Investigation of Notch signalling in *Drosophila* germline stem cell niche

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Chapter 1: general introduction

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List of abbreviations

ALS	Acid-labile subunit
ANK	Ankyrin
AP	Adaptor Protein
aPkc	atypical protein kinase C
AJ	Adherens Junction
AMPk	AMP-activated kinase
Arm	Armadillo
Axn	Axin
β-Gal	β-Galactosidase
Bam	Bag of marbles
Baz	Bazooka
bHLH	Basic Helix Loop Helix
BMP	Bone Morphogenic Protein
Brat	Brain Tumor
BrdU	Bromodeoxyuridine
CAT	Cationic amino acid transporter
Ci	Cubitus interruptus
Cno	Canoe
Cpc	Cap cells
Crb	Crumbs
Dad	Daughter against DPP
DAPI	4',6-diamidino-2-phenylindole
Dl	Delta
Dlg	Disc Large
Dpp	Decapentaplegic
Drosophila	*Drosophila Melanogaster*
Dsh	Disheveled
DSL	Delta/Serrate/Lag2
Dx	Deltex
E(spl)	Enhancer of split
M7-lacZ	E(Spl)m7-lacZ
Mβ-CD2	E(spl)mβ-CD2
Mβ-lacZ	E(spl)mβ1.5-lacZ
Egf	Epidermal growth factor
eIF4A	Eukaryotic initiation factor 4A

En	Engrailed
Ena	Enabled
ESC	Escort cell
Ex	Expanded
Ft	Fat
FC	Follicle cell
Foxo	Forkhead box, sub-group "0"
Fz	Frizzled
FSC	Follicle stem cell
Fu	Fused
Gbb	Glass bottom boat
Glucose-R	Glucose reduced
GMC	Ganglion mother cell
GSC	Germline stem cell
GUK	Guanylate Kinase
JAK	Janus kinase
Hes	Hairy and Enhancer of split
hESCs	human Embryonic stem cells
Hh	Hedgehog
HOPS	Homotypic fusion and vacuole protein forming
Hpo	Hippo
HSC	Haematopoietic stem cell
IGF	Insulin-like growth factor
IIS	Insulin/IGF-like signalling
Ilp	Insulin-like peptide
InR	Insulin-like receptor
IPC	Insulin producing cells
iPS	Induced pluripotent stem cell
IRS	Insulin receptor substrate
ISC	Intestinal stem cell
Lgl	Lethal giant larvae
Mad	Mother against dpp
MAGUK	Membrane-associated guanylate kinase
Mam	Mastermind
Med	Medea
MAPK	Mitogen-activated protein kinase

MSC	Mesenchymal stem cells
N	Notch
NECD	Notch extra-cellular domain
NF1	Neurofibromatosis type I
NICD	Notch Intra-cellular domain
Nos	Nanos
PBS	Phosphate Buffered Saline
Pdk	3-phosphoinositide dependent protein kinase
PH3	Phospho-Histone H3
Pi3K	Phosphoinositide-3 kinase
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
Ptc	Patched
Pten	Phosphatase and tensin
Pum	Pumilio
Pyd	Polychaetoid
Rab	Ras related in brain
Ras	Rat Sarcoma
Rheb	Ras homologue enriched in brain
ROS	Reactive oxygen species
SAC	Sub-apical complex
Sax	Saxophone
Scrib	Scribble
Sdt	Stardust
Ser	Serrate
Shg	Shotgun
SJ	Septate junctions
Sgg	Shaggy
Slif	Slimfast
Sod	Superoxide dismutase
SOP	Sensory organ precursors
STAT	Signal Transducer and Activator of Transcription
Su(dx)	Suppressor of deltex
Su(H)	Suppressor of Hairless
Tam	Tamou
TF	Terminal filament

TGF-β	Transforming growth factor β
Tkv	Thick Veins
Tor	Target of rapamycin
TORC	Tor complex
Trpml	Transient receptor potential cation channel, mucolipin subfamily
Tsc	Tuberous sclerosis complex
Upd	Unpaired
Vkg	Viking
Wg	Wingless
Wt	Wild Type
Wts	Warts
Yki	Yorkie
Yrt	Yurt
ZO-1	Zonula Occludens-1

Abstract

Adult stem cells are vital for tissue maintenance. Stem cell over proliferation results in tumour formation, whilst loss of stem cells causes tissue degeneration and a variety of diseases. Stem cell maintenance and proliferation is regulated through somatic structures called niches. The germline stem cell niche in *Drosophila* ovary has been well defined and it is useful to better understand the interactions between niche and stem cells. Notch signalling is needed for germline stem cell niche creation and maintenance. The aim of this thesis is to better understand both the regulation of Notch signalling during development and its requirement in the adult niche. The first paper, "Reversible regulation of stem cell niche size through dietary control of Notch signalling", revolves around the dynamicity of the niche. The niche is found to respond to diet stimuli and has the ability to be restored. Notch was previously found to be involved in the maintenance of the niche. We found that Notch signalling is altered by diet, and we dissect its different maintenance and recovery roles in the ovary. In the second paper, "ZO-1 controls stem cell niche assembly by acting as an upstream regulator of Deltex-dependent Notch signalling", we show how Notch signalling is finely regulated during niche formation through interplay with the proteins Polychaetoid and Deltex. This paper leads to a better understanding of how the niche is assembled and how Notch signalling is regulated in a context-dependent way. The obtained results from both papers will help understand the dynamics of the model germline stem cell niche, and how Notch signalling is found at the convergence between internal and external stimuli regulating the ovary's response to a changing environment.

Declaration

No portion of work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

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Introduction to thesis and co-author contributions

This thesis is written in the alternative format. Alternative format consists of the results chapter being written in the form of a manuscript ready for submission. The advantages of this format are great, both for the supervisor and the student. The supervisor will have all the data formatted for easy submission in a journal and an already written paper, resulting in a big time saving. Although the paper could be incomplete after submission and need more experiments, it will be easier to add these new data in the already written canvas. The student will be writing as if writing a paper, which is the kind of style he will be using if an academic route is selected. Moreover, the student will have documents with good chances of publication, which are essentials to get a position in our highly competitive post doc world. A third advantage is that if a paper has already been submitted, it can be easily integrated in the thesis without requiring more work, as it is the case for the second paper in this thesis. The first paper, "Reversible regulation of stem cell niche size through dietary control of Notch signalling", shows my main project during these PhD years and is the reason I have chosen this PhD in Martin's lab. I was interested in ageing and stem cell dynamics, and although this main project changed over time considering the results of my experiments and the publication of other groups, it retains the flavour that brought me to this PhD. All the lab work and figure making of this paper has been done by me. The manuscript has been written by me and Martin Baron. For the second paper, "ZO-1 controls stem cell niche assembly by acting as an upstream regulator of Deltex-dependent Notch signalling", I contributed with niche counts, which is the main technique I used during this PhD. My contributions are the following. Figure 1: the entire figure except for panel g and h; Figure 2: for Chart D I contributed four out of ten genotypes; Figure 5: I performed all the interactions with junctional proteins in Chart E.

Chapter 1: general introduction

1.1 Tissue homeostasis and stem cells

Tissue homeostasis refers to the maintenance of tissues in a proper functional and differentiated state after morphogenesis. To maintain proper tissue homeostasis an organism must respond to a plethora of inputs coming from external factors, such as diet and injuries, in conjunction with the numerous signalling pathways present in tissues. Failure to maintain tissue homeostasis leads to degenerative diseases, ageing and tumours. Tissue homeostasis is dynamic and can be summarized into three different processes: tissue renewal; preservation of tissue cells in a proper differentiated state; maintenance of tissue quantity. Through tissue renewal the organism can replace old tissues and create new ones. Tissue renewal is mainly regulated through stem cells, which often reside in defined niches within tissues. Regulation of stem cells and their niches is the main focus of this thesis.

After fertilization a single cell can give rise to an entire organism through embryogenesis. This totipotent cell with the ability to specify each single cell type is an embryonic stem cell (Fuchs and Segre, 2000). Embryonic stem cells can be maintained and expanded in vitro (Thomson, 1998). These amplified cells can be inserted in another embryo, thus creating a chimeric organism where they can contribute to every cell-type (Nichols et al., 1998; Niwa et al., 1998). Moreover, by depriving these cells of Leukaemia inhibitor factor it is possible to see them aggregate in vitro into embryo-like bodies, in which stem cells can differentiate and spawn many cell lineages, including beating heart muscle cells, blood islands, neurons, pigmented cells, macrophages, epithelia and fat-producing adipocytes (Fuchs and Segre, 2000). These experiments demonstrate the capability of an embryonic stem cell to specify all the cell types of an organism.

In the fully developed organism a subset of cells will retain restricted stem cell abilities, becoming adult stem cells (Fuchs and Segre, 2000). Adult stem cells are

specialized cells able, throughout the life of an organism, to either keep their undifferentiated and multi-potent state, or to differentiate into a specialized cell. This property gives to multi-cellular organisms the possibility to self-renew their tissue, replacing damaged cells with new ones or creating new cells to respond to changes in the environment. Adult stem cells are defined by three properties: differentiation, which is the capability to give rise to a progeny of heterogeneous cells; self-renewal, which is the ability to form identical stem cells; homeostatic control, which is the ability to keep under control the first two abilities according to the necessities of the organism (Dalerba et al., 2007). Paradigms of this process are the haematopoietic stem cells (HSCs), which are able to continuously replace for the entire life time of an organism the entire repertoire of differentiated blood cells in adult circulation (Akashi, 2005).

1.1.1 Stem cell regulation

Stem cells have to be maintained over the long life of organisms and this involves several control mechanisms (Morrison and Spradling, 2008). Adult stem cell populations need to be kept in equilibrium between maintenance and differentiation. Excessive self-renewal of stem cells can lead to tumours, whilst exaggerated differentiation will deplete stem cell populations (Ghotra et al., 2009). To avoid depletion, the cell cycle in adult stem cells is tightly regulated and often involves a quiescent state. For example HSC over-proliferation results in stem cell exhaustion (Orford and Scadden, 2008). Inactivation of DNA damage repair systems also can lead to premature stem cell depletion (Ito et al., 2004).

The proliferation versus maintenance equilibrium is achieved through two different strategies: asymmetric cell division or population asymmetry. In the asymmetric cell division strategy, stem cell division always gives rise to one maintained daughter cell, which will retain the functions of a stem cell, and to one differentiated

daughter cell. Stem cells following the population asymmetry strategy do not have a strict selection of which cell will become differentiated and which will self-renew as a stem cell. With this strategy, some stem cells are lost through differentiation while other stem cells maintain the stemness and keep multiplying (Watt and Hogan, 2000). The asymmetric division strategy allows for competition among different stem cells, giving an edge to a stem cell with proliferative advantages or ability to be maintained in the niche (Watt and Hogan, 2000). This could be seen as another system to ensure stem cell vitality, since some wild type (Wt) invertebrate stem cells are regularly displaced from the niche (Margolis and Spradling, 1995). However, it still has not been demonstrated to serve as a damage reduction mechanism (Morrison and Spradling, 2008). An example of the asymmetric division strategy is found in *Drosophila* male and female germline stem cell (GSC) niche (Xie and Spradling, 2000). Most mammalian self-renewing tissues adopt the population asymmetry strategy (Watt and Hogan, 2000).

One mechanism to achieve asymmetric stem cell regulation is through the asymmetric inheritance of proteins, RNAs and organelles between the two daughter cells. In the *Drosophila* neural system, neuroblasts are responsible for the supply of new neurons. Neuroblasts divide into a larger daughter cell that retains the neuroblast function, and a smaller daughter cell named ganglion mother cell (GMC), which will divide once more, differentiating into two neurons. The asymmetry is created through the localization of proteins like Par-3, Par-6 and atypical Protein Kinase C (aPKC) in the apical region of the neuroblast. These proteins direct the axis of polarity and during mitosis are retained by the neuroblast. Thanks to the action of these apical proteins, other proteins like Numb, a tissue-specific repressor of the Notch pathway, Prospero, a transcriptional activator or repressor, and Brain tumor (Brat) are segregated into the GMC and not in the daughter neuroblast (Reichert, 2011).

Another mechanism is to control the plane of stem cell division such that

one daughter cell is pushed further from stem cell self-renewal signals derived from supporting niche cells. This is the case for *Drosophila* female GSCs, which will be described in detail later. Other examples of spindle orientation deciding the destiny of daughter cells include the GSCs of the *Drosophila* testis and *Drosophila* intestinal stem cells (ISCs) (Morrison and Spradling, 2008).

1.1.2 Stem cell niches

Stem cells reside in a structure called the “niche”, defined as: "specific anatomic location that regulates how stem cells participate in tissue generation, maintenance and repair" (Scadden, 2006). The niche concept was first proposed in 1978 (Schofield, 1978). Niches have to be finely regulated to maintain the stem cells. Numerous intrinsic and extrinsic factors converge in the niche to regulate stem cell fate. Different types of niches have been characterized, especially in invertebrates. The germline in *C.elegans* and *Drosophila* is found attached to a well-defined population of somatic cells that produce maintenance signals (Crittenden et al., 2002; Kiger et al., 2000; Scadden, 2006; Xie and Spradling, 2000).

Stem cell niches were originally defined to be a stable and persistent aspect of the tissue anatomy (Ohlstein et al., 2004), but other types of niches have now been identified. For example, *Drosophila* mid-gut stem cells are not in contact with a well-defined niche-like cell type, but adhere to an extracellular matrix. Mid-gut stem cells sit on a basement membrane that divides them from muscle cells, suggesting a niche formed of extra-cellular matrix and non-cellular constituents (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Scadden, 2006). Stem cells can also produce a transient niche with their progenitors that determines stem cell fate (Mathur et al., 2010; Wilson and Kotton, 2008).

In mammals the identification of stem cells is harder than in invertebrates,

as a consequence of the relative rarity of the stem cells in the vastness of the tissues (Morrison and Spradling, 2008). The following stem cell populations and relative niches have been identified in mammals: GSCs lie within the basal cell layer of the seminiferous tubes; epithelial stem cells reside in the bulge of the hair follicle; neural stem cells are found within the lateral ventricle sub-ventricular zone in the central nervous system; muscle stem cells are known as satellite cells and lie under the basal lamina of myofibres, which acts as a niche for the satellite cells (Drummond-Barbosa, 2008; Morrison and Spradling, 2008); the HSC niche comprises a quiescent inducing niche comprised of osteoblasts in the bone marrow, and a proliferating vascular niche (Yin and Li, 2006).

Niches have to be dynamic to respond to injuries requiring stem cell proliferation to be repaired (Voog and Jones, 2010). In the *Drosophila* midgut, ISC division rate increases in response to intestinal injury, stress or ageing. This response is mediated by the secretion of cytokines from differentiated enterocytes, which activates JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) signalling in ISCs (Jiang et al., 2009). During epidermis regeneration new niches are formed. Bulge stem cells migrate from other niches and proliferate in the new ones to aid epidermal regeneration (Voog and Jones, 2010). Satellite cells reside adjacent to muscle fibres and comprise skeletal muscle precursors, which contribute to the repair of damaged muscle fibres (Voog and Jones, 2010). In injured muscles, expression of Notch in satellite cells is sufficient to enhance muscle regeneration. Delta (Dl), a Notch ligand, is expressed during damage response from the myofibre niche, increasing Notch signalling in satellite cells and thus enhancing regeneration (Conboy et al., 2003, 2005).

Adult stem cells are often hard to identify. Histological methods proved to be not efficient and unique markers are rare and often not linked only to stemness functions (Simons and Clevers, 2011). A solid model system used to study the

interactions between the niche and stem cells is the *Drosophila* ovary. The ovary contains two different stem cell populations, GSCs and follicle stem cells (FSCs), whose location in defined niches has been established. *Drosophila* ovary stem cells have been well characterized over the course of the years and numerous markers are available for both the stem cells and the sustaining somatic niches, making it simpler to follow and study these cell populations than other stem cell systems.

1.1.3 Stem cells and ageing

Tissue homeostasis is altered with ageing. If the critically regulated mechanisms that control stem cells fail or become unbalanced the consequences are severe, including age-related pathologies or cancer (Ghotra et al., 2009; Sell, 2004). Stem cell numbers and functionality are known to be affected by age, resulting in an age-dependent decline (Drummond-Barbosa, 2008). As an example of ageing, *Drosophila* GSCs proliferate more slowly and are lost in aged flies (Jones, 2007). Four models for stem cell ageing have been proposed: the intrinsic stem cell ageing model, the niche ageing model, the systemic ageing model and the multidirectional ageing model (Drummond-Barbosa, 2008).

The intrinsic stem cell ageing model states that stem cells intrinsically age, leading to a general systemic ageing. Over-expression of Superoxide dismutase (Sod) in *Drosophila* GSCs extends their lifespan (Pan et al., 2007). Another example is found in HSCs where p16, an important regulator of cellular senescence (Gil and Peters, 2006), is up-regulated in older organisms in response to cell stress (Ito et al., 2006; Janzen et al., 2006). Whilst HSCs transplanted from old Wt animals are exhausted more rapidly than HSCs transplanted from young Wt animals, HSCs from old $p16^{-/-}$ mutants animals outperform HSCs from young $p16^{-/-}$ mutant animals (Janzen et al., 2006).

Another intrinsic process associated with stem cell ageing is telomere

shortening. Telomeres are found at the end of chromosomes and are essential to prevent chromosome damage resulting from shortening of linear chromosomes after replication. Telomere shortening has been found associated to ageing and results in chromosome instability and loss of cell viability (Blasco, 2005). Stem cells, germ cells and the majority of cancer stem cells express high levels of telomerase, which is the enzyme that repairs telomeres (Collins and Mitchell, 2002), suggesting a link between telomeres, ageing and stem cells (Flores et al., 2006). Patients suffering from aplastic anaemia and dyskeratosis congenita lack telomerase activity in HSCs, leading to bone marrow failure (Greenwood and Lansdorp, 2004). While embryonic stem cells express telomerases at high level and some embryonic stem cells lines have been propagated for years without any sign of senescence, adult stem cells express telomerases at lower levels, and stem cell populations like HSCs are known to incur cell senescence. This could suggest that adult stem cells are not truly immortal like embryonic stem cells, even if the telomerase activity enhances their lifespan compared to somatic cells (Krtolica, 2005).

Niche malfunctioning can also result in an ageing phenotype. An example for the niche ageing model is found in older *Drosophila* male GSC niches, where Shotgun (Shg), which is a homolog of classic vertebrate cadherins, and levels of the niche-expressed growth factor Unpaired (Upd) are lowered in old flies. Upd over-expression can rescue the ageing loss of GSCs phenotype (Boyle et al., 2007). In ageing mouse testes, GSCs can be serially transplanted to young individuals, allowing their maintenance for more than three years. Male mice undergo fertility problems due to loss of GSCs between 12 and 24 months of age (Ogawa et al., 2003; Ryu et al., 2006). The niche is thought to be responsible for the mis-maintenance of these stem cells (Drummond-Barbosa, 2008).

The systemic ageing model proposes that a general systemic ageing leads to

ageing of the niche and stem cell. An example that will be further described later in the introduction is the effect of insulin/insulin-like growth factor (IGF)-like signalling (IIS) signalling on *Drosophila* GSC niche, where it maintains cap cell number through Notch signalling (Hsu and Drummond-Barbosa, 2011). Insulin-like peptides (Ilps) expressed in the brain also regulates GSC proliferation in response to nutritional conditions providing an example of stem cell sensing of the nutritional status of the organism (LaFever and Drummond-Barbosa, 2005). Interestingly, dietary restriction has been linked to increased longevity and prolonged stem cell maintenance in a number of organisms (Cerletti et al., 2012; Ramos and Kaeberlein, 2012; Yilmaz et al., 2012). Insulin-like receptor (InR) mutation or ablation of insulin producing cells (IPCs) also extend life span (Giannakou and Partridge, 2007). Another example of possible systemic factors affecting ageing comes from the mouse muscle. In aged muscles, Dl is not expressed at the same level as younger muscles, resulting in impaired regeneration. Muscles from old mice exposed to the systemic environment of a new mouse reactivate cell functions, restoring regeneration (Conboy et al., 2003, 2005).

Thus there are various contributing factors acting on stem cells, their niches, or systemically which affect tissue regeneration in ageing organisms and the multidirectional ageing model proposes that ageing reflects a mixture of all the above models and proposes a general decline of the organism.

1.1.4 Stem cells and cancer

Loss of control of stem cell division can lead to uncontrolled self-renewal, which is a hallmark of cancer. The replicative ageing of stem cells may have evolved as a protection mechanism against cancer, by limiting their ability to replicate. If all the cells in our organism retained the ability to divide, it would be statistically easier to develop cancer (Clarke and Fuller, 2006). So it is not surprising that cancer is being

considered more and more to be a stem cell disease. According to the cancer field theory, cancer may develop if a normal stem cell is placed in an environment that allows for cancer phenotype. The theories of cancer insurgence due to mutations or epigenetic changes state that cancer seems to originate more easily in stem cells (Sell, 2010), possibly because stem cells are the only cell population that persists enough time in the tissues to acquire the required number of mutations to transform in cancer (Alison et al., 2009). Moreover, stem cells' intrinsic self-renewal abilities suggest these kinds of cells require fewer mutations than a normal cell to transform. The cancer stem cells theory is relatively new, and has been described first in 1994 in leukemic stem cells (Lapidot et al., 1994). This theory postulates that cancer arises from stem cells and that cancers have the same kind of cell developmental hierarchy as normal tissues (Sell, 2010). Several examples of cancer stem cells have been found also in solid tumours, where often cells with different proliferative and differentiation capabilities are observed (Dalerba et al., 2007; Ghotra et al., 2009; Visvader and Lindeman, 2008). In the liver, oval cells and hepatic progenitor cells, which are capable of bi-potency, have been found directly associated to cancer in rats and mice (Alison et al., 2009). Cancer stem cells have the characteristics of normal stem cells: quiescence, drug resistance, high DNA damage repair and decreased level of reactive oxygen species (ROS). This ensemble of characteristics makes them difficult to target with conventional drugs (Ghotra et al., 2009). Cancer stem cells do not always originate from a normal stem cell, but may arise also from restricted progenitor or more differentiated cells that thanks to a mutation acquired self-renewal capabilities (Visvader and Lindeman, 2008). An example of this process is found in *Drosophila* larval brain, where cancer stem cells can arise from the de-differentiation of transit-amplifying progenitors cells back to a stem cell-like state (Song and Lu, 2011). Another property of cancer stem cells shared with normal stem cells is their interactions with support cells that can constitute something

resembling a niche. For example, cancer-stimulated mesenchymal stem cells (MSCs) and derived cell types can create a cancer stem cell niche to enable tumour progression (Li et al., 2012a). In some cases mutations have been identified in these support cells which then contribute to cancer development through their activity on associated cancer stem cells (Barcellos-Hoff and Ravani, 2000; Yang et al., 2006; Zhu et al., 2002).

A further similarity between cancer stem cells and normal stem cells is that they may communicate with and in part be supported by adjacent tissues which comprise a kind of niche. Mutations in the niche that mis-regulate it can lead to insurgence of tumours through cancer stem cells. Irradiation of mammary stroma promotes tumour genesis of un-irradiated epithelial cells (Barcellos-Hoff and Ravani, 2000). In the mouse model of the tumour suppressor neurofibroma mutation of Neurofibromatosis type I (NF1), altered mast cells provide tropic factors supporting tumour growth, recruiting glial cells which exhibit increased proliferation, migration and collagen deposits (Yang et al., 2006; Zhu et al., 2002). There are different models that explain the cancer stem cell - niche interaction. The cancer stem cells may be activated by a normal stem cell niche. Alternatively, the modification of a niche can give rise to cancer stem cells. Cancer stem cells may also lead a quiescent niche to become activated or may lead to amplification of the signal from a normal niche (Sell, 2010; Sneddon and Werb, 2007). Targeting the niche instead of the cancer stem cells may be a good way to avoid the aforementioned resistance of the stem cells (Ghotra et al., 2009).

1.1.5 Stem cells and their medical applications

The previous paragraphs showed how stem cell misregulation can create pathological situations. It follows therefore that manipulating or replacing stem cells gives the possibility to be used to treat various age-related and degenerative diseases.

Indeed stem cells are also already directly used to cure diseases that require a boost in regenerative capabilities. Bone marrow transplants, which are a very effective therapy for blood cell diseases, can be considered as a stem cell application. When the bone marrow is transplanted, both the niche and the HSCs are transplanted (Becerra et al., 2011; Till and Mcculloch, 1964). Aged human muscles have less regenerative properties than young ones. Muscle stem cells persist into aged humans, but their responses are inhibited by the ageing niche (Carlson et al., 2009). The decline in muscle stem cell function in humans and mice is caused by an imbalance of Notch and Transforming growth factor β (TGF-β) signalling. It was possible through activation of Notch and mitogen-activated protein kinase (MAPK) to rejuvenate human muscle stem cells in vitro from 70 years old humans, obtaining a similar stress response to human muscle stem cells in vitro from 20 years old humans (Carlson et al., 2009). The MAPK effect was found to occur through activation of Notch signalling (Carlson et al., 2009). The niche has an important role in muscle stem cell transplantation. Preservation of the muscle niche promotes a donor satellite muscle stem cell contribution to muscle regeneration and functional reconstitution (Boldrin et al., 2012). This research opens possibilities to cure atrophy, dystrophy and loss of muscle functions due to trauma (Bareja and Billin, 2013).

Liver regeneration has also been the focus of several studies. Considering experiments done in rodents, many liver diseases appear to be curable through transplantation therapy, including genetic diseases like Wilson's disease, Crigler-Najjar syndrome and tyrosinaemia. Some success in curing these diseases in humans through transplantation has already been achieved (Alison et al., 2009). Also, haematopoietic cells contribute to hepatocyte formation under physiological and pathological conditions (Thorgeirsson and Grisham, 2006), and thus can be used in transplants to enhance liver regeneration. MSCs can be used both to reconstruct organs in vitro or inserted directly

in the organism to promote tissue healing (Becerra et al., 2011). MSCs grafted from bone marrow can reconstruct an irradiated or severely fractured bone (Becerra et al., 2011). MSCs, which were seeded on a de-cellularized human tracheal matrix, re-constructed the trachea in vitro. The trachea was later implanted successfully in a patient (Macchiarini et al., 2008). A similar approach has also been proposed for tissue engineering of articular cartilage. Stem cells from bone marrow and adipose tissues, exposed to TGF-β signalling, can undergo chondrogenic differentiation. These cells could then be used with proper scaffolding to construct cartilage to transplant into patients (Danisovic et al., 2012). MSCs also modulate the injury-related inflammatory response, by interacting with T-cells and dendritic cells and migrating to ischemia and inflammation areas. This suggests a possible use of MSCs to also treat neurodegenerative pathologies (Becerra et al., 2011). As the bone marrow transplant therapy suggests, to have an efficient stem cell-induced regeneration of the tissues, the transplantation of the niche along with the stem cells could prove to be much more efficient than the transplantation of the stem cells on their own (Becerra et al., 2011).

Stem cells in the mammalian intestinal epithelium, if properly treated, are able to self-organize in vitro into crypt-villus organoids, and do not need an epithelium niche for this function (Sato et al., 2009). This study could impact the possibility to create in vitro cellular structures to transplant where needed. A very promising stem cell-based therapy employs induced pluripotent stem cells (iPSs). These stem cells are derived from somatic cells by transfecting them with just four transcription factors. A big advantage of iPSs is that there are no rejection problems, since the cells to be implanted originate from the same patient (Takahashi and Yamanaka, 2006). iPSs are currently being used in animal test models for Huntington disease, along with embryonic stem cells (Benraiss and Goldman, 2011). For the maintenance of pluripotency and promotion of differentiation of iPSs and embryonic stem cells, in vitro

models imitating natural niches are being used (Joddar and Ito, 2013). For example, it is possible for mouse iPSs to differentiate into corneal epithelial-like cells by replicating the environment of a corneal epithelial stem cell niche (Yu et al., 2013). In light of these results with iPSs, understanding the basic functions of a niche could lead to the ability to differentiate iPSs into selected cell-types. These differentiated iPSs could then be used for tissue building and regeneration therapies.

1.2 *Drosophila* ovary as a model for studying tissue renewal

1.2.1 General structure of the ovary

Drosophila can lay up to 90 eggs a day in good food conditions (Drummond-Barbosa and Spradling, 2001). Egg production is achieved through the ovaries, which are structures present in the abdomen of female *Drosophila*. Each *Drosophila* female has 2 ovaries, which are connected through an oviduct (Fig. 1.1). An ovary is composed of 15-20 substructures, called ovarioles (Middleton et al., 2006) (Fig. 1.1). Each ovariole is comprised of a chain of egg-chambers and each egg-chamber is at a different progressive developmental stage (Fig. 1.1). Egg production begins at the anterior tip of each ovariole, where the germarium is found. The germarium hosts the GSCs, the FSCs and their respective niches (Decotto and Spradling, 2005; González-Reyes, 2003) (Fig. 1.2). In the germarium these stem cell populations give rise to germline and somatic cells, which together form the egg-chambers. After being produced, the egg chambers are separated from the germarium (as described in section 1.2.2), but remain linked thanks to stalk cells, which are differentiated follicle cells (FCs). As the egg chambers are pushed away from the germarium they mature, passing through a series of distinct developmental stages (Bate and Arias, 1993). Ovarioles are surrounded by an epithelial sheath and all ovarioles are kept together by the peritoneal sheath to form the ovary.

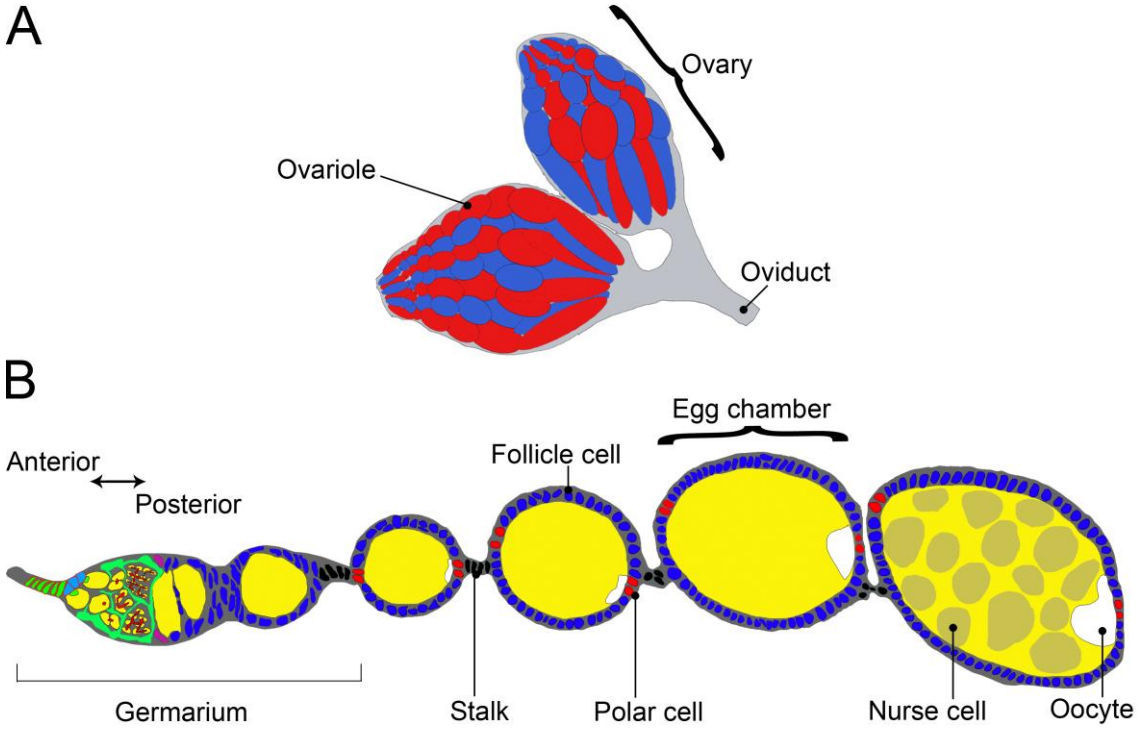

Figure 1.1. Schematic representation of ovary and ovariole
A) Schematic representation of the two ovaries connected by the oviduct, which is the egg laying path. The ovary is made from several sub-units called ovarioles.
B) Schematic representation of a single ovariole. At the anterior tip is found the germarium, the structure where GSCs reside and where egg production is initiated, described in detail in Fig. 1.2. At the posterior of the ovariole are found the egg chambers, each one at a progressively later developmental stage, which are connected by stalks. Each egg chamber while developing will be pushed posteriorly, and will eventually produce a fully developed egg. Each egg chamber is composed of an external layer of FCs surrounding a 16 cell germline cyst. One germline cell will become the oocyte, while the other 15 will become nurse cells that support the oocyte with the production of mRNA, proteins and other materials.

1.2.2 Germarium

The germarium is composed of several different cell populations that influence and regulate each other to produce eggs (Fig. 1.2). At the apical tip there are tightly packed cells called “terminal filament” (TF) cells. In Wt *Drosophila* usually 8-10 TF cells can be found (Xie and Spradling, 2000). The most posterior TF cell contacts another group of cells, called cap cells. There are usually 5-7 cap cells in Wt organisms (Xie and Spradling, 2000). 2-3 GSCs (Song et al., 2002) and 4-6 escort cells (ESC) (Decotto and Spradling, 2005) are anchored to the cap cells. The ESCs also wrap around the GSCs. GSCs divide asymmetrically as one cell (the one that remains in contact with the cap cells) maintains its status as a GSC, while the other differentiates, becoming a cystoblast that moves away posteriorly. The latter undergoes four mitotic divisions to become a cyst of 16 cells (de Cuevas and Spradling, 1998).

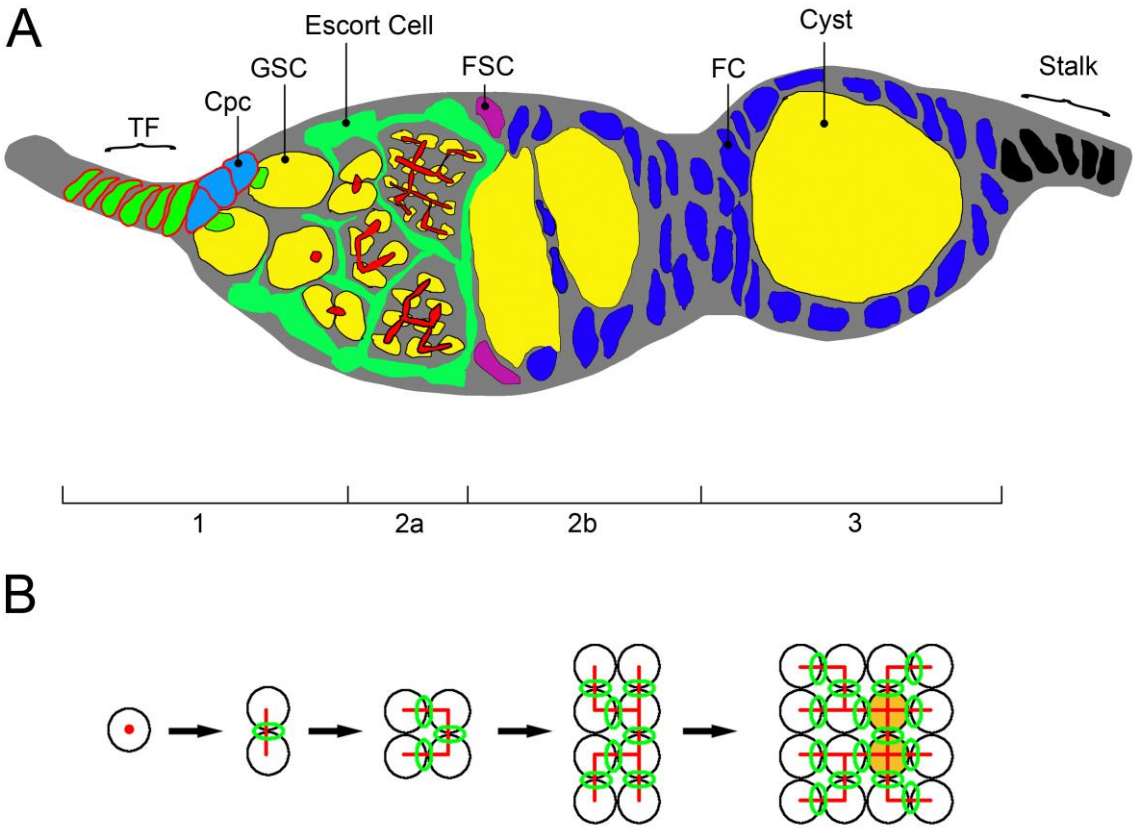

Figure 1.2. Schematic representation of the germarium and germline division
A) On the left side is represented the terminal filament (TF) in green and encircled in red, and the cap cells (Cpc) in pale blue and encircled in red. These two cell populations constitute the GSC niche (yellow with a green dot). The green dot in the GSC is the representation of the spectrosome. ESCs, in green, have the function of surrounding the developing cyst (in yellow) to escort it toward the follicle cells (FC, in blue). The single cell cystoblasts, which are daughter cells of the GSCs that have been displaced from the niche, are marked with a red dot which represents the fusome (derived from the division of the GSC spectrosome). The cystoblast undergoes 4 mitotic divisions, leading to the formation of a 16 cell cyst. The divisions are synchronous, and cytokinesis remains incomplete in these divisions, leaving the cells connected to each other in a defined pattern through ring canals (B in green). This process results in the two oldest cells having four ring canals that connect to four other cells (B in orange). One of these 2 cells becomes the oocyte, while the other cells will become nurse cells (Lilly et al., 2000). Cyst cells are connected by the fusome, a vesicular and cytoskeletal enriched structure (A in red) (de Cuevas et al., 1997). The fusome derives from the GSC spectrosome. When the GSC divides to give birth to two daughter cells, 2/3 of the spectrosome remains in the daughter that will remain as a GSC, while the rest is placed in the daughter cell that will become the cystoblast (de Cuevas et al., 1997). FCs, produced by the Follicle stem cells (FSC, in purple) surround the germline, marking the beginning of the formation of egg chambers. Some FCs develop as stalk cells, which separate each successive egg chamber and others develop as polar cells that are located at the anterior and posterior of each egg chamber (not shown on figure). When the 16 cell cyst is formed, the cell division cycle is arrested, and the 15 cells destined to become nurse cells begin their differentiation. These cells will not divide anymore and support the oocyte, providing mRNA, proteins and organelles through the ring canals (Huynh and St Johnston, 2004).

1.2.3 GSC regulation

1.2.3.1 GSC maintenance and differentiation

The GSCs reside attached to the cap cells. Cell to cell adhesion plays an important part in niche-dependent regulation of GSCs. Without adhesion, important signalling from the niche would not have access to the GSCs. Shg accumulates at the anterior side of the GSC, where the niche is and loss of Shg function leads to loss of GSCs from the niche (Song et al., 2002). The cap cells express and secrete the Bone Morphogenic Protein- (BMP-) related proteins Decapentaplegic (Dpp) and Glass bottom boat (Gbb), which are diffusible ligands (Xie and Spradling, 2000). The BMP-related proteins belong to the TGF-β super-family of growth factors, that is responsible for many biological process in both vertebrates and invertebrates (Hogan, 1996; Kishigami and Mishina, 2005). The BMP signalling pathway is described in Figure 1.3. Dpp travels to the GSCs, where it acts through a cell surface signalling receptor to repress transcription of *bag of marbles* (*bam*), a gene both necessary and sufficient for GSC differentiation (Chen and McKearin, 2003; Song et al., 2004). If Bam is expressed in GSCs, the GSCs differentiate (Gönczy et al., 1997; Ohlstein et al., 2000). When GSCs divide asymmetrically, one daughter cell remains attached to the niche whilst the other daughter cell is displaced. The daughter cell pushed out of the niche receives less niche-derived Dpp (Chen and McKearin, 2003). This reduction in Dpp allows the expression of Bam, leading to differentiation into a cystoblast (Fig1.3) (McKearin and Ohlstein, 1995; Song et al., 2004; Xie and Spradling, 1998). Over-expression of Bam suppresses the tumour phenotype that can be induced by ectopic BMP signalling (Casanueva and Ferguson, 2004).

BMP signalling is controlled on many levels to ensure proper asymmetrical cell fate determination, both on the pathway components (Fig 1.3) and physically. Viking (Vkg), a Type IV collagen can bind Dpp and prevent its diffusion (Wang et al.,

2008b). Flies that are mutant for *vkg* show an increased number of GSCs, resembling the flies with an over-expression of Dpp. The proposed model is that Vkg sequesters Dpp around GSCs within niche, thus not allowing the signal diffusion. If Vkg cannot bind Dpp, the latter will diffuse over a greater area, promoting cell maintenance over differentiation (Wang et al., 2008b). These mechanisms are coupled with a feedback cycle internal to the GSCs to control GSCs differentiation. Bam and Dpp are subject to a feedback loop which helps to precisely define the differentiation of GSCs. When BMP signalling is lowered consequently to the GSC daughter being displaced away from the niche, Bam begins to be expressed, leading through feedback to further repression of BMP signalling and hence further Bam expression (Casanueva and Ferguson, 2004). In order to do so, Bam represses a Nanos (Nos) – Pumilio (Pum) complex. The latter acts as a translational repressor against differentiation mRNAs (Asaoka-Taguchi et al., 1999). Bam prevents Nos accumulation in the GSC (Li et al., 2009b) and binds directly to Pum (Kim et al., 2010). Nos and Pum are required in the maintenance of GSCs. If *pum* or *nos* are mutated, GSCs in adult are lost (Forbes and Lehmann, 1998; Wang and Lin, 2004). One of the Nos and Pum repression targets is *brat*. Brat interacts with Pum to promote differentiation, repressing *Mother against dpp* (*Mad)* expression, and thus inhibiting BMP signalling. Brat thus creates a bi-stable switch for differentiation (Harris et al., 2011) (Fig 1.4). JAK/STAT, a conserved signalling pathway, is also involved in maintenance of GSCs. JAK/STAT signalling regulates Dpp in the cap cells (López-Onieva et al., 2008), thus regulating the GSCs. Dpp regulation is not mediated by Notch signalling (Wang et al., 2008a).

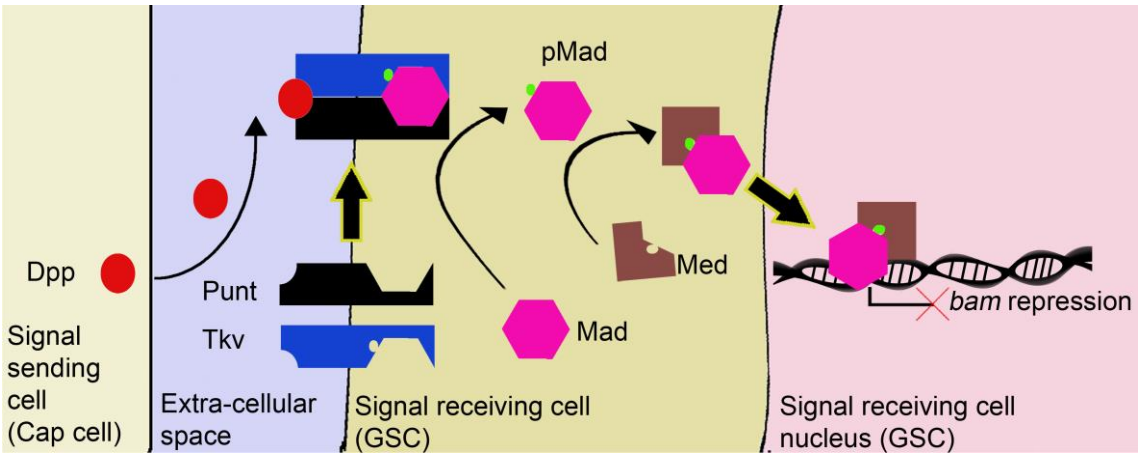

Figure 1.3. Schematic representation of BMP signal
Somatic niche cells express and secrete Dpp and Gbb (Xie and Spradling, 2000). Dpp binds to a receptor complex made by type I and II receptors. Thick veins (Tkv) and Saxophone (Sax) are BMP type I receptors in the GSCs; Punt is a BMP type II receptor. The activated receptor complex phosphorylates and activates Mad (Newfeld et al., 1997). pMad, which is the activated phosphorylated form of Mad, binds with Medea (Med) and translocates to the nucleus where it represses transcription of *bam*, a gene both necessary and sufficient for GSC differentiation (Chen and McKearin, 2003; Song et al., 2004).

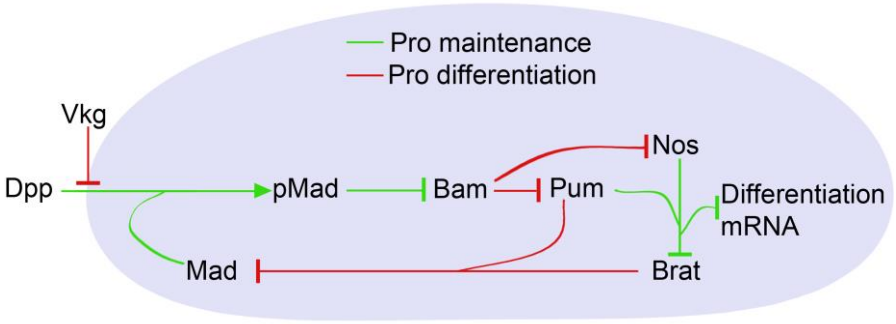

Figure 1.4. Schematic representation of GSC main differentiation and maintenance stimuli

BMP signalling activation leads to the phosphorylation of Mad and consequent repression of *bam* transcription. VKG can bind DPP preventing diffusion to the GSC. Bam can bind both Pum and Nos, blocking the activity of a Pum-Nos complex which prevents the translation of differentiation mRNA. Bam activity leads to the translation of Brat, which can bind with Pum and lead to repression of *Mad* translation, and by binding to Pum prevents Pum binding to Nos, thus enhancing differentiation mRNA translation.

