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miR-125 regulates niche organization in *Drosophila melanogaster* ovary by affecting Notch signaling pathway via its target Tom

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°C	°Celsius
Add	Adducin
bam	bag of marbles
BMP	Bone Morphogenetic Protein
bp	Base Pair
C. elegans	*Caenorhabditis elegans*
CpCs	Cap Cells
DEPC	Diethyldicarbonat
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic Acid
dpp	Decapentaplegic
Drosophila	*Drosophila melanogaster*
E (spl)-C	Enhancer of split Complex
EC	Escort Cell
ecdysone	20-Hydroxyecdysone
En	Engrailed
FSC	follicular stem cell
gbb	Glass bottom boat
GSC	Germline stem cell
h	hour
HYB	hybridization solution
LamC	LaminC
LNA	Locked Nucleic Acid
Lsd1	Lysine-specific demethylase 1
Mad	Mothers against dpp
min	minute
miR	microRNA Molecule
miRNA	microRNA
mRNA	messenger RNA
N^{CA}	Notch Constitutively Active
N^{intra}	Intracellular domain of Notch
nt	Nucleotides
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pMad	Phosphorylated Mothers against Dpp
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA interference
SSCs	Single Spectrosome Containing Cells
Su(H)	Suppressor of Hairless
TF	Terminal Filament cell
TJ	Traffic Jam
TJs	Traffic Jam positive cells
TSA	Tyramide Signal Amplification
UTR	Untranslated Region
WB	Western Block solution

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1 Introduction

Nearly every tissue in an organism needs stem cells for the maintenance of its characteristics. The purpose of stem cells is the replacement of lost cells in the context of natural cell turnover or injury (Kirilly and Xie 2007; Alison et al. 2009). Different tissues host different types of stem cells, however all stem cells share some common characteristics. Firstly, they have the capacity for self-renewal – represented by their ability to replicate asymmetrically. In this course, one of the emerging cells keeps the stem cell characteristics, while the other cell starts to differentiate. Furthermore, stem cells are able to deliver differentiated cells nearly lifelong. For biomedical research stem cells have become an important topic. It can be assumed that the understanding of stem cells and their regulation have the potential to change the treatment of various human diseases dramatically. A possible use in future may be their application in regenerative medicine. Over and above that, it is striking that stem cells have many features in common with cancer stem cells (Reya et al. 2001; Crowe et al. 2004; O'Brien et al. 2010). Putatively, these cancer stem cells are the tumor forming cells of malignant neoplasia. Numerous studies point out the impact on medicine which emerges from the investigation of stem cells and the regulation of their fate (e.g. reviewed in Crowe et al. 2004; O'Brien et al. 2010; Barcellos-Hoff et al. 2013; Tabar and Studer 2014).

Stem cells are normally hosted in a specialized microenvironment, called niche. The niche provides an environment helping the stem cell to maintain its characteristics. The investigation of stem cells, their niches and their interaction *in vivo* helps to understand how stem cell fate is regulated and maintained. However, the *in vivo* investigation of stem cells in their natural environment remains a challenge in most vertebrates as procedures are complicated, expensive and relatively slow. Therefore invertebrate models were used to investigate how the niche communicates with its stem cells. Many of today's insights were obtained using these models. A very popular invertebrate model to study stem cells in their *in vivo* environment is the *Drosophila melanogaster* (*Drosophila*) ovary (Eliazer and Buszczak 2011).

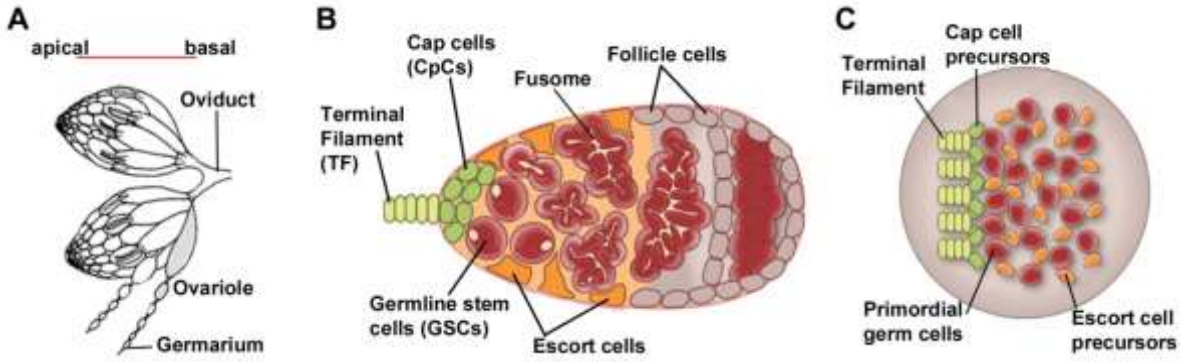

Figure 1: Organization of *Drosophila* ovary, germarium and developing germarium. (A) *Drosophila* females have two ovaries consisting of ovarioles. The germarium is located at the apical tip of each ovariole and hosts the germline stem cells (GSCs). GSCs give rise to egg precursors. During its development the egg progresses from apical to basal along the ovariole. Mature eggs passage the oviduct and enter the uterus before they are ejected. Illustration modified according to Middleton et al. 2006. **(B)** The adult *Drosophila* germarium hosts the germline stem cells (GSCs; red). The GSC niche is formed by terminal filament cells (light green), cap cells (dark green) and possibly escort cells (orange) in contact with GSCs. The escort cells direct the developing cyst during their passage throughout the germarium. Follicle cells (grey) surround germline cysts before their budding off the germarium. Afterwards, they go through further developmental stages along the ovariole. The differentiation process of GSCs can be monitored by morphological changes in the fusome (beige). In GSCs the fusome is round. With progression of differentiation it becomes more branched. Illustration adopted (Eliazer and Buszczak 2011). **(C)** In late larval development, terminal filament cells (light green) are formed. Via Notch pathway they induce neighboring somatic cells to become cap cell precursors (dark green). At the same time, primordial germ cells (red) are present in the developing gonad. They are associated with cap cell precursors and escort cell precursors (orange). Illustration adopted (Eliazer and Buszczak 2011).

1.1 The *Drosophila* germarium is an ideal model system for the investigation of stem cells *in vivo*

Drosophila females have two ovaries, each consisting of around 16 tubular ovarioles. At the apical tip of the ovariole a specialized structure called the germarium is located (Figure 1A). This is the place where the germline stem cells (GSCs) and their niche can be found. The germarium is linked to six to eight developing egg chambers. The most mature egg can be found at the most basal position. A major advantage for investigations within the *Drosophila* germarium is the very defined structure of this organ. All cell types can be easily identified and quantified (Figure 1B).

The germarium hosts two known types of stem cells: germline stem cells (GSCs) and follicular stem cells (FSCs) (Xie 2013). The most prominent niche is the *Drosophila* GSC niche and can be found at the most apical tip of the germarium. The GSC niche hosts two to three GSCs. GSCs are in direct contact with five to seven cap cells (CpCs) and escort cells (ECs). The CpCs are directly attached to eight to ten terminal filament cells (TFs). The GSC niche consists of TFs, CpCs, possibly ECs (Xie 2013) and non-cellular components controlling stem cell fate. The GSCs divide

asymmetrically and give rise to one stem cell staying in contact with the niche and to a differentiating daughter detaching from the niche. This cell undergoes incomplete mitotic division and forms an interconnected 16-cell cyst.

The individual cell types within the germarium can be distinguished from each other easily by location, morphology and several molecular markers (Xie 2013; König and Shcherbata 2013). As an example, LaminC is expressed in both TFs and CpCs, but only CpCs are in direct contact with GSCs. In addition, TFs and CpCs vary in shape: CpCs are small and round whereas TFs are disc-like (Xie 2013). GSCs have an apical oriented single spectrosome and are in contact with CpCs via adherence junctions. The cytoblasts, a differentiated progeny of GSCs, have an adventitious oriented single spectrosome and detached the niche. Later stages of cyst development show branched fusomes (Figure 1B). Because cell types in the *Drosophila* germarium can be identified easily and many precise and easy tools for manipulating gene function in *Drosophila* are available, the *Drosophila* germarium offers an attractive model system to study stem cells in their *in vivo* environment (Kirilly and Xie 2007; Alison et al. 2009; Eliazer and Buszczak 2011; Xie 2013).

1.1.1 The formation of the ovarian niche starts in late larval development

As mentioned, the ovarian niche consists of TFs, CpCs and possibly ECs (Xie 2013). Its formation is poorly understood. The transformation of the primitive gonad into adult ovaries starts during late larval development (Eliazer and Buszczak 2011). Firstly, TFs are formed and draw up structures of up to ten disc-like cells, demarcating individual ovarioles (Figure 1C) (Eliazer and Buszczak 2011). The steroid hormone 20-hydroxyecdysone (ecdysone) or its metabolites were shown to govern the timing of TF formation (Hodin and Riddiford 1998). The formation of CpCs depends on the Notch pathway. TFs signal via the Notch ligand Delta to neighboring somatic cells. In these cells Notch becomes activated and stimulates the somatic cells to become cap cells (Song et al. 2007). It was shown that overexpression of Delta or activated Notch results in formation of ectopic cap cells in adults (Ward et al. 2006; Song et al. 2007).

1.1.2 Many genes and pathways regulate GSC fate in the *Drosophila* germarium

As mentioned above, the characteristics of GSCs are controlled by their microenvironment, namely the niche. Multiple regulatory principles controlling stem cell self-renewal have been uncovered. Physical contacts and diffusible factors control stem cell fate (Alison et al. 2009).

Diffusible factors can act over distances of different lengths to influence cell fate, often by affecting transcription.

Cap cells are regarded to be the most important component of the niche (Xie and Spradling 2000; Ward et al. 2006; Song et al. 2007). They physically contact GSCs via E-cadherin and Integrin mediated cell adhesion complexes (Song et al. 2002). Insulin is required to maintain the E-cadherin expression in CpCs (Hsu and Drummond-Barbosa 2011). E-cadherin levels were shown to determine competitiveness among the GSCs and ensure that only undifferentiated GSCs remain in the niche (Xie 2013). Intrinsic factors (e.g. Rab11, Lis1) were shown to regulate the physical interaction of GSCs and their niche (Xie 2013).

In addition, the niche sends short range signals to the GSCs to maintain their characteristics. Piwi and Yb function in TFs and CpCs is required for the maintenance of GSC self-renewal (Xie 2013). Moreover, major ligands required for GSC self-renewal are Decapentaplegic (dpp) and Glass bottom boat (gbb), two members of the bone morphogenetic protein (BMP) superfamily (Eliazer and Buszczak 2011). Both, dpp and gbb, signal mainly from CpCs to GSCs and cause the phosphorylation of Mothers against dpp (Mad), which binds to Medea. The resulting complex translocate into the nucleus and represses transcription driven by the promoter *bag of marbles* (*bam*). Repression of *bam* by BMP keeps the stem cells in their undifferentiated state (Xie and Spradling 1998; Song et al. 2004). Differentiating Cytoblasts, while moving away from the niche, upregulate their transcription of *bam* by various mechanisms (Eliazer and Buszczak 2011). Different control mechanisms restrict BMP signaling to GSCs only. Additional mechanisms restrict BMP expression to CpCs only. JAK-Stat signaling has been shown to activate the transcription of *dpp* in niche cells and may determine its levels (Xie 2013).

Furthermore, systemic factors modulate the responsiveness of stem cells to niche signals. Insulin, produced by neuroendocrine insulin-producing cells in the brain of the adult fly (Ikeya et al. 2002; Rulifson et al. 2002), has been shown to control GSC proliferation (Kirilly and Xie 2007). Thus, Insulin may link stem cell activities to the nutritional and health status of the fly. In addition, aging affects the function of both GSCs and the niche. This is possibly due to decreased levels of Insulin during aging (Xie 2013).

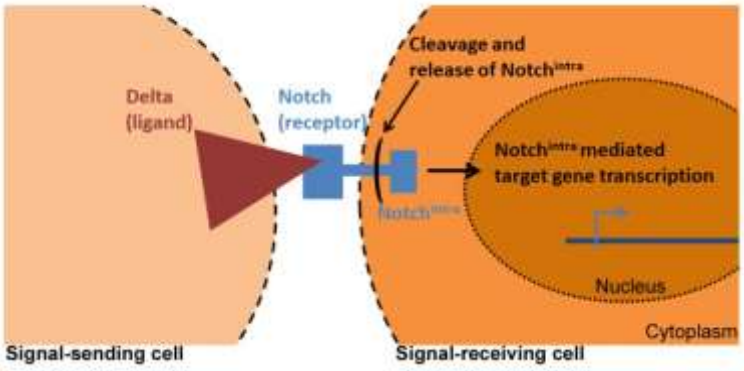

Figure 2: Basic principle of Notch signaling. Key players in Notch signaling are Delta (the ligand) and Notch (the receptor). Both are transmembrane proteins and enable direct cell-cell communication. The signal-sending cell provides activated Delta to activate Notch in the adjacent cell. Binding of Delta to Notch triggers the proteolytic cleavage of the Notch intracellular domain (Notch^{intra}). Notch^{intra} translocates into the nucleus and promotes transcription of Notch target genes. Thus, the cell becomes a signal-receiving cell.

1.1.3 The Notch pathway is a prominent regulator of GSC fate and niche architecture

Conserved signaling pathways are involved in numerous biological processes. Former studies revealed that the GSC niche architecture is regulated by Notch signaling (Ward et al. 2006; Song et al. 2007). As mentioned above, the Notch pathway is involved in niche formation during the development as well.

The evolutionary conserved Notch signaling pathway is a prominent pathway controlling cell communication and cell fate (Lai 2004). Via Notch signaling direct cell-cell communication can be established (Figure 2). It was suggested that a feedback loop exists between the stem cells and their niche cells established via the Notch pathway (Ward et al. 2006), however this was denied by other studies (Hsu and Drummond-Barbosa 2011). It has been reported that forced expression of Delta in the germline or activated Notch in the somatic cells, results in additional CpCs and thereby in an increase in niche size. This increased niche hosts an increased number of GSCs (Ward et al. 2006; Song et al. 2007). It was shown that Notch signaling remains active in adult ovaries, however active Notch signaling is not able to transform somatic cells in adult ovaries to CpCs (Song et al. 2007). Deactivated Notch signaling in adult ovaries results in CpC loss and therewith GSC loss (Song et al. 2007). One study suggests that CpC-expressed Notch ligands are essential for the maintenance of Notch activity in CpCs (Hsu and Drummond-Barbosa 2011). In addition, it was shown that Insulin-like peptides modulate Notch signaling and thereby are required for GSC maintenance and control attachment of GSCs to their niche (Hsu and Drummond-Barbosa 2011).

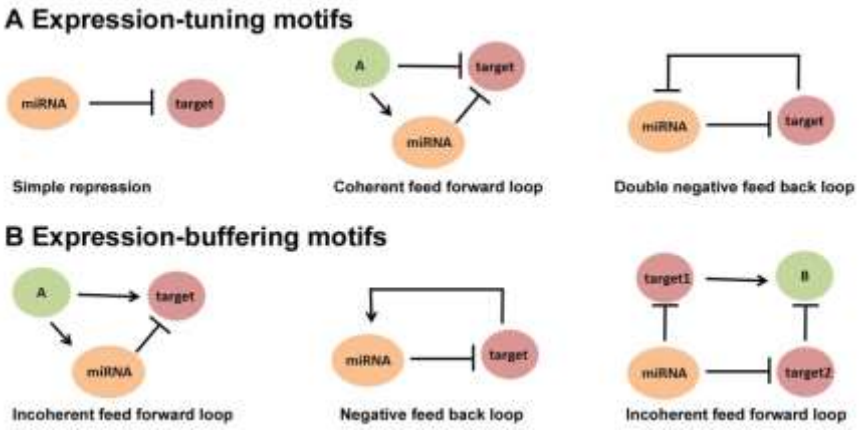

Figure 3: Proposed functions for miRNAs. The functions of miRNAs can be classified according to their function in tuning or buffering expression levels. **(A)** Several motifs are proposed for expression-tuning. Via simple repression the miRNA reduces the expression of its target. Coherent feed forward loops can be described when miRNAs amplify the repression of a target. Double negative feedback loops can be described when miRNA and target repress each other reciprocally. **(B)** Several motifs are proposed for expression-buffering. Incoherent feed forward loops can be described when the miRNA buffers the expression of the target against variations in A. Negative feedback loops can be described when miRNA and the target buffer each other's expression reciprocally from perturbations. Incoherent feed forward loops can be described when target1 and target2 buffer the expression of B against perturbations in the level of the miRNA. Illustration adopted with kind permission (Wu et al. 2009).

