Characterisation of genes involved in early oogenesis in *Drosophila melanogaster*.

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Life Sciences.

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Abstract

Tissue maintenance requires a balance between cell production and cell death. The former is dependent on the activity of stem cells, which in turn are dependent on both extrinsic signals produced by surrounding somatic tissue and intrinsic signals to control their behaviour. Additionally, stem cell activity may be regulated by systemic factors, demonstrating the complexity of stem cell regulation. The ovary of *Drosophila melanogaster* is a useful model for understanding tissue function as production of a viable egg requires the coordination of two different stem cell populations, the germline stem cells and follicle stem cells. In a screen designed to identify genes which regulate early oogenesis in the *Drosophila* ovary, we identified the four candidate genes which are described in the three papers found in this thesis. The first paper demonstrates that two RNA associated proteins, Ataxin 2 binding protein 1 and Gemin3, are essential for germline stem cell and follicle cell production in a Sex lethal dependent manner. The second shows that Glucuronyl transferase I, which is important for regulating the synthesis of key components of the extracellular matrix known as proteoglycans, is able to regulate the activity of several different signalling pathways. Finally, the third paper suggests that Defective proboscis extension response 9, a brain expressed gene involved in the behavioural response to alcohol, is important for regulating both follicle cells and germline stem cells at a systemic level. Taken together, these papers highlight the importance of intrinsic, extrinsic and systemic signalling in regulating stem cell function during *Drosophila* oogenesis.

Declaration

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Introduction to thesis and co-author contributions

This thesis follows the work of a former PhD student, Laura Ponting, in Dr. Baron's lab who carried out a screen of semi-lethal *Drosophila melanogaster* stocks to identify genes which regulate stem cells during oogenesis. From the candidates she identified, three were chosen for further analysis. These can be found in the results chapters in this thesis, which are presented as manuscripts ready for submission. Each results chapter describes the characterisation of one candidate mutant, identifying which genes are responsible for the oogenesis phenotypes observed in each. I will describe how stem cells contribute to tissue homeostasis and what is already known about *Drosophila* oogenesis in the Introduction while the General Discussion will contain a summary of the results, further conclusions and future experiments. Thus the unifying theme of this thesis is the identification of mechanisms which regulate tissue homeostasis and stem cells. Co-author contributions for the results chapters are outlined below.

Results chapter I.

The initial identification of this mutant was carried out by Laura Ponting.

Fig.1 Jump-out, associated PCRs and scoring carried out by Laura Ponting.

Fig.2 I repeated the Bag of Marbles immunofluorescence which was initially carried out by Laura Ponting. All other experiments and images are my own work.

Fig.3 Initial scoring for abnormal ovarioles alone was carried out by Laura Ponting. I repeated the complementation and scored individual phenotypes; Normal, 5n, Compound and Tumorous.

Fig.4 Experiment and images my own work.

Fig.5 Experiment and images my own work.

Fig.6 Experiment and images my own work.

Fig.7 Marian Wilkin carried out the X-Gal staining and scoring of the *Notch* and *Abruptex* alleles over wild type. I scored the interaction of these alleles with $A2bp1^{E03440}$, repeating Marian's experiment as a control. All images are Marian Wilkin's.

Supplemental S1. Experiment and images my own work.

Supplemental S2. Experiment and images my own work.

Supplemental S3. Alessandro Bonfini set up the crosses and carried out the dissection and staining. I scored for the abnormal ovariole phenotype.

Results chapter II.

The initial identification of this mutant was carried out by Laura Ponting.

Fig.1 Experiment and images my own work.

Fig.2 Experiment and images my own work.

Fig.3 Experiment and images my own work.

Fig.4 Experiment and images my own work.

Fig.5 Experiment and images my own work.

Fig.6 Experiment and images my own work.

Fig.7 Experiment and images my own work.

Fig.8 Experiment and images my own work.

Supplemental S1. Experiment and images my own work.

Supplemental S2. Experiment and images my own work.

Supplemental S3. Experiment and table my own work.

Results chapter III.

The initial identification of this mutant was carried out by Laura Ponting.

Fig.1 Scoring and acquisition of images carried out by Laura Ponting, excluding the images in C-F which were repeated by me.