1.2.3.2 Niche maintenance

GSCs are maintained by the niche, but the niche itself needs to be maintained. Notch signalling is the main factor responsible for the maintenance of cap cells. Cap cell numbers decline when Notch signalling is removed in the adult using temperature-sensitive mutations. The number of GSCs followed a similar decrease in these temperature-sensitive mutants (Song et al., 2007). Notch is thought to be cell autonomously required for the maintenance of cap cells (Hsu and Drummond-Barbosa, 2011). Notch and its ligands are both trans-membrane proteins, thus requiring cell to cell contact for the canonical signalling activation. Notch is the receptor of this signalling pathway. The source of the ligand for the maintenance of Notch signalling has not been pinpointed with certainty. GSCs expressing Dl were thought to signal to the niche (Ward, Shcherbata et al. 2006). However, later work has questioned this conclusion (Hsu and Drummond-Barbosa, 2011). Notch signalling has also a role during the development of the niche (Song et al., 2007). Further research on this role may help to understand the dynamics of the adult niche. Notch signalling is sufficient to induce cap cell formation during development. Over-activation of Notch creates larger niches and raises the number of cap cells, which then can support extra GSCs. Over-activation of Notch also leads to the creation of cap cells in ectopic positions, which are able to support GSCs (Hsu and Drummond-Barbosa, 2011; Song et al., 2007). In Wt conditions, the ligand required for the activation of Notch comes from the TF. The cap cell progenitors that come in contact with it become cap cells during late third-instar larval stage and larval-pupa transition stage. It is possible that the adult niche is maintained through ligand-induced signalling coming from the TF. However, not all cap cells contact the TF cells, and TF cells lacking Dl still recruit a few cap cells (Hsu and Drummond-Barbosa, 2011). A ligand-independent mechanism of Notch signal activation will be discussed in detail in chapter 1.4.

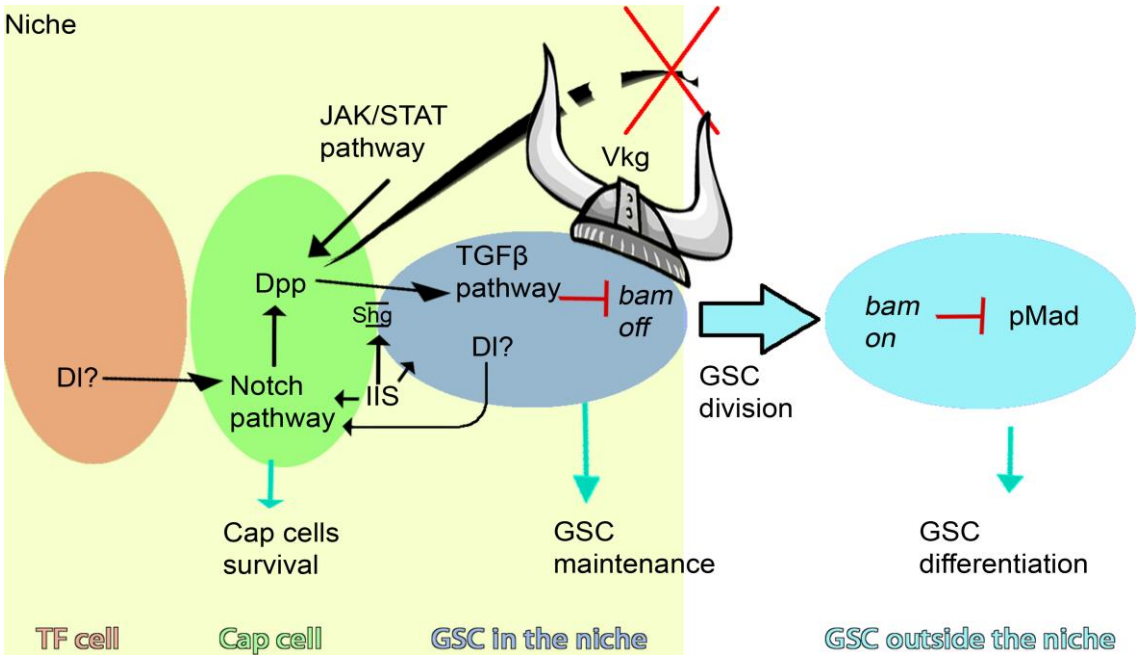

Figure 1.5. Signalling in the GSC niche
The JAK/STAT pathway regulates Dpp expression. Dpp expression activates the TGF-β pathway in the GSCs that leads to the repression of Bam, leading to GSC survival. GSCs and/or TF cells express Dl, which activates the Notch pathway inside the cap cells promoting cap cell survival and consequently GSC maintenance. When the GSC divides, it produces two daughter cells, one that stays inside the niche, and the other that is displaced. The displaced GSC receives a weaker Dpp signal, which can no more reach it because of Vkg, so bam will stop being repressed. This will lead to repression of pMad. IIS regulates the Notch pathway, Shg junctions and GSCs.

1.2.3.3 Role of escort cells

Whilst the cyst is developing it continues to move toward the posterior part of the germarium, through the activity of the ESCs until region 2b. In the anterior part of the germarium the cyst is surrounded by the ESCs. ESCs have been found to be stationary (Kirilly et al., 2011; Morris and Spradling, 2011) and not move along with the cyst and then undergo apoptosis as previously postulated (Decotto and Spradling, 2005). ESCs transfer the cysts using dynamic cytoplasmic processes (Kirilly et al., 2011; Morris and Spradling, 2011). ESCs can adopt different morphologies based on the stage of germline cyst they need to encase. Their processes are short in ESCs that interact with the cystoblast in the anterior part of the germarium, and lengthen in region 2a of the germarium to encase individual 16-cell cysts (Kirilly et al., 2011).

As with cap cells, ESCs seem to require Notch signalling for some of their function. GSCs can associate normally with Notch signalling-defective mutant ESCs lacking *Suppressor of Hairless* (*Su(H)),* which is a key component of the Notch signalling pathway. However, in germaria with *Su(H)*$^{-}$ mutant ESC clones, germline cysts in region 2a of the germarium contain dissociated fusomes resembling spectrosomes. These fusomes appear thicker than normal. ESC morphology itself is reported not to be affected by lack of *Su(H)* (Ward et al., 2006).

Also JAK/STAT signalling controls directly ESC morphology and proliferation: STAT, which is on the receiving side of the signalling, is intrinsically required in the ESCs for their functions and morphology (Decotto and Spradling, 2005).

1.3 Follicle stem cells and egg chambers

1.3.1 Follicle stem cells

Moving posteriorly the cyst, at the boundary between region 2a and 2b of the germarium, becomes surrounded by FCs instead of ESCs. FCs will then move with the cyst as it continues to progress posteriorly forming an egg chamber. The FCs also generate the stalk, which is composed of differentiated FCs that invade between successive cysts becoming stalk cells (Assa-Kunik et al., 2007). The latter separate adjacent egg chambers as they pinch off from the posterior of the germarium. All the somatic cells of the egg chamber are derived from FSCs located at the region 2a/2b boundary of the germarium (Huynh and St Johnston, 2004).

FSCs reside at the junction between region 2a and 2b in the germarium, one stem cell for each side, in fixed non-adjacent niches. FSCs contact the basement membrane, an ESC covering the last region 2a cyst, their own daughter and daughters of the opposite FSC. Shg removal from FSCs results in stem cell loss both in the adult germarium and during development (Song and Xie, 2002). Also integrins, another class of cell-adhesion molecules, have a role in the maintenance of FSCs and their progeny by anchoring FSCs to the basal lamina (O'Reilly et al., 2008).

FSC daughters alternate between the production of cross-migrating cells, which migrate across the anterior face of cyst, and posterior migrating cells, which migrate across the posterior face of the same cyst (Nystul and Spradling, 2007). Notch signalling is required for the cross-migration of FCs, and Dl from the germline is responsible for the activation of Notch signalling (Nystul and Spradling, 2010). When a FSC is lost, the cross-migrating cell is essential for its replacement. When FSCs are lacking Notch, the daughter cells are not able to cross-migrate, and so they cannot replace the other FSC (Nystul and Spradling, 2010).

FSCs are also maintained from signals secreted from the GSC niche.

Hedgehog (Hh) plays an important role in FSC maintenance. The signalling pathway is initiated when the protein encoded by the *hh* gene binds to Patched (Ptc), a trans-membrane protein. Ptc normally restricts the activity of Smoothened, a 7-transmembrane domain protein. Hh is expressed in TF and cap cells (Forbes et al., 1996). Binding of Hh to Ptc releases inhibition of Smoothened activity. Subsequently, this release results in the activation of Cubitus interruptus (Ci), a zinc-finger protein and transcription factor, and to the transcription of target genes (Ingham, 1998). By reducing Hh or Smoothened activity, cyst encapsulation is blocked, whilst Hh signalling stimulates over-proliferation of FCs (Forbes et al., 1996; Zhang and Kalderon, 2001). The excess of somatic FCs results in the presence of extremely long stalks and split cysts containing few nurse cells. The cells in the stalk are not pure stalk cells, indicating that Hh function is required for somatic cell proliferation as opposed to hyperplasia of fully differentiated stalks. Increasing Hh signalling produces more polar cells (Forbes et al., 1996; Zhang and Kalderon, 2001).

Also Wingless (Wg) signalling has a role in FSC maintenance (Song, 2003). In *Drosophila* the diffusible ligand Wg binds to Frizzled (Fz) and Frizzled 2 (Fz2). This results in the phosphorylation of Disheveled (Dsh), a cytoplasmic protein, inhibition of Shaggy (Sgg) and Axin (Axn), stabilization of Armadillo (Arm) and activation of target gene expression (Peifer and Polakis, 2000). In a similar pattern as Hh, Wg is expressed from TF and cap cells; from here it diffuses in the germarium, activating Wg signalling in FSCs. Removal of the Wg intracellular signalling components (Dsh and Arm) from FSCs results in the loss of the FSCs. Interestingly also constitutive Wg signalling obtained by the removal of the negative regulators Sgg and Axn cause FSC loss and somatic FCs over-proliferation (Song, 2003).

Beyond the role in the maintenance of GSCs, BMP signalling has also a role in FSC maintenance: FSCs have the capability to respond to BMP signalling, and Gbb

seems to be the ligand responsible for this activity (Kirilly et al., 2005). FSCs defective for BMP signalling have a shorter lifespan, probably due to their premature differentiation.

1.3.2 Stalk and polar cell specification

FCs enclose the germline cyst, forming the egg chamber. There are three types of FCs. The main body FCs comprise the majority of FCs. Polar cells are specified FCs that acts as signalling centres. For example, lack of polar cells produces egg chambers without stalks (Grammont and Irvine, 2001). There are 2 polar cells in each egg chamber, 1 on each side of the egg chamber adjacent to the anterior and posterior stalk. The stalk cells are specified FCs that connect sequential egg chambers together. Stalk cells and polar cells do not arise from a lineage-restricted kind of FC, but seem to just arise from lineage-unrestricted progenitors in response to specific signalling (Nystul and Spradling, 2010). Polar cell specification starts when FCs are still in region 2b of the germarium, and continues during and after the budding (Nystul and Spradling, 2010). Loss of Notch signalling activity was correlated with a loss of stalk and a compound egg chamber phenotype. Over-expression of active Notch generates a long stalk. FCs lacking Notch have differentiation defects. (Bender et al., 1993; Ruohola et al., 1991; Xu et al., 1992). Loss of Notch results in less polar and stalk cells while over-expression of Notch results in extra polar and stalk cells. Also loss of components of Notch signalling produce similar defects to lack of Notch (Grammont and Irvine, 2001; López-Schier and St Johnston, 2001; Torres et al., 2003). Dl in the germline is responsible for the activation of Notch in the anterior FCs (López-Schier and St Johnston, 2001). Notch and Fringe are cell autonomously required in the FCs for polar cell specification. Fringe is expressed in the polar cell progenitors, raising the level of Notch signalling in these cells. This has the consequence of transforming these Fringe-expressing FCs into polar

cells (Grammont and Irvine, 2001). An early function of the polar cells is to specify the stalk. Polar cells start expressing Dl and activate Notch signalling in the nearby FCs, which will then differentiate into stalk cells. The level of Notch signalling seems to be important for this process. High levels of Notch signalling will result in the formation of polar cells, while low levels of Notch signalling will result in the formation of stalk cells. If Notch levels cannot be kept low in the stalk cells, these will differentiate into polar cells (Assa-Kunik et al., 2007). Another pathway involved in the proper differentiation of the FCs is JAK/STAT, which has an antagonistic activity against Notch signalling. Upd is expressed in polar cells and is required for the differentiation of the stalk (Baksa et al., 2002; McGregor et al., 2002). Upd over-expression in polar cells leads to longer stalks. In early oogenesis Upd from polar cells is capable of inducing STAT activation in stalk cells, but not in the polar cells themselves or in the main body FCs. JAK/STAT seems to have a repressing activity against Notch in stalk cells, to keep Notch signalling low in order to prevent polar cell differentiation (Assa-Kunik et al., 2007). Conversely high Notch signalling in polar cells prevents STAT nuclear localization in polar and main body FCs, thus blocking STAT signalling (Assa-Kunik et al., 2007).

The posterior polar cell is specified by a distinct mechanism. These cells differentiate around 12 hours later than the anterior polar cells of the previous egg chamber. The stalk signals to the subsequent egg chamber and the future posterior polar cells may first differentiate as stalk cells. This transient stalk fate make these cells up-regulate Shg. This stimulates the oocyte to adhere preferentially to these cells, leading to higher Notch signalling when the germline will start expressing Dl, thus leading to polar fate differentiation when Notch levels outcompete STAT levels (Assa-Kunik et al., 2007; González-Reyes and St Johnston, 1998; Torres et al., 2003).

The stacked organization of the stalk also depends on Hh signalling. Contrary to FSC

maintenance, this function of the Hh pathway requires Fused (Fu), which acts in the classical genetic pathway for Hh signal transduction. Fu has been identified as a positive effector of the Hh signalling pathway in *Drosophila*, through interactions with different components of the Hh signalling pathway (Besse et al., 2002).

1.4 The Notch signalling pathway and its regulation

Notch is a trans-membrane protein involved in signalling during many aspects of development including many aspects of oogenesis (Artavanis-Tsakonas et al., 1999). Because of the role of Notch signalling in several important processes, mutations of its signalling pathway often result in cancer. It has also been found that both positively and negatively affecting *Notch* mutations can result in cancer depending on the tissue (Allenspach et al., 2007). Notch is present at the plasma membrane as a heterodimer composed of an extra-cellular domain (NECD) and a membrane-bound intracellular domain. Notch is cleaved in the golgi by a Furin protease at the so called S1 site (Logeat et al., 1998).

The canonical Notch signalling pathway is initiated by binding to Notch of membrane-bound Delta/serrate/Lag2 (DSL) domain ligands, Dl or Serrate (Ser). After the binding with a DSL ligand (Nye et al., 1995; Vässin et al., 1987), the Notch intra-cellular domain (NICD) is cleaved off the full length receptor and travels to the nucleus (Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). In the nucleus NICD binds with the transcription factor Su(H) (Fortini and Artavanis-Tsakonas, 1994; Honjo, 1996; Klein et al., 2000) and other co-factors like Mastermind (Mam) (Kao et al., 1998) to activate transcription of target genes like *Enhancer of split* [*E(spl)*] (Bailey and Posakony, 1995; Kim et al., 1996; Lecourtois and Schweisguth, 1995) (Fig. 1.6). The mechanism that activates the canonical Notch pathway is called trans-activation, meaning that Notch ligands from “signal-sending cells” bind and

activate Notch on the "signal-receiving cell". Notch ligands can also form cis-inhibitory interactions with Notch expressed in the same cell, limiting the zone of Notch activity (Axelrod et al., 1996; Micchelli et al., 1997).

The *E(spl)* complex is a series of genes activated by Notch (Jennings et al., 1994). Every *E(spl)* gene contains at least one binding site for Su(H). Seven genes of this complex (*m3, m5, m7, m8, mβ, mγ*, and *mδ*) encode basic Helix Loop Helix (bHLH) and have over-lapped functions (Delidakis et al., 1991). Despite this, various bHLH genes have a specific expression pattern, each dependent on Notch signalling plus other inputs (de Celis et al., 1996; Cooper et al., 2000; Nellesen et al., 1999).

1.4.1 The functions of Deltex in Notch regulation

An alternate mechanism to activate Notch does not require ligands and involves the trafficking of Notch to the late endosome and requires endosomal fusion to the lysosome. Deltex (Dx) is a RING-domain E3 ubiquitin ligase which can bind to the CDC10/ Ankyrin (ANK) repeats of the NICD via its N-terminal domain (Busseau et al., 1994; Matsuno et al., 1995). Dx promotes Notch signalling, independently of DSL-domain ligands, following its endocytic trafficking to the late endosomes (Hori et al., 2004; Wilkin et al., 2008) using an adaptor protein 3 (AP3-) and homotypic fusion and vacuole protein forming (HOPS-) dependent route. AP complexes recruit cargo to the vesicles (Cowles et al., 1997; Dell'Angelica et al., 1997; Simpson et al., 1997), although it is not known if they directly bind Notch. HOPS complexes work as tethers and mediate endosomal fusion to the lysosome (Seals et al., 2000)**.** Dx has a second role which leads to Notch being retained in the limiting membrane of late endosomes rather than being transferred to the internal compartments of the multivesicular body (Wilkin et al., 2008). From the orientation of Notch in the membrane it is possible to suggest that only the extra-cellular fragment is in contact with the proteolytic enzymes of the

endosome, while the intracellular domain is exposed only to the cytoplasm allowing proteolytic cleavages to release NICD, which can translocate to the nucleus. Mutations in the AP-3 or HOPS complex genes block trafficking of Notch from the early endosome and also prevent Dx-induced signalling. Interestingly these mutations do not affect DSL-domain ligand-induced signalling, suggesting that the mechanisms of activation by the two routes are distinct (Wilkin et al., 2008).

As a null mutation of *dx* has been published (Fuwa et al., 2006), it is clear that Dx is not essential for viability, as fertile homozygous adults are obtained. These adults have Notch loss-of-function phenotypes in the wing (Matsuno et al., 1995)**.** However, the *dx* null mutation is semi-viable and a proportion of *dx* mutants dies during embryogenesis with *Notch*-related phenotypes (Wilkin et al., 2008). Therefore, it is probable that the function of Dx helps boost the potential signal to keep it above a required threshold and make the Notch signalling pathways' function in development more robust.

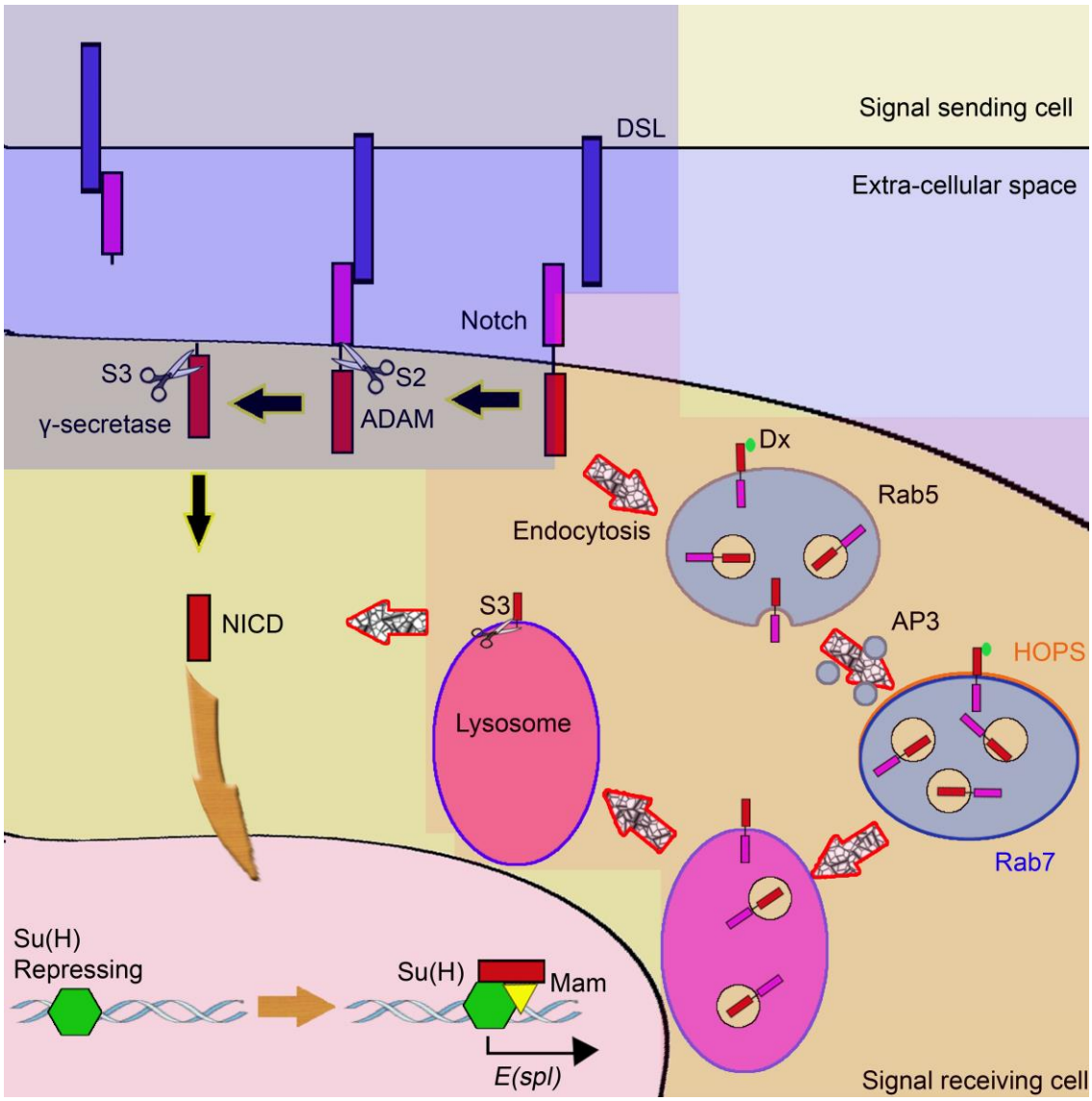

Figure 1.6. Notch Pathway
Notch canonical pathway (blue masked) and endocytic pathway (red masked). In the Notch canonical pathway a DSL ligand, presented from the signal-sending cell, binds to the Notch receptor of the signal-receiving cell. After the binding, Notch undergoes at first an S2 cleavage, mediated by an ADAM metallo-protease. After the S2 cleavage, an S3 cleavage occurs, mediated by a Presenilin-dependent γ-secretase, letting the NICD travel to the nucleus, where it binds to Su(H) and other cofactors like Mam, leading to the expression of genes from the E(Spl) complex. The binding of NICD with Su(H) lifts also the repression activity that Su(H) exercises on other genes. In the endocytic model Notch is endocytosed to the early endosome [marked by Ras (Rat Sarcoma) related in brain (Rab5)] and tagged by Dx. Then Notch is sorted to the late endosomal vesicle (marked by Rab7) through a process mediated by the AP3 complex and HOPS. The Dx tag sorts Notch to the external membrane of lysosome instead of inside the lysosome. This way Notch is not degraded by the lysosome. An S3 cleavage, similar to the one that occurs in the canonical pathway, occurs: this event lets the NICD travel to the nucleus.

1.4.2 Regulation of Notch by cell junctions

Notch signalling in the ovary cap cells has recently been shown to be regulated by the cell junctional protein Zonula occludens-1 protein (ZO-1) (Djiane et al., 2011). Notch has also been found to be regulated by junctional proteins and vice versa, as summarized in table 1.1. To comprehend the dynamics involving Polychaetoid (Pyd), Dx and Notch found in chapter 3, better understanding of the structure of cell junctions and their interactions with Notch and endocytosis is in order.

1.4.2.1 Cell junctions

To subdivide organisms into distinct compartments, epithelial sheets are necessary. Epithelia are sheets of polarized cells with an apical domain that faces external inputs or the lumen of organs, lateral domains that contact the neighbouring epithelial cells and a basal domain anchored to a basal membrane (Laprise and Tepass, 2011). *Drosophila* epithelia are organized through three major junctional organization types: the sub-apical complex (SAC), the Adherens Junction (AJ) and the Septate junction (SJ). The *Drosophila* SAC is localised in a similar location to the vertebrate tight junctions, but functionally the SJ performs a role analogous to the tight junction (Janssens and Chavrier, 2004).

Polarity regulators are organized in functional modules that associate with different membrane proteins (Laprise and Tepass, 2011). At the apical membrane are found different protein complexes. The Par complex is formed of atypical protein kinase C, Par-6, the small GTPase Cdc42 and the scaffolding protein Bazooka (Baz) (Goldstein and Macara, 2007; Suzuki and Ohno, 2006). A second complex built around the trans-membrane protein Crumbs (Crb) comprises Stardust (Sdt), Patj, Lin7, Moesin, Yurt (Yrt) and β-heavy spectrin (Bulgakova and Knust, 2009; Tepass, 2009). AJs consist of cadherins connected to cytoplasmic catenins and have an anchoring function (Banerjee et al., 2006). The catenins are connected to the actin and microtubule network

(Laprise and Tepass, 2011). SJs are found sub-apically to the AJ. SJs limit paracellular diffusion to support the barrier function and selective permeability (Banerjee et al., 2006; Tepass et al., 2001). Around SJs are found proteins regulating basal polarity such as Scribble (Scrib), Lethal giant Larvae (Lgl) and Disc Large (Dlg) (Bilder, 2004). Other regulators are Coracle (Cora), Yrt, Na^+,K^+-ATPase and Neurexin IV (Laprise et al., 2009). Par-1 kinase also supports basal polarity (Benton and St Johnston, 2003). The apical and basal proteins interact with each other to define polarity. For example, aPKC targets Lgl to prevent its apical localization (Betschinger et al., 2003; Hutterer et al., 2004). On the other hand Par-1 phosphorylates Baz to prevent basal localization of the latter (Benton and St Johnston, 2003). Yrt/Cora antagonizes Crb (Laprise et al., 2006, 2009) (Fig. 1.7).

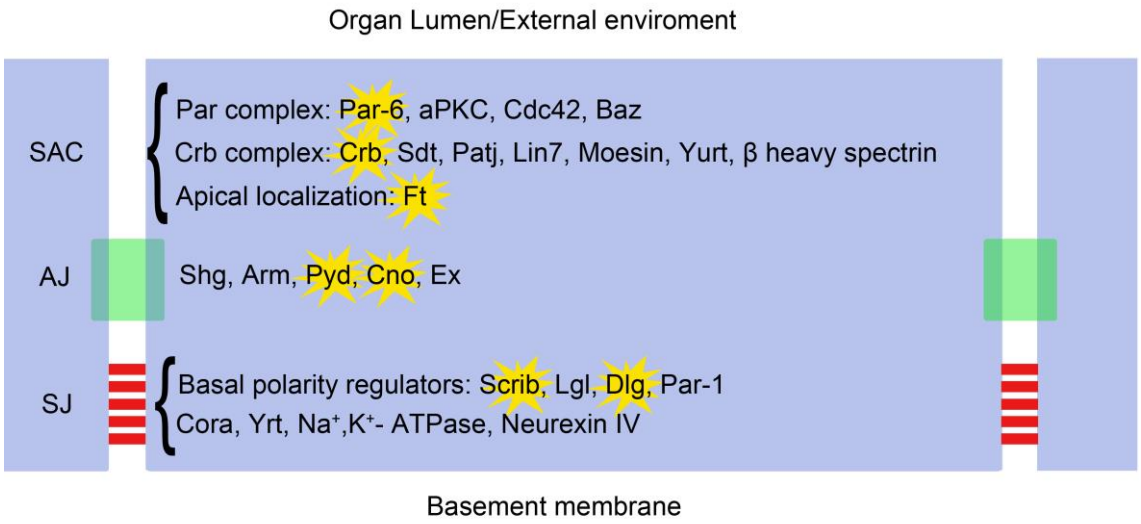

Figure 1.7. Cell junctions in *Drosophila*
A schematic diagram of epithelial cell junctions in *Drosophila*. From apical to basolateral: SAC (sub-apical complex); AJ (Adherens Junction); SJ (Septate Junction). Highlighted in yellow are proteins interacting with the Notch signalling pathway, either directly or indirectly.

Gene (s)	Organism and tissue	Interaction
Crb	*Drosophila*	Crb limits Notch signalling through inhibition of Notch endocytosis (Richardson and Pichaud, 2010) and direct interaction with the γ-secretase complex (Herranz et al., 2006; Laprise, 2011).
Crb	*Danio rerio*	Crb binds directly to Notch and inhibits it (Ohata et al., 2011). This inhibitory activity requires the extracellular domain of Notch (Laprise, 2011; Richardson and Pichaud, 2010).
Crb	*Drosophila* wing	*crb* expression in *Drosophila* wing is regulated by Notch (Herranz et al., 2006).
Cno, Pyd	Drosophila	Canoe (Cno) physically interacts with Notch through its PDZ domain, resulting in Notch signalling inhibition (Carmena et al., 2006). Cno is also associated with Pyd and the actin regulator Enabled (Ena) at the AJs where they colocalize and interact physically and genetically in the regulation of dorsal closure (Choi et al., 2011; Takahashi et al., 1998).
Ft	*Drosophila* embryos	*Fat (ft)* mutants have Notch over-activation-like phenotypes (Marcinkevicius and Zallen, 2013)
Scrib, Dlg	*Drosophila* Ovary	Loss of *scrib* and *dlg* in posterior FCs disrupts Notch signalling (Li et al., 2009a).
Shg	*Drosophila* midgut	Dl is expressed in the ISCs and signals to Notch in daughter cells to promote their differentiation. Shg ensure stable attachment between the two signalling cells resulting in proper Notch signalling activation (Maeda et al., 2008).
β-catenin	*Drosophila* wing discs	Notch associates with β-catenin near the AJs and the complex is rapidly endocytosed promoting the trafficking and degradation of β-catenin in endosomal compartments (Hayward et al., 2005; Sanders et al., 2009).

Table 1.1. Notch and cellular junction components
Examples of Notch interacting with junction components from the literature could help understand the interaction between Notch and Pyd.

1.4.2.2 The structure and function of ZO-1

Drosophila ZO-1 is also known as *tamou* (*tam*) and *pyd*, and it is a member of the membrane-associated guanylate kinase family (MAGUK) (Takahisa et al., 1996). This family of proteins is localised to sites of cell-cell contact, like AJs and synapses, and has multiple protein-protein interaction domains, such as PDZ [the name PDZ comes from the first letter of the first three discovered proteins showing this domain: Post synaptic density protein (Psd95), *Drosophila* Dlg, and ZO-1 domains], SRC homology domains and a domain with homology to the enzyme guanylate kinase (GUK). These domains allow MAGUK proteins to act as a scaffold and organize protein complexes at the plasma membrane, focusing signalling pathways in specialized regions (Dimitratos et al., 1999; Wei and Ellis, 2001). Pyd localizes strongly to sites of cell junctions in both embryos and imaginal disc (Wei and Ellis, 2001) and to the AJ in *Drosophila* pupal retina (Seppa et al., 2008). Pyd regulate the patterning of pupal lattice cells through association with AJ proteins like Shg, β-catenin, and α-catenin. The removal of Pyd from the AJ compromises the tight regulation of the levels of both cell adhesion molecules and junctional proteins in the pupal retina (Seppa et al., 2008) and causes an increase of several AJ-localized proteins including Shg, Arm and Notch (Djiane et al., 2011). Pyd localization to the AJ is controlled by Shg and α-catenin (Seppa et al., 2008). In the wing disc epithelium Pyd co-localizes with Shg (Djiane et al., 2011). Shg and α-catenin seem necessary to build or maintain the AJ and to localize Pyd, but excess of Shg and α-catenin are not sufficient to attract ectopic Pyd (Seppa et al., 2008). Recent work in our lab concentrates on the interactions between Pyd and the Notch pathway (Djiane et al., 2011). Pyd has different effects on Notch in a tissue-specific way. Is has a positive effect on Notch in the development of the peripheral nervous system (Djiane et al., 2011). On the contrary in the GSC niche *pyd* mutants produce an enlargement in the size of the cap cell niche, which is a known result of Notch over-expression, and increased activity of the reporter E(spl)m7-lacZ (Djiane et al., 2011). The mechanisms

of these interactions have not been previously investigated. In turn Pyd seems to be regulated by Suppressor of deltex (Su(dx)). Su(dx) is an E3 ubiquitin ligase belonging to the Nedd4 family that can bind to Notch (Jennings et al., 2007). Su(dx) regulates the sorting of Notch within the endocytic pathway, promoting the transfer of Notch to the internal lumens of the late endosome and lysosome, away from the limiting membrane. It thus acts antagonistically to Dx (Wilkin et al., 2004, 2008). Recent work has suggested that Pyd may be an alternative target for Su(dx). In S2 cells, if expressed on its own, Pyd localizes at the plasma membrane. Su(dx) on its own localizes in small endosomal compartments. If both genes are expressed together, Su(dx) changes its localization to the plasma membrane co-localizing with Pyd. In the GSC niche *su(dx)* mutations can suppress the increased cap cell number dominant phenotype induced by one copy of a mutation of *pyd* (Djiane et al., 2011). In this context then, loss of Su(dx) gives a reduction of Notch signalling rather than an increase as had been observed in the wing. Interestingly the *su(dx)* mutation could not suppress the phenotype of a homozygous null *pyd*, suggesting that the Su(dx) mutation normally acts by increasing Pyd function in the cap cells (Djiane et al., 2011). Therefore Su(dx) might act directly on Notch to down regulate its function in the wing, but act indirectly on Notch through Pyd to up-regulate Notch signalling in the cap cells. In peripheral nervous system development, antagonistic action of Pyd and Su(dx) was also found, but the role of Pyd in this case was to promote Notch signalling (Djiane et al., 2011). It is not understood how Pyd can down-regulate and up-regulate Notch in these different contexts.

In chapter 3 of this thesis we will explore the relationship between Dx and Pyd in the regulation of Notch signalling and endocytosis. Cell adherens proteins can affect or be regulated by endocytosis (Table 1.2). For example β-catenin, which is a component of cadherin-based AJs and the Wnt signalling pathway, is involved in endocytosis and signalling.

Gene (s)	Organism and tissue	Interaction
β-catenin	Various human cell lines	Cadherin-bound β-catenins that accumulate at the perinuclear endocytic recycling compartment upon AJ dissociation, translocate to the nucleus upon Wnt pathway activation. This observation suggests that AJ dissociation may affect the levels of Wnt pathway signalling through an endocytic route (Kam and Quaranta, 2009).
Shg	*Drosophila* embryonic ectoderm	Shg is actively trafficked through endocytosis. Ras (Rat sarcoma) related in brain (Rab)11 mutants lead to disruption of AJs and reduced integrity of the ectoderm. In these mutants Crb is lost before loss of Shg and before AJs are destabilized, whilst Dlg remains cortical. Rab5 mutants had less dramatic effects compared to loss of Rab11. These results show that endocytic trafficking is essential for the maintenance of AJs (Roeth et al., 2009).
Crb	*Drosophila* head, trachea *C.elegans* embryo	Crb controls the delivery of cargos and endocytosis in the apical membrane, thus controlling apical membrane growth (Förster et al., 2010; Richardson and Pichaud, 2010; Shivas et al., 2010).
Crb, Sdt	*Drosophila* Epithelia	Once in the apical membrane Crb needs to interact with Sdt to prevent rapid endocytosis and loss from the membrane (Tepass and Knust, 1993).
Cdc42, Par-6, aPKC, baz	*Drosophila* imaginal epithelia, early embryonic ectoderm	Cdc42, Par-6, and aPKC are required for the endocytosis of AJ components through regulation of actin polymerization, a process that appears to be involved in vesicle scission from the plasma membrane. In the early embryonic ectoderm, Cdc42, Par-6, aPKC, and Baz regulate endocytic trafficking of apical proteins (Georgiou et al., 2008; Harris and Tepass, 2008; Leibfried et al., 2008).
Par-3	*C.elegans*	Eea-1, which is a marker for early endocytosis, localizes to specific puncta. The enrichment in these puncta depends on Par-3 (Andrews and Ahringer, 2007).
Par-6, PKC3/ aPKC	*C.elegans*	Dynamin-1, is enriched and maintained in the anterior cortex in a Par-6 and PKC3/aPKC-dependent manner (Nakayama et al., 2009).
Par module, Cdc-42	*C.elegans*	The anterior Par module and the Rho GTPase Cdc-42 were shown to be required for efficient endocytosis (Balklava et al., 2007).

Table 1.2. Cell junction components and endocytosis
Examples of junction components involved with endocytosis from the literature could help understand how Pyd regulates Notch through endocytosis.

1.5 Nutrition

The availability or lack of nutrients has many documented effects on cells and organisms. In many organisms, including *Drosophila*, calorific restriction extends lifespan (Min and Tatar, 2006). In *Drosophila*, starvation during development results in growth retardation, female sterility and reduced body weight due to fewer and smaller cells (Goberdhan and Wilson, 2003; Partridge et al., 2011).

Ovaries react very quickly to food changes. Flies fed with rich food can lay up to 90 eggs for day. However, if fed with poor food, egg laying falls within 24 hours to only 1.5 eggs per day. Since oogenesis has a high energy requirement, this response may ensure adult survival on poor food (Drummond-Barbosa and Spradling, 2001). Conversely flies switched from poor to rich food increase their egg laying rate within 2 days (Drummond-Barbosa and Spradling, 2001). In order to achieve this astonishing change, the whole egg production system has to slow down or speed up.

1.5.1 Routes to nutrition sensing

The identification of mutants that replicate nutrition effects has been important in uncovering the signalling pathways involved in nutrition responses. For example diet restriction-like phenotypes are characteristic of *chico* mutant flies, which is an insulin receptor substrate (IRS) protein in flies (Böhni et al., 1999; Britton and Edgar, 1998). IIS pathway mutants also have similar phenotypes to diet restriction (Clancy et al., 2001; Tatar et al., 2001). Amino acid restriction extends lifespan and reduces fertility in *Drosophila* (Min and Tatar, 2006). Target of rapamycin (Tor) signalling mediates a signalling pathway that couples amino acid availability to translation initiation and growth. Tor signalling has a similar effect to IIS, in regard to lifespan extension and fecundity (Kapahi et al., 2004). Figure 1.9 shows a map of nutrition-sensing components. Although IIS and Tor signalling are not investigated in

my results, I will provide an overview in the introduction to better understand the dynamics of nutrition in the GSC niche and as a base for future work discussion.

1.5.1.1 Nutrition central regulation

Internal metabolism is regulated centrally in the brain and in the fat body, and locally in the other tissues (Kohyama-Koganeya and Hirabayashi, 2010). In *Drosophila*, the brain contains regions highly specialized for the control of food intake and metabolism (Partridge et al., 2011). Ilps are produced in the brain by IPCs (Geminard et al., 2006). IPCs express Ilp 1,2,3 and 5. Glucose increases IPC activity and intracellular Ca^{2+} (Kréneisz et al., 2010). Intracellular Ca^{2+} increase in IPCs when IPCs are exposed to trehalose, which is the main circulating carbohydrate in insects (Fridell et al., 2009). In mammals pancreatic β cells Ca^{2+} influx as a result of glucose sensing is a critical event in insulin release (Lowell and Shulman, 2005). Thus the insulin secretion mechanism appears to be conserved (Haselton and Fridell, 2010). Genetic ablation of IPCs leads to poor adult survival with growth reduction and increased levels of trehalose (Brogiolo et al., 2001; Rulifson et al., 2002). Starvation affects IPC-derived Ilp 3 and 5 expression (Ikeya et al., 2002). Ilp2 expression, which amounts to 80% of total Ilp in the animal (Buch et al., 2008), is not affected by starvation (Ikeya et al., 2002). Starvation leads to accumulation of Ilps in the IPCs. This accumulation is quickly reversed upon refeeding. Amino acids are a key controller of this event (Géminard et al., 2009). Since the over-expression of Ilp2 in the IPCs does not result in higher Ilp2 secretion (Géminard et al., 2009), it has been speculated that the secretion from the IPCs is regulated from other locations in the fly. The fat body controls Ilps secretion from the IPCs (Géminard et al., 2009). The fat body is the main storage tissue and can also regulate the amount of Ilps in the organism by binding to Ilps through the fly ortholog of the vertebrate IGF-binding protein acid-labile subunit

(ALS) (Arquier et al., 2008). In case of starvation the fat body undergoes autophagy to provide energy. This process is regulated through the Tor pathway (Neufeld, 2010). IIS regulation in the brain is also mediated by Tor signalling. When amino acid import is specifically restricted in fat body cells by genetic manipulation of Slimfast (Slif), which is an amino acid transporter localized at the plasma membrane (Hennig et al., 2006), general IIS is decreased in peripheral tissues. This leads to a systemic reduction of larval growth (Colombani et al., 2003). Mutations in the Tor complex (TORC) or in the amino acid sensor Slif mimic starvation effects on Ilp2 secretion (Géminard et al., 2009). The fat body controls insulin in the brain through the secretion of Upd2. Upd2 activates JAK/STAT signalling in GABAergic neurons that project onto the IPCs. JAK/STAT activation lifts the inhibitory action of GABA neurons on IPCs, permitting the release of Ilps (Rajan and Perrimon, 2012) (Fig. 1.8). IIS and Tor also play a role in the regulation of fat metabolism. Flies on a high fat diet show alteration in IIS/glucose homeostasis. In these flies, initially glucose levels dropped whilst Ilp2 levels rose. After prolonged exposure, glucose levels rise whilst Ilp2 levels drop. The accumulation of lipids in the fat body and in the gut is increased. Also cardiac dysfunctions are noted. These metabolic phenotypes are the hallmark of obesity in mammals too. Systemic inhibition of the Tor pathway prevents fat accumulation and cardiac dysfunctions. Specific inhibition of Tor pathway components [Tsc1-2 (Tuberous sclerosis complex), the ribosomal protein S6 kinase (S6k) and the eukaryotic initiation factor (eIF4E) binding protein Thor] in the fat body were effective in diminishing fat accumulation and heart problems, showing a specific action of Tor signalling in the control of the fat body. Also over-expression of Forkhead box, sub-group "0" (Foxo), which is a negative effector of IIS, in the fat body gives the same results, suggesting that both pathways have a role in regulating the fat body (Birse et al., 2010).

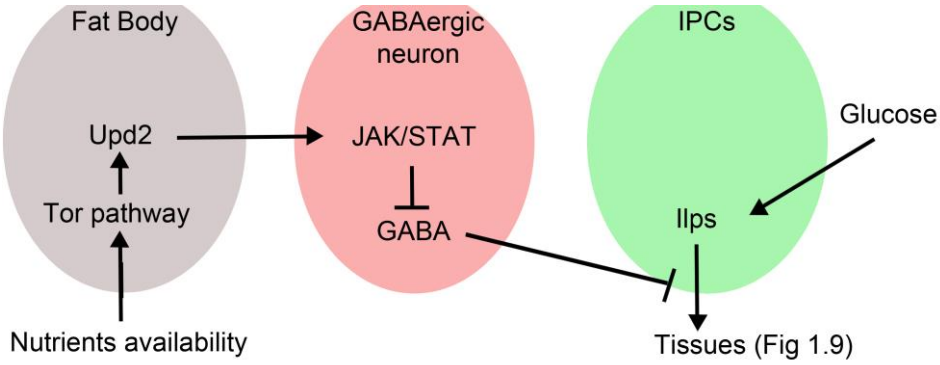

Figure 1.8. Nutrition central regulation
IPCs can sense glucose, but they depend on signals originating in the fat body for Ilps secretion. Nutrient availability is sensed in the fat body through Tor signalling. Tor activation leads to expression of Upd2 from the fat body. Upd2 by activating JAK/STAT signalling in GABAergic neurons blocks the inhibition on IPCs. With the lifted inhibition, IPCs can secrete Ilps activating IIS in tissues.

1.5.1.2 IIS pathway

The control of Ilp levels is only the first step in the regulation of IIS. Ilps binding to the InR cause a series of signalling steps, which are subject to different pathway regulation. Binding of Ilps to InR activates the Phosphoinositide-3 kinase (Pi3k) Dp110 through the IRS Chico or directly without using Chico. Higher availability of Phosphatidylinositol (3,4,5)-triphosphate (PIP3) leads to activation of the kinase, 3-phosphoinositide dependent protein kinase-1 (Pdk1), which in turn activates another kinase, Akt. Akt can be also activated by Pdk2, which is thought to be Tor complex 2 (TORC2) (Sarbassov et al., 2005; Vereshchagina et al., 2008). The Phosphatase and tensin homolog (Pten) antagonizes this process by dephosphorylating PIP3 (Maehama and Dixon, 1998). Activated Akt blocks the inhibiting transcriptional regulator Foxo by preventing its translocation to the nucleus (Van Der Heide et al., 2004; Kramer et al., 2003; Puig et al., 2003), leading to transcription of proliferation genes. IIS can also promote growth through interaction with the Tor signalling pathway (Goberdhan and Wilson, 2003; Partridge et al., 2011).

1.5.1.3 Tor signalling pathway

In *Drosophila* Tor forms two complexes, TORC 1 and 2, with some shared and some distinct components (Wullschleger et al., 2006). Activated TORC1 blocks Thor, which is a translational repressor. TORC1 also activates S6k, a ribosomal kinase, leading to protein production (Goberdhan and Wilson, 2003; Partridge et al., 2011). This mechanism is conserved in mammals, where protein synthesis is promoted if there are enough amino acids in the cell. This effect is achieved through Tor signalling by inhibition of translational repressor Thor and stimulation of S6k (Yan and Lamb, 2012).

Upstream of Tor signalling is found the small GTPase Ras homologue enriched in brain (Rheb), which is a positive regulator of Tor when the two proteins

interact on the lysosomal membrane (Buerger et al., 2006). Rheb is also involved in the formation of endosomes and lysosomes (Saito et al., 2005). Rheb GTP loading is inhibited by Tsc1/2, which thus act as a negative regulator of Tor signalling (Korolchuk et al., 2011; Kwiatkowski and Manning, 2005; Orlova and Crino, 2010; Sancak et al., 2010; Zoncu et al., 2011).

IIS can interact with Tor signalling through Akt. Akt phosphorylates and inhibits Lobe, which was identified as a TORC1 inhibitor (Vander Haar et al., 2007; Sancak et al., 2007). Akt also phosphorylates Tsc2, inhibiting it and thus activating Tor signalling (Cai et al., 2006; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002).