1.2 miRNAs are involved in the regulation of niche fate

The regulation of niches and their stem cells during development and in adults is a complex process. Several principles governing cell fate during these processes were described, but much remains unclear. For many of these processes specific levels of a certain protein are required. Relevant levels can be controlled on transcriptional and post-transcriptional levels. microRNAs (miRNAs) were shown to be important players in translational regulation having implications for controlling stem cell fate and behavior by repressing certain messenger RNAs (mRNAs) in niche cells, stem cells and their differentiating daughter cells (Gangaraju and Lin 2009). miRNAs are proposed to confer signaling robustness by several mechanisms to counteract genetic or environmental perturbations (Inui et al. 2010). miRNAs are expected to be involved in signaling balancing and buffering, and may act as crucial players in numerous feedback loops (for proposed mechanisms see Figure 3). They participate in signaling networks stabilizing tissue patterning by repression of mRNA in cells where this mRNA should not be expressed. The prominent role miRNAs may have is highlighted by the finding of numerous different miRNAs in all known animals and plants. So far, miRBase (Griffiths-Jones 2004) lists 1872 sequences for human miRNAs and 238 sequences for *Drosophila melanogaster* miRNAs (March 2014). A high number of studies reveal that miRNAs are progressively linked to cancer, either as tumor suppressors or oncogenes (Davis-Dusenbery and Hata 2010b; Takahashi et al. 2014).

miRNAs are around 22 nucleotide (nt) non-coding RNAs. They can bind to the 3' untranslated region (UTR) of target mRNAs by imperfect matching to repress translation and stability (Rana 2007). One miRNA can target tens to hundreds of mRNAs due to imperfect matching (Davis-Dusenbery and Hata 2010a). miRNA mediated target repression leads to critical changes in gene expression with perceptible impact on various biological processes. Occasionally, miRNAs have been reported to activate gene expression (Vasudevan et al. 2007).

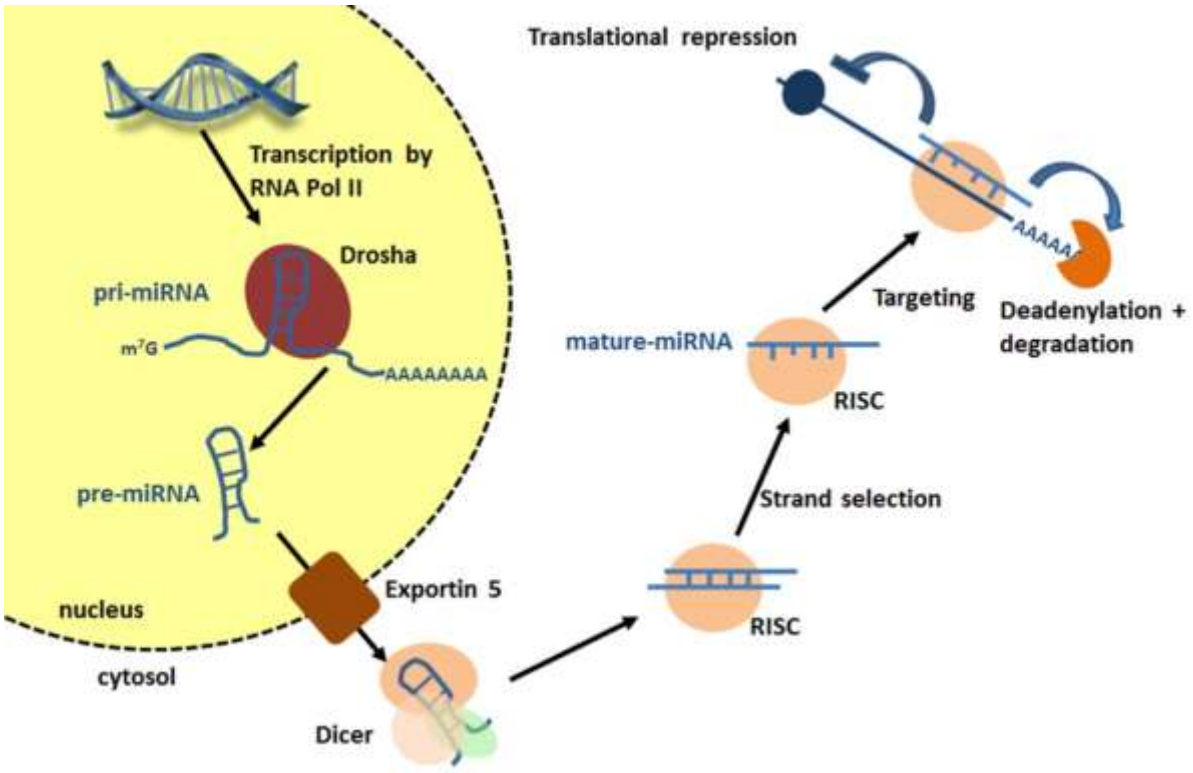

Figure 4: Pathway of miRNA biogenesis. miRNAs are transcribed by RNA Polymerase II as primary miRNA (pri-miRNA). Pri-miRNA is cropped by Drosha to short hairpin-shaped precursor miRNA (pre-miRNA). The pre-miRNA is exported out of the nucleus via Exportin-5. In the cytoplasm, pre-miRNA is processed by Dicer resulting in around 22 nt double-stranded RNA (mature miRNA: passenger strand). Mature miRNA promotes the assembly of the RNA-induced silencing complex **(**RISC) and targets mRNAs to induce gene silencing. This is done by translational repression of respective mRNAs or the promotion of their degradation.

The biogenesis of miRNAs involves multiple steps (Figure 4). Initially, miRNAs are transcribed by RNA polymerase II as primary miRNA (pri-miRNA). In the nucleus, the Drosha complex processes the pri-miRNA in 60-100 nt hairpin-shaped precursor miRNA (pre-miRNA). Via Exportin-5 these pre-miRNAs are exported to the cytoplasm. There they are processed by Dicer resulting in around 22 nt double-stranded RNA containing the mature miRNA strand and the passenger strand (Davis-Dusenbery and Hata 2010a). The mature miRNA promotes the assembly of the RNA-induced silencing complex (RISC). The RISC targets mRNAs and causes their translational repression or promotes their degradation.

Since miRNAs were described for the first time (Lee et al. 1993), numerous studies addressed the question where and how miRNAs may function. Several studies investigated the role of miRNAs in the *Drosophila* ovary (e.g.: Hatfield et al. 2005; Shcherbata et al. 2006; Vagin 2006; Shcherbata et al. 2007; Poulton et al. 2011; Kugler et al. 2013; Huang et al. 2013). It was shown that miRNAs are required for stem cell maintenance, as disruption of miRNA production in GSCs by inactivating miRNA processing enzymes leads to defects in stem cell self-renewal and reduced proliferation (Xie 2013). Some miRNAs have been shown to regulate the cell cycle, others may act downstream of insulin signaling controlling GSC proliferation (Xie 2013). As it was suggested that miRNAs may affect the responsiveness of cells to signaling molecules (Inui et al. 2010), it can be assumed that numerous other miRNAs are involved in tissue establishment and/or maintenance of *Drosophila* germarium architecture and its GSC niche.

1.2.1 The *miR-125* is a putative candidate for regulating niche and GSC fate

The first miRNA described was *lin-4* in *Caenorhabditis elegans* (*C. elegans*) (Lee et al. 1993). Years later, a second miRNA called *let-7* was described (Reinhart et al. 2000; Pasquinelli et al. 2000). Subsequent studies showed that these miRNAs regulate developmental timing in *C. elegans* (Rougvie 2005; Wienholds and Plasterk 2005). Both miRNAs, *let-7* and the fly homolog of *lin-4* (*miR-125*), have been found in *Drosophila* as well. It was shown that both miRNAs are part of a polycystronic locus on the left arm of the second chromosome. The locus is called *let-7-Complex* and encodes for three miRNAs: *let-7*, *miR-100* and *miR-125* (Figure 5) (Sokol et al. 2008). As in *C. elegans*, both *Drosophila* miRNAs, *let-7* and *miR-125*, were shown to regulate timing during development (Caygill and Johnston 2008; Sokol et al. 2008). Their transcription starts in late larval stage, has its peak in pupae while metamorphosis (Pasquinelli et al. 2000; Hutvagner 2001), and depends on ecdysone signaling (Sempere et al. 2002; Chawla and Sokol 2012). Earlier, it was shown that the steroid hormone ecdysone regulates a complex and hierarchical cascade of gene expression triggering the onset of metamorphosis (Thummel 1996). The important function of the miRNAs *let-7* and *miR-125* during *Drosophila* development has been shown as the artificial expression in early larval stages arrests development and leads to death (Caygill and Johnston 2008). Also a total loss of these miRNAs leads to defects in fly function. Mutants lacking miRNAs of the *let-7*-Complex were shown to have various defects in adult functions, including motility and fertility (Caygill and Johnston 2008; Sokol et al. 2008). Strikingly, sequences and developmentally regulation of *let-7* and *miR-125* are conserved among bilat-

erians (Pasquinelli et al. 2000; Sempere et al. 2003). This points to the putatively important function these miRNAs may have in all species. Both miRNAs are present in humans: the miRNA *let-7* is fully conserved and *miR-125* has three homologs.

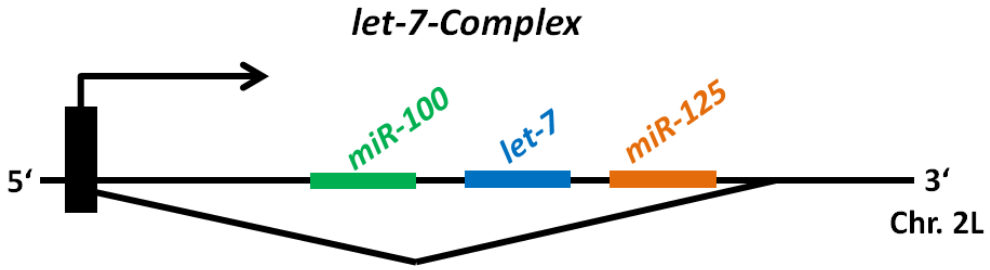

Figure 5: The *Drosophila let-7-Complex* locus. The *Drosophila let-7-C* is located on Chromosome 2 and encodes for three evolutionary conserved miRNAs: *miR-100*, *let-7* and *miR-125*. They are transcribed and regulated together.

In addition to their identified function during development, the miRNAs of the *let-7*-Complex are major examples of miRNAs associated with cancer (Nimmo and Slack 2009). *miR-125* was shown to have tumor-suppressor and tumor-promoter function dependent on the type of tumor (Sun et al. 2013). As cancer is thought to be a disease of stem cells, it is interesting to study the role of the *let-7-Complex* miRNAs in stem cell niche establishment and maintenance. A potential model for this is the GSC niche in the *Drosophila* ovary, as this model is very popular and widely used to study stem cells in their *in vivo* environment (Eliazer and Buszczak 2011).

First hints, that *let-7*-Complex miRNAs may carry out prominent regulatory roles in niche establishment and maintenance, were given by studies showing evidence that transcription of the *let-7*-Complex depends on ecdysone signaling (Sempere et al. 2002). As mentioned above, it was shown before that ecdysone signaling is essential for the initiation of niche formation (Hodin and Riddiford 1998). In addition it was shown that ecdysone signaling regulates niche size during development and controls differentiation of GSC daughters (König et al. 2011).

As already mentioned, the transformation of the primitive gonad into adult ovaries starts during late larval development (Eliazer and Buszczak 2011). At the same time in late larval development, the transcription of *let-7-Complex* miRNAs starts (Pasquinelli et al. 2000; Hutvagner 2001) initiated by ecdysone signaling (Sempere et al. 2002; Chawla and Sokol 2012). Thus, it can be assumed that ecdysone signaling promotes expression of *let-7-Complex* miRNAs in late larval development and thereby may affect niche formation.

This study focuses on the role of *miR-125* and investigates the impact of *miR-125* in establishment and maintenance of *Drosophila* germarium architecture.

1.3 General aims of this study

As mentioned above, it was shown before that ecdysone signaling initiates the formation of the GSC niche in *Drosophila* ovary in late larval development (Hodin and Riddiford 1998). At the same time, the transcription of the *let-7-Complex* miRNAs is activated due to ecdysone signaling (Sempere et al. 2002; Chawla and Sokol 2012). These findings strongly suggest a regulatory role for *let-7-Complex* miRNAs during the development of the GSC niche and in the establishment of *Drosophila* germarium architecture. Up to now, no studies have been performed to investigate the role of these particular miRNAs in processes governing germarium and GSC niche establishment and maintenance. Studies addressing this question may help to understand how miRNAs can be part of the complex mechanisms governing stem cell fate *in vivo*. In addition, they may give general orientation for further studies in mammalian model systems. This study focuses on the role of the *miR-125* only and aims to elucidate its role in the establishment and maintenance of germarium architecture and the GSC niche.

In this study, it is intended to proceed as follows. Firstly, the presence of the *miR-125* needs to be detected in respective cells of the adult *Drosophila* germarium. Secondly, this study aims to investigate the impact of alterations in *miR-125* levels on adult germarium architecture. Thirdly, the proof of a respective target, responsible for the putative effect of *miR-125* in establishment and maintenance of germarium architecture, is a further aim of this study. As it is known, miRNAs act via targeting mRNAs and thereby repress their translation or promote their degradation. The effect miRNAs may have on tissue architecture results downstream of its targets. If applicable, revealing the mechanism of function for *miR-125* may help to link the establishment of the obtained phenotype to processes during development. Thus, this study aims to reveal which general role miRNAs may carry out in stem cell niches and gives orientation for further studies in *Drosophila* and other model organisms.

To sum up, this study aims to address the following questions. Which impact has *miR-125* on the establishment and/or maintenance of the GSC niche in the *Drosophila* germarium? Via which downstream target *miR-125* acts?

2 Methods

2.1 Fly stocks

Stocks of Drosophila melanogaster were raised on standard cornmeal-yeast-agar-medium at 25°C, constant humidity and light dark cycle. When flies were kept at different temperatures this is stated. For crosses, virgin females and males were put together. After 2-3 days the parental generation was removed from the vial. Progeny was collected after hatching and selected for the correct genotype by the help of balancer chromosomes. Oregon R and/or w^{1118} were used as wildtype controls. For ectopic gene expression the Gal4/UAS method was used (Figure 6) (Brand and Perrimon 1993).

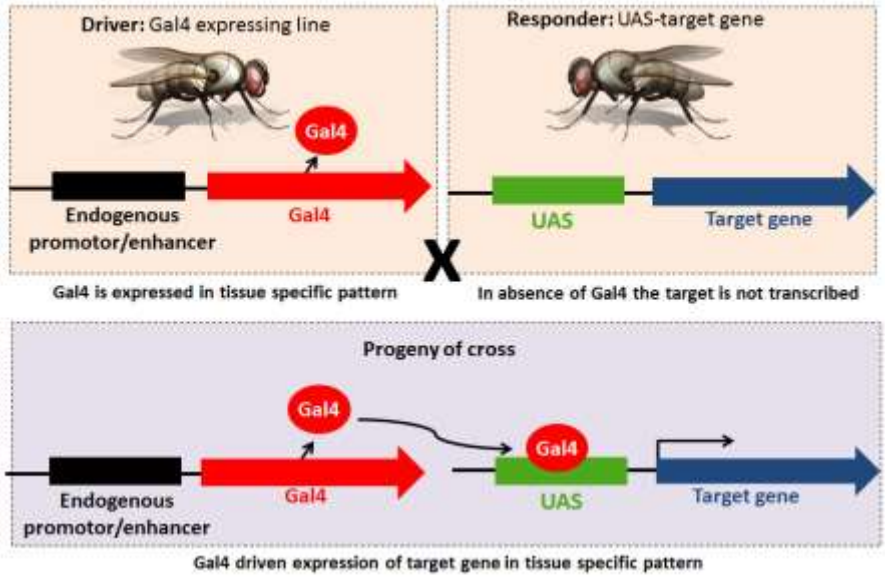

Figure 6: The Gal4 system. The Gal4/UAS system uses two transgenic lines: the driver line expresses the Gal4 protein and the responder line contains a UAS-construct. When both lines are crossed, progeny transcribes the Gal4 protein that can bind to the UAS-Gal4 binding site. Thus target gene transcription is promoted. Thereby, target gene expression can be directed according to the driver line's tissue specific expression pattern.