Fig.2 Jump-out, associated PCRs, RT PCRs and scoring carried out by Laura Ponting.

Fig.3 Complementation analysis carried out by Laura Ponting.

Fig.4 Experiment and images my own work.

Fig.5 Experiment and images my own work.

Fig.6 BLAST search and alignment carried out by me.

Abbreviations

A2BP1	Ataxin2 binding protein 1
AGO1	Argonaute 1
ALDH1	Aldehyde dehydrogenase 1
ATP	Adenosine triphosphate
AX	Abruptex
BAM	Bag of marbles
BGCN	Benign gonial cell neoplasm
BMP	Bone morphogenetic proteins
BRAT	Brain tumour
CB	Cystoblast
cDNA	Complementary dexoyribonucleic acid
CSPG	Chondroitin sulphate proteoglycan
DAD	Daughters against decapentaplegic
DALLY	Development abnormally delayed
DAPI	4'-6-diamidino-2-phenylindole
DA	Daughterless
DCR1	Dicer 1
DEB	Dystrophic epidermolysis bullosa
DIAP1	Drosophila inhibitor od apoptosis
DILP	Drosophila insulin like peptides
DLP	Dally like protein
DNA	Deoxyribonucleic acid
DOM	Domino
DPP	Decapentaplegic
DPR9	Defective proboscis extension response 9
EC	Escort cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FASIII	Fasciclin III
FGF8	Fibroblast growth factor 8
FLP	Flippase
FOX1	Forkhead box 1
FOXO	Forkhead box, subgroup O
FSC	Follicle stem cells
FU	Fused
GAG	Glycosaminoglycan
GAL	Galactose
GBB	Glass bottom boat
GDNF	Glial cell line derived neurotrophic factor
GlcATI	Glucuronyl transferase I
GSC	Germline stem cells
HGPS	Hutchinson-Gilford progeria syndrome
HSPG	Heparin/Heperan sulphate proteoglycans

HH	Hedgehog
HOP	Hopscotch
HP1	Heterochromatin protein 1
Ig	Immunoglobulin
INR	Insulin receptor
ISC	Intestinal stem cells
ISWI	Imitation SWI
JAK	Janus Kinase
KLF4	Krueppel-like factor 4
LIS-1	Lisencephaly-1
LOQS	Loquacious
MAD	Mother's against Dpp
MEL	Maternal effect lethal
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
N	Notch
NOS	Nanos
OCT3/ 4	Octamer binding transcription factor 3/ 4
ODA	Antizyme
ODC	Ornithine decarboxylase
ORB	Oo18 RNA binding protein
PBS Tw	Phosphate buffered saline 0.1% Tween
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3 kinase
piRNA	PIWI interacting ribonucleic acid
PUM	Pumilios
RBP9	RNA binding protein 9
RHO	Rhomboid
RNAi	Ribonucleic acid interference
RRM	RNA recognition motif
RT PCR	Reverse transcription polymerase chain reaction
S6K	p70 ribosomal S6 kinase
SCN	Schnurri
SMA	Spinal muscular atrophy
SMN	Survival motor neuron
SMURF	Smad ubiquitin regulatory factor
SNF	Sans fille
snRNP	Small nuclear ribonucleoproteins
SOX2	Sex determining region Y-box 2
STAT	Signal Transducer and Activator of Transcription
SULF1	Sulfated 1
SXL	Sex lethal
TKV	Thick veins
TOR	Target of rapamycin
UPD	Unpaired
WG	Wingless

XYL	Xylose
YB	fs(1)Yb

Chapter 1. *Introduction*

1 Tissue homeostasis

Although the outward appearance of many multicellular organisms remains relatively consistent over a lifetime, there are few individual cells which survive for that entire period. This discrepancy between an organism's lifespan and that of an individual cell is due to a process known as tissue homeostasis, which ensures that tissues are able to function for a greater length of time than any one individual cell can survive for. One of the major aspects of homeostasis is the elimination of cells which are no longer able to function and their replacement with an equivalent cell type. This is called tissue renewal. It has been estimated that a human being loses, and subsequently replaces, a population of cells almost equal to their body weight each year (Pellettieri and Sanchez Alvarado, 2007).