Apart from IIS, Tsc1/2 also integrates several upstream inputs to regulate growth. Low energy level in the cell results in a decrease of ATP levels and increase in AMP levels. AMP is more sensitive to variations in energy status than ADP because AMP:ATP ratio varies approximately as the square of ADP:ATP ratio (Kahn et al., 2005). Increases in AMP result in activation of AMP-activated kinase (AMPk) (Dasgupta and Milbrandt, 2009; Vincent et al., 2001). In low energy status, AMPk directly phosphorylates Tsc2 (Corradetti et al., 2004; Inoki et al., 2003; Liu et al., 2006), leading to inhibition of Tor signalling. AMPk also phosphorylates Raptor, which is a component of TORC1, thus suppressing Tor activity (Gwinn et al., 2008). Hypoxia leads to energy starvation and consequent inhibition of the Tor pathway. This inhibition can be both Tsc2-dependent through AMPk signalling and Tsc2-independent (Liu et al., 2006). Glucose is an important upstream regulator of this process (Shackelford and Shaw, 2009).

Rheb GTP levels do not to decline following amino acid withdrawal, suggesting that Tor signalling response to amino acid depletion does not come from interactions with IIS but from a distinct pathway (Findlay et al., 2007; Nobukuni et al., 2005). Amino acids can directly control Tor signalling independently of Tsc (Beugnet et

al., 2003; Christie et al., 2002). In *Drosophila,* Minidiscs, which is similar to the catalytic subunit of heterodimeric amino acid transporters, and Slif, which displays a strong homology with amino acid permeases of the cationic amino acid transporter (CAT) family, have been implicated in growth regulation (Colombani et al., 2003; Martin et al., 2000). Amino acid sensing upstream of Tor signalling is also modulated by proton-assisted amino acids transporters (PAT). CG3424 and CG1139 belong to the PAT class, and interact genetically with Tor and IIS components. Mutation in CG3424 results in reduced growth in the fly (Goberdhan et al., 2005). The small GTPases called Rags are another key mediator of amino acid signalling. Amino acids regulate nucleotide loading on Rags, leading to Tor activation (Kim et al., 2008; Sancak et al., 2008). The effect of Rags on Tor is not to directly stimulate it, but to change its localization to lysosomes and late endosomes (Sancak et al., 2008, 2010; Zoncu et al., 2011). This finding is supported by previous literature in yeast which showed the necessity of the lysosome compartment for the recovery from amino acid starvation (Efeyan et al., 2012). This necessity is found also in mammals, where the Vps34 homolog has been implicated in amino acid signalling to mTORC1 (Nobukuni et al., 2005). The change in localization of Tor is supposed to result in activation through the binding to Rheb, which resides in the lysosome (Buerger et al., 2006; Saito et al., 2005; Sancak et al., 2010). Lysosomes in yeast have been found to act as stores of amino acids (Harms et al., 1981; Kitamoto et al., 1988). In an *in vitro* cell free system comprising of lysosomes and purified mTORC1, addition of amino acids was sufficient for the binding of mTORC1 to the lysosomes following Rag activation, suggesting that all the machinery for the intracellular amino acid sensing is found in the lysosomes (Zoncu et al., 2011). An important relationship of Tor with the lysosome is also found in the interactions with transient receptor potential cation channel, mucolipin subfamily (Trpml) and lysoNa$_{ATP.}$ Trpml, a Ca^{2+} vesicular release channel, is required during

autophagy. Loss of Trpml diminishes vesicle fusion leading to lower amino acid intake in the lysosomes, resulting in inhibited activation of TORC1. Feeding a *trpml* mutant with a rich diet reverses the phenotype. TORC1 reciprocally affects Trpml regulating its subcellular localization, thus providing feedback control of autophagy (Venkatachalam et al., 2013). Tor also interacts directly with another lysosomal membrane channel, to regulate its function and consequently the lysosome. The endolysosomal ATP-sensitive Na^+ channel ($lysoNa_{ATP}$) is a complex formed by two-pore channels and mTor, and is regulated in part by Tor localisation. $LysoNa_{ATP}$ becomes more sensitive to ATP inhibition when binding mTor. $LysoNa_{ATP}$ becomes constitutively open upon nutrient removal, which implies also lower ATP levels, and mTor translocation off the lysosome membrane caused by starvation. $LysoNa_{ATP}$ controls the lysosome membrane potential, pH stability and amino acid homeostasis. It seems to be required in regulating fasting endurance (Cang et al., 2013).

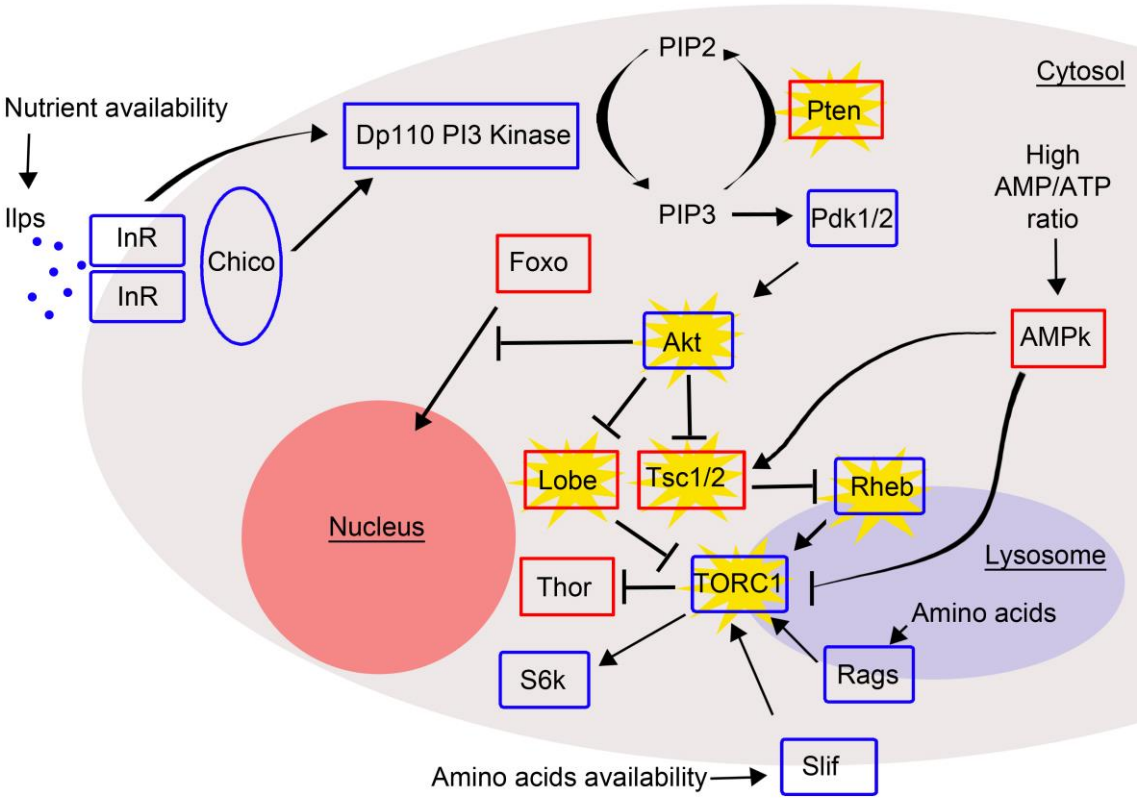

Figure 1.9. Schematic representation of nutrition related pathways in *Drosophila*
Nutrient availability regulates levels of Ilps. Binding of Ilps to InR activates the PI3 Kinase Dp110. This action can be performed through the IRS Chico or directly. Higher availability of PIP3 leads to activation of Pdk1/2, which in turn activates Akt. Pten antagonizes this process by dephosphorylating PIP3. Activated Akt blocks the inhibiting Foxo from entering the nucleus, leading to transcription of proliferation genes. Akt has also a positive action on Tor signalling through inhibition of Tsc and Lobe. Amino acid availability activates Tor signalling pathway through the amino acid transporter Slif. Low energy results in increase of AMP, which activates AMPk. AMPk activates Tsc2 and inhibits Raptor, a component of TORC1. Rags are activated depending on amino acid levels in the lysosome, and can activate Tor. Tor activation blocks Thor, thus leading to gene translation and elicits the activation of S6k (Goberdhan and Wilson, 2003; Partridge et al., 2011). Blue boxed proteins have a positive effect on cell growth. Red boxed proteins have a negative effect on cell growth. In yellow are marked proteins interacting with Notch signalling.

1.5.2 Dietary regulation of stem cells and their niches

Changes in the diet can affect stem cells in different ways. Several studies have investigated the impact of diet on GSCs. In *C.elegans*, IIS promotes germline proliferation through the canonical Pi3K pathway, inhibiting Foxo (Michaelson et al., 2010). In *Drosophila* males GSCs are lost if flies are kept on poor food (McLeod et al., 2010). This effect was reversible upon re-feeding and also through constitutive activation of IIS in the GSCs (McLeod et al., 2010). However, others have shown contrary effects on the male *Drosophila* GSCs (Mair et al., 2010). In the latter case, dietary restriction prevented age-related decline of the GSCs in male *Drosophila*, improving their maintenance. These different outcomes are indicative of how different methods for diet restriction can result in opposite outcomes. In this case (McLeod et al., 2010) used a starvation medium protein-free (10% sucrose/1% agar) and saw a decreased number of GSCs. On the other hand, (Mair et al., 2010) used a diet restriction with less sucrose but supplemented with yeast (5% sucrose and 10% yeast), showing the requirement for dietary proteins in male GSC maintenance. Diet restriction also triggers HSC differentiation. IIS and Tor pathways regulate cell-autonomously the size and activity of the *Drosophila* HSCs to control their growth and maintenance (Benmimoun et al., 2012). In mice, diet restriction delays HSC senescence and sustains HSC activity longer (Chen et al., 2003).

In *Drosophila,* the nutritional sensing that operates globally through the fat body also regulates neuroblast proliferation. Global inactivation of Tor, fat body-specific inactivation of Slif or over-expression of Tsc1/2 all strongly reduce neuroblast proliferation (Sousa-Nunes et al., 2011). In fed larvae, neuroblast inactivation of Tor signalling and inhibition of Pi3K both inhibit reactivation. Alternatively stimulation of Tor or Pi3K leads to neuroblast reactivation (Sousa-Nunes et al., 2011). The Ilps required for neuroblast temporal reactivation come from glial cells. These do not

influence organismal growth, which is controlled through Ilps secreted into the haemolymph by IPCs (Sousa-Nunes et al., 2011). The Tor pathway is also involved in human embryonic stem cells (hESCs). Here the Tor pathway is repressed when maintaining stemness. Expression of active S6k leads to differentiation. Knock-down of inhibitory factors of Tor, like the Tsc complex, leads to differentiation of the hESCs (Easley et al., 2010).

Other nutrient-sensing components have also been linked to stem cell regulation in various tissues. For example AMPk signalling has been shown to regulate neural stem cell proliferation in mice (Dasgupta and Milbrandt, 2009). Mutations in Pten lead to stem cell over-activation in multiple stem cell systems, for example haematopoietic, prostate, neural, intestinal, breast, skin and germ stem cells (Hill and Wu, 2009).

Whilst there are many examples of nutrition affecting stem cells, there are few examples reported of nutrition influencing the niche. One example is the Paneth cells, which are niche cells for human ISCs. Paneth cells respond to diet restriction by augmenting ISC functions through restriction of Tor signalling. These findings show that Tor can regulate stem cell self-renewal in a non-cell autonomous way through the niche (Yilmaz et al., 2012). IIS and Tor pathways also regulate cell autonomously the size and activity of the *Drosophila* haematopoietic niche (Benmimoun et al., 2012).

1.5.3 Diet and ageing influence the GSC niche

Poor diet reduces the number of GSCs positive for division markers such as Phospho-Histone H3 (PH3) and Bromodeoxyuridine (BrdU) labelling (Hsu et al., 2008). Additionally, cysts in region 2a/2b undergo apoptosis (Drummond-Barbosa and Spradling, 2001). The ovary is able to adapt to changes in diet conditions through variations in IIS and Tor signalling (LaFever et al., 2010). IIS can affect the GSCs both

directly and indirectly through the niche. IIS mediates the effect of diet on GSC proliferation during the short term nutrition response (Drummond-Barbosa and Spradling, 2001). *InR* GSC mutant clones proliferate more slowly than their Wt counterparts, indicating that the GSCs respond to Ilps cell autonomously and the niche is not required for their effects on GSC proliferation. Diet can regulate both the G1 and G2 phases of GSC division (Hsu et al., 2008). IIS controls both G1 and G2 phases *in vivo* and in culture. During *Drosophila* development IIS components specifically regulate the length of G1 (Goberdhan and Wilson, 2003). However, in *Drosophila* GSCs IIS regulates maintenance acting only on the G2 phase of the cell cycle. The Ras/MAPK kinase, Pi3K and the transcriptional factor Foxo mediate the effects of IIS, regulating the G2 division phase of GSCs (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). IIS controls the rate of GSC division also in germarium cultured *in vitro*. In the absence of insulin in the culture medium there were few GSC divisions. In contrast when insulin was added there was a big increase in GSC division rates (Morris and Spradling, 2011).

Another role for IIS is the long-term maintenance of GSCs over the lifespan of *Drosophila* and this role is mediated through regulation of niche signalling. In the *Drosophila* ovary ageing results in a progressive decline of egg production (Zhao et al., 2008). This decline is due to reduced GSC division rate and numbers and increased cyst apoptosis (Zhao et al., 2008). The cap cell niche is a more stable population, but still declines slightly after 63 days (Pan et al., 2007). Daughter against dpp (Dad)-lacZ and pMad activity are reduced in aged germaria (Zhao et al., 2008) and increased BMP signalling during the life span of the fly enhances GSC life span and proliferation. Shg levels also decrease with age (Pan et al., 2007). Female flies mutant for InR^{339}/InR^{E19} contain slightly fewer GSCs at eclosion, and subsequently lose them more rapidly than in Wt germaria (Hsu and Drummond-Barbosa, 2009). Remarkably in this maintenance

role, IIS does not have a direct effect on GSCs. Instead the effect of insulin on the GSC goes through the niche, where IIS is regulating Notch signalling (Hsu and Drummond-Barbosa, 2009).. InR^{339}/InR^{E19} mutants flies also eclose with fewer cap cells than Wt control and lose them faster (Hsu and Drummond-Barbosa, 2009). Similar cap cell loss is seen in *Notch* mutants (Ward et al., 2006) and the level of expression of the Notch reporter (E(spl))mβ-CD2 in cap cells and TF cells is also reduced in InR^{339}/InR^{E19} mutants (Hsu and Drummond-Barbosa, 2009).. Expression of an activated form of Notch (Struhl et al., 1993) in the somatic cells of InR^{339}/InR^{E19} mutant flies, prevents the GSC loss phenotype, demonstrating that Notch signalling is acting either downstream or parallel to IIS in the control of cap cell and GSC number (Hsu and Drummond-Barbosa, 2009). InR is cell autonomously required for cap cell maintenance and Notch activation (Hsu and Drummond-Barbosa, 2011). It is not clear how IIS affects Notch but it does not seem to be through control of ligand expression. Ilps do not affect the expression of Dl or Ser in the TF (Hsu and Drummond-Barbosa, 2011). The levels of Dl and Ser reporter lines are also not affected in *chico* mutants (Hsu and Drummond-Barbosa, 2011). One possible predicted outcome of reduced Notch signalling would be reduced BMP pathway signalling since the expression of Dpp has been thought to be controlled by Notch (Ward et al., 2006). However, using a Dad-lacZ reporter line for TGF-β activity, it was possible to see that InR does not control BMP signalling (Hsu and Drummond-Barbosa, 2009).

IIS seems to play a role also in the regulation of cell-cell adhesion, mediated by Shg, as loss of IIS leads to a reduction of Shg expression. Only 21% of InR^{339} cap cells contact GSCs, in comparison to 50% of Wt cap cells (Hsu and Drummond-Barbosa, 2009). Flies with a heterozygous mutation for *shg* present a bigger loss of GSCs than Wt in 63 day old flies. In contrast, over-expression of Shg in GSCs leads to a greater number of GSCs in 63 day old flies (Pan et al., 2007). This effect seems to be in

a parallel pathway to the effects of insulin on Notch. N^{ts2} mutants at 29°C show a decrease in cap cell and GSCs number, but the levels of Shg do not change (Hsu and Drummond-Barbosa, 2011).

Another signalling pathway regulating dietary responses of GSC proliferation is Tor. Tor controls GSC proliferation independently of IIS acting on the G2 phase of cellular cycle. *Tor* mutants have a severe growth delay (LaFever et al., 2010). Tor is also required for GSC maintenance and prevents GSC loss by differentiation (LaFever et al., 2010). Interestingly, mutants for *Tsc*1, a known inhibitor of the Tor pathway (Pan et al., 2004), exhibited a greater loss than *Tor* mutants, showing that an optimal level of signalling is required for GSC maintenance (LaFever et al., 2010). Loss of Tsc1/2 leads to GSC loss due to differentiation. This effect is due to over-activation of Tor signalling (Sun et al., 2010). Tor signalling has been found coupling diet to stem cell functions also in other systems (Yilmaz et al., 2012). Calorific restriction promotes longevity in different organisms, possibly by affecting stem cell systems (Nakada et al., 2011; Simons and Clevers, 2011). For example, in specific mouse strains under calorific restriction new neurons are formed from neural progenitors and HSC decline is prevented (Chen et al., 2003; Ertl et al., 2008). Paneth cells are a key component of the mammalian ISC niche. Under a caloric restriction regime, Tor signalling in Paneth cells is reduced, resulting in an ISC-enhancing effect which is mimicked by rapamycin, a Tor inhibitor. Over-expression of Tor in Paneth cells (but not ISCs) during caloric restriction abolishes the effect on ISCs (Yilmaz et al., 2012). Calorific restriction acts also on other niches. Reduced calorie intake alters the physiology and function of muscle stem cells, enhancing myogenic activity (Cerletti et al., 2012).

1.5.4 Notch and nutrition pathways links

Diet affects cap cells probably through IIS (Hsu and Drummond-Barbosa,

2011). IIS has been shown to regulate GSC proliferation in the *Drosophila* ovary niche and maintains Notch signalling in the cap cell niche. In (Hsu and Drummond-Barbosa, 2011) a direct link between IIS and Notch pathway was reported It was found that IIS affects Notch signalling in cap cells cell-autonomously through a Pi3K- and Foxo-dependent way. It is still not known if IIS affects Notch signalling through regulation of its synthesis, processing or trafficking or cleavage of its intracellular domain upon ligand binding. No dietary regulated effects of IIS and Notch in the niche have been previously reported.

Notch can be directly influenced by diet components. For example, Rat embryonic neural stem cells exposed to folic acid increase Notch-1 and hairy and Enhancer of split (Hes)-5 mRNA and protein expression and cell proliferation (Liu et al., 2010). Furthermore, Notch signalling is linked to several nutrition pathways components in different biological systems, suggesting that nutrition may affect the Notch signalling pathway not just through IIS but also through other nutritional pathways. Multiple interactions between the Notch signalling pathway and Tsc, Rheb, Akt, Lobe and Pten have been reported. Moreover, in some cases Notch signalling has been found to be the one influencing nutritional pathway components (Table 1.3).

Gene(s)	Organism and tissue	Interaction
Tsc1, Rheb	*Drosophila* external sensory organ (SOP)	Inactivation of Tsc1 or over expression of Rheb results in a Notch over-activation phenotype (Karbowniczek et al., 2010).
Tsc2, Rheb	Angiomyolipoma cell lines (Human tumour derived)	This cell line carry a bi-allelic Tsc2 mutation, and exhibits Tsc2- and Rheb- dependent Notch activation (Karbowniczek et al., 2010).
Tsc2	*Tsc2*-null rat cells in a xenograft model	Inhibition of Notch signalling using a γ-secretase inhibitor suppress cell proliferation (Karbowniczek et al., 2010).
Tsc2	Mouse embryonic fibroblasts	Hes-1 protein expression is elevated in mutant cells for *Tsc2*. The activation of Notch seems to go through the induction of the STAT/p63/Jagged signalling cascade (Ma et al., 2010).
Tor, Tsc2	Mouse embryonic fibroblasts	Knock out of Tor from Tsc2 mutant cells results in abolishment of STAT3, p63 and Notch signalling (Ma et al., 2010).
Tsc2	*Drosophila* intestine	Notch-mediated repression of Tsc2 in enteroblasts is required for their proper differentiation into enterocytes (Ma et al., 2010).
Akt	H460 tumour-derived cells	Notch1 can inhibit p53, a tumour suppressor gene, through activation of Akt and consequent Tor signalling (Ma et al., 2010).
Pi3K Akt	T-cell acute lymphoblastic leukaemia	Inhibition of Notch through a γ-secretase inhibitor results in multiple components of the Tor pathway being hypo-phosphorylated (Chan et al., 2007).
Akt	Erythroblastosis oncogene B2 (erB2) transgenic mouse model of breast cancer.	Inhibition of Notch through a γ-secretase inhibitor resulted in decreased phospho-Akt (Efferson et al., 2010).
Tor	Prostate cancer-3 cells	Down regulation of Notch1 decreases Akt phosphorylation and consequent decreases phosphorylation in Tor, Thor and S6k, leading to down regulation of Tor signalling pathway (Wang et al., 2010).
Pi3K	Prostate cancer-3 cells	Inhibition of Pi3K eliminates expression of Notch-1 (Wang et al., 2010).
Akt	Mammalian cells	Akt is an intermediate in the anti-apoptotic pathway mediated by Notch (Perumalsamy et al., 2009).
Lobe	*Drosophila* eye	*Lobe* mutants display a phenotype similar to lacking Notch pathway signalling. Notch seems to promote growth through Lobe (Chern and Choi, 2002).
Pten	Mouse embryonic fibroblasts	Mutant cells for *Pten* have elevated Hes1 protein expression (Ma et al., 2010).
Pten	Mouse retinal neurogenesis	In Pten-deficient neural progenitor cells, NICD was Inefficient in forming transcription activator complexes with Mastermind-like1 (Maml1), resulting in reduced Hes1 levels (Jo et al., 2012).

Akt, Pten	Pancreatic tumours	Inhibition of the Notch pathway leads to tumour suppression. The tumour suppression activity appears to be through inhibition of Akt phosphorylation and activation, and through Pten activation and phosphorylation (Vo et al., 2011).
Pten	Mouse T cells	Loss of Notch signalling led to down-regulation of *Hes1* and rise in *Pten* mRNA expression (Palomero et al., 2007, 2008; Wong et al., 2012).
Pdk1	Mouse T cells	Pdk1-deficient cells cannot respond to Notch signals (Kelly et al., 2007).
Pten	Prostate adenocarcinoma	Retroviral expression of NICD resulted in increased Pten gene expression. CBF-1, which is the Su(H) mammalian homolog and NICD co-factor, was bound to the Pten promoter (Whelan et al., 2009).
Akt, Pten	Human lung tumour cell lines	In 12 out of 13 Akt over-activated tumour lines, the over-activation was due to loss of Pten. Notch was highly activated in the latter cell lines. Inhibition of Notch signalling did not affect Akt activation and cell survival, suggesting in this case that Notch and Akt pathway were working in parallel (Guo et al., 2013).

Table 1.3. Notch signalling and nutritional pathways components
Examples of Notch interacting with different nutrition pathway components from the literature could help understand how nutrition regulates the Notch signalling pathway in the *Drosophila* GSC niche.

1.5.5 Cell junction and growth

The Notch signalling pathway may have a role in cell growth influenced through cell junctions, in particular through interactions with the Hippo (Hpo) signalling pathway. Hpo signalling is a conserved tumour suppressor pathway that regulates levels of cell proliferation and apoptosis. Hpo, Salvador and Mob as tumour suppressors form a complex to stop the translocation to the nucleus of Yorkie (Yki), which is a growth-related transcriptional activator (Boggiano and Fehon, 2012). Expanded (Ex) is bound by Yki and forms a complex with Hpo and Warts (Wts) (Oh et al., 2009). The Hpo pathway shows many interactions with Notch. In the *Drosophila* ovary, Hpo controls polar cell specification. Notch activity is increased in cells mutant for the Hpo pathway component *yki*. Additionally, reduction of Notch activity suppresses polar cell formation in *yki* mutant clones, thus showing that *yki* represses polar cell fate through inhibition of Notch signalling (Chen et al., 2011). In mouse Notch has been linked to the Hpo-Yap pathway, and the two signals seem to influence each other (Barry and Camargo, 2013; Chen et al., 2012b; Li et al., 2012b; Tschaharganeh et al., 2013; Zhou et al., 2011). Several connections between cell junction and growth are reported in table 1.4.

Gene(s)	Organism and tissue	Interaction
Ex, Notch, Shg, Ft, Egf	*Drosophila*	Ex localizes to the AJ, and is an endocytic regulator of Notch, Shg, Ft and Epidermal growth factor (Egf) (Maitra et al., 2006; McCartney et al., 2000).
Yki	Various tissues and organisms	It has been suggested that junctional proteins regulate growth by sequestering Yki to the cell cortex (Boggiano and Fehon, 2012).
Ft, Crb, Ex, Hpo, Wts, Ds	Various tissues and organisms	Both Ft and Crb regulate the sub-cellular location and abundance of Ex, and consequently Hpo signalling. Ft may also regulate the abundance of Wts through Dachsous (Ds), which is an unconventional myosin (Bennett and Harvey, 2006; Chen et al., 2010; Cho et al., 2006; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010; Silva et al., 2006; Willecke et al., 2006).
Crb, Hpo	*Drosophila*	Crb loss and overexpression disrupt Hpo signalling (Genevet and Tapon, 2011; Halder and Johnson, 2011; Pan, 2010).
Crb, Hpo, Notch	*Drosophila* imaginal disc	Crb can act as a tumour suppressor through interactions with either the Hpo pathway (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010) or the Notch pathway (Richardson and Pichaud, 2010).
aPKC, Hpo, Crb	*Drosophila* eye	Over-activation of aPKC disrupts Hpo signalling independently of Crb, and causes overgrowth (Grzeschik et al., 2010).
Lgl, Dlg, Scrib, Hpo	*Drosophila* epithelia	Loss of basal polarity proteins like Lgl, Dlg and Scrib cause excessive growth (Bilder, 2004) and influence Hpo signalling (Chen et al., 2010; Robinson et al., 2010). Their loss also leads to tumours composed of unorganized cell masses, and thus they are known as neoplastic tumour suppressors (Bilder, 2004; Brumby and Richardson, 2005).
Lgl, Hpo	*Drosophila* eye	Loss of Lgl was shown to modulate Hpo signalling. Ex localization was not affected by Lgl loss as it is by Ft and Crb loss. Instead, mis-localization of negative regulators of Hpo such as the Ras-associated family proteins (RASSF) was found (Grzeschik et al., 2010).
Baz, Sdt, Cdc42, Shg, Arm, Ras	*Drosophila*	Loss of apical polarity proteins like Baz, Sdt, Cdc42, Shg and Arm enhances oncogenic effects of activated Ras (Brumby and Richardson, 2005)..
Scrib, Hpo	Various tissues and organisms	Scrib has been linked to Hpo signalling pathway (Chen et al., 2012a; Cordenonsi et al., 2011; Skouloudaki et al., 2009)
Pten	*Drosophila*	Pten has been found to be an important regulator of apical polarity (Chartier et al., 2011; Roch et al., 2010).
AMPk, Lkb1		AMPk and Lkb1, which is upstream of AMPk, are required in epithelial polarity when the cell is under energy stress (Lee et al., 2007; Mirouse et al., 2007).

Table 1.4. Cell junction and growth
Examples of cell junction components interacting with growth.

1.6 Aims

The overall aim of this thesis is to understand the regulation of *Drosophila* GSC and niche population levels. Our aim is to understand both the fine tuning of Notch signalling present during the development of the niche and the dynamicity of the niche required in adult to respond to signalling and environmental inputs. This is reflected in the two papers submitted for publication. The first paper discusses the control of the cap cell niche size in response to nutritional changes and how this is regulated by altered Notch signalling levels. Compared to previous publication from the Drummond Barbosa group, which focus on the impact of diet and insulin on the niche and Notch signalling, the novelty of my first paper is in its discovery and attention to the dynamicity of the niche and exploration of the recovery mechanism through analysis of the Notch signalling pathway in different cell types of the germarium. The second paper describes how Pyd interacts with Dx to affect the source and strength of Notch signalling during the development of the niche to determine niche size. The obtained results will help understand the general mechanism of stem cell and niche maintenance, resulting in future possibilities to understand why niches do not work properly causing cancer and degenerative diseases.

Chapter 2: result part 1

Reversible regulation of stem cell niche size through dietary control of Notch signalling.

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Abstract

Adult stem cells respond to exercise, injury and nutrition through interacting systemic and local signals. Little is known about whether stem cell niches are similarly affected. The *Drosophila* ovary GSC cap cell niche is thought to be post-mitotic and stable over the lifespan of wild type flies. Here we show that the GSC niche is actually a dynamic structure, which can expand and contract in fluctuating dietary conditions. Cap cells are lost when flies are shifted to poor diet and Notch signalling is reduced. Transferring flies to rich food restores Notch signalling, expands the niche and prolongs GSC maintenance in aged flies. These effects are reproduced by transient removal and restoration of Notch signalling using temperature-sensitive mutations. We show that niche size is controlled by spatially distinct Notch functions. Notch acts in cap cells to prevent loss, whereas niche expansion occurs by a Notch-induced transition from adjacent ESCs. Therefore niche plasticity can underlie adaptive environmental responses.

Introduction

Tissue renewal and maintenance fundamentally depend on the activity of stem cells, which provide a pluripotent source to replace lost or damaged tissue. Stem cells need to be tightly controlled through regulation of their proliferation, self-renewal and differentiation. Disruption of this regulation can lead to severe consequences, such as age-related pathologies and cancer (Ghotra et al., 2009; Sell, 2004). This important level of control is typically exerted by adjacent niche cells, which provide anchorage for the stem cells and key regulatory signals. However, it is becoming clear that superposed on this local regulation there are systemic signals that provide adaption to the physiological status of the organism, in ways that are still not well understood. Systemic changes affecting stem cells may mediate the effects of nutrition, including dietary restriction, and exercise on stem cell maintenance and proliferation, with possible consequences for longevity and prolonging a healthy lifespan (Hsu and Drummond-Barbosa, 2011; Partridge et al., 2011). Indeed decline in systemically provided signals could provoke the observed age-related decline of stem cell activity observed in some tissues. Although there has been much work which describes effects of physiologically-dependent regulation of stem cells, less is known regarding the consequences of systemic signals on their underlying niches.

The *Drosophila* ovary is a well-characterised model which has been instrumental in establishing the niche paradigm of stem cell regulation and which is highly responsive to dietary conditions. Previous work has demonstrated a sixty-fold difference in egg laying between rich and poor food conditions (Drummond-Barbosa and Spradling, 2001). The ovary is composed of sub-structures called ovarioles that consist of a chain of egg chambers, each at a different progressive developmental stage. The germ line and somatic cell lineages are derived from two stem cell populations that reside in the germarium, which lies at the anterior tip of each ovariole (Decotto and

Spradling, 2005; González-Reyes, 2003). Cap cells at the anterior tip of the germarium form the niche for the germline stem cells (GSCs), providing anchorage via DE-cadherin (Song et al., 2002). GSCs undergo asymmetrical division to self-renew the GSC, and generate daughter cystoblasts that then undergo 4 cycles of mitosis to form a 16 cell germline cyst (de Cuevas and Spradling, 1998). Escort cells (ESCs), which line the anterior germarium, move the cysts posteriorly using dynamic cytoplasmic processes that invade between successive cysts (Kirilly et al., 2011; Morris and Spradling, 2011). Two follicle stem cells (FSCs) are bilaterally located approximately midway along the germarium and give rise to follicle cells (FC) that encapsulate each cyst to form successive egg chambers. The cap cell niche provides diffusible signals such as Decapentaplegic (Dpp) that regulates the GSC maintenance and Hedgehog, which acts at a distance to regulate FSC proliferation (Forbes et al., 1996; Zhang and Kalderon, 2001).

On protein-poor diets, germline and somatic cell proliferation is reduced and cysts in region 2a/2b undergo apoptosis (Drummond-Barbosa and Spradling, 2001). Systemically derived signals like Insulin-like peptides and the Target of rapamycin (Tor) pathway control the response of the GSCs to nutritional and energetic status (Drummond-Barbosa and Spradling, 2001; LaFever et al., 2010). Systemic signals also play a role in maintaining the niche over the lifespan of the fly (Hsu and Drummond-Barbosa, 2009). Insulin/insulin-like growth factor (IGF)-like signalling (IIS) acts through Notch (N) signalling to maintain a stable niche for long periods of the fly lifespan (Hsu and Drummond-Barbosa, 2009, 2011). Notch signalling is also required during development for the recruitment of cap cells (Song et al., 2007). Although systemic signals are involved in long-term maintenance, there has been no evidence that short-term dietary changes can have an impact on the niche in addition to their direct role in the GSCs. Here we show that dietary restriction can reduce the size of the GSC

niche through reduced Notch signalling, and that the niche has the capability to recover its size if dietary conditions improve. These changes are associated with an effect on GSC maintenance, which overcomes GSC age-related decline. The effect of diet on GSC maintenance has been observed in different species but the underlying cause is not well understood (Conboy et al., 2003; Florian et al., 2012). We find that the niche is a dynamic structure and that niche plasticity is controlled by two distinct Notch functions. Notch is required in the cap cells to prevent their loss, whereas niche recovery is instigated by Notch-stimulated transition of ESCs to cap cells. Such niche plasticity may have implications for understanding formation of pathogenic niches that are associated with cancer stem cells in some human tumours.

Results

Diet reversibly regulates Notch signalling and the size of the GSC niche

We investigated the consequence of culturing wild type (Wt) flies on protein-rich and normal conditions by supplementing standard fly media with live yeast (St+Yeast). We observed no change in cap cell number over a 5 week time course (Fig. 1A,C). GSC numbers showed a slow reduction in numbers evident 24 days after eclosion but this was similar in both rich and poor food conditions (Fig. 1B). Therefore, we aged Wt flies in more restrictive diet conditions on standard medium with reduced glucose (glucose-R) and on agar only. We observed a reduction in niche size in flies kept on glucose-R food, which was apparent 15 days after eclosion (Fig. 1A). GSC numbers declined similarly to other food conditions (Fig. 1B). Thus we find that niche size declines when flies are switched to poor nutritional conditions but the age-dependent decline of GSC numbers is similar in all cases resulting in lower cap cell to GSC ratios in poor food. Flies kept on agar showed a more rapid loss of cap cells

between 3-6 days after eclosion (Fig. 1A,D) and a significant decrease in GSCs compared to all other food conditions (Fig. 1B) We were unable to prolong the latter time course because flies did not survive for longer periods. Thus diet can affect both niche and GSC numbers with progressively more severe effects as nutritional content is reduced.

Ovaries can reversibly adapt egg laying to changes in diet (Drummond-Barbosa and Spradling, 2001). Therefore we wondered if changes in the niche size would also be reversible by changes to the fly culture media (Fig. 1E). Flies were allowed to develop through larval stages on St+Yeast and then shifted to glucose-R food for 15 or 24 days after eclosion. They were then shifted back to St+Yeast for 9 days. In both cases a recovery in cap cell number was observed (Fig. 1F). A similar recovery was observed when flies were first starved for 6 days on agar and then shifted for 9 days on St+Yeast (Fig. 1F). We also observed a corresponding increase in numbers of GSCs as flies were shifted to richer nutrient conditions compared to unshifted flies (Fig. 1G). Thus the normal decline of GSC number that occurs in old flies, in all the tested nutritional conditions, can be overcome by shifting from poor to rich food late in the lifespan.

Since niche size is known to be maintained in the adult through Notch signalling (Hsu and Drummond-Barbosa, 2009; Song et al., 2007; Ward et al., 2006), we investigated if different food conditions could affect the levels of Notch signalling. We first examined the expression pattern of different Notch signal reporter lines since different reporters are known to be very tissue-dependent (Cooper et al., 2000). The E(spl)mβ-CD2 (Mβ-CD2) line has previously been reported to express in the cap cells (Hsu and Drummond-Barbosa, 2009), however we found only expression in the posterior terminal filament cells and not in the cap cells (Fig. 2B). As previously reported the E(spl)m7-lacZ (M7-lacZ) (Bray, 2006; Djiane et al., 2011) construct was

expressed in the cap cells (Fig 2C). E(spl)mβ1.5-lacZ (Mβ-lacZ) (Cooper et al., 2000) was found to express mainly in the ESCs with weak expression in the cap cells (Fig. 2D). Consistent with these signalling patterns we found that Notch was expressed in all three somatic cell types of the niche (Fig. 2A). We found that both the M7-LacZ and Mβ-lacZ reporter lines showed little change over time in flies maintained on St+Yeast. However lower expression of both Notch reporter lines was observed (Fig. 2F,H,I,K) in flies raised on glucose-R food. Interestingly we found that the Mβ-lacZ reporter was more sensitive to loss of live yeast supplementation than M7-LacZ (Fig. S1). We investigated if the loss of Notch signalling on poor food was reversible by shifting flies back to St+Yeast food and found different responses of the two reporters. After shifting to the rich food M7-LacZ expression showed no recovery (Fig. 2H), but Mβ-lacZ signalling levels were rapidly restored (Fig. 2K).

Loss and recovery of cap cells is controlled by altering Notch signalling levels

To investigate further the flexibility of the niche, we used N^{ts1} and N^{ts2} temperature-sensitive mutations of *Notch* (Shellenbarger and Mohler, 1975), which allow normal Notch function at 18^{o}C but produce strong Notch loss-of-function when flies are shifted to 29^{o}C. Flies were shifted from 18^{o}C to 29^{o}C on eclosion and the population sampled at different time points for oogenesis phenotypes. As previously published (Hsu and Drummond-Barbosa, 2011; Song et al., 2007) we observed a decline in cap cell number over time for both *Notch* alleles, while the niche size for Wt controls remained stable (Fig. 3A). *Notch* mutant flies that remained at 18^{o}C also did not show a significant decline in cap cell number. Likewise, we observed a greater decrease in GSC number over time compared to Wt and 18^{o}C controls (Fig 3B). These phenotypes were accompanied by a reduction of ESC invasion between cysts and the

onset of FC packaging phenotypes (Fig. 3D,F), consistent with published data (Ruohola et al., 1991; Xu et al., 1992). Thus lack of adult Notch signalling has an impact on numerous cell types in the germarium, including the cap cells, to maintain their function in the ovary.

We next investigated if the observed N^{ts1} allele phenotypes could be reversed by restoring Notch activity. Adult flies were first cultured at 29^{o}C then reverted to 18^{o}C part way through the time course. When the temperature down-shift was initiated 6 or 9 days into the time course we observed niche recovery (Fig. 4A,B,E). GSC numbers similarly recovered (Fig. 4C,D). Thus Notch has two specific functions in maintaining cap cell number. It prevents loss of cap cells and also allows their replacement. Other ovariole phenotypes affecting cyst packaging were also restored (Fig. 4F).

We investigated if diet could affect cap cells when Notch signalling was removed. We kept N^{ts1} flies at 29^{o}C on standard food and glucose-R food. It was not possible to see a further decrease in niche size, showing that reducing glucose has no additional effect when Notch signalling is already removed (Fig. 4G). Additionally when Notch signalling was restored by shifting to 18^{o}C the niche size did not re-expand to the same extent on glucose-R medium compared to when signalling was restored in St+Yeast (Fig. 4H). The results are compatible with a requirement for Notch for the nutrient modulation of the niche size. The partial recovery of niche size in glucose-R food when Notch activity is restored may reflect a bigger relative effect on Notch signalling of the mutation compared to modulation of Notch by nutrient availability.

Notch prevents niche reduction by signalling in the cap cells

It is possible that the two identified functions of Notch that control niche size reside in the same or different cell types. Thus altered Notch signalling in different

tissues might directly or indirectly contribute to either cap cell loss or niche restoration. *Notch* null cap cell clones generated during development fail to be incorporated into the niche and further decrease over time (Hsu and Drummond-Barbosa, 2011), but it is not clear whether the later loss of cap cells in the adult results only from earlier loss of Notch in cap cells during development. It is unknown whether niche maintenance in the adult only depends on Notch signalling in the cap cells or whether other cell types are involved in supporting cap cell numbers. The cap cell niche is reported to be post mitotic (Xie and Spradling, 1998) ruling out a clone induction approach to investigate cell type requirements that are specific for adult functions rather than for developmental requirements. We therefore adopted a Notch RNAi expression approach to investigate the consequence of removing adult Notch function in different cell types. We first investigated the expression pattern of different Gal4 lines reported to drive expression in the adult ovary by detecting the expression of UAS-CD8-GFP. Only bric-a-brac-Gal-4 (bab1-Gal4-) (Bolívar et al., 2006) driven expression was detected in the cap cells (Fig 5A). bab1-Gal4 also drove expression in anterior ESCs and terminal filament cells although not as strongly as other Gal4 lines. The C306-Gal4 line (Manseau et al., 1997) was found to be expressed in the FC region of the germarium (Fig. 5D). engrailed-Gal4 (en-Gal4) was strongly expressed in all the cells of the terminal filament but not the cap or ESCs (Fig. 5B). C587-Gal4 was expressed in ESCs and in early FC progenitors but no expression was detected in cap cells (Fig 5C). ptc-Gal4 showed a similar expression pattern to C587-Gal4 in the germarium (Fig. 5F). Thus by considering the overlapping and distinctive expression patterns of different Gal4 drivers it is possible to infer the spatial requirements for Notch function for niche and GSC maintenance. In order to mimic the N^{ts} experiments and generate Notch loss-of-function only in the adult, we created fly stocks carrying both the Gal4 driver and a temperature sensitive Gal80 construct ($Gal80^{ts}$ (Matsumoto et al., 1978)). We confirmed Gal4-$Gal80^{ts}$ functionality

by expressing UAS-CD8-GFP at 18°C and 29°C. GFP expression was observed at 29°C but not at 18°C (Fig. 5E,F). Flies were kept at 18°C during development and then switched to 29°C at eclosion and dissected immediately, after 6 days and 15 days. Control flies kept at 18°C showed no phenotypes (data not shown). The separate Gal4-Gal80^{ts} lines and the UAS lines were out-crossed with Wt flies. None of the lines individually showed a phenotype at eclosion (Fig. S2). In the temperature-shifted flies Notch RNAi expressed by different Gal4 drivers resulted in distinct effects on the ovary phenotype (Fig. 5G-K and Fig. S2). en-Gal4 driven Notch RNAi in the terminal filament did not show any phenotypes throughout the timeline (Fig. 5G,H,K). bab1-Gal4 driven Notch-RNAi Val20 caused a strong loss of cap cells, decreasing from 5.1 ± 0.2 to 3.7 ± 0.2 at Day 6 and to 3.2 ± 0.3 at Day 15 (Fig. 5G, I). Reduction of GSC numbers was also observed (Fig. 5H,I). C587-Gal4, ptc-Gal4 and C306-Gal4 all led to reduction of FC encapsulation of egg chambers reflecting Notch functions in the somatic cell lineage (data not shown). C587-Gal4, ptc-Gal4 showed a strong loss of GSCs with both Notch RNAis (Fig 5.H,J, Fig. S2). Expression of Notch RNAi with the FC driver C306-Gal4 also showed significant decline in the number of GSCs (Fig. 5H), suggesting a possible feedback between FCs and GSC maintenance. Despite disruption to other germarium cell types, none of the drivers apart from bab1-Gal4 resulted in loss of cap cells (Fig 5G,J). We also observed no phenotypes when Notch RNAi was driven in the germline with nanos-Gal4 (Fig. 5G), consistent with previous results that failed to find a function for Notch in germline cells (Hsu and Drummond-Barbosa, 2011; Ward et al., 2006). Taking into account the overlapping expression patterns of the Gal4 drivers we conclude that Notch signalling is required only in the cap cells for adult niche maintenance but indirect effects of Notch in other somatic tissues also affect GSC number.

Cap cell death and mitosis do not explain loss and recovery of the niche

The finding that niche size can recover through the expansion of cap cell numbers was surprising because the niche has previously been thought to be post-mitotic and in the Wt essentially stable over much of the fly lifespan. To investigate whether the observed expansion of the niche was due to cap cell mitosis we labelled dividing cells by staining ovaries for the mitosis marker anti-phospho HistoneH3 (PH3). We first repeated the temperature shift experiment to better discern the time line of recovery to ensure anti-PH3 staining was carried out during a time when cap cell numbers were increasing. Niche recovery was already evident 1 day after restoring Notch function by temperature down-shifting (Fig 6A). Ovaries were therefore dissected and stained for PH3 1 day after the temperature shift. However no PH3 labelling was observed in cap cells despite positive staining in the germ line and FC lineages (Fig. 6B). To confirm a lack of cap cell mitosis we also cultured flies on medium containing BrdU, and repeated the experiment. The dissections were performed at the end of the recovery timeline, at Day15 from eclosion. The presence of BrdU in the medium did not affect recovery of the cap cell niche size (Fig. 6C). However, we found no labelling of BrdU in the cap cells. In contrast proliferating germ line cells did incorporate BrdU (Fig. 6D,E). We also investigated whether reduction in cap cell number on loss of Notch signalling was reflected in cell death and could find no evidence for apoptosis in the cap cells (Fig. 6F). We conclude therefore that a transition between cell types rather than proliferation or apoptosis might better explain altered niche sizes in response to altered diet and Notch signalling.

A spatially distinct Notch function drives niche recovery

To investigate which cells might reassign as a cap cell fate to expand the

niche we expressed constitutively active Notch (NICD) in adult flies using the different Gal4 drivers with Gal80^{ts} (Fig. 7). Separately the UAS-NICD line and different Gal4 drivers had no effect on niche size (Fig 7). C587-Gal4- and ptc-Gal4- driven NICD expression resulted in an increase in cap cell number (Fig. 7A,A',B). This NICD-driven increase was again not explained by cap cell division since no BrdU was detected in cap cells after 15 days of labelling (data not shown). In contrast, no increase in cap cell number was noted with en-Gal4, C306-Gal4 or bab1-Gal4 (Fig. 7C-E). The latter was significant because it was the bab1-Gal4 driven expression of Notch RNAi that was capable of reducing niche size, while C587-Gal4 and ptc-Gal4 driven expression had no effect. Since C587-Gal4 and ptc-Gal4 express strongly in the ESCs and not the cap cells and bab1-Gal4 expresses in cap cells and less strongly than other drivers in ESCs we suggest that Notch acts in the cap cells only to prevent their loss from the niche and separately acts in ESCs to recruit additional cap cells to restore normal niche size. This correlates well with the different nutrient shift responses observed for the cap cell- and ESC-expressing M7-LacZ and Mβ-lacZ reporter lines. Thus we conclude that ESCs can act as a latent reservoir of niche cells to provide flexible responses to the physiological status of the fly.