Table 1: Used fly stocks.

Stock	Source	Literature
Oregon R		
w^{1118}		
UAS-miR-125	Gift of Laura A. Johnston	Caygill and Johnston 2008
UAS-Tom RNAi H	Vienna Drosophila RNAi Center #36613	
UAS-Tom RNAi C/CyO	Vienna Drosophila RNAi Center #102652	
E(spl)mß-CD2; UAS-miR-125	generated in these studies	
UASp-Delta-2	gift of Hannele Ruohola-Baker	Jordan et al. 2006
UAS-Notch^{CA}	gift of Hannele Ruohola-Baker	Larkin et al. 1996

let-7-C^{GK1}/CyO; P{W8, let-7-C$^{ΔmiR-125}$}	gift of Nicholas S. Sokol	Sokol et al. 2008
let-7-C^{KO1}/CyO	gift of Nicholas S. Sokol	Sokol et al. 2008
miR-125^{LOF} (*let-7-C^{KO1} /let-7-C^{GK1}; P{W8, let-7-C$^{ΔmiR-125}$}*)	generated in these studies	
UAS-nLacZ, UAS-mCD8::GFP	gift of Frank Hirth	Diaper et al. 2013
E(spl)mβ-CD2/Cyo	gift of Wu Min Deng	Celis et al. 1998
E(spl)mβ-CD2, bab1gal4/TM6	generated in these studies	
bab1Gal4/TM6	Bloomington Stock Center #6803	
let-7-C^{GK1}/CyO	gift of Nicholas S. Sokol	Sokol et al. 2008
actin-Gal4/CyO	Bloomington Stock Center #3954	

LOF-Mutant: The *let-7-C^{KO1}* and *the let-7-C^{GK1}* mutants contain deletions of the *miR-100, let-7* and *miR-125* (Sokol et al. 2008). The transgenic construct introduced in *let-7-C^{GK1}* contains a *Gal4* coding sequence under control of the *let-7* complex promoter (*let-7-C-Gal4*). Animals referred to as *miR-125^{LOF}* have the following genotype: *let-7-C^{KO1} /let-7-C^{GK1}; P{W8, let-7-C$^{ΔmiR-125}$}* (containing *the P{W8, let-7-C$^{ΔmiR-125}$}* transgene that has the rescue construct *P{W8, let-7-C}* which contains a deletion removing the sequence for *miR-125*) (Sokol et al. 2008).

To express transgene constructs in the somatic cells of the germarium, the following drivers were used: *let-7-C^{GK1}* (Sokol et al. 2008) (drives expression in cap cells and escort cells) and bab1Gal4 (drives expression in terminal filament cells and niche). The expression patterns of the driver lines were confirmed by crossing to *UAS-nLacZ, UAS-mCD8::GFP.* For *miR-125* overexpression, *UAS-miR-125* was used. For the downregulation of Tom, *UAS-Tom RNAi H* and *UAS-Tom RNAi C* were used. The effects of the involvement of Notch pathway in the establishment of proper germarium architecture have been investigated by using *UASp-Delta 2* and *UAS-Notch^{CA}*. These crosses were kept at 29°C.

Notch activity: a Notch activity reporter line *E(spl)mβ-CD2* was generated before (Celis et al. 1998). The *E(spl)mβ* promoter and start-site of transcription (E (spl) is a well-characterized direct target of Notch signaling) was combined with the rat CD2 coding sequence. *E(spl)mβ-CD2; bab1Gal4* line has been crossed to *UAS-Tom RNAi H* and *UAS-miR-125* to monitor Notch activity. For control *E(spl)mβ-CD2; bab1Gal4* has been crossed to *w^{1118}*.

2.2 Immunohistochemistry

The flies were anaesthetized on ice-blocks and dissected in phosphate buffered saline (PBS) as described previously (König and Shcherbata 2013). Afterwards, the tissue was fixed while shaking in 4% formaldehyde (Polyscience, Inc.) diluted in PBS for 10 minutes. The staining procedure was performed as described before (König and Shcherbata 2013). The tissue was washed four times in PBT (PBS/ 0.2% Triton X-100) 15 minutes each. Next, the tissue was blocked in PBTB (PBT, 0.2% BSA, 5% Normal Goat Serum, 0.05% Sodium Azide) at room temperature. The primary antibody was added and incubated over night at 4°C. Afterwards, the tissue was washed with PBT four times, 15 minutes each, followed by blocking with PBTB for one hour at room temperature. Then, the secondary antibody was added and incubated for 2.5 hours at room temperature. Next, the samples were washed three times, 15 min each, including one wash containing DAPI (Sigma; 1µg/ml in PBT) to mark the cells' nuclei. After washing, the tissue was transferred in 70% glycerol, 2% NPG, PBS solution and mounted on slides prior to analysis. Pictures were taken by a confocal laser-scanning microscope (Zeiss LSM 700) and analyzed with Zeiss Axio Imager. Pictures were processed using Adobe Photoshop software (CS5).

Table 2: Used antibodies.

Antibody	Raised in	Dilution	Protein recognized	Antibody source
Anti-LaminC	mouse	1:20	Lamin C (marker for cap cells)	Developmental Studies Hybridoma Bank
Anti-Adducin	mouse	1:20	Hts/Adducin-like (marker for spectrosomes and fusomes)	Developmental Studies Hybridoma Bank
Anti-CD2	mouse	1:100	Rat CD2	Biolegend
Anti-Engrailed	mouse	1:20	Engrailed (marker for cap cells)	Developmental Studies Hybridoma Bank
Anti-β-Gal	mouse	1:1000	*E. coli* β-galactosidase	Developmental Studies Hybridoma Bank
Anti-Vasa	rabbit	1:5000	Vasa (marker for germline stem cells)	gift of Jaeckle department
Anti-PH3	rabbit	1:2000	phospho-Histone H3 (marker for mitosis)	Upstate Biotechnology
Anti-pMad	rabbit	1:5000	Phosphorylated Mothers against Dpp (pMAD) (marker for germline stem cells)	D. Vasiliauskas, S. Morton, T. Jessell and E. Laufer
Anti-Traffic jam	guinea pig	1:5000	Traffic Jam (marker for escort cells and cap cells)	D. Godt
Anti-GFP	chicken	1:1000	GFP	Invitrogen

The following secondary antibodies were used: Alexa 488-, Alexa 568- and Alexa 633-conjugated goat anti-mouse, -rabbit, -rat, -guinea pig or -chicken antibodies (1:500, Molecular Probes).

2.3 Quantitative RT-PCR (qRT-PCR)

The procedure was done as already published (Marrone et al. 2012). An appropriate number of CO_2-anaesthetized flies (6 to 10 flies) were put into TRIzol® (Ambion by life technologies™) and grinded. RNA-isolation was performed according to the manufacturer's protocol (PN 15596026.PPS). To determine the levels of miRNA expression, TaqMan MicroRNA assay (Applied Biosystems®) was used. 2S rRNA was used as endogenous control. The following assays were used: let-7 (dme-let-7, Cat. #4427975), miR-100 (dme-miR-100, Cat. #4427975), miR-125 (hsa-miR-125b, Cat. #4427975) and 2S rRNA (2S rRNA, Cat. #4427975). For the determination of mRNA levels Sybr Green master mix (Applied Biosystems®) was used. RpL32 (ribosomal Protein L32) was used as an endogenous control for mRNA. The following primers were used: RpL32 forward - AAGATGACCATCCGCCCAGC, reverse - GTCGATACCCTTGGGCTTGC; Tom forward - ATGTCGTTCATCACACG, reverse - ATGTCGTTCATCACACG. The Reverse Transcription was performed by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) and was performed according to the manufacturer's protocol using RNase Inhibitor (PN 4375575 Rev.E). For the reverse transcription a T100 thermocycler (Bio-Rad) was used. Amplification was done using a StepOne Plus thermocycler (Applied Biosystems®). All reactions were run in triplicate with appropriate blank controls. The one-tailed Student's t-test was used to determine statistical significance of the relative amount of miRNA, error bars represent the standard deviation from the mean.

2.4 LNA *in situ* hybridization for adult ovaries

The LNA (locked nucleic acid) *in situ* hybridization was performed for adult ovaries. Tissue was dissected in cold 1 x PBS. The procedure was performed according to a protocol for LNA in situ hybridization (Kucherenko et al. 2012) which was modified form the original RNA in situ protocol developed by the Berg laboratory (Zimmerman et al. 2013). Ovaries were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA) in 1 x PBS (0.1% DMSO) for 30 min, room temperature. Afterwards, the tissue was washed three times in 1 x PBS. Next, the tissue was gradually dehydrated by washing with 25-50-75-100% ethanol/PBT for 5 min each. The rehydration was done by washing in reverse order of ethanol/PBT concentrations. Next, ovaries were treated

with Proteinase K (3µg/ml, 50mM Tris-HCl, 50mM EDTA) for 15 min. Inactivation was performed by washing two times, 5 min each, in 0.2% glycine solution in 1 x PBS followed by 5min wash in 1 x PBS and two times for 5 min each in PBT. Afterwards, ovaries were post-fixed in 4% PFA in PBT for 30 min. Next, ovaries were washed two times, 5 min each, in PBT (0.1% DEPC). Then, ovaries were washed 3 x in PBT for 5 min each. Next, ovaries were washed in 1:1 PBT/ hybridization solution (1x HYB: 50% Formamide, 5x SSC, 50mg/ml Heparin, 0.1% Tween-20, 100mg/ml salmon sperm DNA, 100mg/ml yeast tRNA) for 5 min. Afterwards, ovaries were washed in 1 x HYB and prehybridized in 1 x HYB for 2-3 h at 54°C shaker. The preheated LNA probe was added in 1:1000 ratio in HYB and was incubated overnight on 59°C shaker. The miRCURY LAN probe was ordered from Exiqon company (hsa-miR-125b #18022-15 5'DIG-TCACAAGTTAGGGTCTCAGGGA-3'-DIG). Afterwards, ovaries were washed in HYB solution (without salmon sperm DNA and yeast tRNA, preheated to 59°C) for 20 min. Next, washing in 1:1 HYB/PBT was performed for 20 min. Ovaries were washed in PBT, 5 x for 5 min at 59°C. Afterwards, samples were blocked in Western Block (WB) solution (Sigma Aldrich) for 1 h and incubated with antibodies against DIG conjugated to HRP (Roche, #11207733910), diluted 1:2000 in WB solution, for 2 h, room temperature. FISH-Tyramide Signal Amplification was performed using TSA Cyanine 3 System (Perkin Elmer©, Inc. #1656398). Ovaries were washed in 1:1 PBT/WB solution, 6 x for 10 min each. Thereafter, samples were incubated in streptavidin-HRP solution (diluted 1:100 in PBR/WBR) for 1 h, room temperature. Next, ovaries were washed in PBT/WB solution, 6 x for 10 min each, next in PBT for 10 min and two times in PBS, 5 min each. Next, the ovaries were incubated for 2 h in Cyanine 3 Tyramide, diluted 1:50 in Amplification Dilutent at room temperature (protected from light). Then, ovaries were washed 6 x in PBS, 10 min each. Afterwards, samples were transferred into mounting medium (70% glycerol, 2% N-propyl gallate, PBS).

2.5 Target scan for *miR-125*

Possible targets for *miR-125* were predicted using TargetScan 6.0 (Kheradpour et al. 2007) and PicTar (Grün et al. 2005).

2.6 Luciferase reporter assay for Tom

The procedure was done as described previously (Yatsenko and Shcherbata 2014). To generate a Tom 3' UTR sensor, an about 300 base pair (bp) region with a possible binding site for *miR-125* was amplified from genomic DNA by polymerase chain reaction. The used primers con-

tained digestion sites for NotI and XhoI (additional bases for enzyme cut are underlined): forward - ATGCGGCCGCGCCTAAACATCGCCAGGATGC, reverse - CGGCTCGAGCGATAGTAACGCTTGATTGTG. Next the fragment was cloned into the psiCHECK-2 vector (Promega) by using NotI and XhoI restriction sites downstream of the *Renilla* luciferase gene. One day after being split 1:6, Drosophila S2R+ cells (DGRC) were seeded to 8 x 10^4 in a 96-well cell culture plate. The transfection took place using the Effectene transfection reagent (Qiagen). The following amounts have been used: 50ng of empty psiCHECK-2 (Promega), 50ng of psiCHECK-2 with the Tom- 3'UTR sensor, 25ng of act-Gal4, 50 ng of the pUAST-miR-125 plasmid (provided by Eric Lai, Bejarano et al. 2012). Around 72 h after transfection, the cells were subjected to the Dual-Glo luciferase assay (Promega). The control reporter (firefly) and *Renilla* luciferase (the altered 3'-UTR experimental reporter) levels have been measured to achieve optimal and stable results. A Wallac 1420 luminometer was used for analysis. For blank subtraction from raw luminescence counts non-transfected cells were used. Counts for control reporter (firefly luciferase) were normalized to experimental reporter (*Renilla* luciferase) counts. This way fold repression of *Renilla* luciferase activity was determined. The determined *Renilla* luciferase activity in the presence of the empty psiCHECK-2 plasmid was subtracted from that in the presence of the psiCHECK-2-Tom-3'UTR plasmid without the presence of transfected miRNA plasmids. Subsequently, *Renilla* luciferase activity was determined with the empty psiCHECK-2 and the psiCHECK-2-Tom- 3'UTR plasmid in the presence of the *miR-125*. The difference between these values was calculated. The difference in luminescence between the psiCHECK-2 plasmid and the psiCHECK-2-Tom-3'UTR plasmid in the presence of the *miR-125* was then normalized to the difference between the psiCHECK-2 plasmid and the psiCHECK-2-Tom-3'UTR plasmid without the *miR-125* to determine the fold reduction caused by the presence of the endogenous *miR-125*. All transfections were done in triplicate to determine an average and standard error of the mean of the data. The Student's two-tailed t-test was used to determine statistical significance.

