Hétié et al. submitted). Ablation was performed by shifting the flies to 31°C for two days. Under this condition, the Matunis lab is able to ablate all the CySCs and early daughters in 100% of the testes.
As there were no other conditions tested, I wanted to figure out whether total ablation is also possible using lower temperatures.
Indeed, it is possible to ablate CySCs and their early daughters at lower temperatures using a longer incubation time, but it is never as effective as ablation at 31°C. This result tells us that complete ablation happens more efficiently when doing it at 31°C.

The fact that ectopic niches occur more often when ablation happened at high temperatures indicates that temperature affects recovery efficiency.
The smaller number of testes having multiple hubs might be due to a less reliable ablation as I never get 100% of testes being completely ablated when using 29°C or 25°C (Table 3). Also it could be that harsher conditions that lead to a more damaged tissue promote the formation of ectopic hubs.

It also has already been known what happens in the ovary when GSCs are ablated. An empty GSC niche (lacking all GSCs) leads to an increase in follicle cell number that start to fill the germarium when GSCs are lost. Subsequently, cap cells go into apoptosis after a few days (Kai and Spradling, 2003). In some cases GSC are replaced by dedifferentiating older germ cells as it is the case in the testis.
Knowing that there is a slight difference between testis and ovary niches when they lose their GSCs, I investigated what happens when I ablated somatic cells in the ovary.

The results indicate that complete escort cell ablation is possible when expressing *Grim* for seven days at 31°C.
It is not surprising that escort cells need a longer time to be ablated than CySCs because they are attached to the basement membrane and they are usually in a quiescent state, which is not the case with CySCs.
The results indicate that ECs first detach from the basement membrane and acquire a round shape before they undergo apoptosis, therefore they need longer time.
Also it is possible that different pro-apoptotic genes might work better in ECs. We know that the apoptotic genes *Reaper* and *Hid* do not work as well as *Grim* in CySCs but it is possible that they can work better in the ovary.
I was able to show that at least a partial ablation of somatic cells happens in the germarium when using the *c587-Gal4* system and the apoptotic gene *Grim*.

The laminC expression of cells that are not CCs or TFCs after incubation at 31°C for several days is either due to unspecific binding of the antibody or there might be a change in gene expression within these cells under the given conditions. It was already published that stalk cells express this marker but not that cells other than CCs, TFCs or stalk cells express the laminC marker (Heck et al. 2012).

As the β-galactosidase expression from the Hh-lacZ reporter is very weak in ECs and they remain very weakly stained even with immunostaining, the results have to be confirmed using the *PZ1444* fly line marks for ECs and FCs.

Unfortunately, I was not able to confirm my results by repeating the experiments with this line because I received the PZ1444 transgenic flies only recently (*PZ1444* is not commercially available).

When germaria lack part of their ECs, I can observe that, when I let them recover for two weeks at 18°C, they get bigger and fatter and they seem to have more germ cells than wild type germaria. Unfortunately, it was not possible to find out whether escort cells are repopulated because there were still some ECs left under these conditions and they are very difficult to quantify using Hh-lacZ. It would be interesting to do the recovery experiment after seven days of ablation where we can be quite sure that all of the ECs are ablated using PZ1444 as a marker.
My result suggests that even after only partial ablation of ECs, the germarium is very disrupted. This indicates that escort cells might play an important role in regulation of germ cell proliferation in the *Drosophila* ovary stem cell niche.
It has already been known that GSCs are required for the maintenance and formation of EC cellular processes and that ECs fail to be replenished when GSCs are lost but nothing is known so far if ECs have an influence on GSC maintenance or differentiation (Kirilly, Wang and Xie, 2011; Morris and Spradling, 2011).
This ablation and recovery phenomenon in the germarium is an interesting topic that needs more investigation in the next few years.