Discussion

The physiological status of an organism including its age and nutrition, transmitted through systemic signals, impacts on the maintenance and activity of stem cells, which determines the lifelong ability to repair and maintain adult tissues (Drummond-Barbosa, 2008). Decline of stem cell activity and inability to repair tissues can contribute to deteriorating function in old age (Drummond-Barbosa, 2008; Ghotra et al., 2009; Sell, 2004; Sharpless and DePinho, 2007). In numerous organisms, dietary restriction has been shown to ameliorate some aspects of aging by mechanisms that are

as yet poorly understood, but may involve systemic effects acting directly on either stem cells or their niches (Angelo et al., 2009; Cerletti et al., 2012; Kim and Shivdasani, 2012; Mair et al., 2010). The *Drosophila* ovary GSC niche has provided an important model system that has been influential in establishing the niche concept of stem cell regulation (Xie and Spradling, 2000). Egg laying is highly sensitive to nutritional status and dramatically slows around 24 hours after shifting to a protein-poor diet through direct effects of IIS on the GSCs (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009). Here we show for the first time that shifting flies from rich to poor food source also reduces the size of the GSC niche by reducing Notch signalling. The GSC niche is assembled during the larval-pupal transition by the recruitment of around 6 cap cells to each terminal filament, a process that requires Notch signalling (Song et al., 2007). Once formed the niche is post mitotic and in Wt flies has been thought to be essentially stable over their lifespan, although there is a slow decline in the numbers of supported stem cells (Pan et al., 2007). The niche stability requires continued Notch signalling over the lifetime of the fly, since mutants of *Notch* lose cap cells. We find that decreasing the nutritional content of culture media results in decreased Notch signalling and reduced niche sizes with increasing severity, or earlier onset, as nutritional conditions become more stringent. Remarkably we find that this cap cell loss is reversible and the niche expands back to initial levels when flies are transferred back to rich food. The reversibility of the niche decline was also demonstrated by using temperature-sensitive mutations to first reduce then restore Notch signalling levels.

Previously it has been shown that Notch signalling is required during development within the cells that are recruited to form the cap cell niche. In the adult, the few *Notch* mutant cap cell clones recruited to the niche were lost more rapidly suggesting a cell-autonomous requirement for niche maintenance (Hsu and Drummond-

Barbosa, 2011). However, in this case all clones were established during the developmental phase, so the observed adult cap cell requirement for Notch might reflect this developmental history. Instead we used *Notch* RNAi expression with a temperature-sensitive Gal80 to investigate exclusively the adult requirement for cap cell maintenance. We showed that expression of RNAi of *Notch* in cap cells led to their loss. In contrast indirect effects of *Notch* loss-of-function on GSC number were seen following expression of *Notch* RNAi in several somatic cell types, suggesting that complex feedback regulation through Notch normally helps to balance germline and somatic cell production in egg chamber formation.

While cap cell maintenance required Notch activity in the cap cells, our results indicate that niche re-expansion involves Notch activity in the adjacent ESCs. Using BrdU labelling and PH3 staining we confirmed that the cap cell niche is post-mitotic and re-expansion of the niche is not accompanied by any evidence of cell division in the niche. In the absence of mitosis of cap cells the most likely cause is a direct transition between the ESC and cap cell fate, supported by the fact that over-expression of NICD in ESCs results in niche enlargement. In this case the ESCs could act as a reservoir of potential niche cells to allow niche capacity to expand on the return of optimal nutritional conditions. This inter-conversion mechanism provides a novel solution for tuning dynamic niche capacity to physiological status. The ability for non-niche cells to be manipulated into acquiring capacity to support stem cells may have implications for understanding the formation of pathogenic niches, which seem to support cancer stem cells in some tumours (Ghotra et al., 2009). The reversibility of this process further suggests such niches may be potential targets for therapeutic intervention.

The response of the niche to nutritional depletion may be an adaptive response to reduce niche capacity and preserve resources in poor conditions, when egg

laying may not be productive for offspring survival. The niche response to reduced nutrient availability takes place over a slower timescale compared to the almost immediate impact on GSC division and egg laying that has previously been described (Drummond-Barbosa and Spradling, 2001). In contrast, restoration of niche size and GSC activity take place on a much shorter timescale. This may reflect a need to further adapt to longer-term exposure to poor environments, but allow rapid up-regulation of egg laying should good conditions be restored. It will be interesting therefore to discern whether these slow and fast responses depend on different nutrient-sensing mechanisms.

It is intriguing to consider the impact of nutritional variation on GSC number. Calorific restriction has been associated with prolonged reproductive lifespan in both invertebrate and vertebrate species and in some cases has also been linked to extended stem cell maintenance (Jones, 2007; Mair et al., 2010; Partridge et al., 2011; Yilmaz et al., 2012). However the mechanisms by which these effects are manifest are not known. In the *Drosophila* ovary we observed a slow decline of GSCs when flies were aged and this decline was similar in all the nutrient conditions tested. However, after keeping flies for extended periods on poor food, the transfer to rich food both restored niche size and overcame the late loss of GSCs that occurred in constant nutritional conditions, whether rich or poor. Thus the nutritional history of the flies affects GSC longevity. It is not yet clear whether this restoration of stem cell capacity reflects parallel mechanisms acting on both the niche and stem cells, or whether changes in the niche alone cause the altered stem cell maintenance. However this latent GSC capacity is only revealed if rich food conditions are restored to old flies, whereupon the increased niche is associated with a rejuvenated stem cell pool. It is intriguing to speculate whether such dietary adaptations of the germline may reflect the early evolutionary origin of the capacity to adjust stem and niche cell activity to nutritional status, a feature which now seems to be a widely conserved aspect of stem

cell regulation.

Materials and Methods

Additional information on crosses, ovaries mounting, cap cells and GSCs scoring, test for Notch reporter lines, Gal4 and UAS constructs can be found in supplemental materials and methods.

Drosophila stocks and culture conditions

Oregon R was used as Wt control. N^{ts1}, N^{ts2} alleles (Shellenbarger and Mohler, 1975) were obtained from Bloomington Stock Centre, Indiana. Notch reporter lines E(spl)m7-LacZ, (Bray, 2006); E(spl)mβ1.5-lacZ (Cooper et al., 2000), E(spl)mβ-CD2 (de Celis et al., 1998) were a gift from Sarah Bray. Transgenic expression was by the Gal4 system (Brand and Perrimon, 1993). Gal4 lines were P{GawB}c587 (C587-Gal4); P{GawB}C306 (C306-Gal4), (Manseau et al., 1997); patched-Gal4 (ptc-Gal4) (Speicher et al., 1994); bric-a-brac-Gal4/TM3 (bab1-Gal4) (Bolívar et al., 2006); P{en2.4-GAL4}e16E (en-Gal4) (Harrison et al., 1995); w^{1118}; P{GAL4::VP16-nos.UTR}CG6325^{MVD1}, P{UASp-GFPS65C-αTub84B}3 (nanos-Gal4) (Van Doren et al., 1998). The Gal80^{ts} stock was P{tubP-GAL80^{ts}} obtained from Bloomington, Indiana. UAS lines were Notch RNAi Valium 20 ((Nval 20) HMS00009 TRiP stock, Harvard) obtained from Bloomington, Notch RNAi GD1112 (Vienna *Drosophila* RNAi Centre). UAS-NICD was obtained from Spyros Artavanis-Tsakonas (Harvard Medical School) and P{UAS-mCD8::GFP.L}LL6 (UAS-CD8-GFP) from the Bloomington stock centre. Fly stocks were maintained at 25°C on standard fly food (72 g/L maize, 79.3 g/L glucose, 50 g/L yeast, 8.5 g/L agar, 0.3% propionic acid and 0.27% Nigipin) unless indicated. For protein-enriched food (St+Yeast), standard food was supplemented with live yeast (Sigma). glucose-reduced food was with standard recipe without added 79.3 g/L glucose. Starvation conditions were obtained on 8.5g/L Agar only.

Immunofluorescence

Primary antibodies used were: guinea pig anti-D4.1.3 (Coracle, 1:5000; gift from R. Fehon, University of Chicago), mouse anti-Lamin C (LC28.26, 1:20; Developmental Studies Hybridoma Bank, DSHB, Iowa), mouse anti-alpha spectrin (3A9 1:10; DSHB), mouse anti Notch (c458.2H, 1:400; DSHB), mouse anti-ratCD2 (MCA154R 1:50; Serotech), mouse anti-β-Galactosidase (β-Gal) (Z3781, 1:250; Promega), rat anti-BrdU (ab6326 1:50; Abcam), rabbit anti-phospho-Histone H3 ser10 (06-570 1:3000; Millipore), goat anti-GFP (Ab5450 1:1000; Abcam), mouse anti-FasIII (7G10 1:20; DSHB). Secondary antibodies used were: donkey anti-guinea pig RRX (706-295-148 1:400; Jackson laboratories), donkey anti-mouse Alexa Fluor 488 (1:1000; invitrogen), donkey anti-mouse Cy3 (115-165-146 1:400; Jackson laboratories), donkey anti-rabbit Alexa Flour 488 (1:1000; Invitrogen), donkey anti-guinea pig Cy5 (706-175-148 1:100; Jackson laboratories), donkey anti-mouse Cy5 (715-175-151 1:100; Jackson laboratories), donkey anti-goat Cy3 (705-165-147 1:400; Jackson laboratories); donkey anti-rat RRX (712-295-153 1:400; Jackson laboratories).

An average of 20 flies for timepoint was anesthetized on ice and pinned with a needle (Fine Science Tools) through the thorax on a Sylgard (Dow Corning) coated plate filled with Grace's insect medium (Sigma). Using forceps (Dumont) the ovaries were removed from the fly. Ovarioles were separated using 0.1 mm diameter insect pins (Minutiens) fixed to forceps. The ovarioles were then transferred into a tube (Eppendorf) and fixed in a medium containing 3% formaldehyde (Polyscience) in 1x Phosphate Buffered Saline (PBS) (Sigma) for 12 minutes. During the incubation with the fixative, the samples were left on a mixer roller (Stuart SRT91). Fixed ovarioles were pelleted at 7000 rpm in a bench-top centrifuge (Eppendorf centrifuge 5415D) for 30 seconds, and then the fixative was removed. PBS with TritonX (Sigma) 0.3 % (PBX) was then added to the ovarioles for 10 minutes. PBX was removed and ovaries were

incubated in blocking solution with 0.5% Normal Donkey serum (NDS) (Jackson laboratories) in PBX for 1 hour. The ovaries were then incubated with the primary antibody diluted in PBS and left overnight on the mixer roller at room temperature. Samples were then washed 5 times in PBX and a secondary antibody diluted in PBS was added overnight. Samples were then washed 5 times in PBX and mounted on microscope slides under a cover slip using Vectashield, containing DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories). Images were captured using an Orca-ER digital camera (Hamamatsu) mounted on an M2 fluorescent microscope (Zeiss). The images were acquired as 0.5µm Z-sections and processed using Volocity software (Perkin Elmer) with deconvolution. Assembly of images was done with Photoshop CS5 (Adobe).

BrdU staining

Ovaries were dissected as previously described but fixed for 20 minutes. Ovaries were denatured into 3M HCl (Sigma) diluted in 0.3% PBX for 15 minutes. HCl was removed, and ovaries were incubated 3 times for 5 minutes in 0.1M Borax (Sigma) to neutralize HCl. After this step, Borax solution was removed and ovaries were incubated in blocking solution with 0.5% NDS in PBX for 1 hour as previously described. Staining and mounting were performed as previously described.

X-Gal staining

Germaria were dissected in PBS, fixed for 20 min in 1.7% glutaraldehyde (Sigma) in PBS, and then washed in PBS. Samples were stained overnight at 37°C in 1 mM $MgCl_2$, 6 mM $K_4FeIICN_6$, 6 mM $K_3FeIICN_6$, and 0.2% X-gal. After washing in PBS, they were then mounted in a solution containing 70% glycerol (Sigma) in PBS.

Apoptosis assay

To detect apoptosis, Apoptag® Fluorescein Direct In situ Apoptosis detection kit (Chemicon International) was used. Dissection and stain procedures were performed as described above.

Statistics

Statistics were performed with SPSS 20.0 (IBM). To analyse differences between data points two-tailed Mann-Whitney U test was used. To compare complete time-courses, the slope coefficient was calculated through linear regression performed with SPSS20.0. Slope coefficients were then compared through independent t test using the following formula: $t = Slope1 - Slope2 / SQR [(SeSlope1)^2 + (SeSlope2)^2]$. To compare X-Gal stain results, the SPSS 20.0 crosstab method with Pearson Chi-Square Asymptotic 2-sided test was used. For graphical representation error bars had to be transformed from standard error to percentage of the standard error in the charts normalized to Day 0. The following formula was used for the transformation: $\%Se = |meanDayX/meanDay0| * SQR [(SeDayX^2/ meanDayX^2) + (SeDay0^2/ meanDay0^2] * 100$

References

Angelo, G., Gilst, M.R. Van, and Van Gilst, M.R. (2009). Starvation protects germline stem cells and extends reproductive longevity in C. elegans. Science (New York, N.Y.) *326*, 954–958.

Bolívar, J., Pearson, J., López-Onieva, L., and González-Reyes, A. (2006). Genetic dissection of a stem cell niche: the case of the Drosophila ovary. Developmental Dynamics : an Official Publication of the American Association of Anatomists *235*, 2969–2979.

Brand, a H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development (Cambridge, England) *118*, 401–415.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nature Reviews. Molecular Cell Biology *7*, 678–689.

De Celis, J.F., Tyler, D.M., and Bray, S.J. (1998). Notch signalling mediates segmentation of the Drosophila leg. Development (Cambridge, England) *125*, 4617–4626.

Cerletti, M., Jang, Y.C., Finley, L.W.S., Haigis, M.C., and Wagers, A.J. (2012). Short-term calorie restriction enhances skeletal muscle stem cell function. Cell Stem Cell *10*, 515–519.

Conboy, I.M., Conboy, M.J., Smythe, G.M., and Rando, T. a (2003). Notch-mediated restoration of regenerative potential to aged muscle. Science (New York, N.Y.) *302*, 1575–1577.

Cooper, M.T., Tyler, D.M., Furriols, M., Chalkiadaki, A., Delidakis, C., and Bray, S.J. (2000). Spatially restricted factors cooperate with notch in the regulation of Enhancer of split genes. Developmental Biology *221*, 390–403.

De Cuevas, M., and Spradling, a C. (1998). Morphogenesis of the Drosophila fusome and its implications for oocyte specification. Development (Cambridge, England) *125*, 2781–2789.

Decotto, E., and Spradling, A.C. (2005). The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. Developmental Cell *9*, 501–510.

Djiane, A., Shimizu, H., Wilkin, M.B., Mazleyrat, S., Jennings, M.D., Avis, J., Bray, S.J., and Baron, M. (2011). Su(dx) E3 ubiquitin ligase-dependent and -independent functions of polychaetoid, the Drosophila ZO-1 homologue. The Journal of Cell Biology *192*, 189–200.

Van Doren, M., Williamson, a L., and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Current Biology : CB *8*, 243–246.

Drummond-Barbosa, D. (2008). Stem cells, their niches and the systemic environment: an aging network. Genetics *180*, 1787–1797.

Drummond-Barbosa, D., and Spradling, A.C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. Developmental Biology *231*, 265–278.

Florian, M.C., Dörr, K., Niebel, A., Daria, D., Schrezenmeier, H., Rojewski, M., Filippi, M.-D., Hasenberg, A., Gunzer, M., Scharffetter-Kochanek, K., et al. (2012). Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. Cell Stem Cell *10*, 520–530.

Forbes, a J., Lin, H., Ingham, P.W., and Spradling, a C. (1996). hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. Development (Cambridge, England) *122*, 1125–1135.

Ghotra, V.P.S., Puigvert, J.C., and Danen, E.H.J. (2009). The cancer stem cell microenvironment and anti-cancer therapy. International Journal of Radiation Biology *85*, 955–962.

González-Reyes, A. (2003). Stem cells, niches and cadherins: a view from Drosophila. Journal of Cell Science *116*, 949–954.

Harrison, D. a, Binari, R., Nahreini, T.S., Gilman, M., and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. The EMBO Journal *14*, 2857–2865.

Hsu, H.-J., and Drummond-Barbosa, D. (2009). Insulin levels control female germline stem cell maintenance via the niche in Drosophila. Proceedings of the National Academy of Sciences of the United States of America *106*, 1117–1121.

Hsu, H.-J., and Drummond-Barbosa, D. (2011). Insulin signals control the competence of the Drosophila female germline stem cell niche to respond to Notch ligands. Developmental Biology *350*, 290–300.

Jones, D.L. (2007). Aging and the germ line: where mortality and immortality meet. Stem Cell Reviews *3*, 192–200.

Kim, T.-H., and Shivdasani, R. a (2012). Stem cell niches: famished Paneth cells, gluttonous stem cells. Current Biology : CB *22*, R579–80.

Kirilly, D., Wang, S., and Xie, T. (2011). Self-maintained escort cells form a germline stem cell differentiation niche. Development (Cambridge, England) *5097*, 5087–5097.

LaFever, L., Feoktistov, A., Hsu, H.-J., and Drummond-Barbosa, D. (2010). Specific roles of Target of rapamycin in the control of stem cells and their progeny in the Drosophila ovary. Development (Cambridge, England) *137*, 2117–2126.

Mair, W., McLeod, C.J., Wang, L., and Jones, D.L. (2010). Dietary restriction enhances germline stem cell maintenance. Aging Cell *9*, 916–918.

Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F., Phan, H., Philp, A.V., Yang, M., Glover, D., Kaiser, K., et al. (1997). GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of Drosophila. Developmental Dynamics : an Official Publication of the American Association of Anatomists *209*, 310–322.

Matsumoto, K., Toh-e, a, and Oshima, Y. (1978). Genetic control of galactokinase synthesis in Saccharomyces cerevisiae: evidence for constitutive expression of the positive regulatory gene gal4. Journal of Bacteriology *134*, 446–457.

Morris, L.X., and Spradling, A.C. (2011). Long-term live imaging provides new insight into stem cell regulation and germline-soma coordination in the Drosophila ovary. Development (Cambridge, England) *138*, 2207–2215.

Pan, L., Chen, S., Weng, C., Call, G., Zhu, D., Tang, H., Zhang, N., and Xie, T. (2007). Stem cell aging is controlled both intrinsically and extrinsically in the Drosophila ovary. Cell Stem Cell *1*, 458–469.

Partridge, L., Alic, N., Bjedov, I., and Piper, M.D.W. (2011). Ageing in Drosophila: the role of the insulin/Igf and TOR signalling network. Experimental Gerontology *46*, 376–381.

Ruohola, H., Bremer, K.A., Baker, D., Swedlow, J.R., Jan, L.Y., and Jan, Y.N. (1991). Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in Drosophila. Cell *66*, 433–449.

Sell, S. (2004). Stem cell origin of cancer and differentiation therapy. Critical Reviews in Oncology/hematology *51*, 1–28.

Sharpless, N.E., and DePinho, R. a (2007). How stem cells age and why this makes us grow old. Nature Reviews. Molecular Cell Biology *8*, 703–713.

Shellenbarger, D.L., and Mohler, J.D. (1975). Temperature-sensitive mutations of the notch locus in Drosophila melanogaster. Genetics *81*, 143–162.

Song, X., Zhu, C.-H., Doan, C., and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science (New York, N.Y.) *296*, 1855–1857.

Song, X., Call, G.B., Kirilly, D., and Xie, T. (2007). Notch signaling controls germline stem cell niche formation in the Drosophila ovary. Development (Cambridge, England) *134*, 1071–1080.

Speicher, S. a, Thomas, U., Hinz, U., and Knust, E. (1994). The Serrate locus of Drosophila and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. Development (Cambridge, England) *120*, 535–544.

Ward, E.J., Shcherbata, H.R., Reynolds, S.H., Fischer, K. a, Hatfield, S.D., and Ruohola-baker, H. (2006). Stem cells signal to the niche through the Notch pathway in the Drosophila ovary. Current Biology : CB *16*, 2352–2358.

Xie, T., and Spradling, A.C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell *94*, 251–260.

Xie, T., and Spradling, A.C. (2000). A niche maintaining germ line stem cells in the Drosophila ovary. Science (New York, N.Y.) *290*, 328–330.

Xu, T., Caron, L. a, Fehon, R.G., and Artavanis-Tsakonas, S. (1992). The involvement of the Notch locus in Drosophila oogenesis. Development (Cambridge, England) *115*, 913–922.

Yilmaz, Ö.H., Katajisto, P., Lamming, D.W., Gültekin, Y., Bauer-Rowe, K.E., Sengupta, S., Birsoy, K., Dursun, A., Yilmaz, V.O., Selig, M., et al. (2012). mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature *486*, 490–495.

Zhang, Y., and Kalderon, D. (2001). Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. Nature *410*, 599–604.

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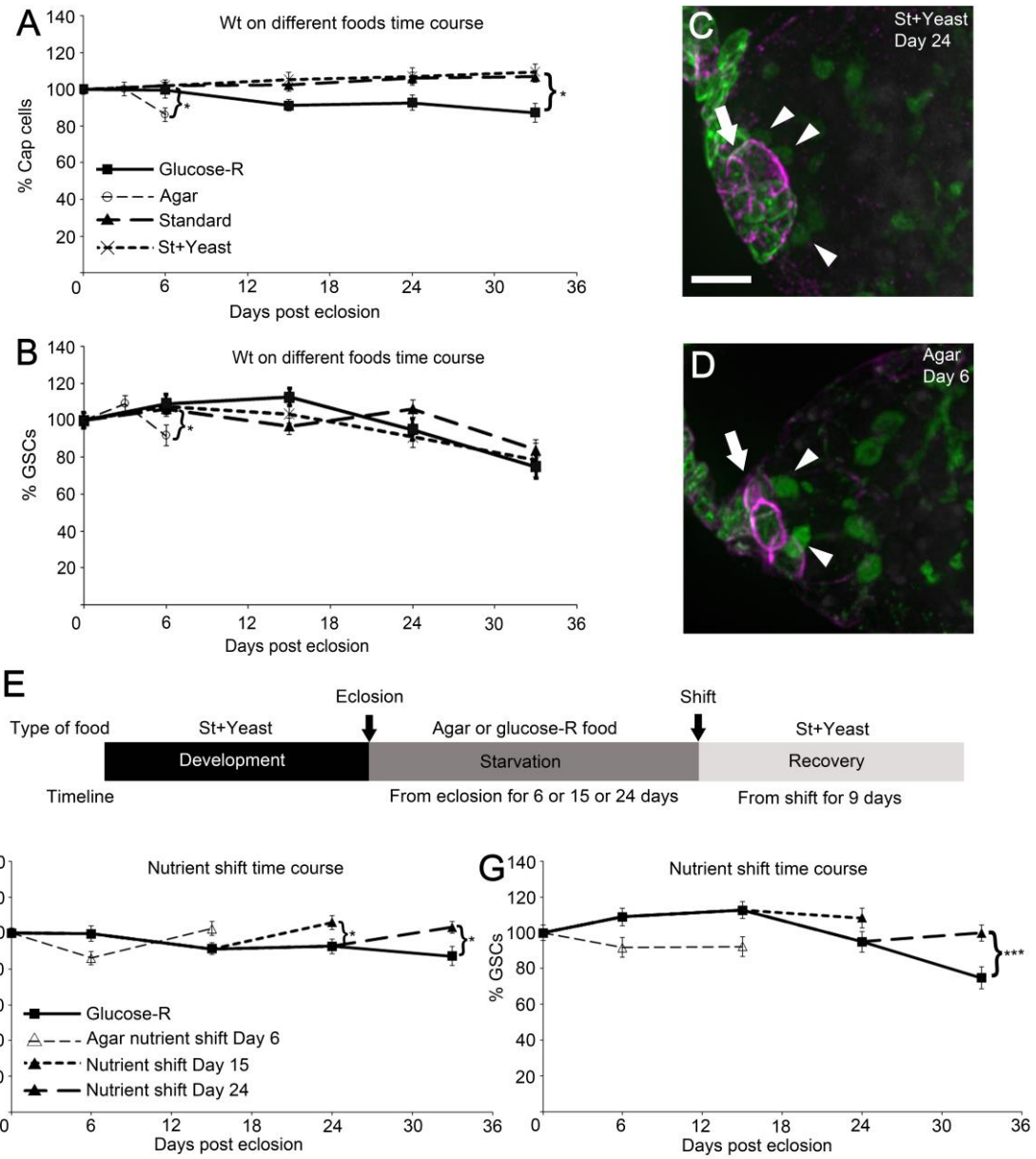
Figures


A
Wt on different foods time course
% Cap cells
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Standard
St+Yeast
Days post eclosion
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Wt on different foods time course
% GSCs
Days post eclosion
C
St+Yeast
Day 24
D
Agar
Day 6
E
Eclosion
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Type of food
St+Yeast
Agar or glucose-R food
St+Yeast
Development
Starvation
Recovery
Timeline
From eclosion for 6 or 15 or 24 days
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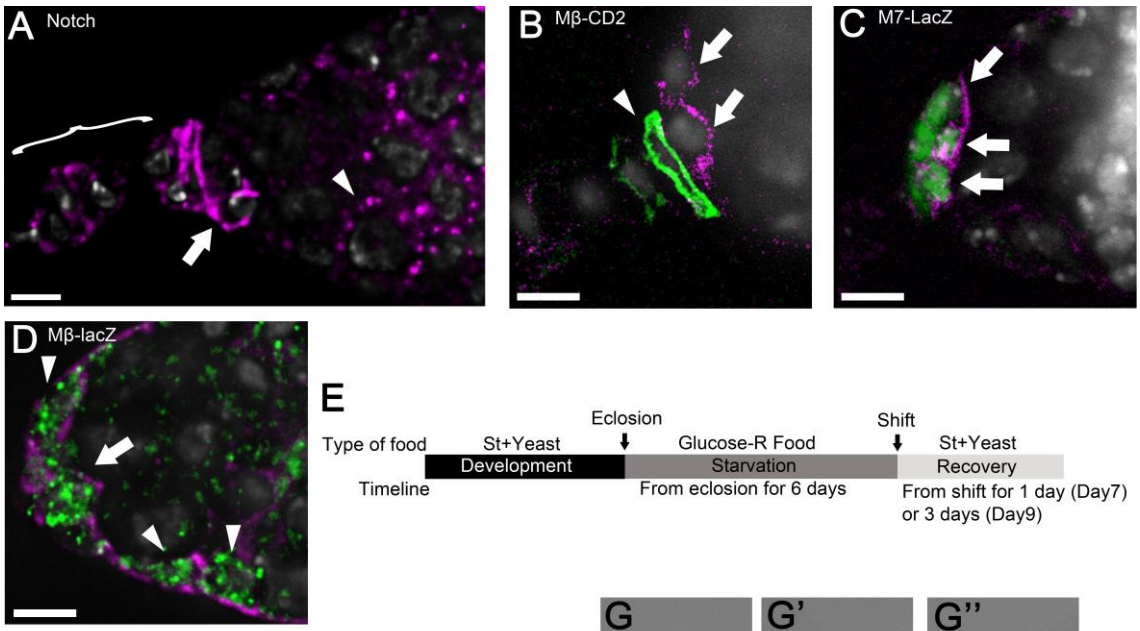
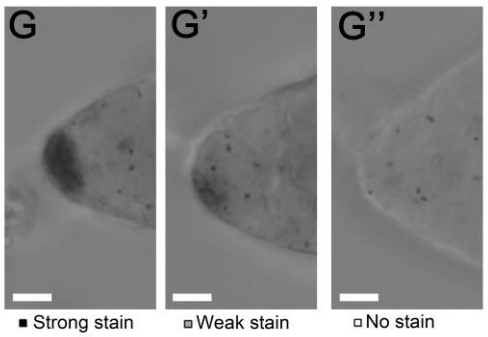
G
Nutrient shift time course
% GSCs
Days post eclosion

Figure 1. Reversible effects of dietary changes on the GSC niche

A) Changes of cap cell number after eclosion in Wt flies kept for up to 33 days after eclosion on: standard food; standard food + yeast (St+Yeast); standard food without added glucose (glucose-R); Agar only. Percentage of cap cells shown normalized to Day 0 (day of eclosion). Cap cell number significantly declines during the course of the timeline in flies on glucose-R and Agar diet compared to all other conditions (*P<0.05, t-test). (B) Changes of GSC number after eclosion in Wt flies kept for up to 33 days after eclosion on different foods as in (A). Percentage of GSCs shown normalized to Day 0 (day of eclosion). GSCs on Agar food significantly reduced at 6 days compared to all other conditions (*P<0.05, Mann-Whitney U test). Error bars are percentage of s.e.m.. Each time point has at least n=40 (C,D) Wt germaria stained with DAPI marking the nuclei (grey), anti-Coracle (magenta) strongly marking cap cell membrane (arrow); anti-LaminC marking cap cells and terminal filament and anti-Spectrin marking distinctive spectrosomes which indicate location of GSCs (arrowhead) (green). Images represent a merged stack of deconvolved optical sections taken through the niche. Scale bar 5 μm. (C) Wt germarium, 24 days post eclosion on St+Yeast showing no decline of niche size. (D) Wt germarium, 6 days post eclosion on agar showing loss of cap cells. (E) Summary of nutrient shift experimental timeline. Larval development was on St+Yeast. After eclosion, flies were kept on poor food for 6 days (on Agar) and 15 or 24 days (on glucose-R). Then flies were moved back to St+Yeast for 9 days. (F,G) Nutrient shift experiment showing changes to cap cells and GSCs. (F) Percentage of cap cells normalized to Day 0. Shifting flies from Agar, or from glucose-R, to St+Yeast recovers cap cell number compared to unshifted flies at the same time point (*P<0.05, Mann-Whitney U test). (G) Percentage of GSCs normalized to Day 0. Shifting flies from Agar, or from glucose-R, to St+Yeast abolishes normal GSC decline. Nutrient shifted Day33 vs. glucose-R Day33 (***P<0.001, Mann-Whitney U test). Error bars are percentage of s.e.m.. Each time point has at least n=40.


A Notch
B Mβ-CD2
C M7-LacZ
D Mβ-lacZ
E
Eclosion
Shift
Type of food
St+Yeast
Glucose-R Food
St+Yeast
Development
Starvation
Recovery
Timeline
From eclosion for 6 days
From shift for 1 day (Day7)
or 3 days (Day9)


F
M7-lacZ expression
% ovarioles
St+Yeast Day3
St+Yeast Day6
St+Yeast Day9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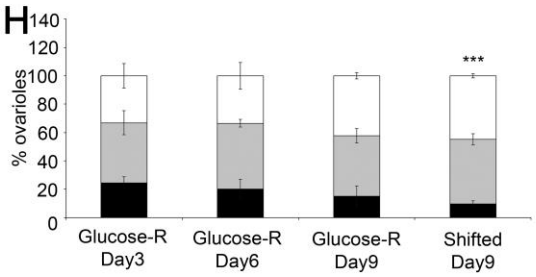
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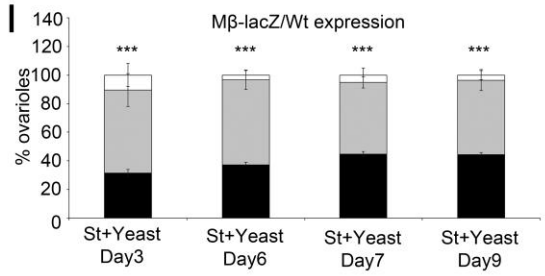
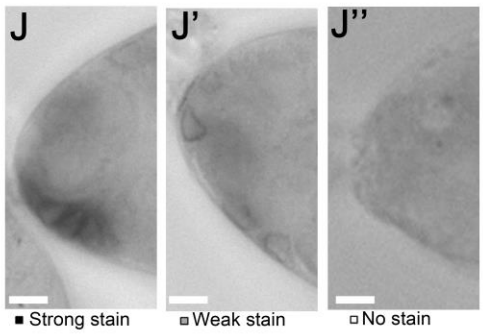
% ovarioles
Glucose-R Day3
Glucose-R Day6
Glucose-R Day9
Shifted Day9


I
Mβ-lacZ/Wt expression
% ovarioles
St+Yeast Day3
St+Yeast Day6
St+Yeast Day7
St+Yeast Day9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Strong stain
Weak stain
No stain

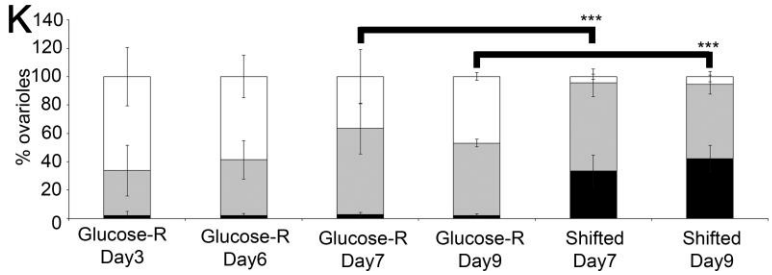
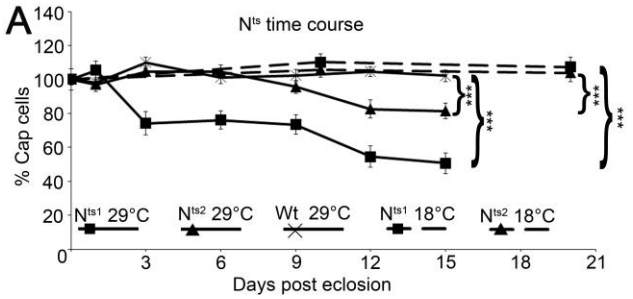

K
% ovarioles
Glucose-R Day3
Glucose-R Day6
Glucose-R Day7
Glucose-R Day9
Shifted Day7
Shifted Day9

Figure 2. Diet affects Notch signalling

Notch (anti-Notch, magenta) is localised throughout the germarium in TF (bracketed region), in cap cells (arrow), and in ESCs seen invading between germline cells (arrowhead). Nuclei marked with DAPI (grey). (B) Mβ-CD2 expression (anti-CD2, green) is found in the posterior TF cell (arrowhead) which contacts the cap cells (arrow). (C) M7-lacZ expression (anti-βGal, green) in the cap cells (arrow). (D) Mβ-lacZ (anti-βGal, green) is expressed in ESCs (arrowheads) and in few cap cells (arrow). In (A-D) DAPI marks the nuclei (grey). (B,D) Anti-Coracle marking cap cells (magenta). (A to D) Scale bars: 5 μm. The germaria (A to C) represent a merged image of several deconvolved optical sections. (E) Summary of nutrient shift experimental timeline. Larval development was on St+Yeast. After eclosion, flies were kept on glucose-R food for 6 days. Then flies were moved back to St+Yeast for 1-3 days. (F) M7-lacZ expression time course on St+Yeast. Stain intensity remains constant. Flies grown on St+Yeast food in F for specified number of days show reduced M7-lacZ expression compared to flies on glucose-R in H (***P<0.001, Chi Square test marked in H**)**. Example of Strong (G), weak (G') and No stain (G'') for M7-lacZ. Scale bars: 5 μm. (H) M7-lacZ time course for nutrient shift experiment. The population shifted to St+Yeast at day 6 and maintained for further 3 days before dissection did not recover M7-lacZ expression. (***P<0.001, Chi Square test for shifted Day9 in H vs. St+Yeast Day9 and St+Yeast Day6 in F). (I) Mβ-lacZ/Wt expression time course on St+Yeast. Stain intensity remains constant. Flies grown on St+Yeast food in I for specified number of days show reduced Mβ-lacZ/Wt expression compared to flies on glucose-R in K (***P<0.001, Chi Square test marked in I**)**. Example of Strong (J), weak (J') and No stain (J'') for Mβ-lacZ/Wt. Scale bars: 5 μm. (K) Mβ-lacZ/Wt time course for nutrient shift experiment. The population shifted to St+Yeast at day 6 and maintained for further 1 or 3 days before dissection recovered Mβ-lacZ/Wt expression (***P<0.001, Chi Square test for shifted Day9 vs. glucose-R Day9; ***P<0.001, Chi Square test for shifted Day7 vs. glucose-R Day7). (F,H,I,K) Error bars are s.e.m from three repeats.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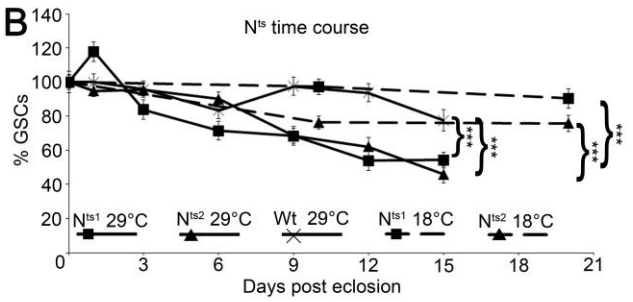
% Cap cells
140
120
100
80
60
40
20
0
Nts1 29°C
Nts2 29°C
Wt 29°C
Nts1 18°C
Nts2 18°C
3
6
9
12
15
18
21
Days post eclosion
***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Days post eclosion
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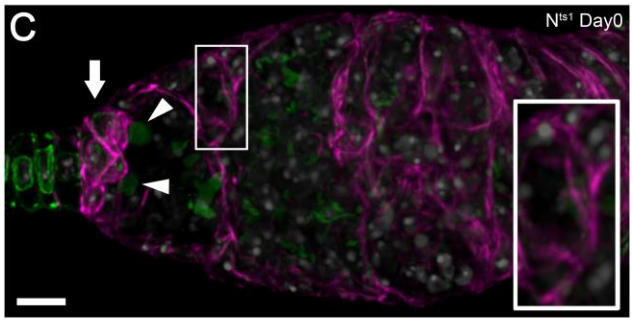
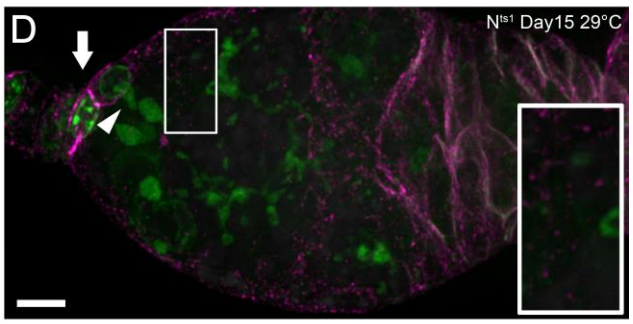

C
Nts1 Day0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Nts1 Day15 29°C


E
Nts1 Day0

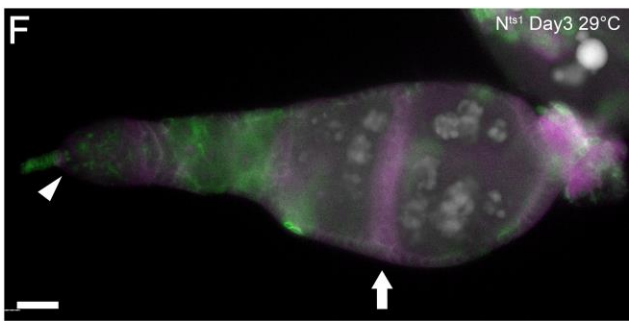

F
Nts1 Day3 29°C

Figure 3. Notch signalling is required in different somatic cell types within the germarium

(A) N^{ts1} and N^{ts2} mutations lead to loss of cap cells after shifting flies to 29°C at eclosion compared to Wt flies and to N^{ts} mutants kept at 18°C (***P<0.001, t-test). Percentage of cap cells shown normalized to Day 0 (day of eclosion). (B) A greater decline in the number of GSCs is observed in N^{ts1} and N^{ts2} at 29 °C compared to Wt flies and to N^{ts} mutants kept at 18°C (P<0.001, t-test). Error bars in (A,B) are percentage of s.e.m.. Each time point has at least n=40. (C-F) ovarioles stained for DAPI marking the nuclei (grey), anti-Coracle marking strongly cap cell membrane and weakly TF, ESCs and FCs (magenta), anti-Spectrin marking the spectrosome (green) and LaminC marking cap cell, TF and stalk nuclear membrane (also in green). The images (C,D) represent a merged stack of deconvolved optical sections. The ovarioles (E, F) are depicted by a single non-deconvolved image. (C) Wt germarium, cap cells (arrow), GSCs (arrowhead) and normal escort invasion between germline cysts highlighted in inset. (D) N^{ts1} germarium after 15 days at restrictive temperature post eclosion showing loss of cap cells (arrow), GSCs (arrowhead) and lack of ESC invasion (inset). Scale bars: 5 μm. (E) N^{ts1} ovariole at eclosion showing a Wt phenotype with egg chambers properly formed and separated by stalk cells (arrow). (F*)* N^{ts1} ovariole at 3 days post eclosion showing fused egg chamber phenotype lacking interconnected stalk (arrow). (E,F) GSC niche indicated with arrowhead. Scale bars: 20 μm.