2.7 TSA *in situ* hybridization in adult ovaries

The procedure of tissue preparation was performed similarly as previously described (Kucherenko et al. 2012). Ovaries were dissected in Drosophila cell culture medium and fixed for 30 min in freshly prepared PBS with 4% paraformaldehyde and 1% DMSO at room temperature. Afterwards, the tissue was washed three times in 1 x PBS. Next, the tissue was gradually dehy-

drated by washing with 25-50-75-100% methanol/PBT for 5 min each. The rehydration was done by washing in reverse order of methanol/PBT concentrations. Next, ovaries were treated with Proteinase K (3µg/ml, 50mM Tris-HCl, 50mM EDTA) for 15 min. The enzyme was inactivated by washing two times, each 5 min, with 0.2% glycine solution in 1 x PBS. This step was followed by 5 min wash in PBS and two times for 5 min each in PBT. Hereafter, brains were post-fixed in PBT with 4% paraformaldehyde for 30 min. Afterwards, ovaries were washed 2 x for 15 min in dH_2O, 3 x in PBT, 1 x in 1:1 PBT/hybridization solution (1x HYB: 50% Formamide, 5x SSC, 50mg/ml Heparin, 0.1% Tween-20, 100mg/ml salmon sperm DNA, 100mg/ml yeast tRNA) and 1 x in HYB, 5 min each. The samples were prehybridized in HYB at 55°C for one hour on the shaker. Afterwards, the preheated DIG-labeled RNA probe was added in a 1:50 ratio, diluted in HYB, and incubated with the ovary sample overnight on 55°C shaker. The RNA probe was produced with a DIG RNA Labeling Kit (SP6/T7) (Roche) following the manufacturer's protocol using T7 RNA polymerase. To obtain the probe against Tom mRNA the coding sequence of Tom (around 500nt) was amplified from genomic DNA. The 5' end of the primers for amplification contained additional nucleotides to introduce a T7-RNA polymerase promoter (additional nucleotides underlined; forward - TAATACGACTCACTATAGGGATGTCGTTCATCACACG, reverse - ATGTCGTTCATCACACG). Next day, the sample was washed at 65°C: 1 x in HYB, 1 x in 1:1 PBT/HYB (20 min each) and 5 x for 5 min in PBT. Next, the samples were blocked in Western Block (WB) solution (Sigma Aldrich) for one hour and incubated with antibodies against DIG conjugated to HRP (Roche, #11207733910), diluted 1:2000 in WB solution, for 2 hours at room temperature. Thereafter, FISH-Tyramide Signal amplification was performed using the TSA Cyanine 3 System (Perkin Elmer©, Inc. #1656398). Afterwards, ovaries were washed in 1:1 PBT/WB 6 x for 10 min each and then incubated in streptavidin-HRP solution (dilution 1:100 in PBR/WBR) for one hour, room temperature. Afterwards, ovaries were washed in PBT/WB solution six times and 10 min each. Next, the sample was washed for 10 min in PBT and two times in PBS, 5 min each. Then, the ovaries were incubated in a solution containing Cyanin 3 Tyramide, diluted 1:50 in Amplification Dilutent for 2 hours at room temperature (protected from light). Next, the samples were washed six times with PBS (10 min each). Finally, ovaries were transferred into mounting medium (70% glycerol, 2% N-propyl gallate, PBS), and mounted on slides prior to analysis.

2.8 Analysis and Statistics

The Analysis of ovarian pictures imaged by confocal microscopy was performed as described (König et al. 2011). Counted ovaries have been chosen randomly. For the determination of the number of cap cells (CpCs), LaminC-positive or Engrailed-positive cells on the apical end of the germarium were counted. LaminC positive CpCs and terminal filament cells (TFs) have been distinguished from each other by location and shaping: small round cells in contact with germline stem cells were counted as CpCs. Germline stem cells and their progeny were identified as Single spectrosome containing cells, positive for Adducin. Germline stem cells have been identified by nuclear accumulation of pMad. Single spectrosome containing cells not in contact with the niche have been counted specially. Average numbers and their average deviations were calculated using Microsoft Excel. For the statistical analysis the two tailed Student's *t*-test was used. Data comparisons were considered statistically significant if the $p < 0.05$. The following levels of significance were used: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3 Results

3.1 *miR-125* is expressed in *Drosophila* germarium

Aim of this study is to analyze the potential role of *miR-125* in the establishment and maintenance of *Drosophila* germarium architecture. Firstly, this study aims to confirm the expression of *miR-125* within the adult *Drosophila* germarium. Therefore the expression pattern of *miR-125* in the adult germarium was characterized. As mentioned in the introduction, *miR-125* is transcribed as part of a polycistronic gene complex , called *let-7-Complex.* This complex consists of three miRNAs (*miR-100*, let-7 and *miR-125*) which are transcribed and regulated together (Sempere et al. 2003; Sokol et al. 2008). A transgenic strain, expressing Gal4 under the control of the endogenous *let-7-Complex* promoter (Sokol et al. 2008), was used to drive expression of the *lacZ* gene encoding β-Gal. The presence of β-Gal was shown in cap cells and escort cells (see Figure 7A). Thus, it was concluded that the *let-7-Complex* is transcribed in the respective cells within the germarium.

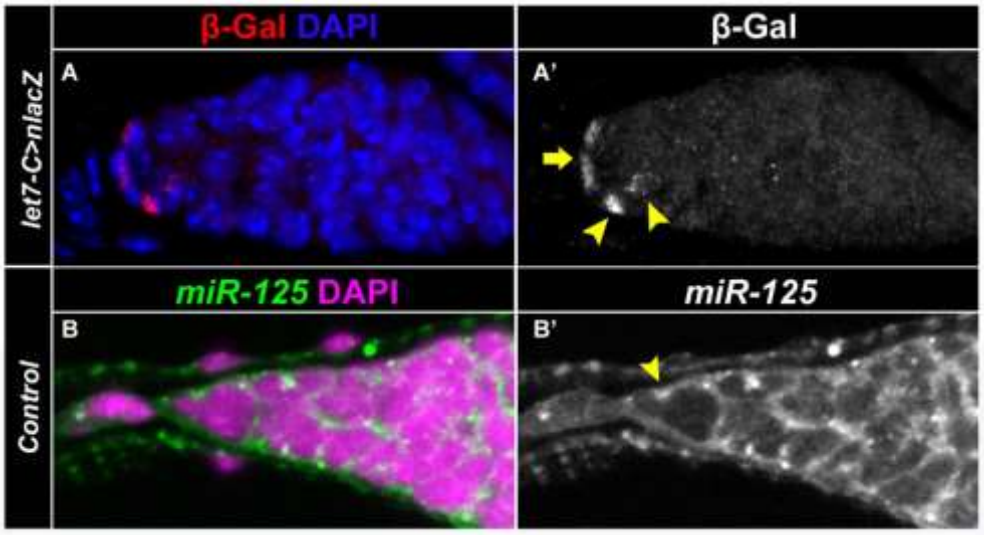

Figure 7: The *let-7-Complex* and *miR-125* are expressed in the germarium of Drosophila. (A) *nlacZ* expression driven by the endogenous promoter of the *let-7-Complex*, shows that the complex is transcribed in niche cells, including cap cells (arrow) and escort cells (arrowheads). **(B)** LNA *in situ* hybridization shows that *miR-125* is expressed in escort cells (arrowhead).

In vivo detection of mature miRNAs can be carried out by *in situ* hybridization using LNA-modified DNA probes (Wienholds 2005; Kloosterman et al. 2006). LNA belongs to a class of RNA analogs showing high hybridization affinity towards small RNA-molecules and thereby can detect the very short forms of mature miRNA. LNA *in situ* hybridization was used to show the presence of *miR-125* in *Drosophila* germarium. It can be shown that *miR-125* is present in simi-

lar cells (Figure 7B) as the *let-7-Complex* is expressed according to the driven expression of β-Gal (Figure 7).

Thus it was shown that the *let-7-Complex*, and thereby *miR-125*, is transcribed in the GSC niche in *Drosophila* ovary. After it has been shown that *miR-125* is present in the *Drosophila* germarium, it was investigated which function *miR-125* may carry out in the establishment and/or maintenance of germarium architecture and its GSC niche.

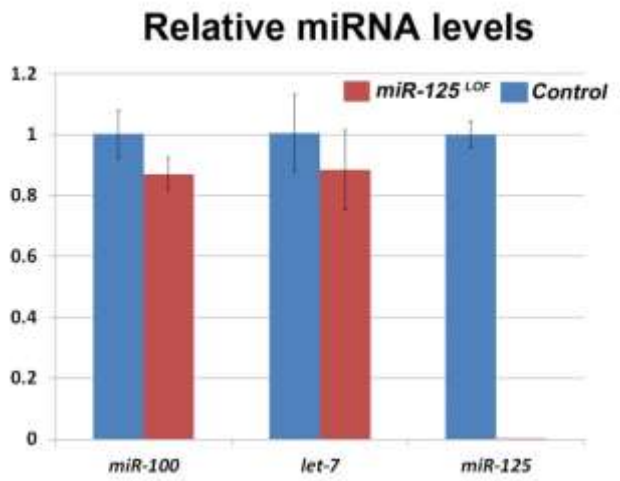

Figure 8: *miR-125* is absent in the *miR-125^{LOF}* mutant. Quantitative qPCR of adult male flies shows that the *miR-125^{LOF}* mutant has similar expression levels of *miR-100* and *let-7* as control flies (w^{1118}), however lacks the expression of *miR-125*. Error bars represent the standard deviation of the mean.

3.2 The loss of *miR-125* leads to an increase in niche size and hosted GSCs

To investigate the role of a particular miRNA, phenotypic analysis can be used. In general this is done by analyzing phenotypes resulting from the loss of an individual miRNA and overexpression of the same miRNA. In this study the phenotype due to the loss of *miR-125* has been investigated first. To generate a *miR-125*^{LOF} mutant, two *let-7-Complex* knockout strains were used: *let-7-C*^{KO1} and *let-7-C*^{GK1} (Sokol et al. 2008). In both strains a lack of *miR-100*, *let-7* and *miR-125* expression can be reported. The mutant used in this study has the rescue construct *P{W8, let-7-Complex$^{\Delta miR-125}$}* (Sokol et al. 2008), deriving from the recue construct *P{W8, let-7-Complex}*, which contains a deletion removing the sequence coding for *miR-125*. The respective *miR-125^{LOF}* mutant has the genotype *let-7-Complex^{KO1} /let-7-Complex^{GK1}; P{W8, let-7-Complex$^{\Delta miR-125}$}*. Quantitative RT-PCR from adult male flies was used to confirm that the *miR-125^{LOF}* mutant is expressing no *miR-125*. Results are shown in Figure 8. Compared with control (w^{1118}), the *miR-125^{LOF}* mutant shows similar expression levels of *miR-100* (*control* 1.00 ± 0.08; *miR-125^{LOF}* 0.87 ± 0.05) and *let-7* (*control* 1.01 ± 0.13; *miR-125^{LOF}* 0.88 ± 0.13). As expected, the mutant lacks the expression of *miR-125* (*control* 1.00 ± 0.05; *miR-125^{LOF}* 0.00 ± 0.00). To analyze niche

phenotypes, several markers were used to mark the different cell types. For somatic cap cells, a main component of the GSC niche, two markers were used: in wildtype cap cells the transcription factor Engrailed (En) and nuclear LaminC (LamC) are coexpressed. GSCs show nuclear accumulation of the phosphorylated transcription factor Mad (pMad). In addition, cells containing single spectrosomes and fusomes can be marked by Adducin (Add). Moreover, cap cells and escort cells can be marked by Traffic jam (TJ). For the analysis of phenotypes, germaria can be dissected and specifically immunostained for the mentioned markers (König and Shcherbata 2013).

Numbers for cap cells (CpCs), germline stem cells (GSCs), single spectrosome containing cells (SSCs) and Traffic jam positive cells (TJs) were counted. It has been shown that numbers for all mentioned celltypes increased significantly (levels of significance ≥ **) in the *miR-125*^{LOF} mutant when compared with control flies (Oregon R crossed to w^{1118}; see Figure 9G). In *control* 5.9 ± 0.3 (n=14) cells have been counted for CpCs and in the *miR-125*^{LOF} mutant 8.1 ± 1.4 (n=19) cells have been counted for CpCs (p-value < 10^{-3}). Thus a 1.4 fold increase in the number of cap cells was observed upon loss of *miR-125*. Strikingly, 84% of all mutant germaria hosted more than 6 cap cells. These data suggest that the loss of *miR-125* is sufficient to induce cap cell markers. Typically, the enlarged mutant niche expands laterally from its apical position in basal direction (see Figure 9B+D). It has been shown before that an increased number of cap cells correlates with an increased number of GSCs (Xie and Spradling 2000; Ward et al. 2006). In this study the 1.4 fold increase in the number of cap cells is corresponding to a 1.8 fold increase in the number of GSCs (*control* 2.6 ± 0.5 (n=11); *miR-125*^{LOF} mutant 4.6 ± 0.5 (n=5); p-value < 10^{-5}). For SSCs a 1.5 fold increase (control 4.1 ± 0.8 (n=14); *miR-125*^{LOF} 6.3 ± 0.9 (n=19); p-value < 10^{-5}) was observed and for TJs a 1.5 fold increase (control 18.5 ± 2 (n=8); *miR-125*^{LOF} 27.5 ± 5.5 (n=8); p-value < 10^{-2}) was observed. To sum up, the data show that the single loss of *miR-125* causes severe changes in the architecture of the *Drosophila* germarium. Strikingly, these changes affect the GSC niche in particular by an increase in its size and a gain in the number of hosted stem cells.

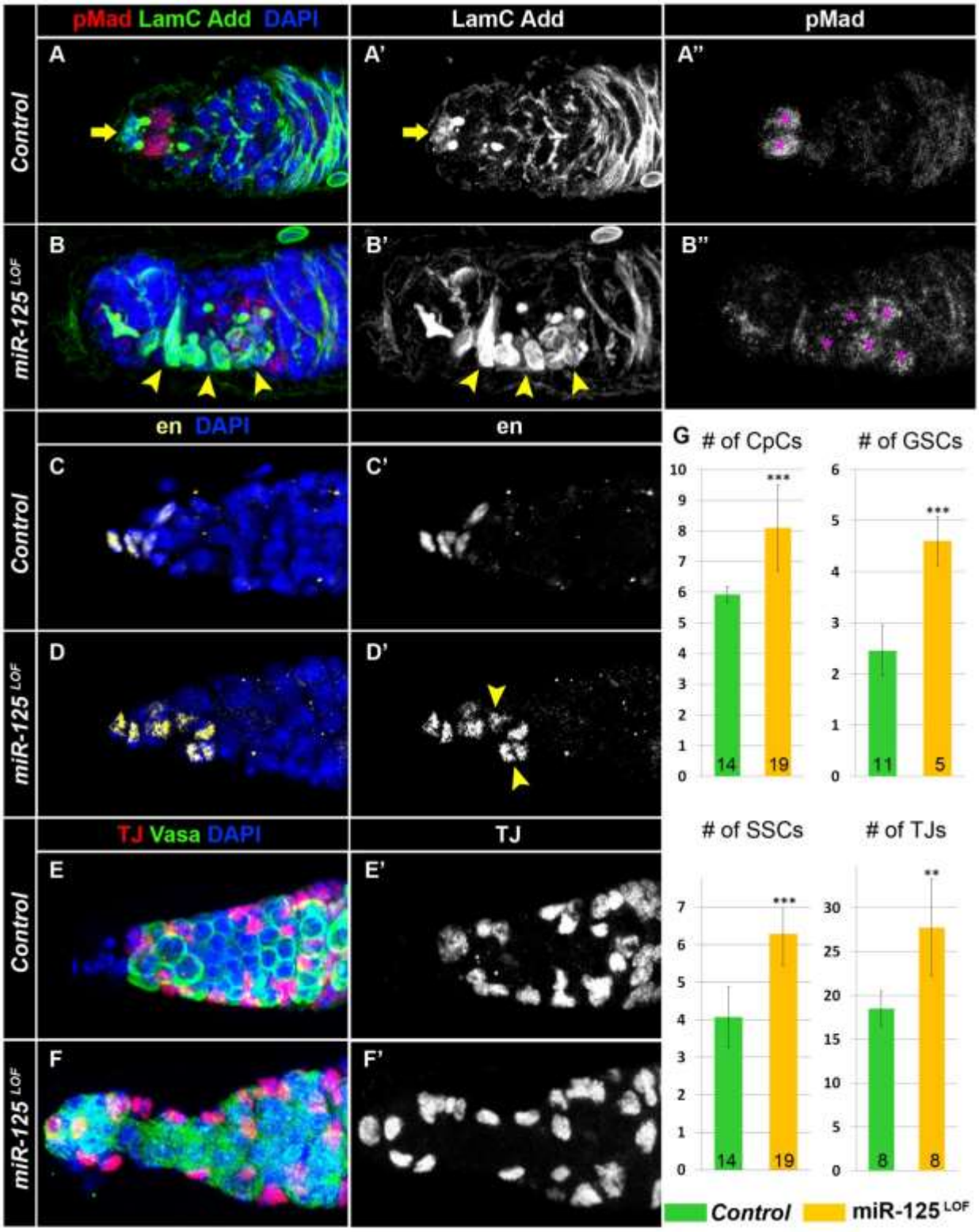

Figure 9: The loss of *miR-125* affects the architecture and organization of the germarium. (A) In control germarium, two GSCs are present. GSCs are marked by the single spectrosome (A'; stained with Adducin, apical dot-like structure) and nuclear accumulation of the stem cell marker pMad (A''; asterisks). The GSCs are directly attached to the niche (marked with LaminC; arrow). **(B)** In contrast to control, the *miR-125^{LOF}* mutant shows an expanded niche in basal direction (arrowheads). The niche cells are marked with LaminC. The expanded niche attracts an increased number of attached GSCs, marked by single spectrosomes (A'; positive for Adducin, dots) and presence of pMad (A''; asterisks). **(C)** Cap cells in the control germarium, marked with Engrailed (en), are located at the apical tip of the germarium. **(D)** In the *miR-125^{LOF}* mutant, the niche shows a higher number of Engrailed positive cells

in comparison to the control flies. The niche expands in basal direction (D'; arrowheads). **(E)** A control germarium is stained against Traffic jam (TJ; marks escort cells and cap cells) and Vasa (marks GSCs and their progeny). **(F)** The *miR-125^{LOF}* mutant shows an increased number of escort cells and cap cells positive for TJ when compared to control flies. **(G)** Statistical analysis for the numbers of counted cap cells (CpCs), germline stem cells (GSCs), single spectrosome containing cells (SSCs) and Traffic jam positive cells (TJs) in *control* and the *miR-125^{LOF}* mutant germaria. All numbers are increased for the *miR-125^{LOF}* mutant when compared to control. Error bars represent average deviations. Significance is calculated using a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3 Overexpression of *miR-125* phenocopies loss of *miR-125*

After having analyzed the phenotype for the mutant lacking *miR-125*, the phenotype obtained by overexpression of *miR-125* was investigated. The two driver lines, used in this study for overexpression of *miR-125,* were *let-7-C^{GK1}* for endogenous overexpression and *bab1Gal4* for ectopic overexpression. The expression pattern for these driver lines was confirmed by crossing to a *UAS-nlacZ* responder line. According to the obtained expression pattern, *let-7-Complex^{GK1}* drives expression of target sequences in cap cells and escort cells and *bab1Gal4* drives expression of target sequences in terminal filament cells, cap cells and escort cells (Figure 10). *Let-7-C^{GK1}* is assumed to be a weaker driver than the *bab1Gal4* driver, as the *bab1Gal4* driver has been proven to be a powerful tool for expression of target sequences in terminal filament, cap cells, escort stem cells and escort cells (Bolívar et al. 2006).