The stem cell niches of *Drosophila* testes and ovaries are known to have a lot of signals and processes in common and also have many differences. To be able to further relate these two systems, I wanted to know if the quiescent cap cells have a similar role as the hub cells in the testis during recovery after ablation, given that hub cells enter the cell cycle.
The first approach was to use EdU staining by feeding the chemical to the flies over a certain period of time. EdU is an S-Phase marker and therefore is able to cover a longer period of the cell cycle when it can be detected.
This stain works really well in the testis but not as well in the ovary niche. Only very few cells get stained with a weak signal. I tried different conditions of recovery and no cap cells or terminal filament cells are observed to enter the cell cycle.

The cells that are stained with EdU are mostly at the basement membrane and do not seem to be GCs. Their shape suggests that these cells are escort cells. Escort cells usually only divide when the EC:GC ratio is not in balance anymore. This would make sense since it seems that recovered germaria acquire a vast number of GCs and therefore their ratio of escort cells to germ cells is increased. To confirm this, the experiment needs to be repeated with either the *PZ1444* fly line where ECs are marked better or with using “Vasa” as a germ cell marker to distinguish somatic cells from GCs.

I also used PH3 staining, an M-Phase marker that acts only for a very short time. With this marker I was able to detect more cycling cells than with EdU. This indicates that the EdU feeding is not a good method for staining the ovary because I should get at least as many EdU positive cells as when doing the experiment with PH3.
Also with PH3 staining I could not find any dividing CCs or TFCs, but I only looked after 14 days of recovery. In the hub, dividing cells can only be detected after three to four days of recovery. So to be really sure that there are no CCs dividing, earlier recovery time points need to be examined.
It was surprising that in both cases (PH3 marker and EdU marker) only a few cells were dividing. I expected far more GCs to be dividing because of the higher amount of germ cells in the germarium when I look after 14 days of recovery. To reveal this, maybe another staining method indicating cell cycle should be used.
An interesting point here is that in some germaria, the PH3 antibody stained two cells in a four cell cyst. This does normally not happen in wild type conditions and indicates that these cells lose their synchrony in replication.
As already mentioned before, it would be interesting to look at later stages of oocyte formation because the loss of synchrony has a distinct phenotype. These ovaries are able to form at least some functional oocytes but they also produce a larger number of nurse cells (Snapp et al. 2004).

6. References

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7.2 Figure References:

Figure 1: Asymmetric division of adult stem cells, Li and Clevers Science 2010;327:542-545; available at: http://www.sciencemag.org/content/327/5965/542/F3.expansion.html; January 13th 2013, 9:15am

Figure 2: Overview of mammalian stem cell niches, Development 2010; available at: http://dev.biologists.org/content/137/10/1586/F2.expansion.html; January 13th 2013, 1:10pm

Figure 3: EM image of *Drosophila* testis niche, image taken by the Matunis Laboratory, 2010

Figure 4: EM image of *Drosophila* ovary niche, Hause, Biocenter, Martin Luther Univ. Halle-Wittenberg; available at: http://www.spp1356.de/project-area-a/reuter; January 15th 2013, 8:00am

Figure 5: UAS-Gal4 system, Elliott and Brand, available at: http://www.springerimages.com/Images/LifeSciences/1-10.1007_978-1-59745-583-1_5-0; January 16th 2013; 2:35pm

Figure 6: Apoptotic pathway, Igney and Krammer, Nature Review Cancer, 2002 Apr;2(4):277-88; available at: http://www.ncbi.nlm.nih.gov/pubmed/12001989; January 19th 2013, 11:45am

Figure 7: Click-it Chemistry EdU Kit, Invitrogen, available at: http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/Click-iT-Detection-Assays/Click-iT-EdU.html; January 22nd 2013, 8:30pm

Figures 8-13: confocal images and X-Gal images taken by Melanie Pieber, Johns Hopkins University, Department of Cell Biology, Dr. Erika Matunis Laboratory; July-February 2013

Figure 8: Testis cartoon, Dr. Erika Matunis Laboratory, 2012
Figure 12: Ovary cartoon, Melanie Pieber, 2013