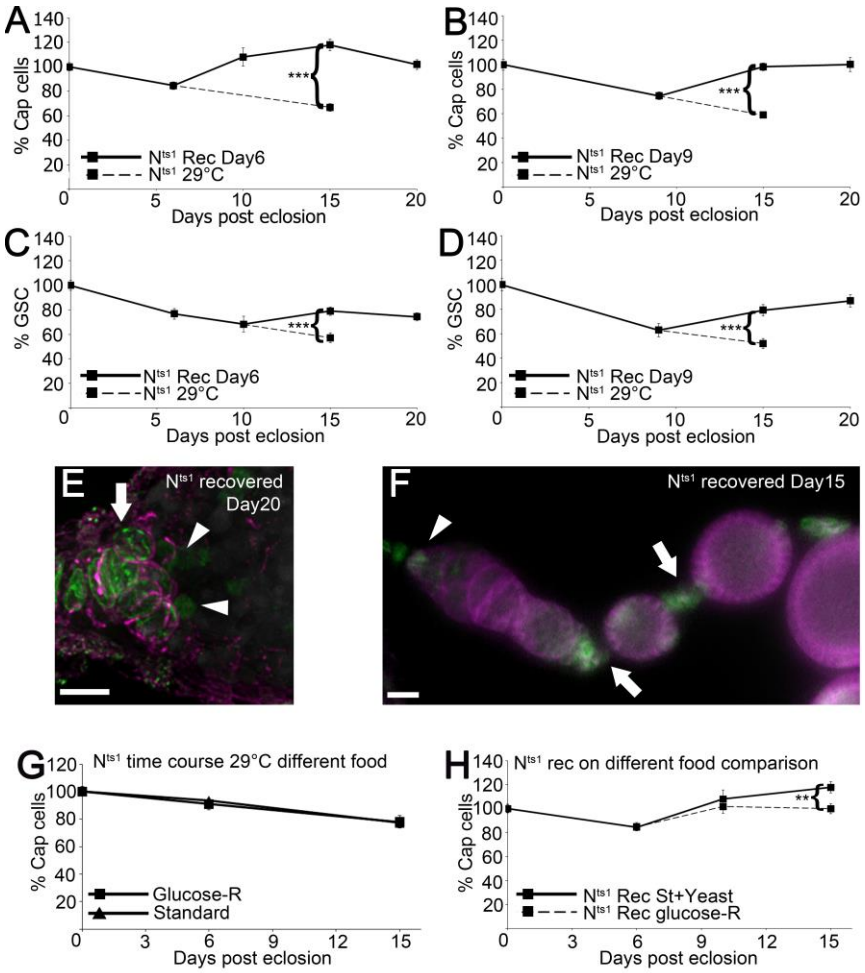
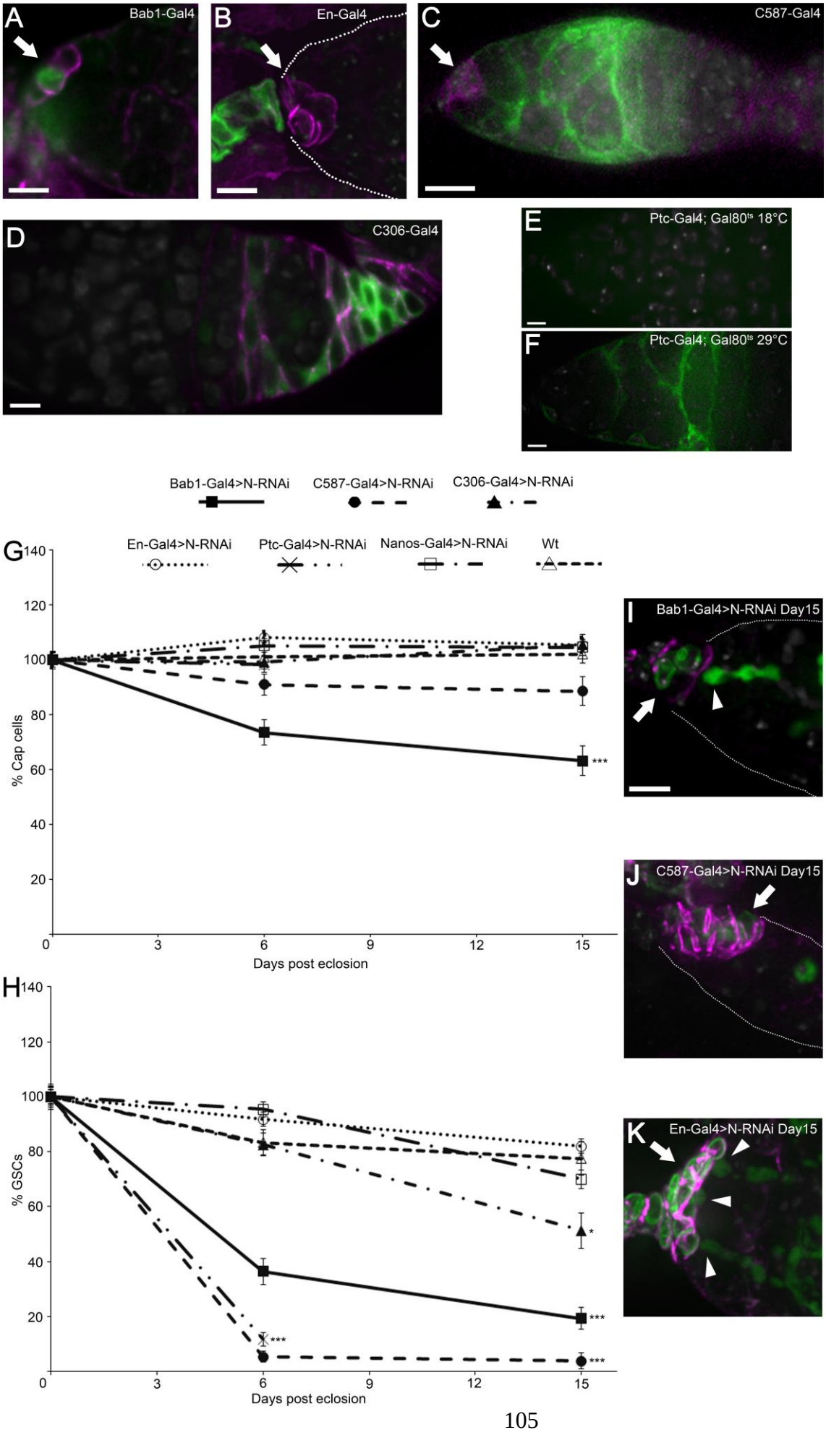


Figure 4. Reversible effects of altered Notch signalling on the niche

(A and B) Temperature shift from 29°C to 18°C reactivates Notch signalling and restore cap cell number. (A) N^{ts1} kept for 6 days post-eclosion at 29°C then restored to 18°C. Cap cell numbers recover at day 15 compared to flies kept at 29°C (***P<0.001, Mann-Whitney U test). (B) N^{ts1} switched to 18°C at 9 days post eclosion still recovers cap cell numbers to levels seen at eclosion compared to unswitched controls (***P<0.001, Mann-Whitney U test). Percentage of cap cells normalized to Day 0. (C) N^{ts1} kept for 6 days at 29°C then restored to 18°C. GSC numbers recover at day 15 compared to flies kept at 29°C (***P<0.001, Mann-Whitney U test). (D) N^{ts1} switched to 18°C at 9 days post eclosion still recovers cap cell numbers to levels seen at eclosion compared to unswitched controls (***P<0.001, Mann-Whitney U test). Percentage of GSCs normalized to Day 0. Error bars (A-D) are percentage of s.e.m. Each time point has at least n=40. (E,F) Ovarioles are labelled for DAPI marking the nuclei (grey), anti-coracle (green) marking strongly cap cell membrane and weakly TF and ESCs (magenta), anti-Spectrin marking the spectrosome and anti-LaminC marking cap cell nuclei (also green). (E) Image is a merged stack of deconvolved optical sections of N^{ts1} niche at 20 days post eclosion following temperature down-shift to 18°C at day 6 showing a recovered niche (arrow) and GSCs (arrowhead). Scale bar: 5 μm. (F) N^{ts1} ovariole at recovered Day 15 showing recovery of chambers, which properly formed and separated by stalk cells (arrow). GSC niche indicated with arrowhead. Scale bar: 10 μm. (G) N^{ts1} showing similar loss of cap cells when maintained at 29°C on standard and glucose-R conditions. (H) N^{ts1} kept for 6 days post eclosion at 29°C then restored to 18°C. On glucose-R food there is less niche expansion after temperature down-shift compared to flies on standard food supplemented with live yeast (**P<0.01 for comparison at day 15, Mann-Whitney U test). Error bars (G,H) are percentage of s.e.m. Each time point has at least n=40.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B
En-Gal4
C
C587-Gal4
D
C306-Gal4
E
Ptc-Gal4; Gal80ts 18°C
F
Ptc-Gal4; Gal80ts 29°C
Bab1-Gal4>N-RNAi
C587-Gal4>N-RNAi
C306-Gal4>N-RNAi
En-Gal4>N-RNAi
Ptc-Gal4>N-RNAi
Nanos-Gal4>N-RNAi
Wt
G
% Cap cells
Days post eclosion
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I
Bab1-Gal4>N-RNAi Day15
J
C587-Gal4>N-RNAi Day15
H
% GSCs
Days post eclosion
*
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K
En-Gal4>N-RNAi Day15

Figure 5. Consequences on the GSC niche of Notch RNAi expression in different cell types of the germarium

(A-F) Expression pattern of UAS CD8-GFP driven by different Gal4 lines. Ovarioles labelled for DAPI marking the nuclei (grey), GFP (green), and anti-Coracle marking strongly cap cell membrane and weakly TF, ESCs and FCs (magenta) (A-C), or (D) anti-FasIII marking FCs (magenta). (A) Bab1-Gal4 drives expression in a subset of cap cells (arrow) and in anterior ESCs. (B) En-Gal4-driven expression in the terminal filament but not cap cells (arrow) or ESCs. (C) C587-Gal4 drives strong expression in ESCs and anterior FC progenitors, but not in cap cells (arrow). (D) C306-Gal4 drives expression in the anterior FC progenitors. (E) $Gal80^{ts}$ inhibits expression of CD8-GFP with Ptc-Gal4 driver at $18^{o}C$, but CD8-GFP is expressed at $29^{o}C$ (F). Ptc-Gal4 drives expression in ESCs and early FC progenitors, similar to c587-Gal4. Scale bars: 5 μm.

(G) Cap cell number decreases when Notch RNAi (Nval 20) is expressed in adult cap cells using Bab1-Gal4 driver but not other Gal4 lines (***P<0.001, t-test). Percentage of cap cells normalized to Day 0. (H) GSCs decrease when Notch RNAi (Nval 20) is expressed with Bab1-Gal4, C587-Gal4 and Ptc-Gal4 (***P<0.001 for all the three lines, t-test) and C306-Gal4 (*P<0.05, t-test). Error bars in G,H are percentage of s.e.m.. Each time point has at least n=40. Similar results were observed with Notch RNAi GD1112 (Fig. S2). (G-H) A complete resume of all samples and controls can be found in fig. S2.

(I-K) Germaria are labelled for DAPI marking the nuclei (grey), anti-Coracle marking strongly cap cell membrane and weakly TF and ESCs (magenta), anti-Spectrin marking the spectrosome (green) and LaminC marking cap cell nuclei (also in green). Images are comprised of merged stacks of deconvolved optical sections. (I) Bab1-Gal4-driven Notch RNAi. Cap cell (arrow) and GSC (arrowhead) number are decreased 15 days post-eclosion. (J) C587-Gal4 driven Notch RNAi. GSC number is decreased 15 days post-eclosion but the cap cell (arrow) number remains constant. (K) en-Gal4-driven Notch RNAi. Cap cell (arrow) and GSC (arrowhead) number are not affected. (I-K) Scale bars: 5 μm.

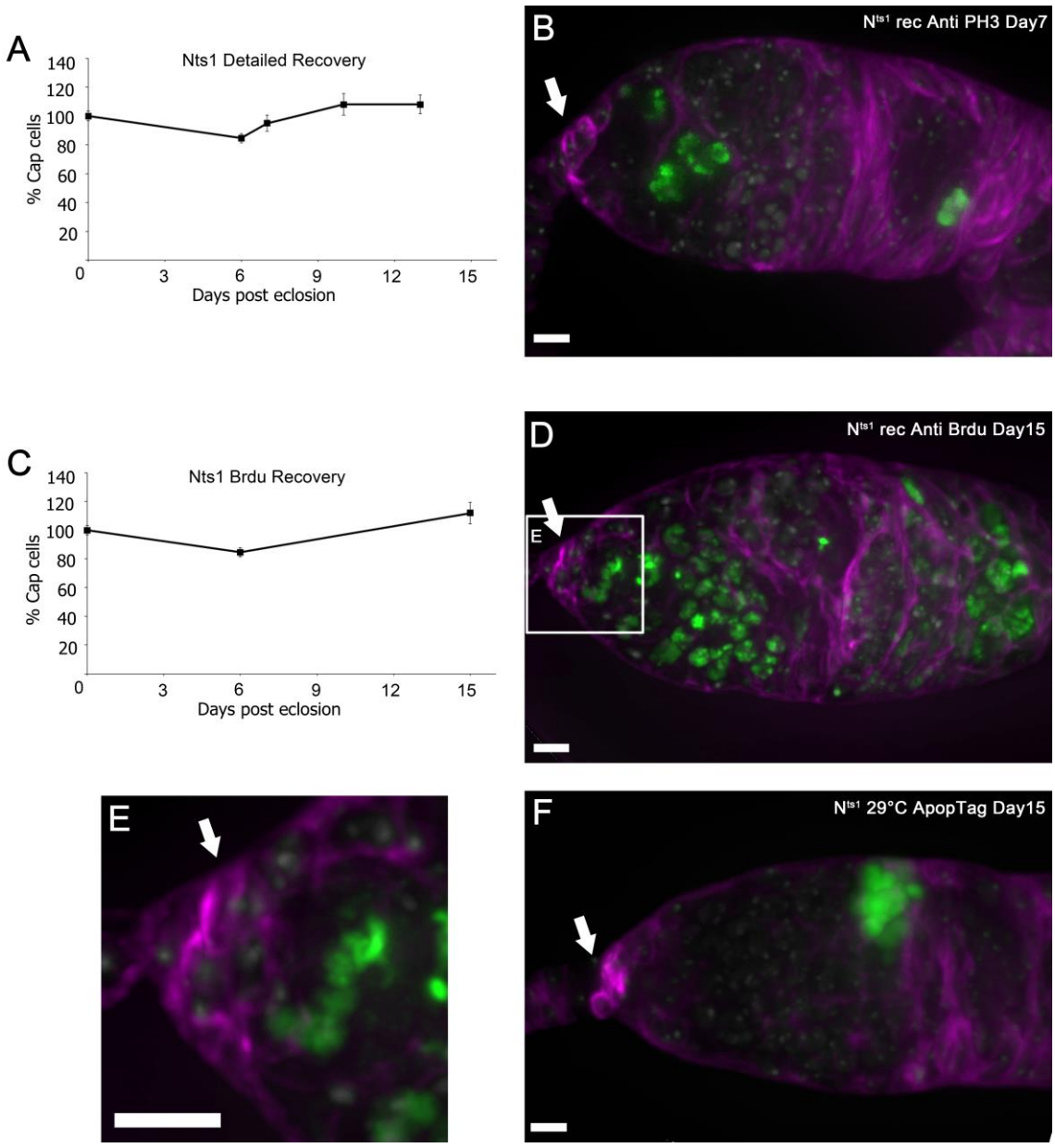


Figure 6. Cap cell mitosis and cell death do not explain changes in cap cell number
(A) Time line of cap cell recovery after temperature down-shift of N^{ts1} flies 6 days post eclosion. Expansion of cap cell niche is already found after 1 day at 18°C. (B) Anti-phospho-histone H3 Ser10 (green) staining was not identified in cap cells (arrow) in flies 7 days post-eclosion (n=239). Germarium also stained with anti-coracle (magenta) and DAPI (grey), also in D-F. (C). In BrdU-fed N^{ts1} flies, niche re-expansion still occurs after temperature downshift at 6 days post-eclosion. (A,C) Percentage of cap cells normalised to day 0. Error bars are s.e.m.. Each time point has at least n=40. (D,E) No anti-BrdU labelling (green) in N^{ts1} cap cells (arrow) 15 days post eclosion after temperature downshift of BrdU-fed flies (n=157). (E) Shows enlarged image of region boxed in (D). BrdU is present in germ line cells but not the cap cells (arrow). (F) Apoptag (green) staining of N^{ts1} germarium 15 days post eclosion at 29°C. No cap cell (arrow) death was observed (n=99) (F). The images (B, D-F) represent merged stacks of deconvolved optical sections. Scale bars (B, D-F), 5 μm.

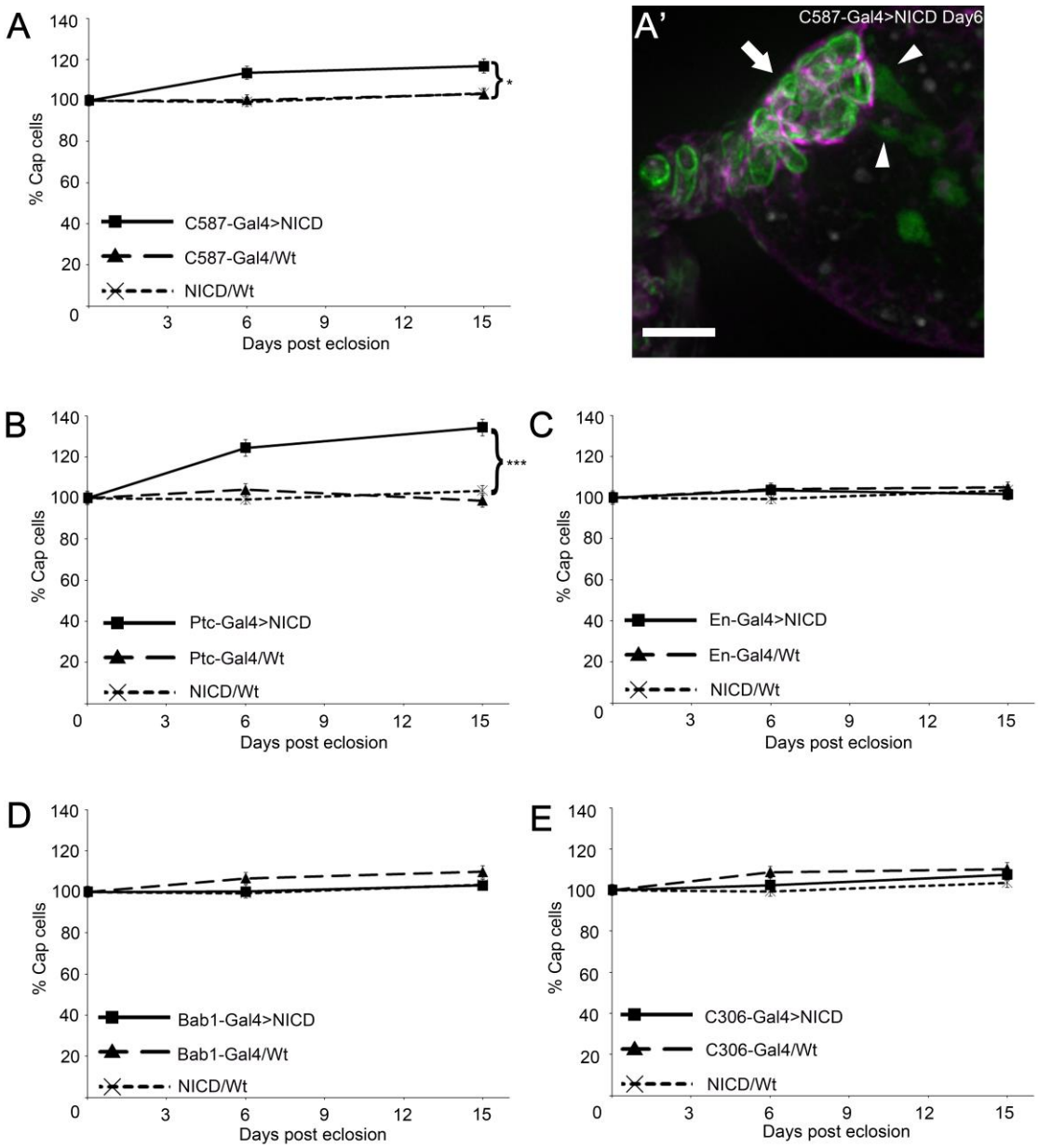


Figure 7. Consequences of NICD expression on niche

(A to E) Percentage of cap cells normalized to Day 0. (A,A') C587-Gal4 driven NICD increases cap cell number compared separate UAS and C587-gal4 line controls (*P <0.05, t-test). (A') The germarium 6 days post eclosion with enlarged niche (arrow) labelled for DAPI (grey), anti-Coracle marking strongly cap cell membrane and weakly TF and ESCs (magenta), anti-Spectrin marking the spectrosome (arrowhead) and anti-LaminC marking cap cell and terminal filament nuclei. Image represents a merged stack of deconvolved optical sections. Scale bar, 5 μm. (B) Ptc-Gal4 driven NICD increases cap cell number compared to separate UAS and Ptc-gal4 line controls (***P <0.001, t-test). En-Gal4 (C), Bab1-Gal4 (D) and C306-Gal4 (E) do not show a significant change in cap cell number (t-test). (A to E) Error bars are s.e.m.. Each time point has at least n=40.

Supplemental materials and methods

Crosses

Crosses were set up in vials using 12 virgin females mated with 12 males of the required genotype. Virgin females are identified by the presence of the dark green meconium in the abdomen region of the fly. Cross-vials are kept at the developing temperature required for the specific experiment, as stated in the experiment description.

Mounting

15 μl of ovaries containing mounting solution are dropped onto a microscopy slide. Ovaries are arranged on the slide under a Stevi SV 11 optical microscope (Zeiss) using the dissecting tools, in order to improve the quality of the slide. Ovaries are then enclosed with a 22x22 mm coverslip (Scientific Laboratory Supplies Ltd) and sealed with nail varnish (Aventor Beauty).

Wing dissection

Wings are dissected from CO2-anaesthetized flies using forceps. Wings are then dried in isopropanol (BDH) and mounted on a slide with a 1:1 mixture of Canadian balsam (Sigma) and methyl salicylate (Fluka). Coverslips are fixed in place with nail varnish.

Notch reporter line testing

Various Notch reporter lines were tested, as reported in Table S1. LacZ lines were assayed with both antibody staining against β-Gal and X-Gal staining. E(spl)mβ-CD2 was tested using an anti-CD2 antibody. For NRE-GFP, GFP expression pattern

was analysed. Notch reporter lines expressing in the germarium were later re-analysed using also anti-coracle as marker to better define expression pattern (Fig. 2).

UAS lines testing

UAS lines for Notch overexpression and Notch RNAis are driven in the wing through dpp-Gal4 and 69B-Gal4 drivers (Table S3). Notch gain-of-function and loss-of-function phenotypes were noted (Fig. S3).

Gal4 lines testing

Gal4 lines are crossed to UAS-CD8-GFP and their expression was surveyed throughout the ovaries (Fig. 5, Table S2). Gal4 lines expressing in the germarium are later re-analysed using also antibodies anti-coracle and anti-fasciclin III as markers to better define expression pattern (Fig. 5).

Scoring of cap cell and GSC numbers

Cap cells are identified by their rounded morphology, strong coracle expression at the cell surface and presence of nuclear membrane-localised LaminC stain. GSCs were identified by anti-Spectrin staining marking the spectrosome of GSCs together with their localisation adjacent to cap cells. Fig. S4 shows an example of cell stained in the germarium, indicating cap cells, escort cells, terminal filament and GSCs.

Scoring of M7-lacZ and Mβ-lacZ

Scoring of M7-lacZ and Mβ-lacZ activity (Fig. 2E-N) required X-Gal stain, as antibody stain against Mβ-lacZ was not strong enough for scoring. The scoring was done visually. Experiments were performed in 3 separate replicas. Experiments are

planned in order to dissect all samples for 1 replica at the same time with the same freshly made solutions to avoid variations in X-Gal stain. Stain intensity was divided in 3 categories: strong, weak, and no stain. The stain intensity was not sharply divided between strong and weak but in a gradation. An intensity threshold was set to divide between the strong and weak stain categories. The same threshold was used for all the different samples.

Supplemental figures

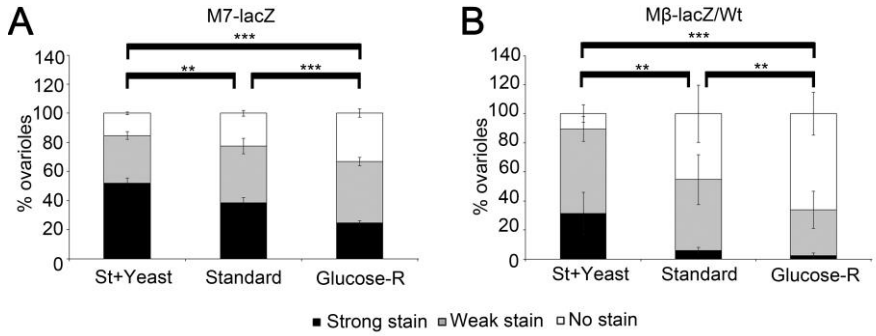
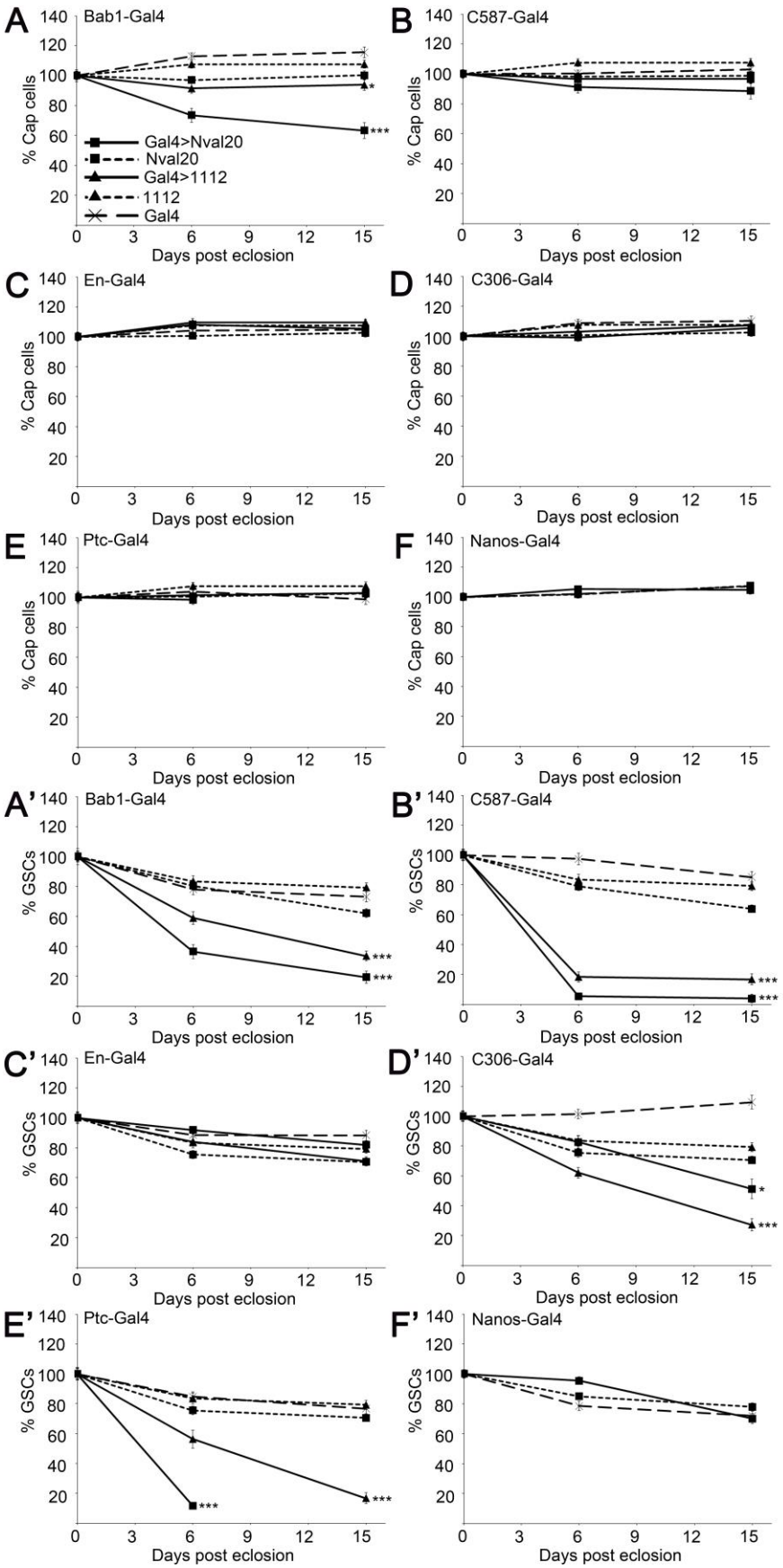


Figure S1. Notch reporter line expression on different food conditions
(A) M7-lacZ expression on standard food is weakly reduced compared to standard food supplemented with yeast (St+Yeast) (**P=0.008, Chi Square test). Flies on glucose-R food show further reduced m7-lacZ expression compared to both flies on standard food and St+Yeast (***P=0.001, Chi Square test). (B) Mβ-lacZ/Wt expression on standard food is reduced compared to St+Yeast (**P=0.008, Chi Square test). Flies on glucose-R food show a further small reduction in Mβ-lacZ/Wt expression compared to flies on standard food (**P=0.002, Chi Square test) and St+Yeast (***P=0.001, Chi Square test). Error bars (A,B) are s.e.m. from three repeats.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En-Gal4
D'
C306-Gal4
E'
Ptc-Gal4
F'
Nanos-Gal4
% Cap cells
% GSCs
Days post eclosion
Gal4>Nval20
Nval20
Gal4>1112
1112
Gal4
*
***

Figure S2. Effects of Notch RNAi in different cell types in the germarium, showing comparison with controls

(A to F) Percentage of cap cells normalized to Day 0. (A) Bab1-Gal4>NRNAis: Cap cell number significantly declines when driving both Nval 20 and 1112 RNAis compared to controls: Bab1-Gal4> Nval 20 vs. Bab1-Gal4, ***P<0.001; Bab1-Gal4> Nval 20 vs. Nval 20, ***P<0.001; Bab1-Gal4> 1112 vs. Bab1-Gal4, *P<0.05; Bab1-Gal4> 1112 vs. 1112, *P<0.05. C587-Gal4 (B), en-Gal4 (C), C306-Gal4 (D), Ptc-Gal4 (E), nanos-Gal4 (F) do not show a significant change in cap cell number. (A' to F') Percentage of GSCs normalized to Day0. (A') Bab1-Gal4>NRNAis: GSC number significantly declines when driving both Nval 20 and 1112 RNAis compared to controls: Bab1-Gal4> Nval 20 vs. Bab1-Gal4, ***P<0.001; Bab1-Gal4> Nval 20 vs. Nval 20, ***P<0.001; Bab1-Gal4> 1112 vs. Bab1-Gal4, ***P<0.001; Bab1-Gal4> 1112 vs. 1112, ***P<0.001. (B') C587-Gal4>NRNAis: GSC number significantly declines when driving both Nval 20 and 1112 RNAis compared to controls: C587-Gal4> Nval 20 vs. C587-Gal4, ***P<0.001; C587-Gal4> Nval 20 vs. Nval 20, ***P<0.001; C587-Gal4> 1112 vs. C587-Gal4, ***P<0.001; C587-Gal4> 1112 vs. 1112, ***P<0.001. (C') en-Gal4>NRNAis: GSC numbers do not decline differently than controls. (D') C306-Gal4>NRNAis: GSC number significantly declines when driving both Nval 20 and 1112 RNAis compared to controls: C306-Gal4> Nval 20 vs. C306-Gal4, ***P<0.001; C306-Gal4> Nval 20 vs. Nval 20, *P<0.05; C306-Gal4> 1112 vs. C306-Gal4, ***P<0.001; C306-Gal4> 1112 vs. 1112, ***P<0.001. (E') ptc-Gal4>NRNAis: GSC number significantly declines when driving both Nval 20 and 1112 RNAis compared to controls: ptc-Gal4> Nval 20 vs. ptc-Gal4, ***P<0.001; ptc-Gal4> Nval 20 vs. Nval 20, ***P<0.001; ptc-Gal4> 1112 vs. ptc-Gal4, ***P<0.001; ptc-Gal4> 1112 vs. 1112, ***P<0.001. (F') nanos-Gal4>NRNAis: GSC numbers do not decline differently than controls. (A to F') Error bars, percentage of s.e.m. Each time point has at least n=40. Statistical analysis: t-test.

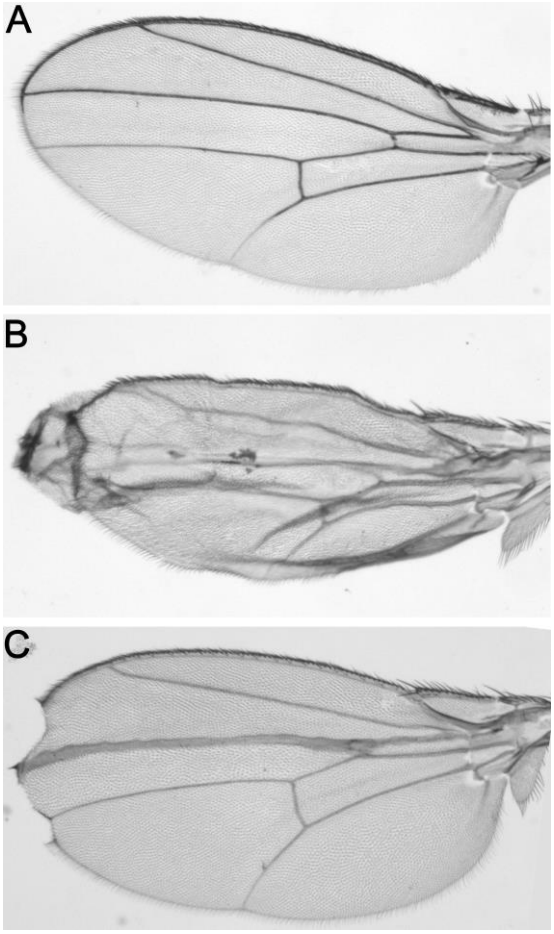

Figure S3. UAS lines expression test in the wing
Several UAS lines were tested in the wing before being used in the ovaries, as reported in Table S3. (A) dpp-Gal4> NFull length at 18°C, showing no visible Notch phenotype. (B) dpp-Gal4>NICD at 18°C, showing Notch gain-of-function phenotype. (C) dpp-Gal4>1112 at 18°C, showing Notch loss-of-function phenotype

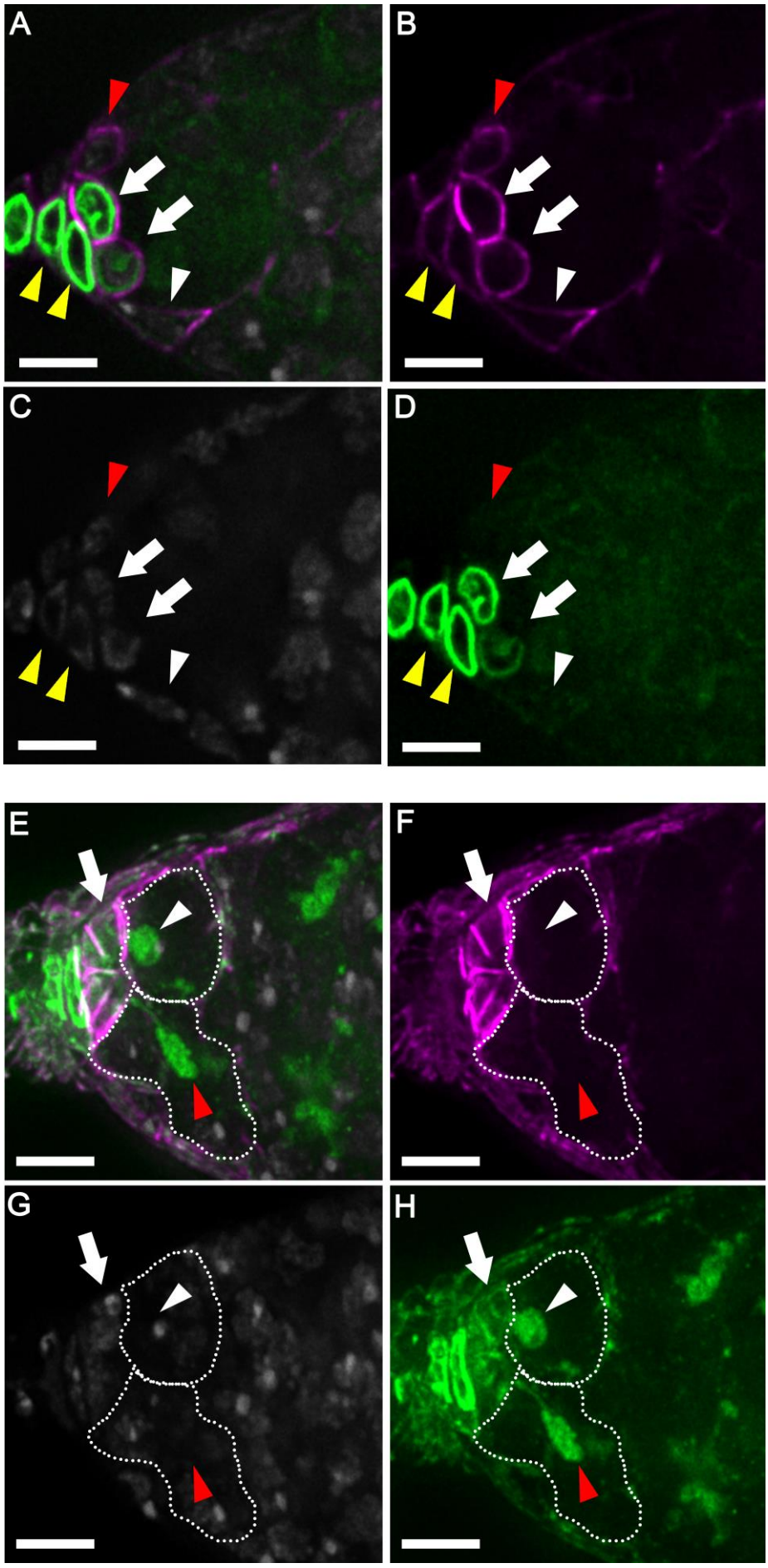
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Figure S4. Cap cell and GSC counting

(A-D) Cap cells (arrow) are identified by their rounded morphology (DAPI marking the nuclei in grey, C), strong coracle (magenta, B) expression at the cell surface and presence of nuclear membrane-localised laminC stain (green, D). ECs (white and red arrowhead) morphology is elongated, often in a triangular-like shape (white arrowhead). The EC marked with the red arrowhead is a good example of EC that could be confused for a cap cell because of its rounded morphology. It is possible to identify it as an EC from the weaker coracle stain (magenta, B) and from the fact that it lacks laminC nuclear stain (green, D). Moreover, germaria are analysed through stacks of picture; in this case, it is possible to see a slightly elongated structure when checking the vertical z-plane. The TF is found at the base of the germarium, anterior of the cap cells. TF cells (yellow arrowheads) have an elongated eye-shape-like morphology (DAPI marking the nuclei in grey, C) and a very strong laminC stain (green, D), stronger than cap cell (arrow) laminC stain. (E-H) GSCs, whose contours are marked with dotted lines, are identified by anti-spectrin staining (green, H) marking the spectrosome (arrowhead) of GSCs and by their localisation adjacent to the niche (arrow). Red arrowhead indicates the elongated spectrosome of a dividing GSC, which is still anchored to the niche.

Supplemental tables

Reporter line	Expression in the germarium	Expression in egg chambers
E(spl)m7-lacZ, gift from Sarah Bray (Bray, 2006)	Cap cells	Stage 2 to 7: strongly in polar cells, weakly in FCs nearby polar cells and in stalk cells.
E(spl)mβ1.5-lacZ, gift from Sarah Bray (Cooper et al., 2000)	Cap cells, ESCs, FCs	Polar cells, basal stalk cells
E(spl)mδ0.5-lacZ, gift from Sarah Bray (Cooper et al., 2000)	No stain	No stain
E(spl) mδ1.9-lacZ, gift from Sarah Bray (Cooper et al., 2000)	No stain	No stain
E(spl)mγ1.9-lacZ, gift from Sarah Bray (Cooper et al., 2000)	No stain	No stain
E(spl)mβ-CD2, gift from Sarah Bray (de Celis et al., 1998)	Basal Tf cell: most posterior Tf cell, contacting the cap cells	Stage 7 to 10: Main body FCs. Stages 2 to 5: Stalk cells adjacent to egg chambers
E(spl)m8-lacZ, gift from Sarah Bray (Kramatschek and Campos-Ortega, 1994)	Weak FC stain	Stalk cells between stage 7 and 8
GBE-Su(H)$_{m8}$-lacZ, gift from Sarah Bray Furriols & Bray, 2001)	FC region	Stage 2 to 7: strongly in polar cells. Stain present also in FCs nearby polar cells; in these cells, stain was stronger than with E(spl)m7-lacZ
w^{1118}; P{w[+m*]=NRE-EGFP}5A (NRE-GFP): 30727 Bloomington stock (Saj et al., 2010)	No stain	Similar pattern to GBE-Su(H)$_{m8}$-lacZ

Table S1. Notch reporter lines expression throughout the ovary

Gal4 line	**Expression in the Germarium**	**Expression in egg chambers**
P{GawB}c587 (C587-Gal4) (Manseau et al., 1997	ESCs, FSCs	No expression
P{GawB}69B (69B-Gal4): 1774 Bloomington stock (Manseau et al., 1997)	No expression	Stage 1-6: stalk cells and some FCs. Stage 7-12: FCs
P{GawB}c306, w^{1118} (c306-Gal4): 3743 Bloomington stock (Manseau et al., 1997)	FCs in region 2b-3	Stage 1-6: stalk cells and some FCs. Stage 7-12: stalk cells and polar cells and FCs nearby polar cells
w^{1118}; P{GawB}c368 (c368-Gal4): 3744 Bloomington stock (Manseau et al., 1997)	No expression	Stage 1-6: stalk cells, polar cells and some FCs. Stage 7-12: stalk cells
Patched-Gal4 (ptc-Gal4) (Speicher et al., 1994)	ESCs, FSCs	From stage 10: FCs
Decapentaplegic-Gal4 (Dpp-Gal4) (Staehling-Hampton et al., 1994)	No expression	From stage 10: possibly FCs
brick-a-brack-Gal4/TM3 (bab1-Gal4): gift from Hillary Ashe (Bolívar et al., 2006)	Terminal filament, cap cells, early ESCs, FCs in region 2b-3	No expression
P{GawB}bab1{Pgal4-2} (bab1old-Gal4): 6803 Bloomington stock (Cabrera et al., 2002)	Terminal filament, FCs in region 2b-3	Stage 1-6: polar cells
*w**; P{en2.4-GAL4}e16E (En-Gal4): 30564 Bloomington stock (Harrison et al., 1995)	Terminal filament	No expression
w^{1118}; P{GAL4::VP16-nos.UTR}CG6325^{MVD1}, P{UASp-GFPS65C-αTub84B}3 (Nanos-Gal4): 7253 Bloomington stock (Van Doren et al., 1998)	No stain, expression verified with Bam-RNAi	No stain, expression verified with Bam-RNAi
E(spl)-GAL4, y^1, w^{1118} [E(spl)-Gal4]: 8226 Bloomington stock	No expression	No expression
y^1, w^{1118}; P{m4-GAL4.Exel}2/CyO (m4-Gal4): 8224 Bloomington stock	No expression	No expression
y^1 ,w^{1118}; P{E(spl)-GAL4.Exel}3/TM3, Sb^1, Ser^1 E(spl)-Gal4/TM3): 8225 Bloomington stock	No Expression	No expression
P{GawB}112A, *w** (112A-Gal4): 7021 Bloomington stock	ESCs, few FCs in region 3	Stage 1-6: stalk cells. Stage 7-12: polar cells and FCs nearby polar cells; migrating border cells
w^*;{w[+mW.hs]=GAL4-arm.S}11 (armadillo-Gal4): 1560 Bloomington stock	No expression	From stage 10: some FCs

w^{*}; P{w[+mW.hs]=GAL4-wg.M}MA1 (wingless-Gal4): 4918 Bloomington stock	No expression	No expression
w^{1118}; P{y[+t7.7] w[+mC]=GMR53A07-GAL4}attP2 (dally-Gal4): 47375 Bloomington stock	No expression	No expression
w^{1118}; P{da-GAL4.w^{-}}3 (Daughterless-Gal4): 8641 Bloomington stock	No expression	No expression

Table S2. Gal4 lines expression throughout the ovary

UAs lines	**69B-Gal4**		**dpp-Gal4**	
	18 °C	25 °C	18 °C	25 °C
Notch RNAi 1112 (1112): 1112GD VDRC stock	Lethal	Lethal	Notch loss-of-function phenotype present	Lethal
Notch RNAi 27228 (27228): 27228GD VDRC stock	Weak Notch loss-of-function phenotype present	Lethal	No phenotype	Notch loss-of-function phenotype present
Notch RNAi 27229/TM3 *Ser*,*Sb* (27229): 27229GD VDRC stock	Notch loss-of-function phenotype present	Lethal	Very weak Notch loss-of-function phenotype present	Notch loss-of-function phenotype present
M26 Notch RNAi/Cyo (M26): gift from Kenji Matsuno	Lethal	Lethal	Weak Notch loss-of-function phenotype present	Notch loss-of-function phenotype present
Notch RNAi Valium 20 (Nval 20): HMS00009 TRiP stock	-	-	Lethal	Lethal
NICD (2nd chromosome): gift from Spyros Artavanis-Tsakonas (Harvard Medical School)	Lethal	Lethal	Lethal	Lethal
NICD (3rd ch.): gift from Spyros Artavanis-Tsakonas (Harvard Medical School)	Lethal	Lethal	Notch gain-of-function phenotype present	Lethal
NFull length: gift from Spyros Artavanis-Tsakonas (Harvard Medical School)	No phenotype	No phenotype	No phenotype	No phenotype

Table S3. UAS lines test in the wing

Supplemental material and methods references

Bolívar, J., Pearson, J., López-Onieva, L., and González-Reyes, A. (2006). Genetic dissection of a stem cell niche: the case of the Drosophila ovary. Developmental Dynamics : an Official Publication of the American Association of Anatomists *235*, 2969–2979.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nature Reviews. Molecular Cell Biology *7*, 678–689.

Cabrera, G.R., Godt, D., Fang, P.-Y., Couderc, J.-L., and Laski, F. a (2002). Expression pattern of Gal4 enhancer trap insertions into the bric à brac locus generated by P element replacement. Genesis (New York, N.Y. : 2000) *34*, 62–65.

De Celis, J.F., Tyler, D.M., and Bray, S.J. (1998). Notch signalling mediates segmentation of the Drosophila leg. Development (Cambridge, England) *125*, 4617–4626.

Cooper, M.T., Tyler, D.M., Furriols, M., Chalkiadaki, A., Delidakis, C., and Bray, S.J. (2000). Spatially restricted factors cooperate with notch in the regulation of Enhancer of split genes. Developmental Biology *221*, 390–403.

Van Doren, M., Williamson, a L., and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Current Biology : CB *8*, 243–246.

Harrison, D. a, Binari, R., Nahreini, T.S., Gilman, M., and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. The EMBO Journal *14*, 2857–2865.

Kramatschek, B., and Campos-Ortega, J.A. (1994). Neuroectodermal transcription of the Drosophila neurogenic genes E(spl) and HLH-m5 is regulated by proneural genes. Development (Cambridge, England) *120*, 815–826.

Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F., Phan, H., Philp, A.V., Yang, M., Glover, D., Kaiser, K., et al. (1997). GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of Drosophila. Developmental Dynamics : an Official Publication of the American Association of Anatomists *209*, 310–322.

Saj, A., Arziman, Z., Stempfle, D., van Belle, W., Sauder, U., Horn, T., Dürrenberger, M., Paro, R., Boutros, M., and Merdes, G. (2010). A combined ex vivo and in vivo RNAi screen for notch regulators in Drosophila reveals an extensive notch interaction network. Developmental Cell *18*, 862–876.

Speicher, S. a, Thomas, U., Hinz, U., and Knust, E. (1994). The Serrate locus of Drosophila and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. Development (Cambridge, England) *120*, 535–544.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, a H., and Hoffmann, F.M. (1994). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A. Cell Growth & Differentiation : the Molecular Biology Journal of the American Association for Cancer Research *5*, 585–593.

Chapter 3: result part 2

ZO-1 controls stem cell niche size by acting as an upstream regulator of Deltex-dependent Notch signalling.

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Running Title: Regulation of Notch by ZO-1

Abstract

Maintenance of the correct stem cell population size is essential for homeostatic tissue renewal and ultimately depends on the capacity of the supporting niche. However, little is known about how niche sizes are determined. Here we show that the assembly of the *Drosophila* germline stem cell niche is promoted by a Deltex-dependent endosomal Notch activation mechanism, which does not require ligands. The junction adaptor protein ZO-1 prevents niche overexpansion by forming a complex with Deltex to suppress Notch endocytosis and signalling. ZO-1 thus acts as the first identified upstream regulator of Deltex. In contrast, ZO-1 promotes ligand-induced Notch signalling by increasing co-clustering of Notch and ligand at cell-cell contacts. ZO-1 thus has a unique capacity to alter both the source and strength of Notch signal activation during tissue assembly and cell-cell junction formation. These novel mechanisms have wider implications for understanding numerous signalling and tumour suppressor activities of ZO proteins.

Introduction

Long-term tissue renewal and maintenance depends on small populations of stem cells that are often anchored in defined locations by specialised cells that comprise stem cell niches. The balance between symmetric and asymmetric cell division maintains a constant stem cell population, however stem cell numbers also depend on the size of the supporting niche. The regulation of niches is a less well-researched aspect of stem cell biology but it is becoming increasingly important to understand effects of aging, nutrition and the interactions between cancer stem cells and their pathogenic niches (Li and Neaves, 2006; Oakley and Van Zant, 2010; Voog and Jones, 2010). In the *Drosophila* ovary the germline stem cell (GSC) niche assembles after the development of the terminal filament stacks that prefigure ovariole formation (Song et al., 2007; Zhu and Xie, 2003). Somatic cells are then recruited and differentiate as cap cells, which exit mitosis and subsequently provide anchorage for the GSCs. Notch (N) receptor signalling is required for recruitment and differentiation of these cap cell precursors (Song et al., 2007), but neither ligand expressed in adjacent terminal filament cells nor the germline stem cells can account for all of the sources of Notch signalling involved (Hsu and Drummond-Barbosa, 2011).