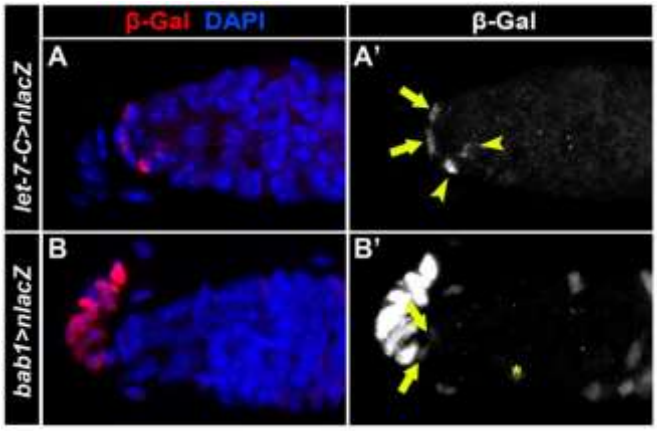

Figure 10: Expression patterns of *bab1Gal4* and *let-7-C^{GK1}* driver lines. (A) *nlacZ* expression driven by the endogenous promoter of the *let-7-Complex*, shows that the complex is transcribed in niche cells, including cap cells (arrow) and escort cells (arrowheads). **(B)** *nlacZ* expression driven by *bab1Gal4,* shows expression in terminal filament cells, cap cells (arrows) and escort cells (asterisk).

Numbers for CpCs, GSCs, SSCs and TJs were counted and compared with respective control counts. In comparison to control, all counted cell types showed a significant increase (levels of significance ≥ **) in their number upon overexpression of *miR-125* (see Figure 11G). For CpCs, overexpression with both drivers resulted in a 1.3 fold increase in comparison to control (*control* 5.9 ± 0.3 (n=14); *let-7-C>miR-125* 7.5 ± 0.8 (n=18); *bab1Gal>miR-125* 7.8 ± 0.5 (n=9); *p*-values: $p < 10^{-5}$ and $p < 10^{-6}$). Strikingly, for endogenous overexpression 89% of all germaria had more than 6 CpCs and for ectopic overexpression all analyzed germaria had more than 6 CpCs. Therefore, the overexpression of *miR-125* with both drivers gave rise to larger niches. Typically,

the niches were expanding laterally from apical in basal direction (see Figure 11B). These enlarged niches were hosting an increased number of GSCs (see Figure 11D). For endogenous overexpression a 1.3 fold increase in the number of GSCs was counted, and for ectopic overexpression a 1.3 fold increase in the number of GSCs was counted (*control* 3 ± 0.5 (n=11); *let-7-C>miR-125* 4.1 ± 0.4 (n=9); *bab1Gal4>miR-125* 4 ± 0.6 (n=7); *p*-values: $p < 10^{-4}$ and $p < 10^{-3}$). These data suggest that isolated overexpression of *miR-125* is sufficient to induce an enlarged GSC niche, hosting an increased number of GSC.

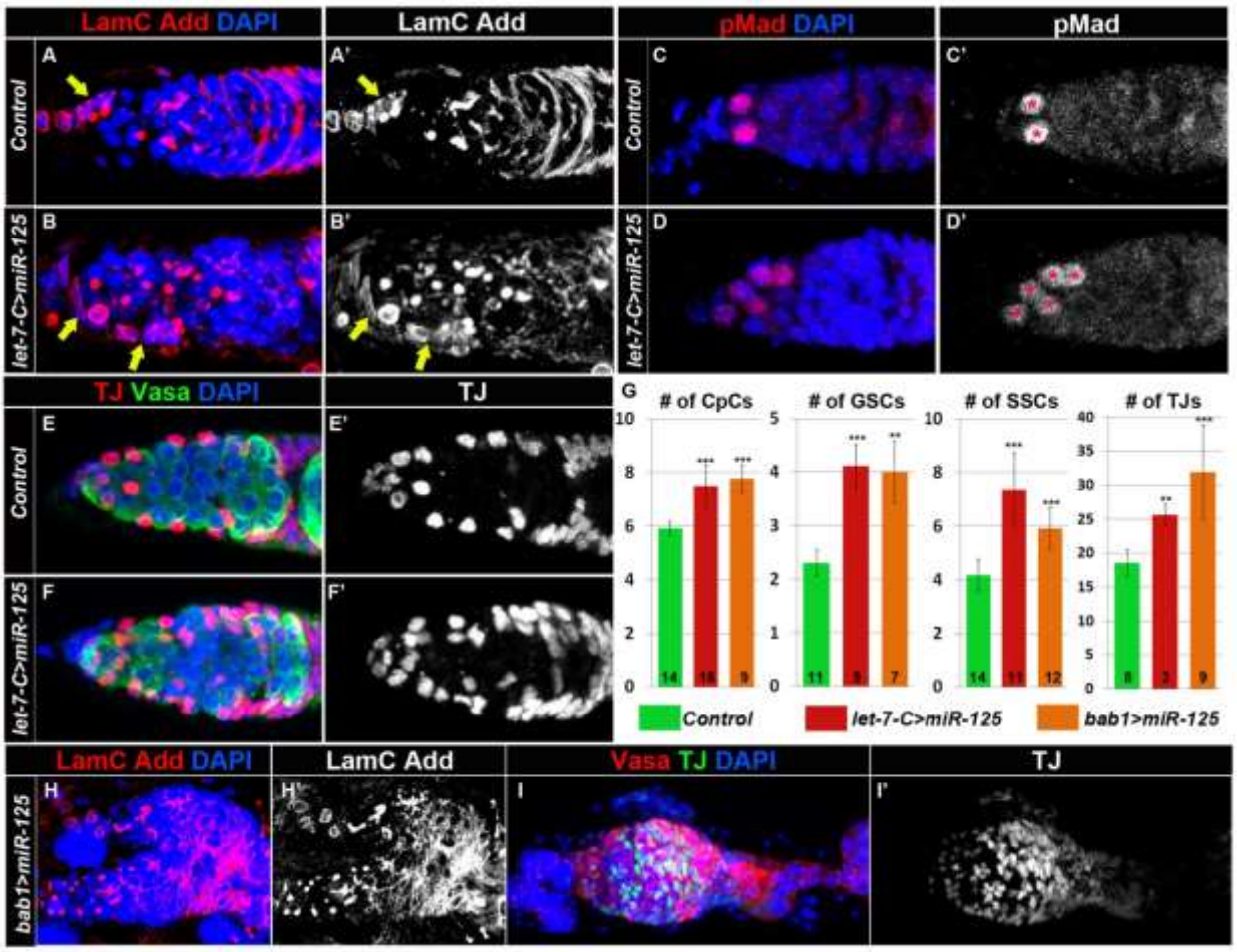

Figure 11: Overexpression of *miR-125* affects the architecture of the germarium. (A) Niche cells in control germarium are marked by LaminC and single spectrosomes are marked by Adducin. The arrow points to the position of the GSC niche (apical tip of the germarium). **(B)** In comparison to control, the size of the niche (arrows) and the number of cap cells are increased when *miR-125* is overexpressed using the *let-7-Complex* endogenous driver. The niche expands laterally from apical to basal direction. **(C)** In the control germarium, two GSCs (asterisks) are attached to the niche. The GSCs are marked by nuclear accumulation of the GSC marker pMad. **(D)** In comparison to control, endogenous overexpression of *miR-125* increases the number of GSCs positive for pMad (asterisks). **(E)** A control germarium is shown. Traffic jam (TJ) positive cells represent escort and cap cells. Vasa marks the germline lineage. **(F)** In comparison to control, an increase in the number of escort and cap cells (positive for TJ) is shown when *miR-125* is overexpressed with the *let-7-Complex* endogenous promoter. **(G)** Statistical analysis for the numbers of counted cap cells (CpCs), germline stem cells (GSCs), single spectrosome containing cells (SSCs) and Traffic jam positive cells (TJs) in control germaria and germaria overexpressing *miR-125* with *let-7-C-Gal4* and *bab1Gal4*. In comparison to control, the number of all cells is increased significantly when *miR-125* is overexpressed. Error

bars represent average deviations. Significance is calculated using a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(H,I)** Ectopic overexpression of *miR-125* with the *bab1Gal4* driver causes severe changes in germarium architecture (fused germaria, increased cyst formation and abnormally shaped germaria lacking egg production). **(H)** A germarium consisting of two apical tips is shown. Both tips seem to fuse in the late regions of the germarium. **(I)** The size of the germarium is increased dramatically due to ectopic overexpression of *miR-125*. A large accumulation of TJ positive cells can be shown (I').

For SSCs a 1.75 fold increase in comparison to control was observed when *miR-125* was over-expressed with its endogenous driver, and for ectopic overexpression a 1.5 fold increase was observed (*control* 4 ± 0.8 (n=14); *let-7-C>miR-125* 7 ± 1.4 (n=11); *bab1Gal4>miR-125* 6 ± 0.8 (n=12); *p*-values: $p < 10^{-5}$ and $p < 10^{-3}$). The number of TJs was increased 1.4 fold upon endogenous overexpression and 1.7 fold upon ectopic overexpression (*control* 18.5 ± 2 (n=8); *let-7-Complex>miR-125* 25.7 ± 1.6 (n=3); *bab1Gal4>miR-125* 31.9 ± 7 (n=9); *p*-value: $p < 10^{-2}$ and $p < 10^{-3}$). In rare cases, strong phenotypes could be observed when *miR-125* was ectopically over-expressed with *bab1Gal4* (see Figure 11H+I). In these cases, the architecture of the whole germarium was abnormal: fused germaria, increased cyst formation and abnormally shaped germaria lacking egg production were observed. One of the examples shows a germarium consisting of two apical tips (see figure 11H). The two tips fuse in later regions of the germarium. It remains elusive if both apical tips host functional GSC niches. The other germarium is enlarged dramatically and shows an abnormal accumulation of TJ positive cells (see Figure 11I).

To test if *miR-125* acts by promoting division of the respective cell types in adult flies, the presence of PH3, a mitosis marker, was tested. In this study no cap cell has been shown to be positive for PH3, neither in control nor in *miR-125* overexpressing flies. Similarly, none of TJ positive cells could be shown to be positive for PH3. Thus, the increased number of cells is most likely not due to a higher rate of divisions in adult flies. Putatively, those changes are mainly due to alterations in developmental processes. Also it is possible that the higher number of these cells is due to transformation from other cell types (e.g. somatic cells) to the counted ones. Both hypotheses need to be tested, but this is not part of the present study. However, staining with anti-PH3 showed that there are more dividing cells within the germarium, which were not positive for TJ. Possibly, this is due to the increased number of functional hosted GSCs giving rise to a larger number of also dividing progeny. As it was shown, the division of the germline depends on various factors, especially on nutritional conditions and the health status of the fly (Kirilly and Xie 2007). Due to a multitude of possible external factors, this increased rate of division

was not investigated further, however may explain that germaria were enlarged in size when *miR-125* was overexpressed.

The data show that isolated overexpression of *miR-125* has a severe influence on germarium architecture. Strikingly, the changes in the germarium architecture copy those observed in the *miR-125*^{LOF} mutant. Both, loss and overexpression of *miR-125*, is sufficient to affect the GSC niche notably by an enlargement in size and an elevation in the number of hosted stem cells. Thus, *miR-125* seems to be an important player in the establishment and/or maintenance of germarium architecture and its GSC niche. To display the mechanism of *miR-125* function, the targets of the miRNA need to be characterized.

Table 3: Tom, a protein involved in the regulation of Notch signaling, is a possible target for *miR-125.* The table shows predicted targets for *miR-125*. Highlighted in red is the target of interest: Tom, a protein involved in the regulation of Notch signaling. Notch signaling was shown to affect germarium architecture.

Target gene name	Biological processes
CG4615	neurogenesis; phagocytosis, engulfment
lab	positive regulation of transcription from RNA polymerase II promoter; embryo development; brain development; brain segmentation
RhoGAP100F	cytoskeletal matrix organization at active zone; maintenance of presynaptic active zone structure
RpL10Ab	mitotic spindle elongation; mitotic spindle organization; centrosome duplication
Tom	sensory organ precursor cell fate determination; sensory organ development; establishment of planar polarity; **Notch signaling pathway**; cell fate specification
CG12932	synaptic transmission; neuromuscular synaptic transmission; regulation of synaptic plasticity; neurotransmitter secretion; adult locomotory behavior; cytoskeletal matrix organization at active zone; maintenance of presynaptic active zone structure; short-term memory; anesthesia-resistant memory
CG13157	unknown
CG13646	amino acid transmembrane transport
CG18265	unknown
CG9518	lateral inhibition
Pglym87	Phosphoglycerate mutase, glycolysis
rab3-GEF	activation of MAPK activity; regulation of apoptotic process; regulation of cell cycle
CG10359	signal transduction
CG12701	mitotic cell cycle; positive regulation of transcription, DNA-dependent; cellularization
CG13908	Glucose transmembrane transport
CG16799	Lysosomal activity, defense response
CG2061	G-protein coupled receptor signaling pathway
CG30084	muscle structure development
CG31700	unknown
CG32432	unknown
CG6175	inter-male aggressive behavior; imaginal disc-derived wing morphogenesis
CG8486	cellular response to mechanical stimulus; mechanosensory behavior
CG9619	unknown

3.4 Tom, a regulator of Notch pathway, is a possible target for *miR-125*

miRNAs can inhibit the translation and/or promote the degradation of target mRNAs, that is why miRNAs are known to be negative regulators of gene expression. To reveal the mechanism of function for *miR-125,* possible targets need to be identified. For target prediction TargetScan 6.2 (Kheradpour et al. 2007) and PicTar (Grün et al. 2005) were used. With TargetScan 23 conserved targets for *Drosophila miR-125* could be found (Table 3), PicTar identified 29 targets. Each target can be associated with a specific biological process; these were ascertained using FlyBase (St. Pierre et al. 2013). In Table 3 possible targets and their involvement in respective biological processes are listed. This study focusses on the possible target Tom (also known as Twin of m4, Barbu), which can be annotated with Notch signaling. As mentioned in the introduction, earlier studies showed that Notch signaling is required for the formation of niches and their maintenance in the *Drosophila* ovary (Xie 2013). Moreover, former work showed that activated Notch in somatic cells leads to additional CpCs and their expansion within the germarium; this in turn leads to an increased number of GSCs (Ward et al. 2006; Song et al. 2007). Similar phenotypes (increase in the number of CpCs and GSCs) were observed in this study for loss and overexpression of *miR-125*. This suggests that *miR-125* acts via Tom and thereby influences Notch signaling to cause the obtained phenotypes.