There are many tissues in humans which undergo constant turnover, with each having different rates of growth. For example, red blood cells only live for approximately one hundred and thirty days (Shemin and Rittenberg, 1946). Also, the lining of the intestine and lung is continually being replaced, epithelial cells in the former lasting five days, and in the latter up to six months (Rawlins and Hogan, 2008). Additionally, there are tissues which undergo cyclic tissue renewal, the best example being the uterus which sheds its lining each month during the menstrual cycle (Ferenczy, 1976; Gargett, 2006). The lifespan of a hair follicle is also cyclic, consisting of a growth phase, a transition phase and a resting period, during which the hair falls out (Blanpain *et al*., 2004). In addition to constant turnover, tissues must also be able to repair themselves, including replacing lost cells, after injury. This means that not only must tissues be able to constantly produce cells, but they must also be able to sense when increased proliferation is required. The best example of this is perhaps seen in wound healing (Singer and Clark, 1999).

The process of replacing a dead cell in a tissue is dependent on the function of adult stem cells. These are undifferentiated cells capable of dividing to produce progeny which will differentiate into an appropriate cell type. All of the examples listed above, taken together, illustrate that tissue renewal is a complex process. Different populations of adult stem cells must function in very diverse environments and must meet the demands of the tissues they find themselves in. Some adult stem cells will divide at a much greater rate and some must divide at a particular time. Additionally, they must produce the appropriate cell type in an appropriate proportion. In order to do this, adult stem cells often rely on signals produced by somatic cells in their environment. Because of their important role in the maintenance of tissues, misregulation of stem cells can lead to many problems, including cancer and ageing. This means understanding the signals which control stem cell behaviour is essential.

2 Stem Cells

2.1 What are stem cells?

Stem cells are multipotent cells, capable of dividing mitotically to produce two daughter cells which will adopt different fates. Usually, one will differentiate while the other will retain stem cell like qualities. There are three classes of stem cells; embryonic stem cells which are produced during the early stages of embryogenesis, adult stem cells which are important for maintaining tissues during an organism's life time and germline stem cells which are essential for producing haploid gametes (Bongso and Richards, 2004). Tissues rely on small populations of adult stem cells to prevent degeneration through homeostatic loss of cells or injury (Slack, 2000). Unlike embryonic stem cells, which are capable of producing any cell type in the body, adult stem cells are less pluripotent and their progeny are limited to a few cell lineages. For example, haematopoetic stem cells are capable of forming all the cellular components of

blood and some cell types found in liver, muscle and central nervous system tissue (Brazelton *et al*., 2000, Ferrari *et al*., 1998, Petersen *et al*., 1999).

2.2 Maintaining stem cell populations

Given the role of stem cells in producing differentiated cells and their subsequent ability to influence a tissue's composition, their behaviour must be tightly regulated. That is, the correct number of stem cells must be maintained and these must proliferate, differentiate and self-renew appropriately. In order to achieve tissue homeostasis, this balance between differentiation and self-renewal must be maintained throughout an organism's life span.

There are two strategies by which a population of stem cells may be maintained; asymmetric division and population self renewal. In the former, stem cells undergo invariant divisions which produce one daughter cell that will go on to differentiate and one daughter cell which will retain a stem cell like fate. This is usually dependent on factors present within the stem cell itself, such as the internal accumulation of cell fate determinants. A good example of this is the localisation of PAR proteins in the *Caenorhabditis elegans* zygote. PAR proteins control the orientation of the mitotic spindle, determining the plane of division and influencing the localisation of other factors required for differentiation (Etemad-Moghadam *et al*., 1995; Morrison and Kimble, 2006). Another example is the asymmetric localisation of Numb during division of neuroblasts in the *Drosophila* embryo (Wakamatsu *et al*., 1999; Lee *et al*., 2006). Alternatively, stem cells may be regulated in a much more stochastic manner. In this instance, stem cell behaviour is determined at the population level. All dividing stem cells in this model have the same potential to differentiate or remain as a stem cell. The result is that some stem cells will undergo symmetric division to produce either two stem cells or two transient amplifying cells or they may undergo asymmetric division,