We have used genetic approaches to identify genes that control niche size in the *Drosophila* ovary. Previously we have shown that mutations of the apical junctional adaptor protein ZO-1, also known as Polychaetoid (Pyd), cause expanded cap cell niches through increased Notch signalling (Djiane et al., 2011), but the mechanism of ZO-1 action is not known. Here we show that loss of the ring finger protein Deltex (Dx) reduces Notch signalling in cap cells, leading to smaller GSC niches and fewer stem cells. Notch signalling can be activated by membrane bound DSL-domain ligands (Kopan and Ilagan, 2009) or alternately by a ligand-independent activation mechanism that requires Notch trafficking to the lysosome. The latter is driven by Dx, which binds

to the Notch intracellular domain (Baron, 2012; Diederich et al., 1994; Hori et al., 2011; Wilkin et al., 2004). We show that full-length ZO-1 acts in a complex with Dx to suppress Dx-induced Notch endocytosis, a mechanism that is modulated by cell density and *in vivo* by several apical junction components. Thus ligand-independent Notch signalling can be subject to upstream regulation in response to external cues mediated through cell-cell junctional organisation. In contrast we find that a specific isoform of ZO-1 promotes ligand-dependent activation of Notch by facilitating receptor-ligand co-clustering at cell-cell contacts. Selection between alternate means to activate Notch can therefore be coupled with formation of apical junction-associated complexes, to maintain appropriate signalling levels during the process of niche assembly. These findings have implications for understanding tumour-suppressor and other signal regulatory functions of ZO proteins in other organisms.

Results

Deltex-dependent Notch activity supports assembly of the GSC niche

In *Drosophila* ovaries we found that *dx* mutants have a reduced niche size and fewer GSCs (Fig. 1a-c,e,f) similar in consequence to removing one copy of *Notch* (Fig. 1d,e,f). Addition of a Dx genomic rescue construct (Dx^{GR}, TX05B) (Busseau et al., 1994) reverted the *dx* phenotypes (Fig. 1e,f). To confirm that *dx* was required in the cap cell niche we expressed *dx*-targeted RNAi using the bab1-Gal4 driver (Bolívar et al., 2006). The latter phenocopied the reduction in niche size seen in *dx* mutants (Fig. 1e). We next investigated a Notch reporter E(spl)M7-lacZ (Djiane et al., 2011) and found reduced signalling in the cap cell niche of *dx* mutants (Fig. 1g,h). Notch signalling has been shown to control niche size during development, as it is involved in the

recruitment of cap cell precursors to the terminal filament. In the adult ovary, Notch also plays a role in niche maintenance (Song et al., 2007). When Notch activity is switched off in the adult using temperature-sensitive mutants the size of the niche declines over time due to loss of cap cells (Song et al., 2007). To determine the contribution of *dx* to these distinct Notch functions adults were dissected immediately after eclosion and at various adult ages up to 15 days. In *dx* null mutants the niche size was reduced in newly eclosed flies, but remained stable as adult flies aged (Fig. 1i). Thus loss of *dx* specifically reduces cap cell recruitment and does not affect subsequent adult niche maintenance. Flies heterozygous for a null allele of *Notch* also eclosed with a similarly reduced niche size, which also did not further decline with age (Fig.1i). Hence niche formation in development is more sensitive to a reduction of Notch signalling activity than is the subsequent adult niche maintenance, making the contribution of Dx more critical during this developmental phase.

ZO-1 and Dx regulate cap cell recruitment to the GSC niche

ZO-1 has previously been identified as a negative regulator of Notch signalling in the cap cells of the *Drosophila* ovary GSC niche. *ZO-1 (pyd)* mutants display an increased Notch signalling level, increased niche size, and consequently increased numbers of GSCs (Djiane et al., 2011). Having established the requirement for Dx-dependent Notch signalling in the niche we next investigated genetic interactions with *ZO-1* mutants. When the latter are combined with a null mutation of *dx*, we found that the expanded niche phenotype of *ZO-1* mutants was suppressed (Fig. 2a,b,d). This suggests that Dx acts downstream or parallel to ZO-1 in regulating Notch signalling. Dx-induced Notch signalling is suppressed by mutations of HOPS (homotypic fusion and vacuole protein sorting) complex proteins (Wilkin et al., 2008), which are required for late endosomal-lysosomal fusion. However, disruption of the HOPS complex has no

effect on ligand-induced Notch signalling (Wilkin et al., 2008). A viable hypomorphic allele of the HOPS component *carnation (car)* also rescued the expanded *ZO-1* niche phenotypes (Fig. 2c,d). Thus ZO-1 normally opposes a Notch signal in the GSC niche that depends on late endosome-lysosome trafficking.

ZO-1 suppresses Dx-induced Notch signalling

In the wing imaginal disc, Notch signals at the boundary of the dorsal and ventral wing compartments to induce *wingless* expression and regulate wing margin formation. Notch signalling can be induced independently of its ligands by the overexpression of Dx (Hori et al., 2004; Wilkin et al., 2008). In the wing imaginal disc this results in increased *wingless* expression resulting in ectopic extra wing margin sensory bristles in the adult wing (Fig. 3a,b,e,f). When Dx is overexpressed in a *ZO-1* mutant background Notch signalling is strongly upregulated, reflected in increased ectopic margin and *wingless* expression (Fig. 3c,g). When Dx is expressed in a *car* mutant background, Notch is endocytosed but accumulates in a prelysosomal compartment, and the ectopic Notch signalling is suppressed (Wilkin et al., 2008). Wing margin notches are also observed which are due to a downregulation of endogenous Notch signalling because of the removal of Notch from the cell surface and therefore away from ligand access (Wilkin et al., 2008). In contrast disruption of the HOPS complex has no effect on Notch signalling when induced by ligand expression (Wilkin et al., 2008). Thus the requirement for HOPS components is diagnostic for the lysosomal Notch activation mechanism. Expressing Dx in a *car, ZO-1* double mutant background blocked ectopic Notch signalling and produced wing notches (Fig. 3c,i). Thus wild type ZO-1 normally suppresses the lysosome-dependent Notch activation mechanism that is induced by Dx.

ZO-1 complexes with Dx and downregulates Notch endocytic trafficking

To investigate whether ZO-1 directly affects Dx-induced Notch signalling we used a luciferase reporter based cell culture assay for Notch signalling. ZO-1 is an alternatively spliced multidomain protein comprising of three PDZ domains, a guanylate kinase domain, and a C-terminal proline-rich domain (Fanning and Anderson, 2009) (Fig. 3j). The proline-rich domain regulates the stability and cell localisation of ZO-1 (Djiane et al., 2011) and together with an alternatively spliced exon-6 (Ex6) is necessary for ZO-1 to upregulate Notch signalling in peripheral nervous system (PNS) development (Djiane et al., 2011). However, in the cell culture signalling assay, full length ZO-1 and constructs lacking either the proline-rich region (ZO-1ΔPro) or Ex6 (ZO-1ΔEx6) (Fig. 3j) were able to downregulate Dx-induced Notch activity (Fig. 3k**)**.

Dx promotes Notch signalling by first inducing Notch endocytosis and secondly causing Notch to be retained on the outer limiting membrane of the late endosome/lysosome, where it can be activated by proteolytic cleavages ultimately leading to Presenilin-dependent release of the Notch intracellular domain (Wilkin et al., 2008). Thus the Dx-induced signal can potentially be blocked at many different steps in the Notch trafficking pathway. To explore further the relationship between Dx and ZO-1, we examined their interaction in S2 cells. When expressed in S2 cells Dx localises in cytoplasmic vesicular structures and is not found at the plasma membrane (Fig. 4a). When coexpressed with ZO-1 some Dx puncta colocalised at the cell surface with full-length ZO-1 and ZO-1ΔEx6 but not ZO-1ΔPro (Fig. 4b-d). As previously observed (Djiane et al., 2011) the ZO-1ΔPro construct was more localised to the cytoplasm (Fig. 4d). To determine whether ZO-1 and Dx exist in a complex we used a coimmunoprecipitation assay and found that ZO-1 coprecipitates with Dx (Fig. 4e). This physical interaction required the Pro-rich C-terminal of ZO-1 but did not require

Ex6, consistent with the cellular colocalisation data (Fig. 4b,d). To investigate the mechanism by which ZO-1 blocks Dx-induced Notch signalling we performed a Notch endocytosis assay using antibodies to the Notch extracellular domain in a pulse-chase labelling assay on live cells. We found that Notch, ZO-1 and Dx colocalize at the cell membrane and ZO-1 expression blocks Notch endocytosis (Fig. 4f,l). This suppression of Notch trafficking did not require Ex6 (Fig. 4i,l). In contrast ZO-1ΔPro failed to block Dx-induced endocytosis (Fig. 4j,l). The latter was surprising given that the ZO-1ΔPro construct efficiently downregulated Dx-induced Notch signalling (Fig. 3k). Instead we found ZO-1ΔPro itself induced Notch endocytosis (Fig. 4l) and prevented Dx from causing the retention of Notch on the outer limiting membrane of the Rab7 positive late endosome (Fig. 4m-o). This ZO-1ΔPro effectively sequestered Notch from cytoplasmic access. Thus different constructs of ZO-1 are able to block Dx-induced signalling at various points in the endocytic pathway. We expressed Dx in wing imaginal discs of *ZO-1* null larvae and confirmed that Notch was efficiently endocytosed in Dx-expressing cells (Fig. 4p,q).

Dx-induced Notch signalling can be regulated through cell-cell contacts

Mammalian ZO-1 has previously been shown to mediate cell-density dependent regulation of transcription factor localisation to the nucleus (Balda and Matter, 2000; Kausalya et al., 2004; Siliciano and Goodenough, 1988). We wondered whether Dx-induced Notch signalling would be similarly modulated. ZO-1 was only strongly localised to the cell membrane at high cell densities (Fig. 5a,b). At low cell density we found full-length ZO-1 expression was less effective at reducing Dx-induced Notch endocytosis and did not significantly suppress Dx-induced Notch signalling (Fig. 5a-d). In contrast ZO-1ΔPro blocked Notch signalling in both low and high cell density

cultures, consistent with its different mechanism of action (Fig. 5d). The results suggested that cell junction assemblies may act as upstream regulators of Dx-induced signalling. We were unable to disrupt cell-cell contacts using RNAi in cell culture and therefore we investigated *in vivo* whether other apical junction complex components affected Dx-activation of Notch, using the wing overexpression assay (Fig. S1). Canoe is the *Drosophila* homologue of mammalian AF6 which binds ZO-1 and in *Drosophila* functionally interacts with both ZO-1 and Notch (Carmena et al., 2006; Choi et al., 2011). Par-6 has been found in mammalian breast cancer cells to antagonise ZO-1 function (Viloria-Petit et al., 2009). Crumbs is a transmembrane protein located in the apical membrane domains of epithelial cells and with associated proteins, including Par-6, regulates cell polarity (Fletcher et al., 2012; Tepass, 2012). Crumbs together with Fat also regulates Hippo signalling (Cho et al., 2006; Robinson et al., 2010), the latter itself linked to Notch regulation (Yu et al., 2008), and Crumbs has recently been characterised as a binding partner for ZO-1 (Ivarsson et al., 2011). RNAi targeting these junctional components in wing discs showed a variety of interactions with overexpressed Dx, reflecting increased and decreased Notch signalling (Fig. S1). We next tested for genetic interactions of corresponding mutations with the dominant expanded niche phenotype of the ZO-1 null mutant, pyd^{147} (Fig. 5e). There was no genetic interaction with *shotgun*, however mutant alleles of *fat, par6, canoe* and *crumbs* all reduced the *pyd* mutant phenotype. Furthermore, the viable fat^{1} and $canoe^{c156}$ alleles themselves displayed a reduced niche size compared to wild type flies. Interestingly RNAi against *fat* in S2 cell culture assay also resulted in a small but significant reduction of Dx-induced Notch signalling (Fig. S1). Together the results indicate that various components of cell-cell junctions can have differential consequences on ligand-independent Notch signalling.

ZO-1 facilitates Notch-ligand coclustering and promotes ligand-dependent Notch activation

Although our results strongly point to a regulatory role of ZO-1 on ligand-independent Dx-driven Notch signalling it was important to rule out an explanation that the effects of ZO-1 might be due to a downregulation of ligand-dependent signalling. We therefore investigated the consequence of ZO-1 expression on ligand-induced signalling in S2 cells. In contrast to its effect on Dx-induced Notch activation we found that full-length ZO-1 increased ligand-induced signalling (Fig. 6a). This activity required both Ex6 and the Pro-rich domain. This contrasts with the effect on Dx-induced signalling where Ex6 was not required, suggesting that a separate mechanism is involved. We demonstrated that ZO-1 could form a complex with Notch in the absence of Dx. Coimmunoprecipitation depended on the presence of the Ex6 and Pro-rich domains but did not require any Notch sequence C-terminal to the ankyrin-like repeats (Fig. 6b,c). Therefore we investigated whether ZO-1 acted at cell junctions to stabilise ligand-receptor contacts. When Notch- and ligand- expressing cells are cocultured the cells aggregate together through junctions comprised of coclustered Notch-ligand complexes (Fig. 7a-e). We found that coexpression of ZO-1 with Notch increased the junction length of both Notch-Serrate and Notch-Delta contacts (Fig. 7a,b,e and Fig. S2). This function of ZO-1 was also dependent on the presence of the Ex6 and Pro-rich domains (Fig. 7c-e), consistent with coimmunoprecipitation data (Fig 6B,C). This positive role on Notch signalling may therefore explain the function of ZO-1 to suppress ectopic sensory bristle formation, which also requires Ex6 (Djiane et al., 2011). Thus ZO-1 has distinct and opposing effects on Notch signalling initiated by the ligand-dependent and independent mechanisms. Taken together our results show that increased Notch signalling observed in ZO-1 mutant ovary niches is best explained by the ability of ZO-1 to act as the first identified upstream regulator of the ligand-independent, lysosomal Notch activation mechanism. ZO-1 is therefore so far unique as a Notch

regulator that not only controls Notch signalling levels but also selects between alternate means of its activation (summarised schematically in Fig. 7f).

Discussion

The niche plays a critical role in providing anchorage and signalling necessary for the precise control of stem cell populations, allowing long-term maintenance and repair of adult tissues. The size of the niches that provide support and signalling functions for stem cells is a key factor in determining stem cell numbers (Calvi et al., 2003; Djiane et al., 2011; Zhang et al., 2003). Little is known regarding the factors that determine niche size however. The *Drosophila* ovary GSC niche has been a key model system in establishing the niche concept. Development of the niche requires Notch signalling for recruitment and differentiation of cap cells that provide anchorage for the GSCs. Here we highlight the importance of Dx as a protein required for full niche assembly. Dx mediates a distinct, ligand-independent source of Notch activation, which unlike the ligand-stimulated form requires Notch trafficking to the late endosome/lysosome (Hori et al., 2011; Wilkin et al., 2008). We propose that this alternate Dx-dependent Notch activation mechanism enables sufficient cells to commit to the cap cell fate when cell junctions and ligand-receptor contacts are not well established. In turn we found that Dx signalling is itself kept in check by the junctional adaptor protein ZO-1, preventing inappropriate over-expansion of the niche. ZO-1 is a conserved scaffold adaptor protein that associates with adherens, tight and septate junctions (Fanning and Anderson, 2009) and is highly enriched around the surface of cap cells (Djiane et al., 2011). We found that mutations in several genes linked to apical junction regulation, cell polarity and growth affected both *ZO-1* mutant phenotype and Dx-induced signalling. Tissue assembly and junctional organisation can therefore be coupled to Notch signal regulation to modulate niche size. Indeed the ability of ZO-1 to

block Dx-induced Notch endocytosis and signalling in S2 cells depended on cell density. Thus cells can sense their local environment through ZO-1 to regulate Notch independently of its ligands. A number of downstream regulators of Dx-induced Notch signalling have been identified previously that control the trafficking destination of Notch subsequent to its Dx-induced endocytosis (Hori et al., 2011; Mukherjee et al., 2005; Wilkin et al., 2008). However, it has not hitherto been clear whether the Dx-regulated signal is also sensitive to external cues. To our knowledge ZO-1 is the first identified upstream regulator of the late endosomal-dependent Notch activation mechanism that is associated with Dx activity.

This work therefore provides a new view of how Notch, acting as a sensor to both patterning information and cellular architecture, can integrate ligand-induced signalling with other types of input during tissue morphogenesis. Stem cell niches are often comprised of small populations of specialised cells, which occupy defined locations in tissues. While there has been much work published which demonstrates the importance of junctions between the niche and the stem cell in the regulation of the latter's maintenance and fate (Marthiens et al., 2010), there has been little research addressing the role of contacts between niche cells and morphology of the niche. Our work suggests more attention should be paid to understanding how cell-cell contacts are affecting niche function and capacity. Indeed the specialised spindle-shaped subset of osteoblast cells, which provide the niche for haematopoietic stem cells, are an example of unexplored junctional diversity as a defining characteristic of the niche (Calvi et al., 2003; Zhang et al., 2003).

We identified two additional means by which ZO-1 can regulate cell signalling in an isoform-specific manner. ZO-1 which lacked the C-terminal Pro rich-domain was still able to block Dx-induced signalling without preventing Notch endocytosis. This was achieved by promoting the internalisation of Notch from the

limiting membrane of the late endosome into the multivesicular body. We also found a separate action of ZO-1 to promote ligand-induced Notch activation, which was associated with an ability to form a complex with Notch and promote its coclustering with ligands at cell junctions. This distinct activity thus explains why *ZO-1* mutants cause decreased Notch signalling in the peripheral nervous system (Chen et al., 1996; Djiane et al., 2011) instead of increased signalling as observed in the cap cell niche. The positive role of ZO-1 on ligand-induced signalling required the alternately spliced Ex6 domain that was dispensable for ZO-1 mediated downregulation of Dx-induced activity. This isoform specificity means that that the promotion of Notch-ligand coclustering cannot just be a consequence of the suppression of Dx activity. The Ex6 positive ZO-1 isoform may instead regulate the lateral movement of Notch into or out of these coclusters. Interestingly in mammalian cells, a similar role for ZO-1 has been proposed in gap junction assembly to regulate lateral hemi-channel movement between adjacent membrane domains (Rhett et al., 2011).

The new ways by which ZO-1 can regulate Notch, identified in this study, have a wider impact for understanding the mechanisms by which ZO-1 can interact with other signalling pathways affecting cell fate, proliferation and tumour formation. For example, ZO-1 promotes embryonic stem cell differentiation through regulation of STAT, ERK and BMP signalling (Xu et al., 2012), all of which can be regulated by endosomal trafficking (Pálfy et al., 2012). Interestingly ZO-1 acts on BMP signalling, as it does with Notch, by suppressing non-canonical and promoting canonical BMP signalling mechanisms. Thus ZO-1 may have a more general role in switching between alternate developmental signals during tissue assembly. A well-characterised example of a ZO-1-regulated signal is that of ZONAB, a transcription factor that controls cell proliferation (Balda and Matter, 2000; Balda et al., 2003; Georgiadis et al., 2010). As we have shown with Notch signalling, ZO-1 downregulates ZONAB in a cell density-

dependent manner by localising it to the cell surface. This activity may be linked to a tumour suppressor function for ZO-1, which is also associated with epithelial-mesenchymal transition, a key feature of cancer progression (Georgiadis et al., 2010; Hoover et al., 1998; Martin et al., 2004; Polette et al., 2007; Reichert et al., 2000; Viloria-Petit et al., 2009). The finding that Notch, an important oncogene, can be regulated similarly is thus of considerable interest, suggesting that ZO-1 loss in tumours may simultaneously compromise cell contact-dependent control of proliferation and cell fate.

Methods

Drosophila Stocks

All experiments were performed at 25°C on standard *Drosophila* culture medium apart from RNAi expression in the ovary which was performed at 29°C. Transgenic overexpression and RNAi knockdown were performed using the dpp-Gal4 (Staehling-Hampton et al., 1994), or bab1-Gal4 (gift from A. González-Reyes). Other fly stocks were UAS-Dx (Wilkin et al., 2008) (Gift from K. Matsuno), $dx^{JF02367}$, dx^{V21042}, cno^{C156}, $cno^{MI00782}$, car^{1}, $crb^{KG05098}$, $crbj^{1B5}$, fat^{1}, N^{55E11}, shg^{k03401} (Bloomington Stock Center), Dx^{GR} TX05B (Busseau et al., 1994), dx^{152} (Fuwa et al., 2006), *E(spl)m7-lacZ* (Djiane et al., 2011), $par\text{-}6^{29VV}$ (Jones and Metzstein, 2011), pyd^{147}, pyd^{180} (Djiane et al., 2011), pyd^{tamou} (gift from R. Ueda); *yw* was used as wild type.

S2 cell culture

S2 cells were grown at 25°C in Schneider's medium (Invitrogen), with 10% FBS (Hyclone) and antibiotics. S2 cells were transfected using Effectene (Qiagen).

Constructs: pUAST-ZO-1-GFP (full length, ΔEx6, and ΔPro) as described previously (Djiane et al., 2011). ZO-1 constructs were also subcloned into pMT (Invitrogen), removing GFP and inserting a C-terminal V5-tag. pMT-Notch-YFP, pMT-Notch, pMT-NotchΔIntra-YFP, pMT-Notch-Ank7-YFP derived from pUASTNotch-YFP (gift of K. Matsuno). pMT-NotchΔIntra-YFP and pMT-Notch-Ank7-YFP were generated by removing the intracellular domain, or the region after Ankyrin repeat 7 respectively. pMT-Venus-Deltex, and pMT-Dx-V5 were derived from pUAST-Venus-Deltex (gift from K. Matsuno). Other vectors were pUAST-EYFP-Rab7, (gift from M.P. Scott); pUAST-Ser (Cordle et al., 2008) and pMT-GAL4 (DGRC).

Immunocytochemistry and histology

In situ hybridization, X-gal staining and immunolocalisation in 3rd instar imaginal discs were described previously (Djiane et al., 2011; Wilkin et al., 2004). Ovarioles were immunostained as previously described (Song et al., 2007), except primary antibody incubation was at room temperature. S2 cells were grown on poly-lysine, (Sigma) coated cover slips. Immunostaining was performed at room temperature. Cells were fixed in 4% formaldehyde (Polysciences) for 30 mins, rinsed in PBS, permeabilised in 0.2% Triton X-100 /PBS, and blocked 1 hr in 3% skimmed milk/PBS, then incubated with primary antibody in blocking solution for 2 hrs, washed in PBS before 1hr incubation with secondary antibody. Tissue and cell preps were washed in PBS and mounted in Vectashield with DAPI (Vector labs). Primary antibodies: rat anti-Dx (14A, 1:200, ref. 13), mouse anti-Notch extracellular domain (C458.2H, 1:200, DSHB); mouse anti-Notch intracellular domain (C17.9C6, 1:1000, DSHB); rabbit anti-V5 (1:1000, Bethyl laboratories); goat anti-Serrate (1:1000, Santa Cruz); guinea pig anti-Coracle (D4.1.3, 1:5000, R. Fehon); mouse anti-Lamin C (LC28.26, 1:20, DHSB) and mouse anti-αSpectrin (3A9, 1:10, DSHB). For Notch

uptake assay, S2 cells grown on coated cover slips were incubated with C458.2H for 15 mins on ice, washed with ice cold S2 medium and chased 60 mins at 25°C. Cells were fixed, permeabilised, and stained as above. Aggregation assay with Notch- and ligand- expressing cells was as described (Cordle et al., 2008). Images captured using Volocity (Perkin Elmer) with an Orca-ER digital camera (Hamamatsu) mounted on an M2 fluorescent microscope (Zeiss). Deconvolution of 0.5μm optical sections was performed with 3 nearest neighbours using Openlab (Improvision), or by iterative deconvolution (Volocity) and processed in Photoshop (Adobe). Distance measurements were performed with ImageJ.

Immunoprecipitation and Western blotting

For coimmunoprecipitation experiments, S2 cells were grown in 6-well dishes and transfected with pMT plasmids. $CuSO_4$ was added after 24 hr to induce expression and after a further 24 hrs cells were homogenised in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1mM $CaCl_2$ and protease inhibitor cocktail (EDTA-free Complete; Roche), and cleared by centrifugation. The lysate was incubated with 10µl GFP-TRAP agarose (Chromotek) for 1 hr at 4ºC, and washed 4 times in lysis buffer. Bound proteins were eluted with sample buffer, run on 3-8% Nupage Gels (Invitrogen) and Western blotted with rabbit anti-ZO-1 (Djiane et al., 2011) (1:10000), mouse anti-V5 (1:5000; Invitrogen) or rabbit anti-GFP (1:20000; ImmunoKontact).

Notch Luciferase Reporter Assay

S2 cells in 12-well dishes were transfected with pMT plasmids, NRE:Firefly (gift from S. Bray) and Actin:Renilla (gift from G.Merdes). After 24 hrs cells were re-suspended and seeded into white 96-well plates (Nunc #136101) and $CuSO_4$ was added

after a further 24 hrs. For cell density and ligand experiments S2 cells were transfected in 12-well dishes, $CuSO_4$ added after 2 days and cells re-seeded into white 96-well plates at the indicated density, or on top of fixed Delta-expressing S2 cells (S2-Mt-Dl; DGRC). 24hrs after induction, luciferase activity was assayed with Dual-Glo Luciferase (Promega), quantified by luminometer (Berthold) and Firefly/Renilla ratio calculated for triplicate samples. Experiments repeated a minimum of three times.

Statistical Methods

Quantified data expressed as mean +/- s.e.m. For luciferase assays, signalling was normalised to a control as indicated in figures. Statistical significance was determined by two-tailed Mann Whitney U test, utilising SPSS software (SPSS Inc.)

References

Balda, M.S., and Matter, K. (2000). The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. The EMBO Journal *19*, 2024–2033.

Balda, M.S., Garrett, M.D., and Matter, K. (2003). The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. The Journal of Cell Biology *160*, 423–432.

Baron, M. (2012). Endocytic routes to Notch activation. Seminars in Cell & Developmental Biology 6–11.

Bolívar, J., Pearson, J., López-Onieva, L., and González-Reyes, A. (2006). Genetic dissection of a stem cell niche: the case of the Drosophila ovary. Developmental Dynamics : an Official Publication of the American Association of Anatomists *235*, 2969–2979.

Busseau, I., Diederich, R.J., Xu, T., and Artavanis-Tsakonas, S. (1994). A member of the Notch group of interacting loci, deltex encodes a cytoplasmic basic protein. Genetics *136*, 585–596.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841–846.

Carmena, A., Speicher, S., and Baylies, M. (2006). The PDZ protein Canoe/AF-6 links Ras-MAPK, Notch and Wingless/Wnt signaling pathways by directly interacting with Ras, Notch and Dishevelled. PloS One *1*, e66.

Chen, C.M., Freedman, J. a, Bettler, D.R., Manning, S.D., Giep, S.N., Steiner, J., and Ellis, H.M. (1996). Polychaetoid is required to restrict segregation of sensory organ precursors from proneural clusters in Drosophila. Mechanisms of Development *57*, 215–227.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R., and Irvine, K.D. (2006). Delineation of a Fat tumor suppressor pathway. Nature Genetics *38*, 1142–1150.

Choi, W., Jung, K., Nelson, K.S., Bhat, M.A., Beitel, G.J., Peifer, M., and Fanning, A.S. (2011). The single Drosophila ZO-1 protein Polychaetoid regulates embryonic morphogenesis in coordination with Canoe/afadin and Enabled. Molecular Biology of the Cell *22*, 2010–2030.

Cordle, J., Johnson, S., Tay, J.Z.Y., Roversi, P., Wilkin, M.B., de Madrid, B.H., Shimizu, H., Jensen, S., Whiteman, P., Jin, B., et al. (2008). A conserved face of the Jagged/Serrate DSL domain is involved in Notch trans-activation and cis-inhibition. Nature Structural & Molecular Biology *15*, 849–857.

Diederich, R.J., Matsuno, K., Hing, H., and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development (Cambridge, England) *120*, 473–481.

Djiane, A., Shimizu, H., Wilkin, M.B., Mazleyrat, S., Jennings, M.D., Avis, J., Bray, S.J., and Baron, M. (2011). Su(dx) E3 ubiquitin ligase-dependent and -independent functions of polychaetoid, the Drosophila ZO-1 homologue. The Journal of Cell Biology *192*, 189–200.

Fanning, A.S., and Anderson, J.M. (2009). Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. Annals of the New York Academy of Sciences *1165*, 113–120.

Fletcher, G.C., Lucas, E.P., Brain, R., Tournier, A., and Thompson, B.J. (2012). Positive feedback and mutual antagonism combine to polarize Crumbs in the Drosophila follicle cell epithelium. Current Biology : CB *22*, 1116–1122.

Fuwa, T.J., Hori, K., Sasamura, T., Higgs, J., Baron, M., and Matsuno, K. (2006). The first deltex null mutant indicates tissue-specific deltex-dependent Notch signaling in Drosophila. Molecular Genetics and Genomics : MGG *275*, 251–263.

Georgiadis, A., Tschernutter, M., Bainbridge, J.W.B., Balaggan, K.S., Mowat, F., West, E.L., Munro, P.M.G., Thrasher, A.J., Matter, K., Balda, M.S., et al. (2010). The tight junction associated signalling proteins ZO-1 and ZONAB regulate retinal pigment epithelium homeostasis in mice. PloS One *5*, e15730.

Hoover, K.B., Liao, S.Y., and Bryant, P.J. (1998). Loss of the tight junction MAGUK ZO-1 in breast cancer: relationship to glandular differentiation and loss of heterozygosity. The American Journal of Pathology *153*, 1767–1773.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., and Matsuno, K. (2004). Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development (Cambridge, England) *131*, 5527–5537.

Hori, K., Sen, A., Kirchhausen, T., and Artavanis-Tsakonas, S. (2011). Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. The Journal of Cell Biology *195*, 1005–1015.

Hsu, H.-J., and Drummond-Barbosa, D. (2011). Insulin signals control the competence of the Drosophila female germline stem cell niche to respond to Notch ligands. Developmental Biology *350*, 290–300.

Ivarsson, Y., Wawrzyniak, A.M., Wuytens, G., Kosloff, M., Vermeiren, E., Raport, M., and Zimmermann, P. (2011). Cooperative phosphoinositide and peptide binding by PSD-95/discs large/ZO-1 (PDZ) domain of polychaetoid, Drosophila zonulin. The Journal of Biological Chemistry *286*, 44669–44678.

Jones, T.A., and Metzstein, M.M. (2011). A novel function for the PAR complex in subcellular morphogenesis of tracheal terminal cells in Drosophila melanogaster. Genetics *189*, 153–164.

Kausalya, P.J., Phua, D.C.Y., and Hunziker, W. (2004). Association of ARVCF with zonula occludens (ZO)-1 and ZO-2: binding to PDZ-domain proteins and cell-cell adhesion regulate plasma membrane and nuclear localization of ARVCF. Molecular Biology of the Cell *15*, 5503–5515.

Kopan, R., and Ilagan, M.X.G. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell *137*, 216–233.

Li, L., and Neaves, W.B. (2006). Normal stem cells and cancer stem cells: the niche matters. Cancer Research *66*, 4553–4557.

Marthiens, V., Kazanis, I., Moss, L., Long, K., and Ffrench-Constant, C. (2010). Adhesion molecules in the stem cell niche--more than just staying in shape? Journal of Cell Science *123*, 1613–1622.

Martin, T. a, Watkins, G., Mansel, R.E., and Jiang, W.G. (2004). Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer. European Journal of Cancer (Oxford, England : 1990) *40*, 2717–2725.

Mukherjee, A., Veraksa, A., Bauer, A., Rosse, C., Camonis, J., and Artavanis-Tsakonas, S. (2005). Regulation of Notch signalling by non-visual beta-arrestin. Nature Cell Biology *7*, 1191–1201.

Oakley, E.J., and Van Zant, G. (2010). Age-related changes in niche cells influence hematopoietic stem cell function. Cell Stem Cell *6*, 93–94.

Pálfy, M., Reményi, A., and Korcsmáros, T. (2012). Endosomal crosstalk: meeting points for signaling pathways. Trends in Cell Biology *22*, 447–456.

Polette, M., Mestdagt, M., Bindels, S., Nawrocki-Raby, B., Hunziker, W., Foidart, J.-M., Birembaut, P., and Gilles, C. (2007). Beta-catenin and ZO-1: shuttle molecules involved in tumor invasion-associated epithelial-mesenchymal transition processes. Cells, Tissues, Organs *185*, 61–65.

Reichert, M., Müller, T., and Hunziker, W. (2000). The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of beta-catenin/Tcf/Lef signaling. The Journal of Biological Chemistry *275*, 9492–9500.

Rhett, J.M., Jourdan, J., and Gourdie, R.G. (2011). Connexin 43 connexon to gap junction transition is regulated by zonula occludens-1. Molecular Biology of the Cell *22*, 1516–1528.

Robinson, B.S., Huang, J., Hong, Y., and Moberg, K.H. (2010). Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein Expanded. Current Biology : CB *20*, 582–590.

Siliciano, J.D., and Goodenough, D. a (1988). Localization of the tight junction protein, ZO-1, is modulated by extracellular calcium and cell-cell contact in Madin-Darby canine kidney epithelial cells. The Journal of Cell Biology *107*, 2389–2399.

Song, X., Call, G.B., Kirilly, D., and Xie, T. (2007). Notch signaling controls germline stem cell niche formation in the Drosophila ovary. Development (Cambridge, England) *134*, 1071–1080.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, a H., and Hoffmann, F.M. (1994). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A. Cell Growth & Differentiation : the Molecular Biology Journal of the American Association for Cancer Research *5*, 585–593.

Tepass, U. (2012). The apical polarity protein network in Drosophila epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. Annual Review of Cell and Developmental Biology *28*, 655–685.

Viloria-Petit, A.M., David, L., Jia, J.Y., Erdemir, T., Bane, A.L., Pinnaduwage, D., Roncari, L., Narimatsu, M., Bose, R., Moffat, J., et al. (2009). A role for the TGFbeta-Par6 polarity pathway in breast cancer progression. Proceedings of the National Academy of Sciences of the United States of America *106*, 14028–14033.

Voog, J., and Jones, D.L. (2010). Stem cells and the niche: a dynamic duo. Cell Stem Cell *6*, 103–115.

Wilkin, M.B., Carbery, A., Fostier, M., Aslam, H., Mazaleyrat, S.L., Higgs, J., Myat, A., Evans, D.A.P., Cornell, M., and Baron, M. (2004). Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. Current Biology : CB *14*, 2237–2244.

Wilkin, M.B., Tongngok, P., Gensch, N., Clemence, S., Motoki, M., Yamada, K., Hori, K., Taniguchi-kanai, M., Franklin, E., Matsuno, K., et al. (2008). Drosophila HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway. Developmental Cell *15*, 762–772.

Xu, J., Lim, S.B.H., Ng, M.Y., Ali, S.M., Kausalya, J.P., Limviphuvadh, V., Maurer-Stroh, S., and Hunziker, W. (2012). ZO-1 regulates Erk, Smad1/5/8, Smad2, and RhoA activities to modulate self-renewal and differentiation of mouse embryonic stem cells. Stem Cells (Dayton, Ohio) *30*, 1885–1900.

Yu, J., Poulton, J., Huang, Y., and Deng, W. (2008). The hippo pathway promotes Notch signaling in regulation of cell differentiation, proliferation, and oocyte polarity. PloS One *3*, e1761.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.-G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836–841.

Zhu, C.-H., and Xie, T. (2003). Clonal expansion of ovarian germline stem cells during niche formation in Drosophila. Development (Cambridge, England) *130*, 2579–2588.

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Author contributions

MB conceived the project; HS, SW, MBW, AB, YH, DA, MB performed the experiments., HS, SW, MBW, AB, MB constructed the figures and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests

Figures

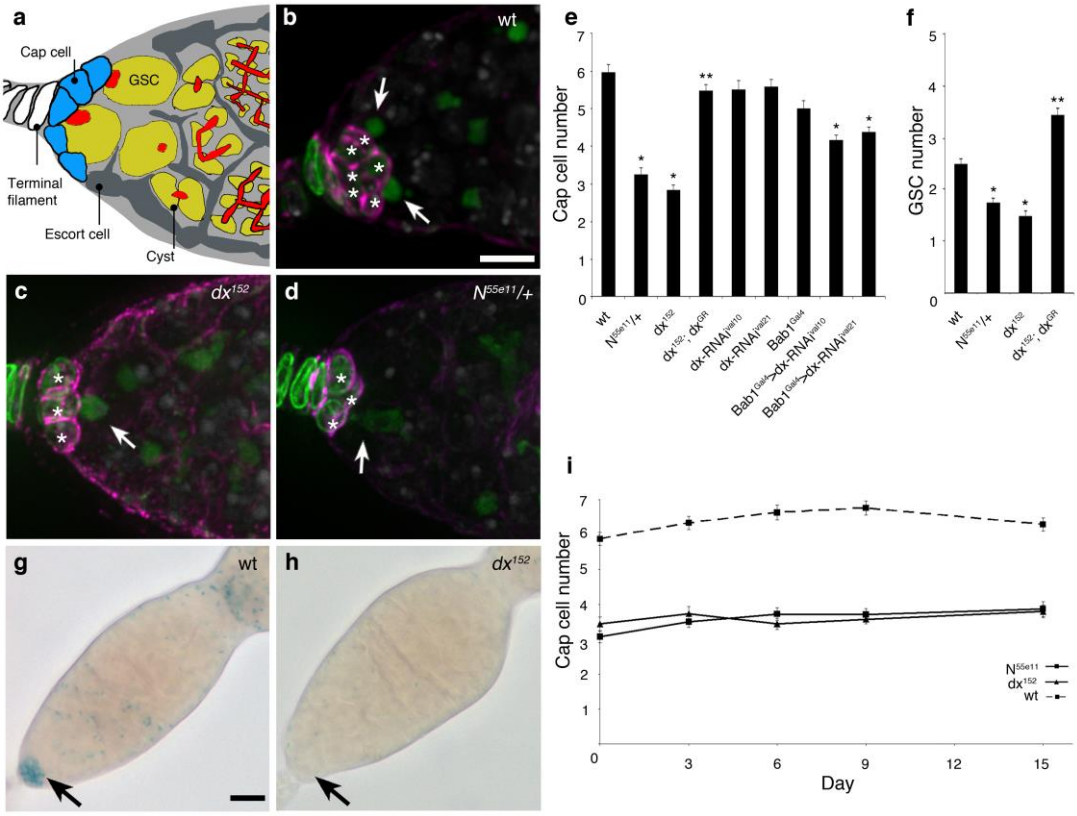

Figure 1. Dx regulates size of the GSC niche
(**a**) Diagram of the *Drosophila* GSC niche showing terminal filament (white), cap cells (blue), and escort cells (dark grey). GSCs (yellow) are attached to cap cells and contain the spectrosome at their anterior membrane (red). Dividing cysts (yellow) show the branching fusome (red) extending through their cytoplasm. (**b-d**) Germaria labelled for DAPI (grey), anti-coracle (magenta) strongly marking cap cell membrane and weakly terminal filament and escort cells; anti-α–Spectrin marking the GSC spectrosome (arrows, green) and laminC weakly marking cap cells but more strongly the TF (also green). Images (**b-d**) represent a merged stack of deconvolved layers. (**b**) Wt niche. (**c**) dx^{152} and (**d**) N^{55e11}/+ niches showing reduced number of cap cells (asterisks) and GSCs compared to Wt. (**b-d**) Scale bar, 5 μm. (**e**) Mean numbers of cap cells. dx^{152} and N^{55e11}/+ have a reduced number of cap cells compared to Wt. dx^{152} phenotype is rescued by genomic rescue construct. Bab1-Gal4 driven expression of *dx*-RNAi also reduces cap cell number. (**f**) Mean numbers of GSCs. dx^{152} and N^{55e11}/+ have reduced number of GSCs compared to Wt. Phenotype of dx^{152} is rescued by the genomic rescue construct. (**e,f**) *P<0.0001 compared to wild type; **P<0.0001 compared to dx^{152}. Statistical significance determined by two-tailed Mann Whitney U test. (**g,h**) *E(spl)m7*-lacZ expression seen in Wt cap cells (arrow) (**g**), is reduced in dx^{152} (**h**), scale bar, 10μm. (**i**) Mean cap cell number for dx^{152} and N^{55e11}/+ remains stable over time in adult flies, suggesting niche formation during development is more sensitive to reduction in Notch signalling compared to during adult niche maintenance. (**e,f,i**) Data displayed as means of a minimum of 20 ovarioles, error bars are s.e.m.

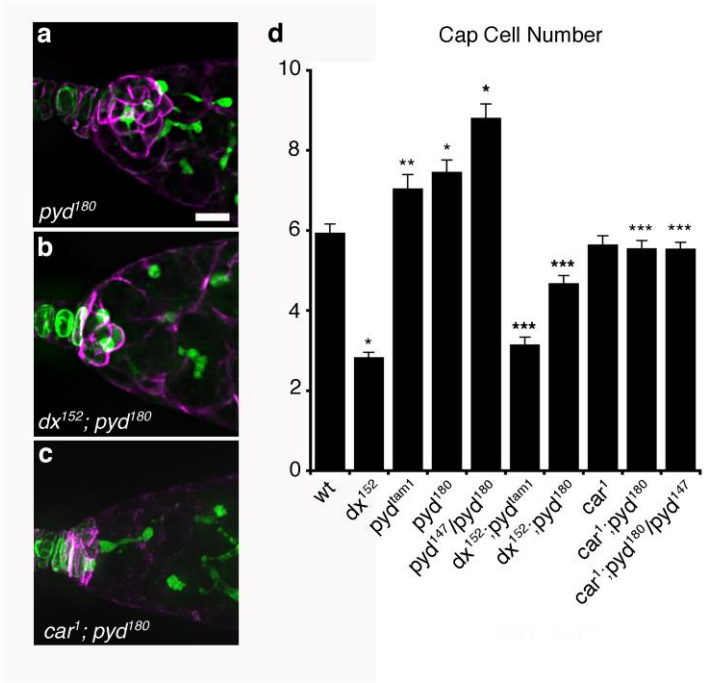

Figure 2. ZO-1 antagonises Dx function

(**a-c)** shows the GSC niche region of ovarioles stained for anti-Coracle (magenta), anti-Lamin C (green), and anti-α-spectrin (also in green, marking the GSC spectrosomes). (**a**) The ZO-1 mutant *pyd*^{180} has an enlarged GSC niche, and an increased number of GSCs. This is reduced in flies additionally mutant for *dx*^{152} (**b**), or the hypomorphic *car*^{1} (**c**). The scale bar in **a** represents 5μm in **a**-**c**. (**d**) Mean cap cell numbers. *dx*^{152} has fewer, and *pyd* mutants have more cap cells than wild-type. *dx*^{152}, *pyd* double mutants have reduced numbers of cap cells compared to *pyd* mutants alone. *car*^{1} also prevents the *pyd* niche expansion phenotype. Data displayed as mean of a minimum of 20 ovarioles per genotype, error bars are s.e.m. Statistical significance determined by two tailed Mann-Whitney U test. *P <0.0001 compared to wild type; **P=0.032 compared to wild type; ***P<0.0001 compared to respective *pyd* mutants alone.

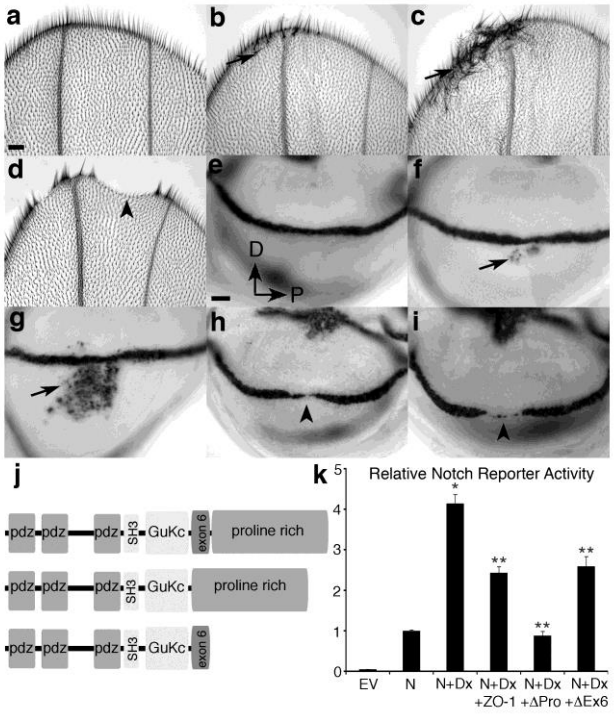

Figure 3. ZO-1 suppresses Notch signalling induced by Dx expression
(**a**) Adult distal wing tip showing a wild-type (Wt) margin. (**b**) Ectopic margin bristles (arrows) induced by overexpression of Dx with dpp-Gal4, in a wild-type background, or (**c**) in a pyd^{147}/pyd^{180} null. (**d**) Notching (arrowhead) associated with overexpression of Dx in a car^{1} mutant. (**e-i**) Late third instar wing imaginal discs stained for *wingless mRNA* expression to report Notch activation. In a Wt disc (**e**) there is a clear line of *wingless* expression demarcating the dorsal/ventral boundary. (**f-i**) Dx overexpressed along the A/P axis using the dpp-Gal4 driver. (**f**) Mild ectopic *wingless* expression (arrow) induced in wild type background. (**g**) Enhanced *wingless* expression in *pyd* null mutant. (**h**) Overexpression of Dx in a car^{1} mutant or (**i**) in a car^{1}, *pyd* double mutant, leads to a loss of *wingless* (arrow heads). Scale bar in **a** represents 40μm in **a-d**. Scale bar in **e** represents 20μm in **e-i**. (**j**) Forms of ZO-1 used. ZO-1 and ZO-1ΔEx6 correspond to variants ZO-1 PB and ZO-1 PC respectively (FlyBase). ZO-1ΔPro is truncated C-terminal to "exon 6". (**k**) S2 cells were transfected with empty vector (EV) or with Notch (N) + Dx and constructs of ZO-1 as indicated. Normalised mean Notch reporter activity is relative to cells expressing N only. ZO-1, ZO-1ΔEx6 and ZO-1ΔPro reduce Dx-induced Notch signalling in cell culture. *P<0.001 compared with N only; **P<0.002 compared to N+Dx. Statistical significance determined by two-tailed Mann Whitney U test. Error bars are s.e.m. Experiments were performed a minimum of three times each with technical triplicates.