Tom is a member of the Bearded family, so far this family has been found in insects only (Lai et al. 2000b; Lai et al. 2005; Schlatter and Maier 2005). All encoded proteins of this family are very small (70-218 amino acids in *Drosophila*) and are known to antagonize Notch signaling (Lai et al. 2000a). Bearded family members interact with the E3 ubiquitin ligase Neuralized (Bardin and Schweisguth 2006; Fontana and Posakony 2009), operating in the signal sending cells. Upon their ubiquitination, the Notch ligands Delta and Serrate are internalized; this step is essential for their activation (Lai et al. 2001; Yeh et al. 2001; Pitsouli 2005). The activated ligand can bind and interact with Notch resulting in the cleavage of the intracellular domain of Notch and its translocation into the nucleus. In the nucleus, interaction with the transcriptional factor Suppressor of Hairless (Su(H)) takes place and downstream targets are transcribed (Tien et al. 2009), among which is the *Bearded complex* (Lai et al. 2000a). This way lateral inhibition can be promoted: the cell receiving Notch signaling inhibits the activation of own ligands (Bardin and Schweisguth 2006). Overexpression of Tom has been shown to block Neuralized dependent Notch signaling by competitive inhibition (Bardin and Schweisguth 2006). It can be considered

that *miR-125* inhibits Tom and thereby promotes Neuralized dependent activation of the Notch signaling pathway.

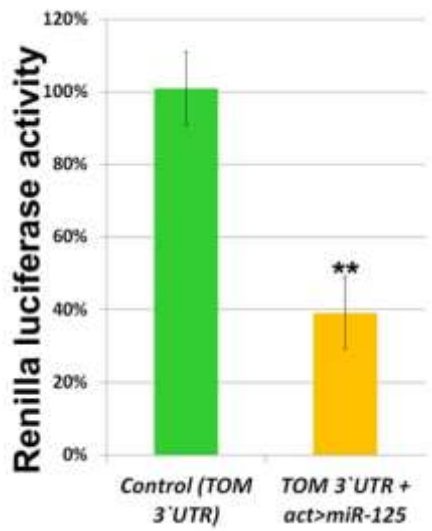

Figure 12: *miR-125* targets *Tom 3'UTR in vitro.* Ectopic expression of *miR-125* can downregulate *Tom-3'UTR* luciferase reporter *in vitro*. Relative downregulation of luciferase activity (average ± standard error of the mean): Control 1.01 ± 0.1; *act>miR-125* 0.39 ± 0.14. Significance is calculated using a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To validate that Tom can be a direct target of *miR-125,* a luciferase assay was performed *in vitro*. The assay is based on *miR-125*'s binding to its specific target site in the Tom *3' UTR*. Binding would repress the production of the reporter protein luciferase and reduce the measured luciferase activity in comparison to control (Kuhn et al. 2008). The obtained data show that the luciferase reporter activity was reduced 2.6 fold upon overexpression of *miR-125* (*control* 1.01 ± 0.1; act>*miR-125* 0.39 ± 0.14; *p*-value: $p<10^{-2}$; see Figure 12). Thus, Tom levels can be directly regulated by *miR-125*.

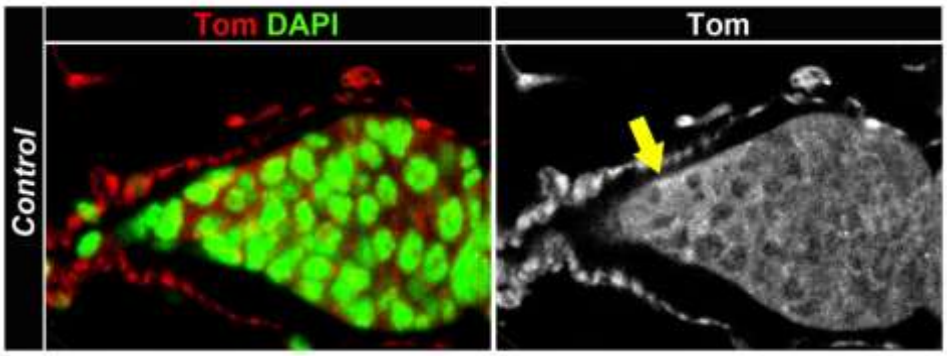

Figure 13: mRNA of Tom is present in the germarium. TSA *in situ* hybridization shows that Tom mRNA is present in escort cells (arrow).

3.5 Downregulation of Tom by RNAi phenocopies loss and overexpression of *miR-125*

The working hypothesis is that the observed phenotypes in *Drosophila* germarium are due to downregulation of Tom by *miR-125*. To verify this hypothesis, the presence of Tom mRNA has been shown in the area of interest by *in situ* hybridization using Tyramide Signal Amplification

(TSA). Obtained results show that Tom mRNA can be detected in escort cells (see Figure 13). For phenotypic validation of Tom as a target of *miR-125*, RNA interference (RNAi) has been used. The knockdown of Tom mRNA via TomRNAi can be expected to phenocopy Tom mRNA knockdown via *miR-125*. The Vienna *Drosophila* RNAi Center lists two different lines for TomRNAi: *UAS-TomRNAi C* and *UAS-TomRNAi H*. Both lines were tested for their ability to downregulate Tom mRNA. The *actin* driver line (ubiquitous expression) was crossed to the respective TomRNAi lines. Resulting Tom mRNA levels in adult male flies were measured by quantitative RT-PCR and compared with control (w^{1118}; see Figure 14). *UAS-TomRNAi H* has been shown to downregulate the level of Tom mRNA with a higher effect (relative level in comparison to control: *control* 1.00 ± 0.04; *act>TomRNAi H* 0.45 ± 0.01; *act>TomRNAi C* 0.70 ± 0.04; *p*-values: $p<9.1\text{x}10^{-3}$ and $p<1.5\text{x}10^{-3}$). Thus, only *UAS-TomRNAi H* was used in this study and is referred to as *UAS-TomRNAi*. For phenotypic analysis, this TomRNAi line was crossed to the endogenous driver line of the *let-7-Complex* resulting in TomRNAi expression in the same cells where *miR-125* is normally expressed. To verify the drawn hypothesis, obtained results are expected to phenocopy *miR-125* overexpression with the endogenous *let-7-Complex* driver line.

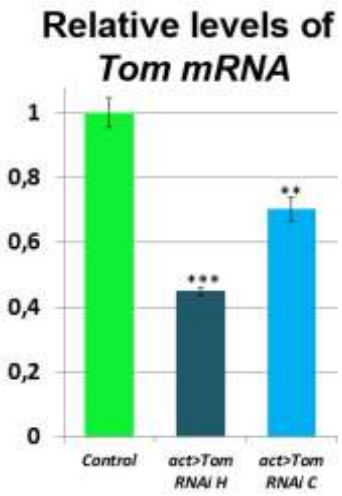

Figure 14: Tom can be downregulated using RNAi lines. Ubiquitous expression of TomRNAi, using *actGal4* (*act>TomRNAi*), significantly downregulates Tom mRNA in the total RNA obtained from whole flies. The TomRNAiH-line is shown to downregulate the level of Tom mRNA more effective. *Control* 1.00 ± 0.04; *act>Tom RNAiH* 0.45 ± 0.01; *act>TomRNAiC* 0.70 ± 0.04. Error bars represent the standard deviation of the mean. Significance is calculated using a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Numbers of CpCs, GSCs, SSCs and TJs were counted and compared with respective control counts and to *miR-125* overexpression by its endogenous *let-7-Complex* driver. Data show that expression of TomRNAi leads to a significant (levels of significance ≥ **) increase in the number of all counted cell types when compared with control, similarly to results achieved for overexpression of *miR-125* (see Figure 15G). Expression of TomRNAi and overexpression of *miR-125* lead to an increased number of CpCs (*control* 5.9 ± 0.3 (n=13), *let-7-C>miR-125* 7.5 ± 0.8 (n=16); *let-7-C>TomRNAi* 7.3 ± 0.8 (n=4); *p*-values: $p<10^{-5}$ and $p<10^{-2}$). The niche expands laterally from

apical to basal direction (compare Figure 15B'+C'). The expanded niche hosts a higher number of GSCs (compare Figure 15B''+C''; *control* 2 ± 0.2 (n=11), *let-7-C>miR-125* 4 ± 0.4 (n=9); *let-7-C>TomRNAi* 3 ± 0.4 (n=4). *p*-values: $p<10^{-4}$ and $p<10^{-2}$). The numbers of SSCs and TJs increase (SSCs: control 4 ± 0.6 (n=14), *let-7-C>miR-125* 7 ± 1.4 (n=11); *let-7-C>TomRNAi* 6 ± 0.8 (n=4); *p*-values: $p<10^{-5}$ and $p<10^{-2}$*;* TJs: control 18.5 ± 2 (n=8),), *let-7-C>miR-125* 25.7 ± 1.6 (n=3); *let-7-C>TomRNAi* 26.3 ± 1.5 (n=10); *p*-values: $p<10^{-2}$ and $p<10^{-5}$).

Obtained data show that expression of TomRNAi phenocopies *miR-125* overexpression: both lead to an expansion of the GSC niche and an increase in the number of hosted GSCs. Moreover, number of SSCs and TJs are elevated in both cases. This data strongly suggest that *miR-125* acts via downregulation of Tom.

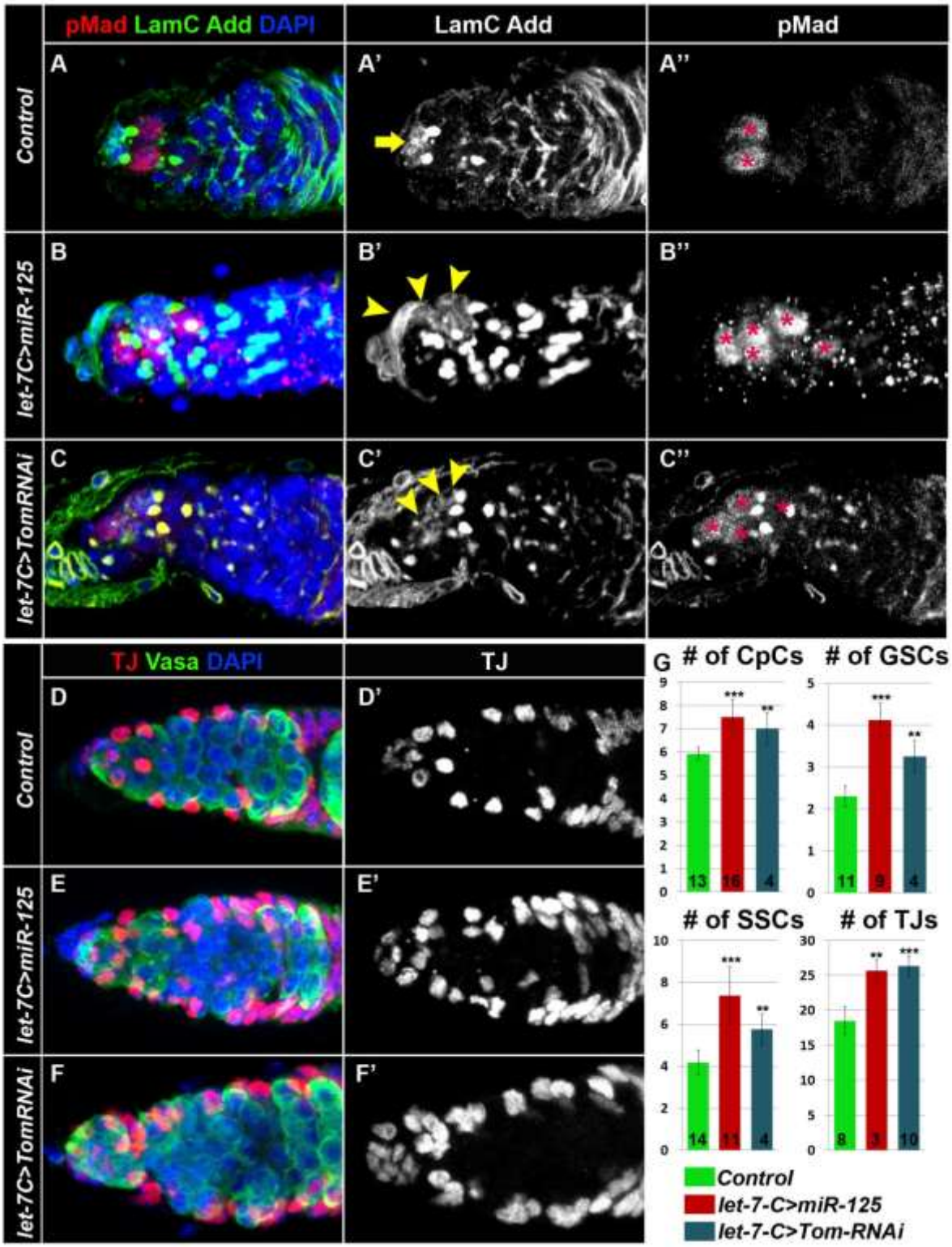

Figure 15: Misexpression of Tom affects the germarium architecture similarly to *miR-125* overexpression. (A) In control germarium, niche cells are marked by LaminC (LamC) and single spectrosomes are marked by Adducin (Add). The arrow (A') points to the position of the niche at the apical tip of the germarium. Two germline stem cells (GSCs) are attached to the niche and show nuclear accumulation of the GSC marker pMad (A''; asterisks). **(B)** In comparison to control, the number of cap cells (CpCs) is increased when *miR-125* is overexpressed by the *let-7-Complex* endogenous driver. The niche expands laterally from apical to basal direction (A'; arrowheads). The expanded niche attracts an increased number of GSCs, marked by nuclear accumulation of pMad (A''; asterisks). **(C)**

TomRNAi expression using the *let-7-Complex* endogenous promoter leads to similar phenotypes as observed for overexpression of *miR-125*. Germaria show higher number of CpCs, the niche expands laterally from apical to basal direction (C'; arrowheads). The expanded niche hosts an increased number of GSCs, marked by pMad (A''; asterisks). **(D)** A control germarium is shown. Traffic jam (TJ) positive cells represent escort and cap cells. Vasa marks the germline lineage. **(E)** In comparison to control, the numbers of TJ positive cells increase when *miR-125* is overexpressed using the *let-7-Complex* driver. **(F)** In comparison to control, *TomRNAi* expression using the *let-7-Complex* driver leads to a gain in the number of TJ positive cells. Similar phenotypes can be obtained when *miR-125* is endogenously overexpressed (compare to E). **(G)** Statistical analysis for the numbers of counted cap cells (CpCs), germline stem cells (GSCs), single spectrosome containing cells (SSCs) and Traffic jam positive cells (TJs) in control germaria, germaria with endogenous overexpression of *miR-125* and *Tom-RNAi* expression using the *let-7-C* driver. The data show that expression of *TomRNAi* phenocopies endogenous overexpression of *miR-125*. In comparison to control, both lead to a significant increase in the numbers of all counted cell numbers. Error bars represent average deviations. Significance is calculated using a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6 Overexpression of *miR-125* is able to modify Notch activity pattern, and alteration in Notch signaling show similar phenotypes as loss and overexpression of *miR-125*

Tom has been shown to inhibit Neuralized-dependent endocytosis of Delta (Bardin and Schweisguth 2006). This endocytosis of the ligand is essential for its activation and only activated Delta can bind to Notch and can promote Notch signaling. Correspondingly, up- or downregulation of Tom is expected to have an effect on the activity of Notch signaling. Notch signaling can be monitored by using Notch reporter lines. In this study *E(spl)mβ-CD2/Cyo* (Celis et al. 1998) is used. The *Enhancer of split complex* (*E (spl)-C*) is a well-characterized direct target of Notch signaling. For the used line, the *E(spl)mβ* promoter and start-site of transcription was combined with the rat CD2 coding sequence. Accordingly, using the *E(spl)mβ-CD2/Cyo* line Notch active cells were detected by immunohistochemistry using antibodies against rat CD2 protein. In this study, a reporter line was additionally generated containing the *bab1Gal4* driver. The obtained reporter line was crossed to *UAS-miR125* (*E[spl]:CD2/+; bab1>miR-125*). For control *E(spl)mβ-CD2; bab1Gal4* has been crossed to w^{1118}. This way, Notch activity was monitored for ectopic overexpression of *miR-125*. The genotype *E[spl]:CD2/+; bab1>miR-125* was selected because ectopic overexpression of *miR-125* with *bab1Gal4* has been shown to provoke the strongest phenotypes (see Figure 11G+15G). Respective germaria were stained with TJ, for identification of cap cells and escort cells, and CD2 to monitor Notch activity (see Figure 16A+B). Based on this staining, the number of cells positive for both TJ and CD2 was counted, representing the cap or escort cells receiving Notch signaling. Data show that the number of cells positive for both markers increases upon ectopic overexpression of *miR-125* (Figure 16C; *control* 0.5 ± 0.5 (n=11); *E[spl]:CD2/+; bab1>miR-125* 2.9 ± 1.4 (n=16); $p<10^{-3}$). Thus, it was shown that Notch activity can be altered due to overexpression of *miR-125*.