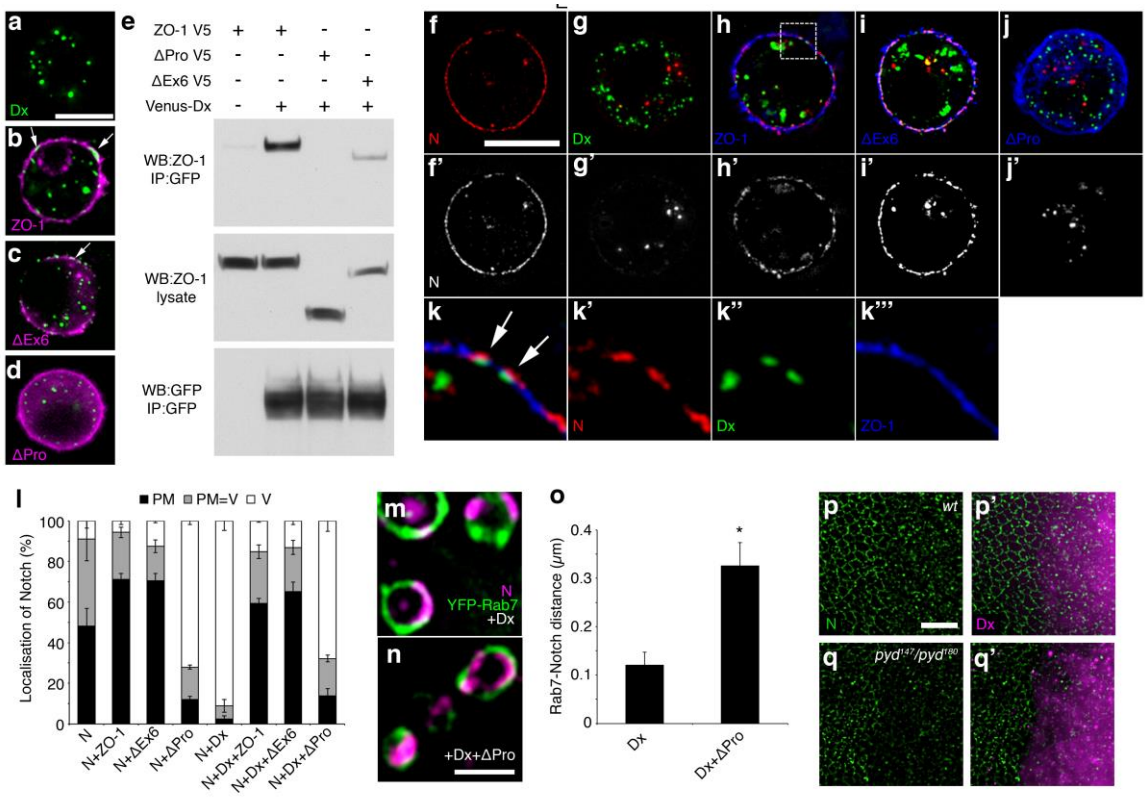

Figure 4. Regulation of Notch endocytosis by ZO-1 interaction with Dx

(**a-d**) Recruitment of Dx to plasma membrane by ZO-1 in S2 cells. S2 cells were transfected with venus-Dx (green) (**a**) or Dx and V5-tagged ZO-1 constructs (magenta): full length ZO-1 (**b**), ΔEx6 (**c**), and ΔPro (**d**). Arrows in **b** and **c** indicate Dx puncta at the plasma membrane. Scale bar, 10μm. (**e**) Coimmunoprecipitation of Dx and ZO-1. S2 cells were transfected as indicated. Venus-tagged Dx was immunoprecipitated using GFP-TRAP beads and detected using anti-GFP. ZO-1 proteins in the lysate, and those that coprecipitated with Venus-Dx, were detected using anti-ZO-1. (**f-k**) Notch uptake assay with ZO-1 variants. (**f-j**) Merged images showing immunostained Notch (red in **f-j**, grey scale in **f**'-**j'**), Dx (green), and ZO-1 (blue). Box in **h** is magnified in (**k-k'''**) to show colocalisation of Notch, Dx and ZO-1 at plasma membrane (arrows in **k**). Scale bar; 10μm. (**l**) Notch ECD antibody localisation after 1 hour chase in each cell was visually scored as plasma membrane (PM), vesicular (V), or intermediate (PM=V). Data shows mean % localisation from three separate experiments, error bars are s.e.m. Each experimental repeat quantified Notch localisation in a minimum of 80 cells. Cells transfected with Notch (N) only or N+ Dx together with different ZO-1 constructs. *P<0.001 for mean % vesicular localisation compared to N only. **P<0.001 for mean % vesicular localisation compared to N +Dx. Statistical significance determined by two tailed Mann Whitney U test. (**m-o**) Notch localisation on S2 cell late endosome labelled by EYFP-Rab7. (**m**) Following Dx expression endocytosed Notch (magenta) is retained at limiting membrane of late endosome (EYFP-Rab7, green). (**n**) ΔPro coexpression with Dx diverts Notch inside the Rab7 endosome. Scale bar, 1μm. **(o)** Quantified internalisation of Notch into late endosomal lumen. Mean distance between Notch localisation and EYFP-Rab7. *P<0.001 for Dx+ΔPro compared to Dx, n>66. Statistical significance determined by two tailed Mann Whitney U test, error bars are s.e.m. (**p,q**) Overexpression of Dx (**p',q'**, magenta) in a wild type (**p**) or pyd^{147}/pyd^{180} background (**q**) caused clearance of Notch (green) from the apical junction region. Scale bar (**p**), 10μm.

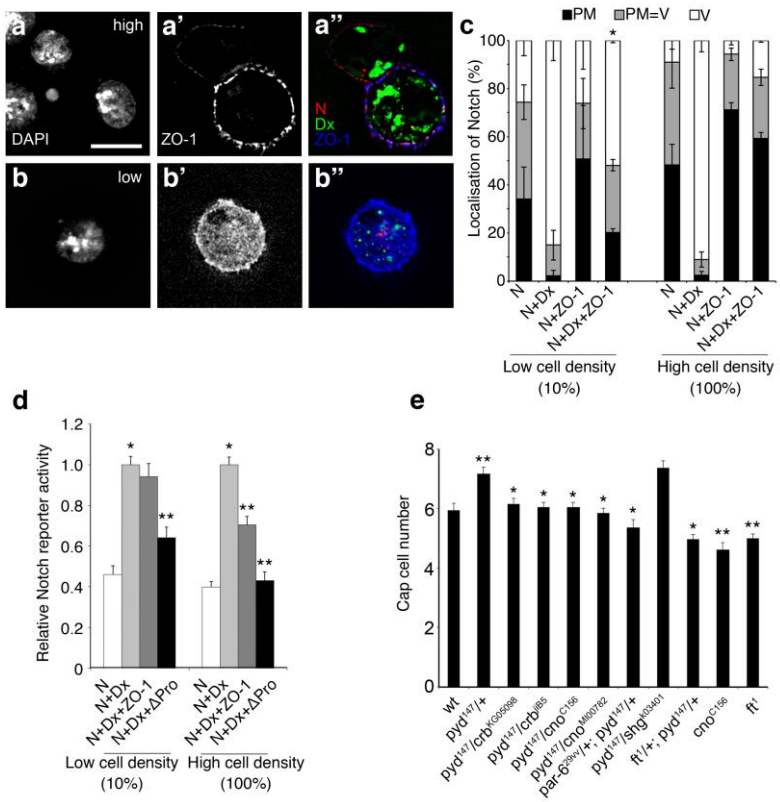

Figure 5. Cell-cell contacts regulate Dx-induced Notch signalling
(**a-c**) Transfected cells were kept confluent or diluted (1:10) for 24 hours before Notch uptake assay at high (**a**) and low (**b**) cell density. DAPI and ZO-1 staining are shown in (**a,b**) and (**a',b'**) respectively. Merged staining of Notch (red), Dx (green), and ZO-1 (blue) are shown in (**a''** and **b''**). Scale bar, 10μm. (**c**) Quantified Notch endocytosis. Notch ECD antibody localisation in each cell was scored as plasma membrane (PM), vesicles (V), or intermediate (PM=V). Data shows mean % localisation from three separate experiments, error bars are s.e.m. For each experimental repeat Notch localisation quantified in a minimum of 80 cells. ZO-1 blocks Dx-induced Notch endocytosis at high cell density but is less effective at low cell density. *P<0.001 for mean % vesicular localisation compared to N+Dx+ZO-1 at high cell density. Statistical significance determined by two tailed Mann Whitney U test. (**d**) ZO-1 regulation of Notch signalling depends on cell density. S2 cells were transfected as indicated and seeded at low or high density. Normalised mean Notch reporter activity is shown relative to cells expressing Notch and Dx. *P<0.001 compared to N only. **P<0.002 compared to N+Dx. Experiments were performed a minimum three times, each with technical triplicates. Error bars are s.e.m. (**e**) Mean cap cell numbers from minimum of 40 ovarioles. *P<0.001 compared to *pyd*^{147}/+; **P<0.002 compared to Wt. Statistical significance determined by two-tailed Mann Whitney U test. Error bars are s.e.m.

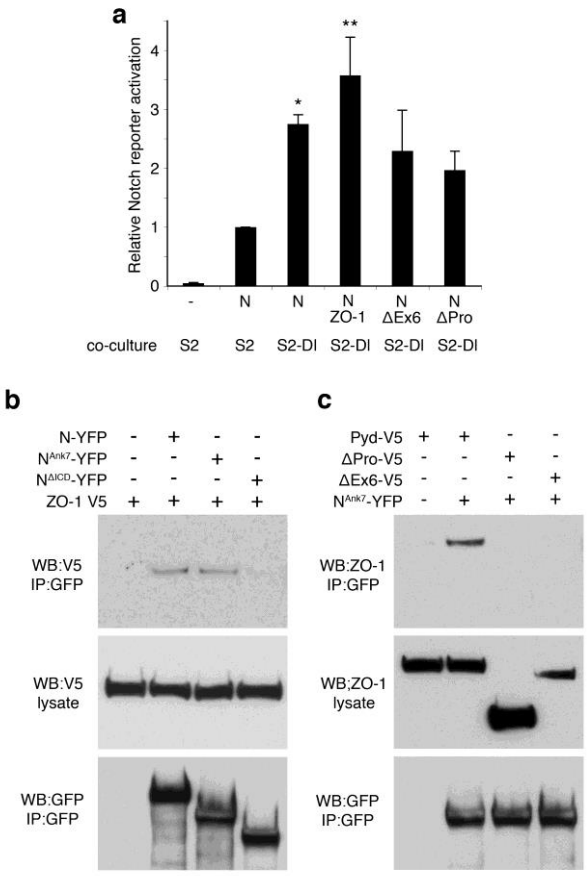

Figure 6. ZO-1 promotes ligand-induced Notch signalling
(a) Luciferase assay in S2 cells showing regulation of ligand-induced Notch activation by ZO-1. Mean normalised Notch reporter activity is shown relative to cells expressing Notch (N) cocultured with non-expressing S2 cells. S2 cells transfected with N and ZO-1 constructs as indicated. Only full-length ZO-1 increases ligand-induced Notch signalling. Experiments were performed three times, each with technical triplicates. Error bars are s.e.m. *P<0.001 compared to N with blank S2 co-culture. **P<0.001 compared to N with Dl-S2 co-culture. Statistical significance determined by two tailed Mann Whitney U test. (**b**,**c**) Notch and ZO-1 coimmunoprecipitate. YFP-tagged Notch proteins were immunoprecipitated using GFP-TRAP beads and detected using anti-GFP. V5-tagged ZO-1 proteins in the lysate, and those that coprecipitated with Notch proteins, were detected using anti-ZO-1 or anti-V5.

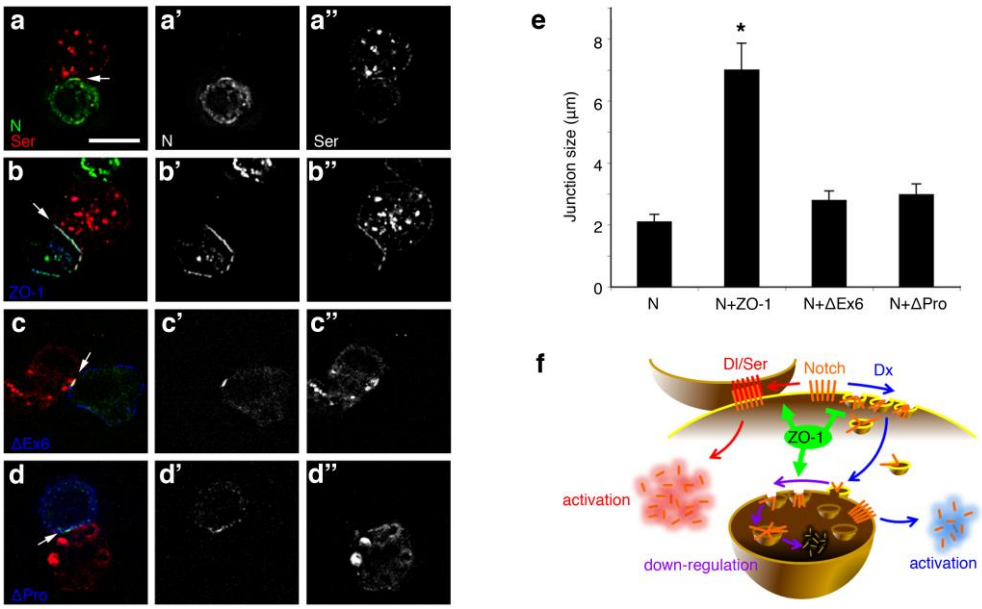

Figure 7. ZO-1 regulates coclustering of Notch and ligands at cell-cell contacts
(**a-e**) Notch-Ser coclustering was observed when Notch-expressing cells and Ser-expressing cells were mixed (arrows). Coclustering was promoted when Notch-cells were expressing GFP-tagged ZO-1, but not ΔEx6 or ΔPro. (**a-d**) merged pictures of Notch (green), Ser (red), and ZO-1 (blue). Notch staining (**a'-d'**) and Ser staining (**a''-d''**) shown separately (grey scale). Scale bar; 10μm. (**e**) Mean junction length of Notch/Ser interface. Cells transfected with Notch (N) and ZO-1 constructs as indicated. Error bars are s.e.m. *P<0.001 compared with N only. A minimum of 27 junctions scored for each combination. Statistical significance determined by two tailed Mann Whitney U test. (**f**) Summary of ZO-1 regulatory interactions with Notch. Notch can be activated by ligands or through Dx-induced endocytosis. Activation by the latter route depends on retention of Notch on the outer surface of the late endosome. ZO-1 promotes Notch-ligand interactions and downregulates Dx-induced Notch endocytosis. A secondary effect of ZO-1 promotes internalisation of Notch from the outer endosomal membrane into the internal compartments, sequestering it from the cytoplasm and preventing its activation.

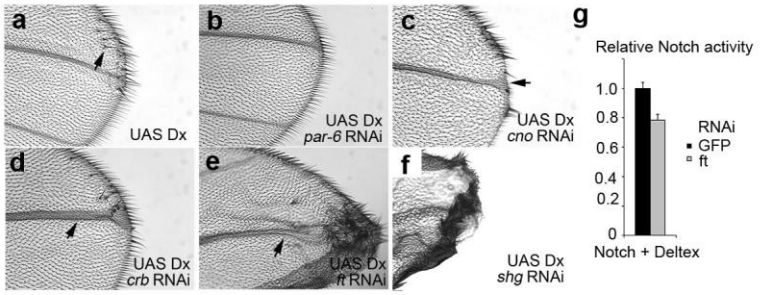

Figure S1. Cell-Cell junction components regulate Dx-induced Notch signalling
(**a**) Dpp-Gal4 driven expression of Dx results in weak ectopic wing margin (arrow), which is suppressed by coexpression with RNAi *par-6* (**b**). Coexpression with RNAi against *cno* (**c**) or *crb* (**d**) additionally produces notches or L3 vein thickening (arrows), characteristic of Notch loss-of-function. (**e**) Coexpression of Dx with *ft* RNAi promotes Notch activity resulting in enhanced ectopic margin, but also results in broadened L3 vein (arrow). (**f**) Coexpression of Dx with *shg* RNAi promotes Notch activity resulting in enhanced ectopic margin and wing outgrowths. *Drosophila* UAS-RNAi lines, obtained from Bloomington stock centre were: *cno*^{HMS00239}*; crb* ^{JF02777}*; ft* ^{JF03245}*; par-6* ^{HMS01410}*; shg* ^{JF02769}. (**g**) Ft regulates Dx-induced Notch signalling in S2 cells. S2 cells were transfected with Notch and Dx together with NRE:FL and Actin:RL. One day later transfected cells were serum-starved for 1hr, then re-suspended and seeded into white 96-well plates together with 1µg dsRNA per well against either GFP (control) or *ft* (BKN21241; Sheffield RNAi Screening Facility). Four hours later, serum was supplemented back to 10%. After 2 days Copper was added to induce pMT expression and 24hrs later the luciferase assay was performed. Normalised mean Notch reporter activity is shown relative to GFP control. Experiment was repeated six times, **P=0.01 two tailed Mann Whitney U-test, error bars are s.e.m.

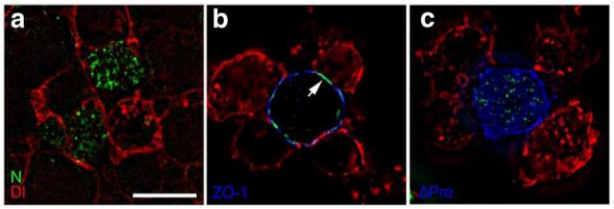

Figure S2. Increased Notch-Delta junction size resulting from ZO-1 coexpression
S2 cells expressing Notch with internally inserted GFP tag (gift of S. Artavanis-Tsakonas). Cells expressing Notch-GFP only (**a**) and Notch-GFP with ZO-1-V5 (**b**) or Notch and ΔPro-V5 (**c**) were mixed with S2-Dl cells, resulting in formation of Notch-Delta junctions. Cells were stained as for Notch/Serrate cell aggregation assay (Fig. 7, main text). Only full-length ZO-1 increases the length of the Notch/Delta contact sites (arrow). Scale bar, 10 μm. Notch staining (GFP autofluorescence, green); Dl staining (red) using mouse anti-Delta extracellular domain, (C594.9B, DSHB) at 1:1000; V5-tagged ZO-1immunostained (blue) with rabbit anti-V5 (1:1000, Bethyl laboratories).

Chapter 4: general discussion

The *Drosophila* ovary offers a good model to study the regulation of stem cell niches, with its well-defined stem cell populations and niches and the availability of genetic approaches to identify stem cell-related phenotypes. In this thesis I focus my attention on the influence of Notch signalling on GSCs and their niche. Notch signalling shows different functions in the regulation of the GSC niche. In the two papers presented in this thesis it is shown that Notch regulates the size of the niche in two ways. During development, it recruits cap cells to determine the size of the niche at eclosion. During adult life, it regulates niche size in response to nutritional cues.

Notch could be seen as a sensor that can integrate multiple inputs from cell architecture and organismal physiology. A common feature of the two papers presented in this thesis is that numerous inputs additional to ligand-stimulated activation can modulate signalling output of the Notch receptor. During development, the cell junctional component ZO-1/Pyd controls Notch endocytosis and signal activation via an endocytic activation pathway initiated by Dx. This determines the size of the developed niche at eclosion. During adult life, Notch signalling is controlled by dietary responses, allowing reversible contraction of the niche. The latter highlights the presence of a mechanism to adjust Notch signalling through systemically derived signals. Such mechanisms are becoming increasingly evident in the control of stem cells and their niches in other organisms including humans and thus our findings may be more widely applicable.

4.1 Links between nutrition sensing and Notch signalling

In the first paper of this thesis it is shown that Notch signalling in the adult niche is dietary dependent. Loss and restoration of Notch signalling correlated with variations in niche size in response to shifting from poor to rich food. To further consolidate the relationship between nutrition and Notch signalling, we could test if

expressing NICD with C587-Gal4; Gal80^{ts} in flies on a glucose-reduced diet would overcome the loss of cap cells due to nutrition restriction.

It was not determined which diet components are involved in this response. Further experiment could help understand if glucose has a specific role in the cap cell decline or if the decrease in total calories is responsible for the cap cell decline. Moreover, which nutritional sensing mechanisms are involved in this response is not clear. Apart from IIS which influences the GSCs and their niche (Hsu and Drummond-Barbosa, 2009), there are a number of possible routes by which nutritional status may regulate Notch, as described in the introduction. All of these nutritional sensing pathways are intricately connected to each other and survey different nutrition components.

4.1.1 Different nutrition components or total caloric count?

Different studies found contrary results on of the impact of dietary restriction on stem cells as illustrated in the introduction for the male *Drosophila* GSCs. Some stem cells seem to be affected positively by diet restriction, while others seem to be affected negatively. The precise dietary conditions seem to be important in determining the observed outcome. Diet restriction lies in a specific food interval. Above this interval it is not possible to see the life expansion, but the fertility is high. Below the interval it is not possible to see the increase in lifespan and the fertility is reduced (Chapman and Partridge, 1996; Chippindale et al., 1993).

In our study we do not yet know if a specific component of the diet and nutrition-related pathway is affecting the niche and GSCs, or if the effects depend on the total amount of calories. In general, studies on lifespan show the importance of the role of specific components rather than the overall caloric contribution (Tatar, 2011). Studies relative to life expansion and fecundity show that methionine can restore full

fecundity to flies on caloric restriction without affecting *Drosophila* lifespan. Other nutrients have no effect and essential amino acids have the same effect as with full-fed flies, raising fecundity but lowering lifespan (Grandison et al., 2009). At least for the regulation of GSCs, proteins seem to have an essential effect.

In our nutrition paper we show dietary effects on both the niche and the GSCs. It is possible that the niche and GSCs have different diet requirements for their regulation. Moreover, even the maintenance function of Notch in cap cells and the recovery function of Notch in the ESCs could be subject to regulation through different nutrient conditions. M7-lacZ, which is expressed in cap cell, does not show a big reduction from the St+Yeast compared to standard food. The reduction in stain intensity mostly occurs when comparing flies on standard food versus glucose-reduced food. In contrast, Mβ-lacZ expression shows a sharp decrease already on standard food compared to St+Yeast, possibly suggesting that its expression could be more sensitive to variations in protein levels. The regulation of the GSCs may be further complicated by the fact that the functioning of Notch in several different somatic cell types in the ovary is shown to non-autonomously affect GSC numbers in the niche.

Additional experiments will be required to uncover the different means by which the niche and GSCs are regulated after both nutrients down and up-shift. A first approach could be to add different nutrients to the water/agar food, for example or glucose or yeast or specific amino acids, and check for changes in niche size, GSC number and Notch reporter lines activity. This experiment permits us to dissect particular nutrient needs in the regulation of cap cell size and the Notch signalling pathway activity. A second approach could be to dilute standard food instead of removing only glucose as done in the nutrition paper. This experiment could tell us if the important nutritional component for the cap cells is glucose or if it is a reduction in total caloric uptake. The diet component specific approach might allow for a more

rigorous study of diet requirements compared to the dilution approach. There are some controversies about the quantification of the energy continent of diet, due to differences between theoretical energy content of a diet and the actual biological content. The theoretical energy content describes how many calories a certain food should contain, derived from bomb calorimetric studies. The actual biological content quantify how much of that food is available to the animal, and this varies with the animal physiology, age and genotype (Piper et al., 2005). Because of this difference, we might see no effect because the flies could eat more to compensate for the more diluted food (Tatar, 2011). As a control for compensatory feeding we could check flies food uptake, which can be estimated in different ways: dye uptake, fecal deposits, proboscis extension behaviour, body weight, radiotracers and calorimetry (Tatar, 2011). Many results show that there can be compensatory feeding, although flies on diluted food still ingest less calories than flies on rich food (Tatar, 2011).

4.1.2 Nutritional pathways involved

Previous work by other groups (mainly the Drummond-Barbosa group) does not show an effect of diet on the size of the GSC niche. This may be because most studies have compared protein-rich and protein-poor diets equivalent to our standard medium with and without live yeast supplementation. I also did not observe any effect on niche size when comparing these conditions. It was necessary to also withdraw supplementary glucose from the medium to observe the niche reduction. Since amino acids, rather than glucose, regulate insulin release in flies then this might suggest other sensing mechanisms might be involved, for example AMPk-dependent regulation. It is noteworthy that removing glucose was not sufficient to reduce cap cell number if live yeast supplementation was present, which might suggest a role for IIS or Tor signalling. Therefore, although IIS remains a prime candidate for mediating dietary effects on

Notch in the niche, other possible links between nutrients and Notch signalling need to be explored. It is indeed possible that multiple nutrition-sensing routes are involved with additive responses to glucose and amino acid provision.

Future work needs to address whether any of these components in different nutrient-sensing pathways have a role in the diet-regulated response of Notch signalling. A first approach could be to repeat the timeline experiment with flies on glucose-reduced food done in the nutrition paper, but using mutants for components of nutrition pathways, such as *Tor*, *Tsc1/2*, *Rheb*, *InR*, *chico*. We could then survey the effects of these mutants on the GSCs and their niche. Based on previous publications (Hsu and Drummond-Barbosa, 2009, 2011), we expect either faster decrease in the number of cap cells or higher decrease in the same timeframe. To better relate Notch to nutrition pathways in the *Drosophila* germarium we could also test the aforementioned mutants effect on the expression of the Notch reporter lines E(spl)m7-lacZ and E(spl)mβ1.5-lacZ. This experiment could also give us clue about a possible tissue specific action of certain nutrition pathways. Moreover, to identify in which cell types the nutrition components are required I could use RNAi against components of nutrition pathways with the different Gal4 expression. A similar approach could be used to rescue the loss of Notch signalling or cap cells in starvation conditions, using overexpression of key components of the IIS rather than RNAi. For instance, overexpression through the Gal4 system of an activated form of InR or of Ilps could rescue cap cell loss and Notch reporter signalling loss on flies kept on a diet restricted medium. The same experiments could be done with components of the Tor signalling pathway. For the *Drosophila* GSC niche system, it is also possible that nutrition may exert its influence on cells contacting the niche which act as signal donor cells, changing ligand expression there to influence Notch in the niche, as described in table 1.3 for mouse embryonic fibroblasts (Ma et al., 2010). It would be also helpful to survey signal reporters for the different nutrient

sensing pathways in the different nutritional conditions. A PI3 reporter line (tubulin-GPH) that changes its localization from the cytoplasm/nuclei to the plasma membrane (Britton et al., 2002) has already been used in ovaries, although not in the cap cell region (Hsu and Drummond-Barbosa, 2009). Another possible usable reporter line for IIS is an antibody against Foxo. During normal IIS, Foxo is phosphorylated and found in the cytoplasm. During reduced IIS, Foxo remains un- phosphorylated and localizes to the nucleus (Bauer et al., 2007; Fridell et al., 2009).

4.1.3 Defining recovery mechanism

In the nutrition paper we show the dynamicity of the cap cell niche and its capability to recover. We propose a model in which ESCs can transform in cap cells when needed. To consolidate this model we require proof that some cap cells were once ESCs. In order to do so we could perform a cell lineage analysis by constitutively activating the expression of UAS-GFP in ESCs, regardless of their becoming cap cells during the recovery. In order to do so we will need to drive UAS-FLP in ESCs using C587-Gal4 in flies also containing UAS-GFP and Actin>CD2>Gal4 (Pignoni and Zipursky, 1997). Flipping out CD2 will constitutively activate Actin-Gal4 only in the cells which were expressing C587-Gal4. Thus ESCs will be able to express GFP even if C587-Gal4 expression may cease after transforming into cap cells. A $Gal80^{ts}$ construct would be also necessary to prevent the expression during development. As readout of this experiment we will have to look for cap cells expressing GFP, which in this experimental setup can happen only if ESCs converted into cap cells. A similar method has already been used recently in the germarium to analyse ESCs dynamics (Morris and Spradling, 2011).

In the nutrition paper we found that the diet induced recovery affects also GSC maintenance. The normal decline of GSC number that occurs in old flies, in all the

tested nutritional conditions, can be overcome by shifting from poor to rich food late in the lifespan. It would be interesting to characterize this effect further by expanding the recovery time for more than the 9 day recovery used in the nutrition paper, to test if the effect on the GSCs is long lasting. As we also tested the nutrition recovery from two different time points (15 days and 24 days from eclosion), it would be interesting to see if the effect on GSCs is still taking place if the nutritional recovery starts later (33 days from eclosion).

4.2 Further work for Pyd paper

In the second paper of this thesis it is found that Pyd controls Notch signalling by restricting Notch endocytosis. Pyd affects Notch endocytosis through a complex with Dx, down-regulating Notch endocytosis and its endocytic-dependent activation. Pyd acted oppositely on two distinct forms of Notch signalling. Pyd suppress ligand-independent Notch activation by Dx and promotes ligand-dependent Notch activation by facilitating Notch ligand coclustering at cell junctions. These activities explain contrary *pyd* mutant phenotypes in the ovary GSC niche and the peripheral nervous system to respectively cause over-activation and reduced Notch signalling. Several other genetic interactions with various junctional organisation components are identified to affect niche size, suggesting a link between cell junction organisation and composition and Notch regulation.

So far our work on the interaction between ZO-1/Pyd and Notch identified mechanisms in cell culture and showed that these mechanisms are consistent with phenotypes and genetic interactions observed *in vivo* in the GSC niche. Further work is required to elucidate if the mechanisms proposed are actually taking place in the cap cells in a way similar to S2 cells. One first assumption that was made is that Pyd acts on the cap cell in a cell-autonomous way, but this is not certain although Pyd stains

strongly cap cells (Djiane et al., 2011). Cell-autonomous effects are shown for Dx through the usage of Dx RNAi expressed in cap cells. It could be shown in the same way for Pyd by expressing Pyd RNAi in the cap cells during development and scoring the number of cap cells. Another experiment could be to check if the localization of Notch in the cap cells is affected in *dx* and *pyd* mutant. Cap cells are small and have a very limited cytosol, so it may be hard to find the subcellular localization of Notch. Still, it should be possible to assess the levels of Notch protein at the plasma membrane, and use this as an indicator of endocytosis. Considering the model presented in the Pyd paper, we would expect to find more Notch protein at the cell surface in *dx* mutants, since without Dx Notch would not be endocytosed as well as in Wt cap cells. On the contrary, we would expect to find less Notch protein at the cell surface in *pyd* mutants. Finally, we could assess the levels of Notch also in a double mutant for *dx* and *pyd,* as the one used in cap cell counts. In this fly experiment, *dx* loss should be able to revert a possible effect of *pyd* mutants on cap cells, as already seen for cap cell numbers.

A further direction of investigation could be to fully characterise the nature and location of cell-cell junctions in cap cells and the localisation of Pyd within these different junctions. The nature of cap cells has not been properly investigated previously but it is known that ZO-1/Pyd is enriched in these cells (Djiane et al., 2011). It is interesting that cap cells have junctions with three different cell types: other cap cells; terminal filament cells; GSCs. Cadherin-based junctions are known to be important at the cap cell/GSC interface to retain GSCs in the niche, but little is known regarding the nature and requirement for other junctions. Immunostaining of different junctional proteins in the cap cells could clarify the nature of these junctions, and analysis of junctional mis-organisation following mutation of different components might clarify the regulation of junctions and their impact on Notch.

4.3 Conclusions

There is considerable overlap in the regulation of cell growth and polarity, junctional components and nutrition-sensing pathways. Organisms seem to converge and funnel a great deal of inputs through fine regulation of conserved pathways, like for example with Hpo signalling and as it is becoming increasingly clear, Notch. The ability of Notch to signal through ligands or other endocytic-dependent mechanisms further adds to the potential for cross talk with other inputs. Endocytosis is an important process by which cells interface with the environment. It allows cells to sense their physiological surrounding, to control signalling levels and to control the composition of the cell surface and the way the cell interacts with its encircling environment. Future research will have to investigate further this integrative fine regulation. Mutations in the essential components of major developmental pathways are often lethal, so it is possible that many diseases we see are caused by mis-regulation of these fine controlling components. Understanding mechanisms of cross talk between environmental inputs with developmental signalling may provide ways to understand numerous risk factors associated with various diseases and their outcomes and provide ways to intervene beneficially in novel ways.

General introduction and discussion references

Akashi, K. (2005). Lineage promiscuity and plasticity in hematopoietic development. Annals of the New York Academy of Sciences *1044*, 125–131.

Alison, M.R., Islam, S., and Lim, S. (2009). Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly. The Journal of Pathology *217*, 282–298.

Allenspach, E.J., Maillard, I., Aster, J.C., and Pear, W.S. (2007). Notch signaling in cancer. Cancer Biology & Therapy *1*, 466–476.

Andrews, R., and Ahringer, J. (2007). Asymmetry of early endosome distribution in C. elegans embryos. PloS One *2*, e493.

Arquier, N., Géminard, C., Bourouis, M., Jarretou, G., Honegger, B., Paix, A., and Léopold, P. (2008). Drosophila ALS regulates growth and metabolism through functional interaction with insulin-like peptides. Cell Metabolism *7*, 333–338.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science (New York, N.Y.) *284*, 770–776.

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K., and Kobayashi, S. (1999). Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. Nature Cell Biology *1*, 431–437.

Assa-Kunik, E., Torres, I.L., Schejter, E.D., Johnston, D.S., and Shilo, B.-Z. (2007). Drosophila follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. Development (Cambridge, England) *134*, 1161–1169.

Axelrod, J.D., Matsuno, K., Artavanis-Tsakonas, S., and Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by dishevelled. Science (New York, N.Y.) *271*, 1826–1832.

Bailey, a M., and Posakony, J.W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. Genes & Development *9*, 2609–2622.

Baksa, K., Parke, T., Dobens, L.L., and Dearolf, C.R. (2002). The Drosophila STAT protein, stat92E, regulates follicle cell differentiation during oogenesis. Developmental Biology *243*, 166–175.

Balklava, Z., Pant, S., Fares, H., and Grant, B.D. (2007). Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. Nature Cell Biology *9*, 1066–1073.

Banerjee, S., Sousa, A.D., and Bhat, M.A. (2006). Organization and function of septate junctions: an evolutionary perspective. Cell Biochemistry and Biophysics *46*, 65–77.

Barcellos-Hoff, M.H., and Ravani, S.A. (2000). Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Research *60*, 1254–1260.

Bareja, A., and Billin, A.N. (2013). Satellite cell therapy -- from mice to men. Skeletal Muscle *3*, 2.

Barry, E.R., and Camargo, F.D. (2013). The Hippo superhighway: signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. Current Opinion in Cell Biology 1–7.

Bate, M., and Arias, A.M. (1993). The Development of Drosophila melanogaster (Cold Spring Harbor Laboratory Press).

Bauer, J.H., Chang, C., Morris, S.N.S., Hozier, S., Andersen, S., Waitzman, J.S., and Helfand, S.L. (2007). Expression of dominant-negative Dmp53 in the adult fly brain inhibits insulin signaling. Proceedings of the National Academy of Sciences of the United States of America *104*, 13355–13360.

Becerra, J., Santos-Ruiz, L., Andrades, J. a, and Marí-Beffa, M. (2011). The stem cell niche should be a key issue for cell therapy in regenerative medicine. Stem Cell Reviews *7*, 248–255.

Bender, L.B., Kooh, P.J., and Muskavitch, M.A.T. (1993). Complex function and expression of Delta during Drosophila oogenesis. Genetics *133*, 967–978.

Benmimoun, B., Polesello, C., Waltzer, L., and Haenlin, M. (2012). Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in Drosophila. Development (Cambridge, England) *139*, 1713–1717.

Bennett, F.C., and Harvey, K.F. (2006). Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. Current Biology : CB *16*, 2101–2110.

Benraiss, A., and Goldman, S. a (2011). Cellular therapy and induced neuronal replacement for Huntington's disease. Neurotherapeutics : the Journal of the American Society for Experimental NeuroTherapeutics *8*, 577–590.

Benton, R., and St Johnston, D. (2003). Drosophila PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. Cell *115*, 691–704.

Besse, F., Busson, D., and Pret, A.-M. (2002). Fused-dependent Hedgehog signal transduction is required for somatic cell differentiation during Drosophila egg chamber formation. Development (Cambridge, England) *129*, 4111–4124.

Betschinger, J., Mechtler, K., and Knoblich, J.A. (2003). The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature *422*, 326–330.

Beugnet, A., Tee, A.R., Taylor, P.M., and Proud, C.G. (2003). Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. The Biochemical Journal *372*, 555–566.

Bilder, D. (2004). Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes & Development *18*, 1909–1925.

Birse, R.T., Choi, J., Reardon, K., Rodriguez, J., Graham, S., Diop, S., Ocorr, K., Bodmer, R., and Oldham, S. (2010). High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in Drosophila. Cell Metabolism *12*, 533–544.

Blasco, M. a (2005). Telomeres and human disease: ageing, cancer and beyond. Nature Reviews. Genetics *6*, 611–622.

Boggiano, J.C., and Fehon, R.G. (2012). Growth control by committee: intercellular junctions, cell polarity, and the cytoskeleton regulate Hippo signaling. Developmental Cell *22*, 695–702.

Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865–875.

Boldrin, L., Neal, A., Zammit, P.S., Muntoni, F., and Morgan, J.E. (2012). Donor satellite cell engraftment is significantly augmented when the host niche is preserved and endogenous satellite cells are incapacitated. Stem Cells (Dayton, Ohio) *30*, 1971–1984.

Boyle, M., Wong, C., Rocha, M., and Jones, D.L. (2007). Decline in self-renewal factors contributes to aging of the stem cell niche in the Drosophila testis. Cell Stem Cell *1*, 470–478.

Britton, J.S., and Edgar, B. a (1998). Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. Development (Cambridge, England) *125*, 2149–2158.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., and Edgar, B. a. (2002). Drosophila's Insulin/PI3-Kinase Pathway Coordinates Cellular Metabolism with Nutritional Conditions. Developmental Cell *2*, 239–249.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Current Biology : CB *11*, 213–221.

Brumby, A.M., and Richardson, H.E. (2005). Using Drosophila melanogaster to map human cancer pathways. Nature Reviews. Cancer *5*, 626–639.

Buch, S., Melcher, C., Bauer, M., Katzenberger, J., and Pankratz, M.J. (2008). Opposing effects of dietary protein and sugar regulate a transcriptional target of Drosophila insulin-like peptide signaling. Cell Metabolism *7*, 321–332.

Buerger, C., DeVries, B., and Stambolic, V. (2006). Localization of Rheb to the endomembrane is critical for its signaling function. Biochemical and Biophysical Research Communications *344*, 869–880.

Bulgakova, N. a, and Knust, E. (2009). The Crumbs complex: from epithelial-cell polarity to retinal degeneration. Journal of Cell Science *122*, 2587–2596.

Busseau, I., Diederich, R.J., Xu, T., and Artavanis-Tsakonas, S. (1994). A member of the Notch group of interacting loci, deltex encodes a cytoplasmic basic protein. Genetics *136*, 585–596.

Cai, S.-L., Tee, A.R., Short, J.D., Bergeron, J.M., Kim, J., Shen, J., Guo, R., Johnson, C.L., Kiguchi, K., and Walker, C.L. (2006). Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. The Journal of Cell Biology *173*, 279–289.

Cang, C., Zhou, Y., Navarro, B., Seo, Y.-J., Aranda, K., Shi, L., Battaglia-Hsu, S., Nissim, I., Clapham, D.E., and Ren, D. (2013). mTOR Regulates Lysosomal ATP-Sensitive Two-Pore Na(+) Channels to Adapt to Metabolic State. Cell *152*, 778–790.

Carlson, M.E., Suetta, C., Conboy, M.J., Aagaard, P., Mackey, A., Kjaer, M., and Conboy, I. (2009). Molecular aging and rejuvenation of human muscle stem cells. EMBO Molecular Medicine *1*, 381–391.

Carmena, A., Speicher, S., and Baylies, M. (2006). The PDZ protein Canoe/AF-6 links Ras-MAPK, Notch and Wingless/Wnt signaling pathways by directly interacting with Ras, Notch and Dishevelled. PloS One *1*, e66.

Casanueva, M.O., and Ferguson, E.L. (2004). Germline stem cell number in the Drosophila ovary is regulated by redundant mechanisms that control Dpp signaling. Development (Cambridge, England) *131*, 1881–1890.

De Celis, J.F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C., and Bray, S.J. (1996). Functional relationships between Notch, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal development. Development (Cambridge, England) *122*, 2719–2728.

Cerletti, M., Jang, Y.C., Finley, L.W.S., Haigis, M.C., and Wagers, A.J. (2012). Short-term calorie restriction enhances skeletal muscle stem cell function. Cell Stem Cell *10*, 515–519.

Chan, S.M., Weng, A.P., Tibshirani, R., Aster, J.C., and Utz, P.J. (2007). Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. Blood *110*, 278–286.

Chapman, T., and Partridge, L. (1996). Female fitness in Drosophila melanogaster: an interaction between the effect of nutrition and of encounter rate with males. Proceedings. Biological Sciences / The Royal Society *263*, 755–759.

Chartier, F.J.-M., Hardy, É.J.-L., and Laprise, P. (2011). Crumbs controls epithelial integrity by inhibiting Rac1 and PI3K. Journal of Cell Science *124*, 3393–3398.

Chen, D., and McKearin, D. (2003). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. Current Biology : CB *13*, 1786–1791.

Chen, C., Schroeder, M.C., Kango-Singh, M., Tao, C., and Halder, G. (2012a). Tumor suppression by cell competition through regulation of the Hippo pathway. Proceedings of the National Academy of Sciences of the United States of America *109*, 484–489.

Chen, C.-L., Gajewski, K.M., Hamaratoglu, F., Bossuyt, W., Sansores-Garcia, L., Tao, C., and Halder, G. (2010). The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in Drosophila. Proceedings of the National Academy of Sciences of the United States of America *107*, 15810–15815.

Chen, H.-J., Wang, C.-M., Wang, T.-W., Liaw, G.-J., Hsu, T.-H., Lin, T.-H., and Yu, J.-Y. (2011). The Hippo pathway controls polar cell fate through Notch signaling during Drosophila oogenesis. Developmental Biology *357*, 370–379.

Chen, J., Astle, C.M., and Harrison, D.E. (2003). Hematopoietic senescence is postponed and hematopoietic stem cell function is enhanced by dietary restriction. Experimental Hematology *31*, 1097–1103.

Chen, L., Qin, F., Deng, X., Avruch, J., and Zhou, D. (2012b). Hippo pathway in intestinal homeostasis and tumorigenesis. Protein & Cell *3*, 305–310.

Chern, J.J., and Choi, K.-W. (2002). Lobe mediates Notch signaling to control domain-specific growth in the Drosophila eye disc. Development (Cambridge, England) *129*, 4005–4013.

Chippindale, A.K., Leroi, A.M., Kim, S.B., and Rose, M.R. (1993). Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. Journal of Evolutionary Biology *6*, 171–193.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R., and Irvine, K.D. (2006). Delineation of a Fat tumor suppressor pathway. Nature Genetics *38*, 1142–1150.

Choi, W., Jung, K., Nelson, K.S., Bhat, M.A., Beitel, G.J., Peifer, M., and Fanning, A.S. (2011). The single Drosophila ZO-1 protein Polychaetoid regulates embryonic morphogenesis in coordination with Canoe/afadin and Enabled. Molecular Biology of the Cell *22*, 2010–2030.

Christie, G.R., Hajduch, E., Hundal, H.S., Proud, C.G., and Taylor, P.M. (2002). Intracellular sensing of amino acids in Xenopus laevis oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. The Journal of Biological Chemistry *277*, 9952–9957.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., and Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science (New York, N.Y.) *292*, 104–106.

Clarke, M.F., and Fuller, M. (2006). Stem cells and cancer: two faces of eve. Cell *124*, 1111–1115.

Collins, K., and Mitchell, J.R. (2002). Telomerase in the human organism. Oncogene *21*, 564–579.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., Léopold, P., Le, P., and Valrose, U.M.R.C. (2003). A nutrient sensor mechanism controls Drosophila growth. Cell *114*, 739–749.

Conboy, I.M., Conboy, M.J., Smythe, G.M., and Rando, T. a (2003). Notch-mediated restoration of regenerative potential to aged muscle. Science (New York, N.Y.) *302*, 1575–1577.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., and Rando, T. a (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature *433*, 760–764.

Cooper, M.T., Tyler, D.M., Furriols, M., Chalkiadaki, A., Delidakis, C., and Bray, S.J. (2000). Spatially restricted factors cooperate with notch in the regulation of Enhancer of split genes. Developmental Biology *221*, 390–403.

Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frasson, C., Inui, M., Montagner, M., Parenti, A.R., Poletti, A., et al. (2011). The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. Cell *147*, 759–772.

Corradetti, M.N., Inoki, K., Bardeesy, N., DePinho, R.A., and Guan, K. (2004). Regulation of the TSC pathway by LKB1: evidence of a molecular link between

tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes & Development *18*, 1533–1538.

Cowles, C.R., Odorizzi, G., Payne, G.S., and Emr, S.D. (1997). The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. Cell *91*, 109–118.

Crittenden, S.L., Bernstein, D.S., Bachorik, J.L., Thompson, B.E., Gallegos, M., Petcherski, A.G., Moulder, G., Barstead, R., Wickens, M., and Kimble, J. (2002). A conserved RNA-binding protein controls germline stem cells in Caenorhabditis elegans. Nature *417*, 660–663.

De Cuevas, M., and Spradling, a C. (1998). Morphogenesis of the Drosophila fusome and its implications for oocyte specification. Development (Cambridge, England) *125*, 2781–2789.

De Cuevas, M., Lilly, M. a, and Spradling, a C. (1997). Germline cyst formation in Drosophila. Annual Review of Genetics *31*, 405–428.

Dalerba, P., Cho, R.W., and Clarke, M.F. (2007). Cancer stem cells: models and concepts. Annual Review of Medicine *58*, 267–284.