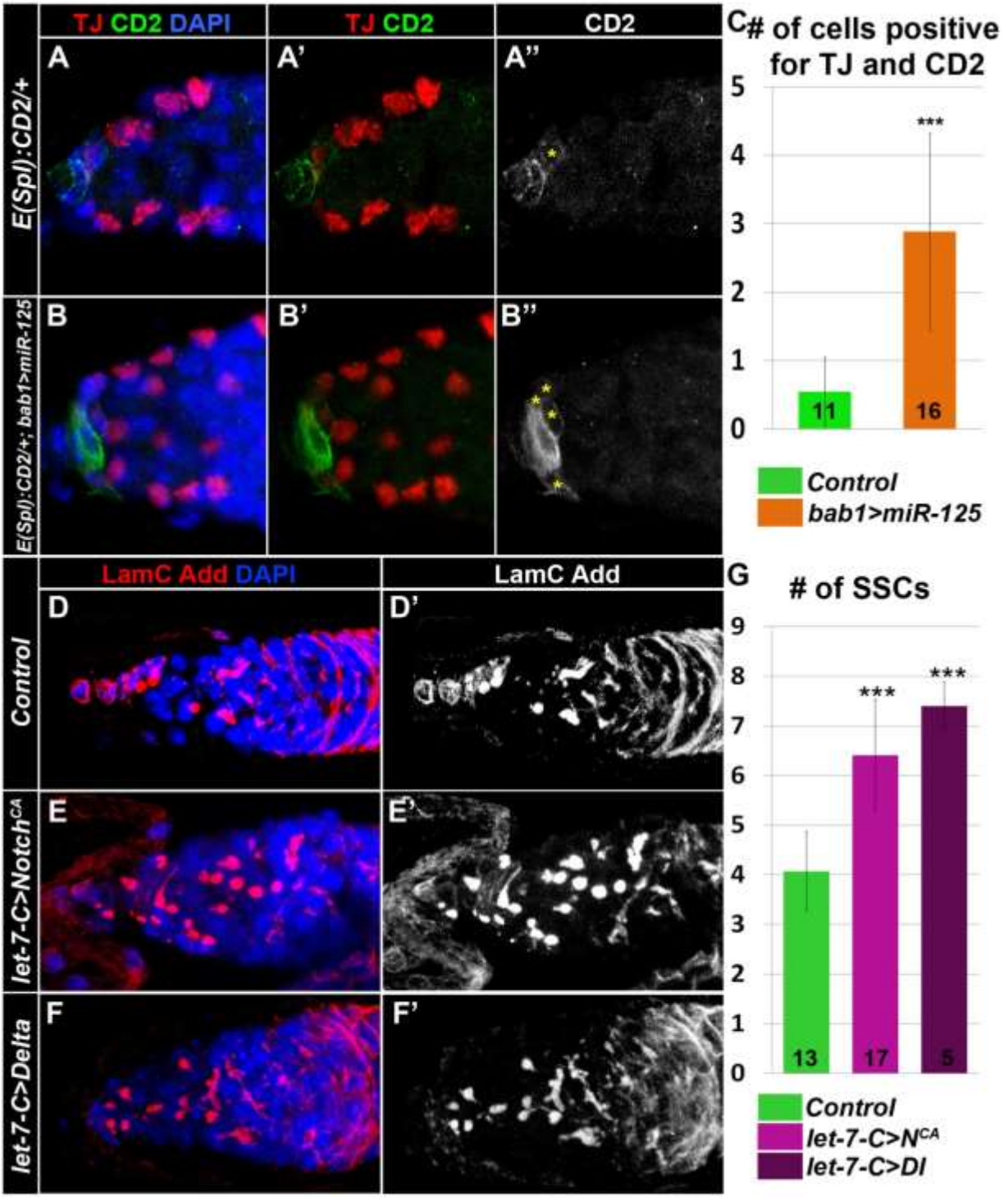

Figure 16: *miR-125* affects Notch pathway activity in *Drosophila* germarium. (A) Notch activity can be monitored by expression of a Notch reporter (CD2). Cap and escort cells are marked by Traffic jam (TJ). In control germarium, Notch is active in terminal filament and cap cells. Cells positive for both TJ and CD2 are marked by asterisks. **(B)** In contrast to control, Notch activity can be detected in a higher number of cap cells (asterisks) upon *miR-125* overexpression with *bab1Gal4*. **(C)** The number of cap cells and/or escort cells positive for both TJ and the Notch reporter (positive for CD2) is increased upon ectopic overexpression of *miR-125* with *bab1Gal4*. **(D)** In control germarium, the niche is marked by LaminC (LamC) and single spectrosomes are marked by Adducin (Add). **(E)** In comparison to control, expression of constitutively active Notch by the *let-7-Complex* promoter (*let-7-C>Notch^{CA}*) leads to an increase of germarium size and a higher number of SSCs, marked by Adducin (E'; white dots). **(F)** Overexpression of Delta by the *let-7-Complex* promoter (*let-7-C>Delta*) leads to similar phenotypes as observed for expression

of constitutively active Notch. An increase in germarium size and an increase in the number of single spectrosome containing cells, marked by Adducin (B'; white dots), is shown. **(G)** Statistical analysis for the number of SSCs in control and overexpression of N^{CA} and Delta by the *let-7-Complex* promoter shows that the number for SSCs is increased in both cases. **(C,G)** Error bars represent average deviations. Significance is calculated using a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

After it was shown that *miR-125* alters the Notch activity pattern, it was tested if direct alterations in the Notch pathway cause similar phenotypes as observed for loss and overexpression of *miR-125*. A reduced level of Tom leads to less inhibition of Neuralized and thus to an increased activation of Delta; this in turn leads to an increase in Notch signaling. Therefore, similar results are expected when Delta or Notch constitutively active (N^{CA}) are overexpressed using the *let-7-Complex* driver. Respective germaria were stained for Adducin and LaminC to mark SSCs and CpCs (Figure 16D-F). Only cells positive for Adducin were counted due to a not working anti-LaminC antibody. Data show that the number of SSCs was elevated for both overexpressed targets (*control* 4 ± 0.6 (n=13); *let-7-C> N^{CA}* 6.4 ± 1.1 (n=17); *let-7-C> Delta* 7.4 ± 0.5 (n=5); *p*-values: $p<10^{-4}$ and $p<10^{-5}$). These numbers correspond to SSC numbers shown for loss of *miR-125* (6.3 ± 0.9 (n=19)) and overexpression of *miR-125* (*let-7-C>miR-125* 7 ± 1.4 (n=11); *bab1>miR-125* 6 ± 0.8 (n=12)).

In summary, obtained data strongly suggests that *miR-125* regulates niche organization via its target Tom by affecting Notch signaling pathway. Loss or overexpression of *miR-125* is sufficient to enlarge the number of cap cells and hosted germline stem cells similar to alterations of Notch signaling (Ward et al. 2006; Song et al. 2007). The obtained results propose the *miR-125* as a strong regulator and stabilizing factor for the function of the Notch signaling pathway.

4 Discussion

This study aimed to address the following questions: Which impact has *miR-125* on the establishment and/or maintenance of the GSC niche in the *Drosophila* germarium? Via which downstream target is the putative effect of *miR-125* established? Both questions will be discussed in the following sections. In addition, it will be discussed whether the revealed mechanisms may enable crosstalk between different signaling pathways, whether they can be transferred to vertebrate models and whether they have impact on medicine.

4.1 *miR-125* is needed for the proper development of the *Drosophila* germarium and its GSC niche

This study showed for the first time that *miR-125* has an impact on the establishment of the GSC niche in the *Drosophila* ovary. Loss or overexpression of *miR-125* was shown to be sufficient to enlarge the number of cap cells and hosted GSCs (see Figure 9+11). Putatively, the observed phenotypes for the *miR-125*^{LOF} and overexpression of *miR-125* are due to the requirement of *miR-125* during development.

In this study, the *bab1Gal4* driver (Bolívar et al. 2006) and the *let-7-Complex* driver (Sokol et al. 2008) were used for overexpression of *miR-125*. Based on expression patterns, both drivers are active in adult cap cells (see Figure 10). It was shown that cap cells do not divide upon endogenous overexpression of *miR-125* in the adult fly. It still needs to be tested, whether escort cells can transform to cap cells in adult and therefore give rise to the increased number of counted CpCs. As the present study showed that *miR-125* affects the Notch signaling pathway (see Figure 16), the proposed transformation would result from altered Notch signaling. Thus, the increased number of CpCs in the adult *Drosophila* germarium may possibly result from escort cell transformation upon aberrant Notch signaling in adult. However, results of a former study contradict this hypothesis (Song et al. 2007). Referring to this former study, Notch signaling is not able to enforce a transformation from somatic cell to cap cell in adult organisms.

As niche cells in the adult organism do not divide upon overexpression of *miR-125,* and escort cell transformation upon aberrant Notch signaling was not shown (Song et al. 2007), *miR-125* may affect developmental processes and thereby increases the number of niche cells. As illustrated, the impact of *miR-125* on the establishment of germarium architecture and the GSC niche during development can be considered to be relevant. Further support of this hypothesis

can be found in the following chapter of the discussion. There an outline is given of how altered Notch signaling can disorganize the known processes governing *Drosophila* germarium development.

4.2 *miR-125* alters Notch signaling

This study shows that *miR-125* affects the conserved Notch signaling pathway via its target Tom (see Figure 15+16). As mentioned in the introduction, Notch signaling is known to be involved in the formation of the *Drosophila* GSC niche (Eliazer and Buszczak 2011). Alterations in Notch signaling have similar effects on niche architecture as perturbations in the level of *miR-125* have in this study (Ward et al. 2006; Song et al. 2007). This finding strongly supports the hypothesis that the phenotypic effect upon overexpression or loss of *miR-125* is established via alterations in Notch signaling.

The Notch pathway has been shown to be a prominent regulator of cell fate during development throughout all Metazoa (Lai 2004). Notch signaling promotes cells to be different from one another depending on whether they are Notch receiving or Notch sending cells. Numerous studies in *Drosophila* indicate that there is nearly no tissue which's development does not depend on Notch signaling. Disturbances in the activation of the Notch pathways have severe consequences for the affected organism (developmental defects, adult pathologies). Due to the severe consequences emerging from altered Notch signaling, the organism needs to control Notch signaling strictly. miRNAs may be part of such control mechanisms. Several miRNAs were shown or proposed to target Notch target genes like the Bearded family (Stark et al. 2003; Lai et al. 2005). This study shows that *miR-125* is an additional regulator of Notch signaling (see Figure 16). An illustration of the proposed mechanism of function is given in Figure 17.

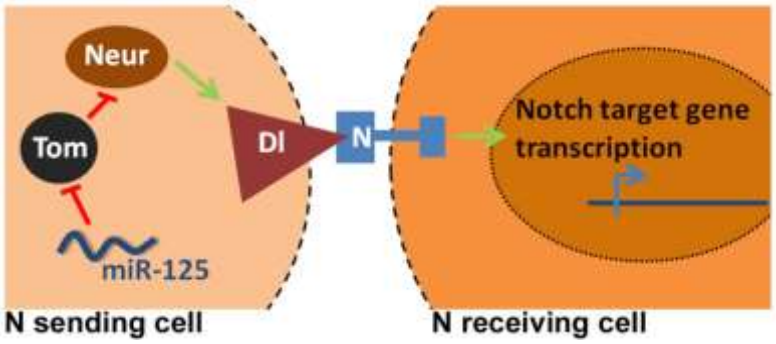

Figure 17: Proposed mechanism of function for the *miR-125*. *miR-125* targets the mRNA of Tom, a member of the Bearded family, and thereby reduces the Tom level. Tom inhibits the Neuralized (Neur) dependent activation of the Notch ligand Delta (Dl). Activated Delta can signal to adjacent cells via binding to the Notch receptor (N). Cells receiving Notch signaling activate the transcription of Notch target genes. Thus, *miR-125* can influence Notch activity.

4.2.1 *miR*-125 may stabilize Notch dependent lateral inhibition

Cases were described in which driven activation and failure of Notch have opposite effects (Lai 2004). In this study, loss of *miR-125* and overexpression of *miR-125* were shown to have the same phenotypic effects on the architecture of the GSC niche in the *Drosophila* ovary. Both alterations lead to an increase in the number of niche cells and hosted GSCs (see Figure 9+11). The presumptive reason for this can be found in *miR-125*'s mechanism of function. This study shows that *miR-125* acts via targeting Tom, a member of the Bearded family. This family is known to inhibit Neuralized dependent internalization of Delta by preventing interaction of Delta with Neuralized (Bardin and Schweisguth 2006). Thereby the Notch pathway is affected. A former publication (Bardin and Schweisguth 2006) suggests that Bearded family members are involved in Notch pathway mediated lateral inhibition in sensory organ precursor cell (SOP) determination and proposes a transcriptional feedback loop in proneural clusters. This feedback loop may work as described in the next section (compare Figure 18). In Notch receiving cells, active Su(H) promotes the expression of the *Bearded-Complex,* and its products (e.g. Tom) prevent Neuralized-dependent activation of Delta. Thereby, cells receiving Notch signaling cannot be Notch sending cells due to a lack of activated ligands. On the other hand, in the Notch sending cell, Su(H) is in its non-active state and represses the transcription of Bearded family members. This in turn leads to a higher rate of Neuralized-dependent activation of ligands and keeps the cell in its signal sending state.

Bearded family members are suspected to intensify weak differences in the activity of Delta signaling between two different cells (Bardin and Schweisguth 2006). This way, Bearded family members support cells to pick up their fate: to be a Notch signaling sending cell or a Notch signaling receiving cell. Therewith Bearded family members establish and stabilize lateral inhibition. As it was shown in this study, *miR-125* targets Tom, a member of the mentioned Bearded family, and thereby fine-tunes the level of Tom. Thus, *miR-125* may stabilize the process of lateral inhibition. Figure 18 shows an illustration of the proposed Notch pathway mediated lateral inhibition stabilized by the *miR-125*.

Lateral inhibition is known to be involved in numerous processes during development. Notch signaling, as a way of cell-cell interaction, has been shown to be a fundamental pathway for the establishment of lateral inhibition (Lai 2004). Data obtained in this study, allows proposing that *miR-125* is a crucial player in the establishment and/or maintenance of this inhibition process

by fine-tuning the level of Tom (see Figure 18). Both loss and overexpression of *miR-125* may destabilize processes establishing and maintaining lateral inhibition. A misbalance in lateral inhibition may lead to the loss of lateral inhibition. Therefore loss and overexpression of *miR-125* may result in similar phenotypes. The similarity in the obtained phenotypes shows that the disruption of the lateral inhibition is crucial for the establishment of the obtained phenotypes, without paying regard to the fact which side of the sending and receiving machinery is responsible for the disruption of the equilibrium conditions.