Danisovic, L., Varga, I., Zamborsky, R., and Böhmer, D. (2012). The tissue engineering of articular cartilage: cells, scaffolds and stimulating factors. Experimental Biology and Medicine (Maywood, N.J.) *237*, 10–17.

Dasgupta, B., and Milbrandt, J. (2009). AMP-activated protein kinase phosphorylates retinoblastoma protein to control mammalian brain development. Developmental Cell *16*, 256–270.

Decotto, E., and Spradling, A.C. (2005). The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. Developmental Cell *9*, 501–510.

Delidakis, C., Preiss, A., Hartley, D.A., and Artavanis-Tsakonas, S. (1991). Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of Drosophila melanogaster. Genetics *129*, 803–823.

Dell'Angelica, E.C., Ohno, H., Ooi, C.E., Rabinovich, E., Roche, K.W., and Bonifacino, J.S. (1997). AP-3: an adaptor-like protein complex with ubiquitous expression. The EMBO Journal *16*, 917–928.

Dimitratos, S.D., Woods, D.F., Stathakis, D.G., and Bryant, P.J. (1999). Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family. BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology *21*, 912–921.

Djiane, A., Shimizu, H., Wilkin, M.B., Mazleyrat, S., Jennings, M.D., Avis, J., Bray, S.J., and Baron, M. (2011). Su(dx) E3 ubiquitin ligase-dependent and -independent functions of polychaetoid, the Drosophila ZO-1 homologue. The Journal of Cell Biology *192*, 189–200.

Drummond-Barbosa, D. (2008). Stem cells, their niches and the systemic environment: an aging network. Genetics *180*, 1787–1797.

Drummond-Barbosa, D., and Spradling, A.C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. Developmental Biology *231*, 265–278.

Easley, C.A., Ben-Yehudah, A., Redinger, C.J., Oliver, S.L., Varum, S.T., Eisinger, V.M., Carlisle, D.L., Donovan, P.J., and Schatten, G.P. (2010). mTOR-mediated activation of p70 S6K induces differentiation of pluripotent human embryonic stem cells. Cellular Reprogramming *12*, 263–273.

Efeyan, A., Zoncu, R., and Sabatini, D.M. (2012). Amino acids and mTORC1: from lysosomes to disease. Trends in Molecular Medicine *18*, 524–533.

Efferson, C.L., Winkelmann, C.T., Ware, C., Sullivan, T., Giampaoli, S., Tammam, J., Patel, S., Mesiti, G., Reilly, J.F., Gibson, R.E., et al. (2010). Downregulation of Notch pathway by a gamma-secretase inhibitor attenuates AKT/mammalian target of rapamycin signaling and glucose uptake in an ERBB2 transgenic breast cancer model. Cancer Research *70*, 2476–2484.

Ertl, R.P., Chen, J., Astle, C.M., Duffy, T.M., and Harrison, D.E. (2008). Effects of dietary restriction on hematopoietic stem-cell aging are genetically regulated. Blood *111*, 1709–1716.

Findlay, G.M., Yan, L., Procter, J., Mieulet, V., and Lamb, R.F. (2007). A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. The Biochemical Journal *403*, 13–20.

Flores, I., Benetti, R., and Blasco, M. a (2006). Telomerase regulation and stem cell behaviour. Current Opinion in Cell Biology *18*, 254–260.

Forbes, a, and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of Drosophila germline stem cells. Development (Cambridge, England) *125*, 679–690.

Forbes, a J., Lin, H., Ingham, P.W., and Spradling, a C. (1996). hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. Development (Cambridge, England) *122*, 1125–1135.

Förster, D., Armbruster, K., and Luschnig, S. (2010). Sec24-dependent secretion drives cell-autonomous expansion of tracheal tubes in Drosophila. Current Biology : CB *20*, 62–68.

Fortini, M.E., and Artavanis-Tsakonas, S. (1994). The suppressor of hairless protein participates in notch receptor signaling. Cell *79*, 273–282.

Fridell, Y.-W.C., Hoh, M., Kréneisz, O., Hosier, S., Chang, C., Scantling, D., Mulkey, D.K., and Helfand, S.L. (2009). Increased uncoupling protein (UCP) activity in Drosophila insulin-producing neurons attenuates insulin signaling and extends lifespan. Aging *1*, 699–713.

Fuchs, E., and Segre, J. a (2000). Stem cells: a new lease on life. Cell *100*, 143–155.

Fuwa, T.J., Hori, K., Sasamura, T., Higgs, J., Baron, M., and Matsuno, K. (2006). The first deltex null mutant indicates tissue-specific deltex-dependent Notch signaling in Drosophila. Molecular Genetics and Genomics : MGG *275*, 251–263.

Geminard, C., Arquier, N., Layalle, S., Bourouis, M., Slaidina, M., Delanoue, R., Bjordal, M., Ohanna, M., Ma, M., Colombani, J., et al. (2006). Control of Metabolism and Growth Through Insulin-Like Peptides in Drosophila. Diabetes *55*, S5–S8.

Géminard, C., Rulifson, E.J., and Léopold, P. (2009). Remote control of insulin secretion by fat cells in Drosophila. Cell Metabolism *10*, 199–207.

Genevet, A., and Tapon, N. (2011). The Hippo pathway and apico-basal cell polarity. The Biochemical Journal *436*, 213–224.

Georgiou, M., Marinari, E., Burden, J., and Baum, B. (2008). Cdc42, Par6, and aPKC regulate Arp2/3-mediated endocytosis to control local adherens junction stability. Current Biology : CB *18*, 1631–1638.

Ghotra, V.P.S., Puigvert, J.C., and Danen, E.H.J. (2009). The cancer stem cell microenvironment and anti-cancer therapy. International Journal of Radiation Biology *85*, 955–962.

Giannakou, M.E., and Partridge, L. (2007). Role of insulin-like signalling in Drosophila lifespan. Trends in Biochemical Sciences *32*, 180–188.

Gil, J., and Peters, G. (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nature Reviews. Molecular Cell Biology *7*, 667–677.

Goberdhan, D.C.I., and Wilson, C. (2003). The functions of insulin signaling: size isn't everything, even in Drosophila. Differentiation; Research in Biological Diversity *71*, 375–397.

Goberdhan, D.C.I., Meredith, D., Boyd, C. a R., and Wilson, C. (2005). PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids. Development (Cambridge, England) *132*, 2365–2375.

Goldstein, B., and Macara, I.G. (2007). The PAR proteins: fundamental players in animal cell polarization. Developmental Cell *13*, 609–622.

Gönczy, P., Matunis, E., and DiNardo, S. (1997). bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis. Development (Cambridge, England) *124*, 4361–4371.

González-Reyes, A. (2003). Stem cells, niches and cadherins: a view from Drosophila. Journal of Cell Science *116*, 949–954.

González-Reyes, a, and St Johnston, D. (1998). The Drosophila AP axis is polarised by the cadherin-mediated positioning of the oocyte. Development (Cambridge, England) *125*, 3635–3644.

Grammont, M., and Irvine, K.D. (2001). fringe and Notch specify polar cell fate during Drosophila oogenesis. Development (Cambridge, England) *128*, 2243–2253.

Grandison, R.C., Piper, M.D.W., and Partridge, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature *462*, 1061–1064.

Greenwood, M.J., and Lansdorp, P.M. (2004). Telomeres, telomerase, and hematopoietic stem cell biology. Archives of Medical Research *34*, 489–495.

Grzeschik, N. a, Parsons, L.M., Allott, M.L., and Richardson, H.E. (2010). Lgl/aPKC and Crb regulate the Salvador/Warts/Hippo pathway. Fly *4*, 288–293.

Guo, Y., Du, J., and Kwiatkowski, D.J. (2013). Molecular dissection of AKT activation in lung cancer cell lines. Molecular Cancer Research : MCR *4396*.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. Molecular Cell *30*, 214–226.

Vander Haar, E., Lee, S.-I., Bandhakavi, S., Griffin, T.J., and Kim, D.-H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nature Cell Biology *9*, 316–323.

Halder, G., and Johnson, R.L. (2011). Hippo signaling: growth control and beyond. Development (Cambridge, England) *138*, 9–22.

Harms, E., Gochman, N., and Schneider, J.A. (1981). Lysosomal pool of free-amino acids. Biochemical and Biophysical Research Communications *99*, 830–836.

Harris, K.P., and Tepass, U. (2008). Cdc42 and Par proteins stabilize dynamic adherens junctions in the Drosophila neuroectoderm through regulation of apical endocytosis. The Journal of Cell Biology *183*, 1129–1143.

Harris, R.E., Pargett, M., Sutcliffe, C., Umulis, D., and Ashe, H.L. (2011). Brat promotes stem cell differentiation via control of a bistable switch that restricts BMP signaling. Developmental Cell *20*, 72–83.

Haselton, A.T., and Fridell, Y.-W.C. (2010). Adult Drosophila melanogaster as a model for the study of glucose homeostasis. Aging *2*, 523–526.

Hayward, P., Brennan, K., Sanders, P., Balayo, T., DasGupta, R., Perrimon, N., Arias, A.M., and Martinez Arias, A. (2005). Notch modulates Wnt signalling by associating with Armadillo/beta-catenin and regulating its transcriptional activity. Development (Cambridge, England) *132*, 1819–1830.

Van Der Heide, L.P., Hoekman, M.F.M., and Smidt, M.P. (2004). The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. The Biochemical Journal *380*, 297–309.

Hennig, K.M., Colombani, J., and Neufeld, T.P. (2006). TOR coordinates bulk and targeted endocytosis in the Drosophila melanogaster fat body to regulate cell growth. The Journal of Cell Biology *173*, 963–974.

Herranz, H., Stamataki, E., Feiguin, F., and Milán, M. (2006). Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of gamma-secretase activity. EMBO Reports *7*, 297–302.

Hill, R., and Wu, H. (2009). PTEN, stem cells, and cancer stem cells. The Journal of Biological Chemistry *284*, 11755–11759.

Hogan, B.L. (1996). Bone morphogenetic proteins in development. Current Opinion in Genetics & Development *6*, 432–438.

Honjo, T. (1996). The shortest path from the surface to the nucleus: RBP-J kappa/Su(H) transcription factor. Genes to Cells : Devoted to Molecular & Cellular Mechanisms *1*, 1–9.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., and Matsuno, K. (2004). Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development (Cambridge, England) *131*, 5527–5537.

Hsu, H.-J., and Drummond-Barbosa, D. (2009). Insulin levels control female germline stem cell maintenance via the niche in Drosophila. Proceedings of the National Academy of Sciences of the United States of America *106*, 1117–1121.

Hsu, H.-J., and Drummond-Barbosa, D. (2011). Insulin signals control the competence of the Drosophila female germline stem cell niche to respond to Notch ligands. Developmental Biology *350*, 290–300.

Hsu, H.-J., LaFever, L., and Drummond-Barbosa, D. (2008). Diet controls normal and tumorous germline stem cells via insulin-dependent and -independent mechanisms in Drosophila. Developmental Biology *313*, 700–712.

Hutterer, A., Betschinger, J., Petronczki, M., and Knoblich, J. a (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during Drosophila embryogenesis. Developmental Cell *6*, 845–854.

Huynh, J.-R., and St Johnston, D. (2004). The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte. Current Biology : CB *14*, R438–49.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Current Biology : CB *12*, 1293–1300.

Ingham, P.W. (1998). Transducing Hedgehog: the story so far. The EMBO Journal *17*, 3505–3511.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.-L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nature Cell Biology *4*, 648–657.

Inoki, K., Zhu, T., and Guan, K.-L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577–590.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N., et al. (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature *431*, 997–1002.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y., et al. (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nature Medicine *12*, 446–451.

Janssens, B., and Chavrier, P. (2004). Mediterranean views on epithelial polarity. Nature Cell Biology *6*, 493–496.

Janzen, V., Forkert, R., Fleming, H.E., Saito, Y., Waring, M.T., Dombkowski, D.M., Cheng, T., DePinho, R. a, Sharpless, N.E., and Scadden, D.T. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature *443*, 421–426.

Jennings, B., Preiss, a, Delidakis, C., and Bray, S.J. (1994). The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the Drosophila embryo. Development (Cambridge, England) *120*, 3537–3548.

Jennings, M.D., Blankley, R.T., Baron, M., Golovanov, A.P., and Avis, J.M. (2007). Specificity and autoregulation of Notch binding by tandem WW domains in suppressor of Deltex. The Journal of Biological Chemistry *282*, 29032–29042.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B. a (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343–1355.

Jo, H.S., Kang, K.H., Joe, C.O., and Kim, J.W. (2012). Pten coordinates retinal neurogenesis by regulating Notch signalling. The EMBO Journal *31*, 817–828.

Joddar, B., and Ito, Y. (2013). Artificial niche substrates for embryonic and induced pluripotent stem cell cultures. Journal of Biotechnology.

Jones, D.L. (2007). Aging and the germ line: where mortality and immortality meet. Stem Cell Reviews *3*, 192–200.

Kahn, B.B., Alquier, T., Carling, D., and Hardie, D.G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metabolism *1*, 15–25.

Kam, Y., and Quaranta, V. (2009). Cadherin-bound beta-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between beta-catenin pools. PloS One *4*, e4580.

Kao, H.-Y.Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C.R., Evans, R.M., and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes & Development *12*, 2269–2277.

Kapahi, P., Zid, B.M., Harper, T., Koslover, D., Sapin, V., and Benzer, S. (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Current Biology : CB *14*, 885–890.

Karbowniczek, M., Zitserman, D., Khabibullin, D., Hartman, T., Yu, J., Morrison, T., Nicolas, E., Squillace, R., Roegiers, F., and Henske, E.P. (2010). The evolutionarily conserved TSC/Rheb pathway activates Notch in tuberous sclerosis complex and Drosophila external sensory organ development. The Journal of Clinical Investigation *120*, 93–102.

Kelly, A.P., Finlay, D.K., Hinton, H.J., Clarke, R.G., Fiorini, E., Radtke, F., and Cantrell, D. a (2007). Notch-induced T cell development requires phosphoinositide-dependent kinase 1. The EMBO Journal *26*, 3441–3450.

Kiger, A.A., White-Cooper, H., and Fuller, M.T. (2000). Somatic support cells restrict germline stem cell self-renewal and promote differentiation. Nature *407*, 750–754.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., and Guan, K.-L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. Nature Cell Biology *10*, 935–945.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., and Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. Nature *382*, 133–138.

Kim, J.Y., Lee, Y.C., and Kim, C. (2010). Direct inhibition of Pumilo activity by Bam and Bgcn in Drosophila germ line stem cell differentiation. The Journal of Biological Chemistry *285*, 4741–4746.

Kirilly, D., Spana, E.P., Perrimon, N., Padgett, R.W., and Xie, T. (2005). BMP signaling is required for controlling somatic stem cell self-renewal in the Drosophila ovary. Developmental Cell *9*, 651–662.

Kirilly, D., Wang, S., and Xie, T. (2011). Self-maintained escort cells form a germline stem cell differentiation niche. Development (Cambridge, England) *5097*, 5087–5097.

Kishigami, S., and Mishina, Y. (2005). BMP signaling and early embryonic patterning. Cytokine & Growth Factor Reviews *16*, 265–278.

Kitamoto, K., Yoshizawa, K., Ohsumi, Y., and Anraku, Y. (1988). Dynamic aspects of vacuolar and cytosolic amino acid pools of Saccharomyces cerevisiae. Journal of Bacteriology *170*, 2683–2686.

Klein, T., Seugnet, L., Haenlin, M., and Martinez Arias, a (2000). Two different activities of Suppressor of Hairless during wing development in Drosophila. Development (Cambridge, England) *127*, 3553–3566.

Kohyama-Koganeya, A., and Hirabayashi, Y. (2010). The Drosophila 7-pass transmembrane glycoprotein BOSS and metabolic regulation: What Drosophila can teach us about human energy metabolism. (Elsevier Inc.).

Korolchuk, V.I., Saiki, S., Lichtenberg, M., Siddiqi, F.H., Roberts, E. a, Imarisio, S., Jahreiss, L., Sarkar, S., Futter, M., Menzies, F.M., et al. (2011). Lysosomal positioning coordinates cellular nutrient responses. Nature Cell Biology *13*, 453–460.

Kramer, J.M., Davidge, J.T., Lockyer, J.M., and Staveley, B.E. (2003). Expression of Drosophila FOXO regulates growth and can phenocopy starvation. BMC Developmental Biology *3*, 5.

Kréneisz, O., Chen, X., Fridell, Y.-W.C., and Mulkey, D.K. (2010). Glucose increases activity and Ca2+ in insulin-producing cells of adult Drosophila. Neuroreport *21*, 1116–1120.

Krtolica, A. (2005). Stem cell: balancing aging and cancer. The International Journal of Biochemistry & Cell Biology *37*, 935–941.

Kwiatkowski, D.J., and Manning, B.D. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. Human Molecular Genetics *14 Spec No*, R251–8.

LaFever, L., and Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science (New York, N.Y.) *309*, 1071–1073.

LaFever, L., Feoktistov, A., Hsu, H.-J., and Drummond-Barbosa, D. (2010). Specific roles of Target of rapamycin in the control of stem cells and their progeny in the Drosophila ovary. Development (Cambridge, England) *137*, 2117–2126.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature *367*, 645–648.

Laprise, P. (2011). Emerging role for epithelial polarity proteins of the Crumbs family as potential tumor suppressors. Journal of Biomedicine & Biotechnology *2011*, 868217.

Laprise, P., and Tepass, U. (2011). Novel insights into epithelial polarity proteins in Drosophila. Trends in Cell Biology *21*, 401–408.

Laprise, P., Beronja, S., Silva-Gagliardi, N.F., Pellikka, M., Jensen, A.M., McGlade, C.J., and Tepass, U. (2006). The FERM protein Yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size. Developmental Cell *11*, 363–374.

Laprise, P., Lau, K.M., Harris, K.P., Silva-Gagliardi, N.F., Paul, S.M., Beronja, S., Beitel, G.J., McGlade, C.J., and Tepass, U. (2009). Yurt, Coracle, Neurexin IV and the Na(+),K(+)-ATPase form a novel group of epithelial polarity proteins. Nature *459*, 1141–1145.

Lecourtois, M., and Schweisguth, F. (1995). The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. Genes & Development *9*, 2598–2608.

Lecourtois, M., and Schweisguth, F. (1998). Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. Current Biology : CB *8*, 771–774.

Lee, J.H., Koh, H., Kim, M., Kim, Y., Lee, S.Y., Karess, R.E., Lee, S.-H., Shong, M., Kim, J.-M., Kim, J., et al. (2007). Energy-dependent regulation of cell structure by AMP-activated protein kinase. Nature *447*, 1017–1020.

Leibfried, A., Fricke, R., Morgan, M.J., Bogdan, S., and Bellaiche, Y. (2008). Drosophila Cip4 and WASp define a branch of the Cdc42-Par6-aPKC pathway regulating E-cadherin endocytosis. Current Biology : CB *18*, 1639–1648.

Li, H.-J., Reinhardt, F., Herschman, H.R., and Weinberg, R. a (2012a). Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signaling. Cancer Discovery *2*, 840–855.

Li, Q., Shen, L., Xin, T., Xiang, W., Chen, W., Gao, Y., Zhu, M., Yu, L., and Li, M. (2009a). Role of Scrib and Dlg in anterior-posterior patterning of the follicular epithelium during Drosophila oogenesis. BMC Developmental Biology *9*, 60.

Li, Y., Minor, N.T., Park, J.K., McKearin, D.M., and Maines, J.Z. (2009b). Bam and Bgcn antagonize Nanos-dependent germ-line stem cell maintenance. Proceedings of the National Academy of Sciences of the United States of America *106*, 9304–9309.

Li, Y., Hibbs, M.A., Gard, A.L., Shylo, N.A., and Yun, K. (2012b). Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. Stem Cells (Dayton, Ohio) *30*, 741–752.

Lilly, M.A., de Cuevas, M., and Spradling, A.C. (2000). Cyclin A associates with the fusome during germline cyst formation in the Drosophila ovary. Developmental Biology *218*, 53–63.

Ling, C., Zheng, Y., Yin, F., Yu, J., Huang, J., Hong, Y., Wu, S., and Pan, D. (2010). The apical transmembrane protein Crumbs functions as a tumor suppressor that regulates Hippo signaling by binding to Expanded. Proceedings of the National Academy of Sciences of the United States of America *107*, 10532–10537.

Liu, H., Huang, G.-W., Zhang, X.-M., Ren, D.-L., and X Wilson, J. (2010). Folic Acid supplementation stimulates notch signaling and cell proliferation in embryonic neural stem cells. Journal of Clinical Biochemistry and Nutrition *47*, 174–180.

Liu, L., Cash, T.P., Jones, R.G., Keith, B., Thompson, C.B., and Simon, M.C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. Molecular Cell *21*, 521–531.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N.G., and Israël, A. (1998). The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proceedings of the National Academy of Sciences of the United States of America *95*, 8108–8112.

López-Onieva, L., Fernández-Miñán, A., and González-Reyes, A. (2008). Jak/Stat signalling in niche support cells regulates dpp transcription to control germline stem cell maintenance in the Drosophila ovary. Development (Cambridge, England) *135*, 533–540.

López-Schier, H., and St Johnston, D. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. Genes & Development *15*, 1393–1405.

Lowell, B.B., and Shulman, G.I. (2005). Mitochondrial dysfunction and type 2 diabetes. Science (New York, N.Y.) *307*, 384–387.

Ma, J., Meng, Y., Kwiatkowski, D.J., Chen, X., Peng, H., Sun, Q., Zha, X., Wang, F., Wang, Y., Jing, Y., et al. (2010). Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade. The Journal of Clinical Investigation *120*, 103–114.

Macchiarini, P., Jungebluth, P., Go, T., Asnaghi, M.A., Rees, L.E., Cogan, T. a, Dodson, A., Martorell, J., Bellini, S., Parnigotto, P.P., et al. (2008). Clinical transplantation of a tissue-engineered airway. Lancet *372*, 2023–2030.

Maeda, K., Takemura, M., Umemori, M., and Adachi-Yamada, T. (2008). E-cadherin prolongs the moment for interaction between intestinal stem cell and its progenitor cell to ensure Notch signaling in adult Drosophila midgut. Genes to Cells : Devoted to Molecular & Cellular Mechanisms *13*, 1219–1227.

Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. The Journal of Biological Chemistry *273*, 13375–13378.

Mair, W., McLeod, C.J., Wang, L., and Jones, D.L. (2010). Dietary restriction enhances germline stem cell maintenance. Aging Cell *9*, 916–918.

Maitra, S., Kulikauskas, R.M., Gavilan, H., and Fehon, R.G. (2006). The tumor suppressors Merlin and Expanded function cooperatively to modulate receptor endocytosis and signaling. Current Biology : CB *16*, 702–709.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Molecular Cell *10*, 151–162.

Marcinkevicius, E., and Zallen, J. a (2013). Regulation of cytoskeletal organization and junctional remodeling by the atypical cadherin Fat. Development (Cambridge, England) *140*, 433–443.

Margolis, J., and Spradling, A. (1995). Identification and behavior of epithelial stem cells in the Drosophila ovary. Development (Cambridge, England) *121*, 3797–3807.

Martin, J.F., Hersperger, E., Simcox, a, and Shearn, a (2000). Minidiscs Encodes a Putative Amino Acid Transporter Subunit Required Non-Autonomously for Imaginal Cell Proliferation. Mechanisms of Development *92*, 155–167.

Mathur, D., Bost, A., Driver, I., and Ohlstein, B. (2010). A transient niche regulates the specification of Drosophila intestinal stem cells. Science (New York, N.Y.) *327*, 210–213.

Matsuno, K., Diederich, R.J., Go, M.J., Blaumueller, C.M., and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development (Cambridge, England) *121*, 2633–2644.

McCartney, B.M., Kulikauskas, R.M., LaJeunesse, D.R., and Fehon, R.G. (2000). The neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation. Development (Cambridge, England) *127*, 1315–1324.

McGregor, J.R., Xi, R., and Harrison, D.A. (2002). JAK signaling is somatically required for follicle cell differentiation in Drosophila. Development (Cambridge, England) *129*, 705–717.

McKearin, D., and Ohlstein, B. (1995). A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. Development (Cambridge, England) *121*, 2937–2947.

McLeod, C.J., Wang, L., Wong, C., and Jones, D.L. (2010). Stem cell dynamics in response to nutrient availability. Current Biology : CB *20*, 2100–2105.

Micchelli, C. a, and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature *439*, 475–479.

Micchelli, C.A., Rulifson, E.J., and Blair, S.S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. Development (Cambridge, England) *124*, 1485–1495.

Michaelson, D., Korta, D.Z., Capua, Y., and Hubbard, E.J.A. (2010). Insulin signaling promotes germline proliferation in C. elegans. Development (Cambridge, England) *137*, 671–680.

Middleton, C.A., Nongthomba, U., Parry, K., Sweeney, S.T., Sparrow, J.C., and Elliott, C.J.H. (2006). Neuromuscular organization and aminergic modulation of contractions in the Drosophila ovary. BMC Biology *4*, 17.

Min, K.-J., and Tatar, M. (2006). Restriction of amino acids extends lifespan in Drosophila melanogaster. Mechanisms of Ageing and Development *127*, 643–646.

Mirouse, V., Swick, L.L., Kazgan, N., St Johnston, D., and Brenman, J.E. (2007). LKB1 and AMPK maintain epithelial cell polarity under energetic stress. The Journal of Cell Biology *177*, 387–392.

Morris, L.X., and Spradling, A.C. (2011). Long-term live imaging provides new insight into stem cell regulation and germline-soma coordination in the Drosophila ovary. Development (Cambridge, England) *138*, 2207–2215.

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598–611.

Nakada, D., Levi, B.P., and Morrison, S.J. (2011). Integrating physiological regulation with stem cell and tissue homeostasis. Neuron *70*, 703–718.

Nakayama, Y., Shivas, J.M., Poole, D.S., Squirrell, J.M., Kulkoski, J.M., Schleede, J.B., and Skop, A.R. (2009). Dynamin participates in the maintenance of anterior polarity in the Caenorhabditis elegans embryo. Developmental Cell *16*, 889–900.

Nellesen, D.T., Lai, E.C., and Posakony, J.W. (1999). Discrete enhancer elements mediate selective responsiveness of enhancer of split complex genes to common transcriptional activators. Developmental Biology *213*, 33–53.

Neufeld, T.P. (2010). TOR-dependent control of autophagy: biting the hand that feeds. Current Opinion in Cell Biology *22*, 157–168.

Newfeld, S.J., Mehra, A., Singer, M.A., Wrana, J.L., Attisano, L., and Gelbart, W.M. (1997). Mothers against dpp participates in a DDP/TGF-beta responsive serine-threonine kinase signal transduction cascade. Development (Cambridge, England) *124*, 3167–3176.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, a (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379–391.

Niwa, H., Burdon, T., Chambers, I., and Smith, a. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & Development *12*, 2048–2060.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., et al. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proceedings of the National Academy of Sciences of the United States of America *102*, 14238–14243.

Nye, J.S., Kopan, R., and Kpan, R. (1995). Developmental signaling. Vertebrate ligands for Notch. Current Biology : CB *5*, 966–969.

Nystul, T., and Spradling, A. (2007). An epithelial niche in the Drosophila ovary undergoes long-range stem cell replacement. Cell Stem Cell *1*, 277–285.

Nystul, T., and Spradling, A. (2010). Regulation of epithelial stem cell replacement and follicle formation in the Drosophila ovary. Genetics *184*, 503–515.

O'Reilly, A.M., Lee, H.-H., and Simon, M. a (2008). Integrins control the positioning and proliferation of follicle stem cells in the Drosophila ovary. The Journal of Cell Biology *182*, 801–815.

Ogawa, T., Ohmura, M., Yumura, Y., Sawada, H., and Kubota, Y. (2003). Expansion of murine spermatogonial stem cells through serial transplantation. Biology of Reproduction *68*, 316–322.

Oh, H., Reddy, B.V.V.G., and Irvine, K.D. (2009). Phosphorylation-independent repression of Yorkie in Fat-Hippo signaling. Developmental Biology *335*, 188–197.

Ohata, S., Aoki, R., Kinoshita, S., Yamaguchi, M., Tsuruoka-Kinoshita, S., Tanaka, H., Wada, H., Watabe, S., Tsuboi, T., Masai, I., et al. (2011). Dual roles of Notch in regulation of apically restricted mitosis and apicobasal polarity of neuroepithelial cells. Neuron *69*, 215–230.

Ohlstein, B., and Spradling, A. (2006). The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature *439*, 470–474.

Ohlstein, B., Lavoie, C.A., Vef, O., Gateff, E., and McKearin, D.M. (2000). The Drosophila cystoblast differentiation factor, benign gonial cell neoplasm, is related to DExH-box proteins and interacts genetically with bag-of-marbles. Genetics *155*, 1809–1819.

Ohlstein, B., Kai, T., Decotto, E., and Spradling, A. (2004). The stem cell niche: theme and variations. Current Opinion in Cell Biology *16*, 693–699.

Orford, K.W., and Scadden, D.T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nature Reviews. Genetics *9*, 115–128.

Orlova, K. a, and Crino, P.B. (2010). The tuberous sclerosis complex. Annals of the New York Academy of Sciences *1184*, 87–105.

Palomero, T., Sulis, M.L., Cortina, M., Real, P.J., Barnes, K., Ciofani, M., Caparros, E., Buteau, J., Brown, K., Perkins, S.L., et al. (2007). Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nature Medicine *13*, 1203–1210.

Palomero, T., Dominguez, M., and Ferrando, A.A. (2008). The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. Cell Cycle (Georgetown, Tex.) *7*, 965–970.

Pan, D. (2010). The hippo signaling pathway in development and cancer. Developmental Cell *19*, 491–505.

Pan, D., Dong, J., Zhang, Y., and Gao, X. (2004). Tuberous sclerosis complex: from Drosophila to human disease. Trends in Cell Biology *14*, 78–85.

Pan, L., Chen, S., Weng, C., Call, G., Zhu, D., Tang, H., Zhang, N., and Xie, T. (2007). Stem cell aging is controlled both intrinsically and extrinsically in the Drosophila ovary. Cell Stem Cell *1*, 458–469.

Partridge, L., Alic, N., Bjedov, I., and Piper, M.D.W. (2011). Ageing in Drosophila: the role of the insulin/Igf and TOR signalling network. Experimental Gerontology *46*, 376–381.

Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. Science (New York, N.Y.) *287*, 1606–1609.

Perumalsamy, L.R., Nagala, M., Banerjee, P., and Sarin, a (2009). A hierarchical cascade activated by non-canonical Notch signaling and the mTOR-Rictor complex regulates neglect-induced death in mammalian cells. Cell Death and Differentiation *16*, 879–889.

Pignoni, F., and Zipursky, S.L. (1997). Induction of Drosophila eye development by decapentaplegic. Development (Cambridge, England) *124*, 271–278.

Piper, M.D.W., Mair, W., and Partridge, L. (2005). Counting the calories: the role of specific nutrients in extension of life span by food restriction. The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences *60*, 549–555.

Potter, C.J., Pedraza, L.G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nature Cell Biology *4*, 658–665.

Puig, O., Marr, M.T., Ruhf, M.L., and Tjian, R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes & Development *17*, 2006–2020.

Rajan, A., and Perrimon, N. (2012). Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. Cell *151*, 123–137.

Ramos, F.J., and Kaeberlein, M. (2012). Ageing: A healthy diet for stem cells. Nature *486*, 477–478.

Reichert, H. (2011). Drosophila neural stem cells: cell cycle control of self-renewal, differentiation, and termination in brain development. Results and Problems in Cell Differentiation *53*, 529–546.

Richardson, E.C.N., and Pichaud, F. (2010). Crumbs is required to achieve proper organ size control during Drosophila head development. Development (Cambridge, England) *137*, 641–650.

Robinson, B.S., Huang, J., Hong, Y., and Moberg, K.H. (2010). Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein Expanded. Current Biology : CB *20*, 582–590.

Roch, F., Polesello, C., Roubinet, C., Martin, M., Roy, C., Valenti, P., Carreno, S., Mangeat, P., and Payre, F. (2010). Differential roles of PtdIns(4,5)P2 and phosphorylation in moesin activation during Drosophila development. Journal of Cell Science *123*, 2058–2067.

Roeth, J.F., Sawyer, J.K., Wilner, D. a, and Peifer, M. (2009). Rab11 helps maintain apical crumbs and adherens junctions in the Drosophila embryonic ectoderm. PloS One *4*, e7634.

Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science (New York, N.Y.) *296*, 1118–1120.

Ruohola, H., Bremer, K.A., Baker, D., Swedlow, J.R., Jan, L.Y., and Jan, Y.N. (1991). Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in Drosophila. Cell *66*, 433–449.

Ryu, B.-Y., Orwig, K.E., Oatley, J.M., Avarbock, M.R., and Brinster, R.L. (2006). Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells (Dayton, Ohio) *24*, 1505–1511.

Saito, K., Araki, Y., Kontani, K., Nishina, H., and Katada, T. (2005). Novel role of the small GTPase Rheb: its implication in endocytic pathway independent of the activation of mammalian target of rapamycin. Journal of Biochemistry *137*, 423–430.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R. a, Kang, S. a, Spooner, E., Carr, S. a, and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Molecular Cell *25*, 903–915.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science (New York, N.Y.) *320*, 1496–1501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell *141*, 290–303.

Sanders, P.G.T., Muñoz-Descalzo, S., Balayo, T., Wirtz-Peitz, F., Hayward, P., and Arias, A.M. (2009). Ligand-independent traffic of Notch buffers activated Armadillo in Drosophila. PLoS Biology *7*, e1000169.

Sarbassov, D.D., Guertin, D. a, Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science (New York, N.Y.) *307*, 1098–1101.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., et al. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature *459*, 262–265.

Scadden, D.T. (2006). The stem-cell niche as an entity of action. Nature *441*, 1075–1079.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7–25.

Schroeter, E.H., Kisslinger, J. a, and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature *393*, 382–386.

Seals, D.F., Eitzen, G., Margolis, N., Wickner, W.T., and Price, a (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. Proceedings of the National Academy of Sciences of the United States of America *97*, 9402–9407.

Sell, S. (2004). Stem cell origin of cancer and differentiation therapy. Critical Reviews in Oncology/hematology *51*, 1–28.

Sell, S. (2010). On the stem cell origin of cancer. The American Journal of Pathology *176*, 2584–494.

Seppa, M.J., Johnson, R.I., Bao, S., and Cagan, R.L. (2008). Polychaetoid controls patterning by modulating adhesion in the Drosophila pupal retina. Developmental Biology *318*, 1–16.

Shackelford, D.B., and Shaw, R.J. (2009). The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nature Reviews. Cancer *9*, 563–575.

Shivas, J.M., Morrison, H. a, Bilder, D., and Skop, A.R. (2010). Polarity and endocytosis: reciprocal regulation. Trends in Cell Biology *20*, 445–452.

Silva, E., Tsatskis, Y., Gardano, L., Tapon, N., and McNeill, H. (2006). The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. Current Biology : CB *16*, 2081–2089.

Simons, B.D., and Clevers, H. (2011). Strategies for homeostatic stem cell self-renewal in adult tissues. Cell *145*, 851–862.

Simpson, F., Peden, a a, Christopoulou, L., and Robinson, M.S. (1997). Characterization of the adaptor-related protein complex, AP-3. The Journal of Cell Biology *137*, 835–845.

Skouloudaki, K., Puetz, M., Simons, M., Courbard, J.-R., Boehlke, C., Hartleben, B., Engel, C., Moeller, M.J., Englert, C., Bollig, F., et al. (2009). Scribble participates in Hippo signaling and is required for normal zebrafish pronephros development. Proceedings of the National Academy of Sciences of the United States of America *106*, 8579–8584.

Sneddon, J.B., and Werb, Z. (2007). Location, location, location: the cancer stem cell niche. Cell Stem Cell *1*, 607–611.

Song, X. (2003). wingless signaling regulates the maintenance of ovarian somatic stem cells in Drosophila. Development *130*, 3259–3268.

Song, X., and Xie, T. (2002). DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila ovary. Proceedings of the National Academy of Sciences of the United States of America *99*, 14813–14818.

Song, Y., and Lu, B. (2011). Regulation of cell growth by Notch signaling and its differential requirement in normal vs. tumor-forming stem cells in Drosophila. Genes & Development *25*, 2644–2658.

Song, X., Zhu, C.-H., Doan, C., and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science (New York, N.Y.) *296*, 1855–1857.

Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., McCarthy, J.J., and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development (Cambridge, England) *131*, 1353–1364.

Song, X., Call, G.B., Kirilly, D., and Xie, T. (2007). Notch signaling controls germline stem cell niche formation in the Drosophila ovary. Development (Cambridge, England) *134*, 1071–1080.

Sousa-Nunes, R., Yee, L.L., and Gould, A.P. (2011). Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila. Nature *471*, 508–512.

Struhl, G., and Adachi, a (1998). Nuclear access and action of notch in vivo. Cell *93*, 649–660.

Struhl, G., Fitzgerald, K., and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell *74*, 331–345.

Sun, P., Quan, Z., Zhang, B., Wu, T., and Xi, R. (2010). TSC1/2 tumour suppressor complex maintains Drosophila germline stem cells by preventing differentiation. Development (Cambridge, England) *137*, 2461–2469.

Suzuki, A., and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. Journal of Cell Science *119*, 979–987.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takahashi, K., Matsuo, T., Katsube, T., Ueda, R., and Yamamoto, D. (1998). Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in Drosophila morphogenesis. Mechanisms of Development *78*, 97–111.

Takahisa, M., Togashi, S., Suzuki, T., Kobayashi, M., Murayama, A., Kondo, K., Miyake, T., and Ueda, R. (1996). The Drosophila tamou gene, a component of the activating pathway of extramacrochaetae expression, encodes a protein homologous to mammalian cell-cell junction-associated protein ZO-1. Genes & Development *10*, 1783–1795.

Tatar, M. (2011). The plate half-full: status of research on the mechanisms of dietary restriction in Drosophila melanogaster. Experimental Gerontology *46*, 363–368.

Tatar, M., Kopelman, a, Epstein, D., Tu, M.P., Yin, C.M., and Garofalo, R.S. (2001). A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science (New York, N.Y.) *292*, 107–110.

Tepass, U. (2009). FERM proteins in animal morphogenesis. Current Opinion in Genetics & Development *19*, 357–367.

Tepass, U., and Knust, E. (1993). Crumbs and stardust act in a genetic pathway that controls the organization of epithelia in Drosophila melanogaster. Developmental Biology *159*, 311–326.

Tepass, U., Tanentzapf, G., Ward, R., and Fehon, R. (2001). Epithelial cell polarity and cell junctions in Drosophila. Annual Review of Genetics *35*, 747–784.

Thomson, J. a. (1998). Embryonic Stem Cell Lines Derived from Human Blastocysts. Science *282*, 1145–1147.

Thorgeirsson, S.S., and Grisham, J.W. (2006). Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. Hepatology (Baltimore, Md.) *43*, 2–8.

Till, J.E., and Mcculloch, E. a (1964). Repair Processes in Irradiated Mouse Hematopoietic Tissue. Annals of the New York Academy of Sciences *114*, 115–125.

Torres, I.L., López-Schier, H., and St Johnston, D. (2003). A Notch/Delta-dependent relay mechanism establishes anterior-posterior polarity in Drosophila. Developmental Cell *5*, 547–558.

Tschaharganeh, D.F., Chen, X., Latzko, P., Malz, M., Gaida, M.M., Felix, K., Ladu, S., Singer, S., Pinna, F., Gretz, N., et al. (2013). Yes-Associated Protein Upregulates Jagged-1 and Activates the NOTCH Pathway in Human Hepatocellular Carcinoma. Gastroenterology.

Vässin, H., Bremer, K.A., Knust, E., and Campos-Ortega, J.A. (1987). The neurogenic gene Delta of Drosophila melanogaster is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. The EMBO Journal *6*, 3431–3440.

Venkatachalam, K., Wong, C., and Montell, C. (2013). Feast or famine: role of TRPML in preventing cellular amino acid starvation. Autophagy *9*, 98–100.

Vereshchagina, N., Ramel, M.-C., Bitoun, E., and Wilson, C. (2008). The protein phosphatase PP2A-B' subunit Widerborst is a negative regulator of cytoplasmic

activated Akt and lipid metabolism in Drosophila. Journal of Cell Science *121*, 3383–3392.

Vincent, O., Townley, R., Kuchin, S., and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes & Development *15*, 1104–1114.

Visvader, J.E., and Lindeman, G.J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nature Reviews. Cancer *8*, 755–768.

Vo, K., Amarasinghe, B., Washington, K., Gonzalez, A., Berlin, J., and Dang, T.P. (2011). Targeting notch pathway enhances rapamycin antitumor activity in pancreas cancers through PTEN phosphorylation. Molecular Cancer *10*, 138.

Voog, J., and Jones, D.L. (2010). Stem cells and the niche: a dynamic duo. Cell Stem Cell *6*, 103–115.

Wang, X., and Proud, C.G. (2009). Nutrient control of TORC1, a cell-cycle regulator. Trends in Cell Biology *19*, 260–267.

Wang, Z., and Lin, H. (2004). Nanos maintains germline stem cell self-renewal by preventing differentiation. Science (New York, N.Y.) *303*, 2016–2019.

Wang, L., Li, Z., and Cai, Y. (2008a). The JAK/STAT pathway positively regulates DPP signaling in the Drosophila germline stem cell niche. The Journal of Cell Biology *180*, 721–728.

Wang, X., Harris, R.E., Bayston, L.J., and Ashe, H.L. (2008b). Type IV collagens regulate BMP signalling in Drosophila. Nature *455*, 72–77.

Wang, Z., Li, Y., Banerjee, S., Kong, D., Ahmad, A., Nogueira, V., Hay, N., and Sarkar, F.H. (2010). Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-kappaB signaling pathways. Journal of Cellular Biochemistry *109*, 726–736.

Ward, E.J., Shcherbata, H.R., Reynolds, S.H., Fischer, K. a, Hatfield, S.D., and Ruohola-baker, H. (2006). Stem cells signal to the niche through the Notch pathway in the Drosophila ovary. Current Biology : CB *16*, 2352–2358.

Watt, F.M., and Hogan, B.L. (2000). Out of Eden: stem cells and their niches. Science (New York, N.Y.) *287*, 1427–1430.

Wei, X., and Ellis, H.M. (2001). Localization of the Drosophila MAGUK protein Polychaetoid is controlled by alternative splicing. Mechanisms of Development *100*, 217–231.

Whelan, J.T., Kellogg, A., Shewchuk, B.M., Hewan-Lowe, K., and Bertrand, F.E. (2009). Notch-1 signaling is lost in prostate adenocarcinoma and promotes PTEN gene expression. Journal of Cellular Biochemistry *107*, 992–1001.

Wilkin, M.B., Carbery, A., Fostier, M., Aslam, H., Mazaleyrat, S.L., Higgs, J., Myat, A., Evans, D.A.P., Cornell, M., and Baron, M. (2004). Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. Current Biology : CB *14*, 2237–2244.

Wilkin, M.B., Tongngok, P., Gensch, N., Clemence, S., Motoki, M., Yamada, K., Hori, K., Taniguchi-kanai, M., Franklin, E., Matsuno, K., et al. (2008). Drosophila HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway. Developmental Cell *15*, 762–772.

Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C.-L., Tao, C., Zhang, X., and Halder, G. (2006). The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. Current Biology : CB *16*, 2090–2100.

Wilson, A.A., and Kotton, D.N. (2008). Another notch in stem cell biology: Drosophila intestinal stem cells and the specification of cell fates. BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology *30*, 107–109.

Wong, G.W., Knowles, G.C., Mak, T.W., Ferrando, A.A., and Zúñiga-Pflücker, J.C. (2012). HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRβ-selected mouse thymocytes. Blood *120*, 1439–1448.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471–484.

Xie, T., and Spradling, A.C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell *94*, 251–260.

Xie, T., and Spradling, A.C. (2000). A niche maintaining germ line stem cells in the Drosophila ovary. Science (New York, N.Y.) *290*, 328–330.

Xu, T., Caron, L. a, Fehon, R.G., and Artavanis-Tsakonas, S. (1992). The involvement of the Notch locus in Drosophila oogenesis. Development (Cambridge, England) *115*, 913–922.

Yan, L., and Lamb, R.F. (2012). Amino acid sensing and regulation of mTORC1. Seminars in Cell & Developmental Biology *23*, 621–625.

Yang, F.-C., Chen, S., Clegg, T., Li, X., Morgan, T., Estwick, S. a, Yuan, J., Khalaf, W., Burgin, S., Travers, J., et al. (2006). Nf1+/- mast cells induce neurofibroma like phenotypes through secreted TGF-beta signaling. Human Molecular Genetics *15*, 2421–2437.

Yilmaz, Ö.H., Katajisto, P., Lamming, D.W., Gültekin, Y., Bauer-Rowe, K.E., Sengupta, S., Birsoy, K., Dursun, A., Yilmaz, V.O., Selig, M., et al. (2012). mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature *486*, 490–495.

Yin, T., and Li, L. (2006). The stem cell niches in bone. The Journal of Clinical Investigation *116*, 1195–1201.

Yu, D., Chen, M., Sun, X., and Ge, J. (2013). Differentiation of mouse induced pluripotent stem cells into corneal epithelial-like cells. Cell Biology International *37*, 87–94.

Zhang, Y., and Kalderon, D. (2001). Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. Nature *410*, 599–604.

Zhao, R., Xuan, Y., Li, X., and Xi, R. (2008). Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila. Aging Cell *7*, 344–354.

Zhou, D., Zhang, Y., Wu, H., Barry, E., Yin, Y., Lawrence, E., Dawson, D., Willis, J.E., Markowitz, S.D., Camargo, F.D., et al. (2011). Mst1 and Mst2 protein kinases restrain intestinal stem cell proliferation and colonic tumorigenesis by inhibition of Yes-associated protein (Yap) overabundance. Proceedings of the National Academy of Sciences of the United States of America *108*, E1312–20.

Zhu, Y., Ghosh, P., Charnay, P., Burns, D.K., and Parada, L.F. (2002). Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science (New York, N.Y.) *296*, 920–922.

Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., and Sabatini, D.M. (2011). mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science (New York, N.Y.) *334*, 678–683.