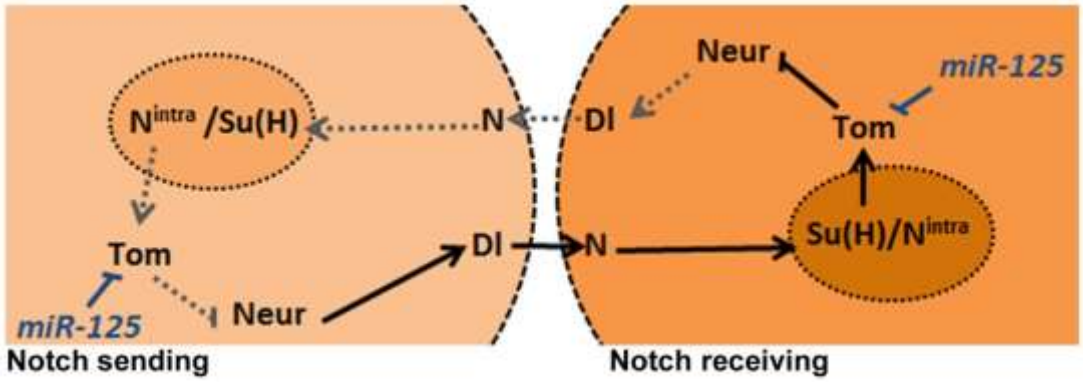

Figure 18: Proposed role of *miR-125* in Notch pathway mediated lateral inhibition. Lateral inhibition can be mediated by the Notch pathway and involves a feedback loop. Neuralized (Neur) and Bearded family members, e.g. Tom, were proposed to be part of such a feedback loop (Bardin and Schweisguth 2006). In cells receiving Notch signaling, the intracellular domain of Notch (N^{intra}) and Su(H) activates the transcription of Notch target genes, among them is Tom. Tom can inhibit the Neuralized dependent activation of the Notch ligand Delta. This way, a cell receiving Notch signaling prevents to be a Notch sending cell at the same time. As the Notch signaling receiving cell does not present activated Delta, the neighboring Notch sending cell does not receive Notch signaling and thereby does not transcribe Bearded family members. This results in unrestricted Neuralized dependent activation of Delta and keeps the cell in the Notch sending state. Thus, lateral inhibition is established and maintained. *miR-125* is involved in this process by fine-tuning the levels of Tom. Figure inspired by Bardin and Schweisguth 2006.

4.2.2 The underlying developmental mechanism remains unclear

It was shown that the formation of cap cells can only take place in late larval development and early pupal stages (Song et al. 2007). Active Notch signaling was shown to be sufficient for the induction of cap cells by TFs. By now, it remains elusive at which exact time point the transcription of *miR-125* is needed for the error-free running of the underlying developmental processes. The reason for this is that the tissue specific expression patterns for the *let-7-Complex* in the ovary have not been described during development. However, it can be assumed that the developmental expression for the *let-7-Complex* partially coincides with the expression for the *bab1Gal4* driver line. Figure 10 shows that both drivers, *bab1Gal4* and the *let-7-Complex* driver, have similar expression patterns in adult. As obtained phenotypes upon overexpression of *miR-*

125 with both drivers show similar characteristics (see Figure 11G), it can be assumed that expression patterns for both drivers may overlap partially during development as well.

In contrast to the developmental expression patterns for the endogenous driver of the *let-7-Complex*, the developmental expression patterns for the *bab1Gal4* have been described already (Godt and Laski 1995; Sahut-Barnola et al. 1995). The transcription of *bab* is firstly reported in late larval development and marks the developing TFs. However, to prove the idea of both drivers co-expression, a more detailed analysis of expression patterns for both drivers during development is needed.

Developing TFs were shown to signal via the Notch ligand Delta to adjacent somatic cells and induce them to become cap cells (Song et al. 2007). It can be assumed that the expression of *miR-125* in the TFs downregulates the present Tom levels and ensures that TFs can maintain their Notch signaling sending state to induce CpCs. Putatively, the perpetuation of this signal receiving and sending state is essential for the proper development of the GSC niche. A loss of *miR-125* in developing TFs may elevate Tom levels and thereby reduce the presence of activated Delta. Possibly, TFs need to be Notch signaling sending cells to maintain their TF state. TFs losing their Notch sending status may become CpCs as well. An overexpression of *miR-125* may increase the level of active Delta and enhance the induction of CpCs. Thus, both loss and overexpression of *miR-125* can result in additional CpCs.

4.2.3 *miR-125* helps to regulate Notch signaling spatially and temporally

As reviewed (Lai 2004), the highly conserved Notch pathway has been shown to be an important regulator of cell fate during development and in adult organisms. The finding that *miR-125* can contribute to the regulation of Notch signaling via targeting Tom may help to understand how Notch signaling is regulated in a tissue dependent manner. According to its expression patterns, *miR-125* fine-tunes the level of Tom. *miR-125*'s loss or overexpression shows phenotypic consequences for the respective tissue. Former studies revealed the presence of other miRNAs targeting the Notch pathway (Stark et al. 2003; Lai et al. 2005; Inui et al. 2010; Poulton et al. 2011), among them are *miR-7*, *miR-4* and *miR-79* targeting Bearded family members. A miRNA regulating Notch signaling does not influence Notch signaling in the whole organisms, but in specific areas according to its own expression patterns and the presence of its target proteins. Thus, miRNAs help to regulate Notch signaling in a certain spatial and temporal

pattern, and thereby help to established and maintain the functional complexity of an organism. This study proposes the *miR-125* as one of these players to generate the proper spatial and temporal pattern of Notch signaling.

4.3 *miR-125* links ecdysone signaling to the Notch pathway

As mentioned in the introduction, former studies indicated that miRNAs of the *let-7-Complex* play a major role during *Drosophila* development. It was shown that miRNAs of the *let-7-Complex* regulate timing during *Drosophila* development. Their loss results in various defects in adult functions, including motility and fertility (Caygill and Johnston 2008; Sokol et al. 2008). So far, there are only a few developmental processes associated with miRNAs of the *let-7-Complex*. No specific developmental process was associated predominantly to the *miR-125* by now. This study demonstrated the first time, which effects the deregulation of *miR-125* has on the formation of specific tissue architecture. As it was shown, deregulation of *miR-125* is sufficient to induce additional CpCs and GSCs within the *Drosophila* germarium (see Figure 9+11). Putatively, this happens during development (see above).

As mentioned in the introduction, the formation of the ovarian GSC niche is poorly understood. The formation of the adult ovarian niche starts in late larval development (Eliazer and Buszczak 2011) and coincides with the upregulation of the miRNAs of *let-7-Complex* (Pasquinelli et al. 2000; Hutvagner 2001). During development, precise levels of *miR-125* help to define the GSC niche size as it was shown already for ecdysone signaling (König et al. 2011). Former studies showed that the transcription of *let-7-Complex* miRNAs is triggered by ecdysone (Sempere et al. 2002; Chawla and Sokol 2012). Data obtained in this study suggest that ecdysone may participate in the definition of niche size partially via regulating the levels of *miR-125*. Thus, *miR-125* may establish crosstalk between the *Drosophila* steroid hormone ecdysone and the evolutionary conserved Notch pathway. Thereby the temporal regulation of Notch signaling can be influenced.

4.4 The revealed mechanism of function for *miR-125* in *Drosophila* cannot be transferred to mammals

As mentioned in the introduction, *miR-125* is a highly conserved miRNA from nematode to humans. In *Drosophila* one version of *miR-125* is present, in human there are three homologs*:* *hsa-miR-125a*, *hsa-miR-125b-1* and *hsa-miR-125b-2*. All three human homologs were shown to

be involved in a wide variety of cellular processes, like cell differentiation, proliferation and apoptosis (Sun et al. 2013). Deregulation of human *miR-125* is linked to many diseases such as cancer. Here, *miR-125* can have tumor-suppressor and tumor-promoter function according to the type of tumor. Hence, there is a strong need to understand how *miR-125* works in the vertebrate context.

The validated target of *Drosophila miR-125*, Tom, or its homolog is not detectable in the genome of vertebrates. This does not necessarily mean that there is no Tom-like protein in vertebrates because the functional motifs are too short for reliable detection of homologs by sequence alignments (Bardin and Schweisguth 2006). The interaction partner of Tom, Neuralized, is conserved in mammals; however seems to have no function linked to the Notch pathway (Koo et al. 2007). Accordingly, it is not appropriate to transfer the proposed mechanism of function for *Drosophila miR-125* to mammalian *miR-125*. This goes along with the hypothesis that many miRNAs are eminently well conserved, but its targets are not (Chen and Rajewsky 2007).

Even so, insight obtained by this study may have medical impact. What was shown is that deregulation of a single miRNA is able to affect important signaling pathways, such as the Notch pathway, in a visible manner. As reviewed (Lai 2004), aberrant Notch signaling is linked to various kinds of human diseases, among them are all kinds of cancer and developmental defects. Each single new finding can help to understand how aberrant Notch signaling develops and how this may lead to the severe consequences observed in mammalian systems. Moreover, this study provides evidence that steroid hormones may crosstalk with prominent signaling pathways as the Notch pathway via miRNAs (see above).

4.5 GSC niche expansion upon aberrant *miR-125* expression may help to understand the formation of mammalian tumors

As mentioned in the introduction, miRNA levels are frequently aberrant in the context of cancer (Takahashi et al. 2014). There are discussions about how *Drosophila* can serve as a model for cancer, because flies in general lack the vast *in situ (*abnormal cell grow in their normal place) overproliferation that is characteristic for mammalian tumors (Potter et al. 2000). However, the use of *Drosophila* as a model for cancer research increased within the last years and may give valuable insight in underlying biological processes (Rudrapatna et al. 2012).

This study deals with the *miR-125* in the *Drosophila* ovary. Data show that aberrant levels of *miR-125* lead to an increase in GSC niche size and a higher number of hosted GSCs (see Figure 9+11). Small GSC tumors in the *Drosophila* have been described already (Eliazer et al. 2011). The authors identified tumors by counting GSC-like cells containing single spectrosomes, and counted tenfold more cells upon loss of the histone lysine-specific demethylase 1 (Lsd1). The counted cell type may correspond to SSCs counted in this study. However, this study could not even show duplication in the number of SSCs upon aberrant *miR-125* levels. Hence, it is not conformable to identify phenotypes in this study as small GSC tumors.

Even when the identification of tumors was negative in this study, an increase in GSC niche size upon aberrant *miR-125* levels was shown. Based on numerous studies, a model for cancer development has been proposed that suggests a 'precancer niche'. The subsequent expansion and maturation of the 'precancer niche' may promote tumors and their progression (Barcellos-Hoff et al. 2013). The investigation of niche expansion in model organisms like *Drosophila* helps to understand the underlying processes in mammals. This study shows that *miR-125* can enforce the expansion of the GSC niche during development by modifying Notch signaling. Former studies highlighted already the role of Notch signaling in different stem cell systems (Ward et al. 2006; Song et al. 2007; Giachino and Taylor 2014; Mourikis and Tajbakhsh 2014; Liu et al. 2013). It was shown, that alterations in Notch signaling can contribute to tumor formation (Aster 2014), but the underlying mechanisms remain unknown for now. Data obtained in this study propose *miR-125* as a regulator of Notch signaling. Alterations in Notch signaling, e.g. due to aberrant levels of corresponding miRNAs, can enforce the expansion of stem cell niches. Thus, this study proposes aberrant miRNA levels as promoters of niche expansion and tumor formation.

Moreover, phenotypes obtained in this study can be screened whether they meet the medical definition for neoplasia. In the simplest meaning, neoplasia is defined as an abnormal mass of tissue resulting from abnormal growth or division of cells (Kumar and Robbins 2007). This study has shown that the numbers of all counted cell types (CpCs, GSCs, SSCs, TJs) were increased significantly upon overexpression of *miR-125* (see Figure 11G). The increase in the total number of cells within the germarium is accompanied by an increase in germarium size. As the numbers for all counted cell types increased significantly in comparison with control flies, obtained phenotypes may meet the simplest definition of neoplasia. Partially, the increased size of these

germaria may depend on the increased number of CpCs. Putatively, CpCs attract an additional number of GSCs. Most likely, additional GSCs show division similar to control GSCs, as it was demonstrated already (Song et al. 2007). This in turn may lead to the increased number of SSCs representing GSC progeny. Thus, the increased size of the germaria may be caused mainly by the increase number of CpCs. However, the increased number of TJs also contributes to the increase of germarium size, but their enhanced formation cannot be explained by now. Putatively, they also form during the development.

Strikingly, in rare cases of ectopic overexpression of *miR-125* using the *bab1Gal4* driver, extreme phenotypes were observed (for examples see Figure 11H+I). Phenotypes comparable to Figure 11I can be suspected to show characteristics of neoplasia due to dysregulation of cell division. These germaria are dramatically enlarged and show high accumulation of TJ positive cells. However, it remains elusive at which certain time point those enormous accumulations of cells emerge. These findings suggest that ectopic expression of *miR-125* increases the likelihood for the formation of abnormal cell masses within the germarium, as they were found extremely rarely but persistently.

As mentioned in the beginning of this section, tumors in *Drosophila* lack the characteristics tumors normally show in the mammalian system. Therefore, it is hard to classify changes in *Drosophila* tissue architecture as tumors or to compare them with tumors in the mammalian system. However, it can be seen that the increase in germarium size is mainly due to the expansion of a stem cell niche. As mentioned above, the expansion of a 'precancer niche' is assumed to promote tumors and their progression in the mammalian system. Thus, at least a parallel between the observed changes in *Drosophila* tissue architecture and the development of mammalian tumors can be drawn.

4.6 Summarized

miR-125 is needed for the proper development of the *Drosophila* germarium and its GSC niche. The role of *miR-125* in the adult *Drosophila* germarium remains unclear. Via targeting Tom, a member of the Bearded family, *miR-125* alters the conserved Notch signaling. Putatively, the phenotypic effect of aberrant *miR-125* levels is due to the disruption of processes stabilizing Notch dependent lateral inhibition. Moreover, this study proposes *miR-125* has a regulator of spatial and temporal patterning of Notch signaling. As expression of *miR-125* is triggered by the

steroid hormone ecdysone, the revealed mechanism of function proposes miRNAs as linkage between steroid hormones and prominent signaling cascades.

The revealed mechanism of function for *miR-125* cannot be transferred to mammals. However, the data may help to understand how Notch signaling can be regulated and how alterations in Notch signaling, causing various diseases, may develop. Moreover, the expansion of the GSC niche by aberrant expression of *miR-125* shows similarities with a proposed mechanism of tumor formation and progression.

5 Abstract

For the maintenance of tissue homeostasis upon injury or natural cell turnover, a constant source of new cells is needed. Usually these cells are delivered by tissue specific adult stem cells. The stem cells reside in a specialized microenvironment, called the niche. This niche provides an environment controlling adult stem cell proliferation and maintenance. Defects in the formation of the niche may result in stem cell loss or overproliferation, and can lead to a lack of tissue regeneration or cancer formation. A better understanding of stem cells in their natural environment may help to understand the development of certain diseases, such as cancer, and may change their treatment. Previously, microRNAs were shown to participate in stem cell regulation *in vivo.* This study investigated the role of microRNA *miR-125* (part of the conserved *let-7-Complex*) in the *Drosophila melanogaster* germarium. The *Drosophila* germarium hosts germline stem cells (GSCs) and is an ideal model system to study the interaction between stem cells and their niche. In this study, we show that *miR-125* participates in the establishment of proper GSC niche. Strikingly, the GSC niche was expanded upon loss or overexpression of *miR-125* and hosted an increased number of stem cells. This developmental niche expansion upon aberrant *miR-125* levels shows similarities to the proposed expansion of 'precancer niches' promoting the formation and progression of mammalian tumors. We have found that *miR-125* targets Tom, a member of the Bearded family, involved in the regulation of the highly conserved Notch pathway. As the transcription of *miR-125* is triggered by ecdysone, a steroid hormone in the fly, *miR-125* can be proposed as a linkage between steroid hormones and the prominent Notch pathway. Thus, *miR-125* may help to regulate Notch signaling spatially and temporally.

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