producing unequal daughters. The decision is made randomly but all three of the outcomes of stem cell division occur at an equal frequency, leading to a stable population of stem cells (Snippert and Clevers, 2011). This means that the lifespan of a single stem cell in this model is not predictable but the behaviour of the population of stem cells is. Examples of this are seen in epithelial tissues like the epithelial stem cells of the mammalian small intestine. In this instance, Leucine-rich repeat-containing G-protein coupled receptor 5-positive stem cells found in intestinal crypts divide and give rise to enterocytes, goblet cells, paneth and enteroendocrine cells. If a population of epithelial stem cells within a crypt are tagged with different coloured fluorescent markers, over time, the crypt becomes dominated by one colour. This suggests most of the stem cells have been lost and the remaining cells in the tissue are the progeny of a single stem cell (Snippert and Clevers, 2011). A similar example is seen in mammalian testis. Spermatogonia, which give rise to transit amplifying cells that will eventually become spermatids, are randomly lost and replaced by their neighbours (Klein *et al*., 2010).

2.3 Intrinsic and extrinsic signalling in stem cells

Many components play a role in regulating stem cell self renewal and differentiation. These can be intrinsic, which are required cell-autonomously, or extrinsic, which are non-cell autonomous and act in the local microenvironment that the stem cell resides in. Additionally, systemic factors such as hormones are able to co-ordinate stem cell behaviour over large distances.

Examples of intrinsic factors that play roles in regulating stem cell behaviour are Numb and Prospero. These are required for the differentiation of *Drosophila* neuroblasts which undergo division to produce a ganglion mother cell and a replacement neuroblast. The former requires the localisation of the PAR complex to the

apical membrane, the latter requires Brain tumour (BRAT), Numb and Prospero to be localised to the basal membrane. The mitotic spindle must align itself correctly in relation to the position of these protein complexes at the surface membrane. Aurora A kinase mutants have misaligned mitotic spindles and poor localisation of Numb, leading to symmetric division of neuroblasts. The result is an over abundance of neuroblasts (Lee *et al*., 2006). Another feature of stem cells is the expression of telomerase, which is required to extend telomeres. These are structures found at the end of each chromosome and consist of repeating sequences. In cells in which telomerase is not active, telomeres become gradually shorter with each replication since polymerase is unable to replicate the ends of deoxyribonucleic acid (DNA). Telomerase deficient mice show atrophy of tissues which have a high cell turnover and they also have shortened telomeres (Blasco, 2007)**.**

Epigenetic modifications of chromatin also play a role in regulating stem cells. These include acetylation and methylation of histones, which are protein complexes that associate with DNA. It has been shown that mammalian embryonic stem cells undergo significant chromatin remodeling; markers for heterochromatin, such as Heterochomatin Protein -1 (HP1), are dispersed in embryonic stem cells, and form progressively more punctuate foci as the stem cells divide and begin to differentiate. This suggests that the genome has more heterochromatin and, consequently, more transcriptionally restrained regions in differentiating cells (Meshorer *et al*., 2006). It is also known that differentiated cells can be epigenetically reprogrammed into pluripotent stem cells, indicating the importance of epigenetics in stem cell behaviour (Kim *et al*., 2011).

A final example of the importance of intrinsic factors in regulating the fate of a stem cell's progeny is seen in the proteins required to induce pluripotency in somatic cells. Using a mouse embryonic stem cell-specific factor fused with an antibiotic resistance gene, Takahashi and Yamanaka, (2006) were able to demonstrate that mouse

embryonic fibroblasts can be reverted into an embryonic stem cell-like state using only four transcription factors; Octamer-binding transcription factor 3/4 (OCT3/4), Krueppel-like factor 4 (KLF4), Sex determining region Y-box 2 (SOX2) and c-Myc (Takahashi and Yamanaka, 2006). With the use of the Lysine 4 Histone 3 demethylation inhibitor, tranylcypromine, it is possible to induce pluripotency in mouse embryonic fibroblasts with the aid of OCT4 alone (Li *et al*., 2011). In addition to the transcription factors listed above, micro ribonucleic acids (miRNAs) are intrinsic factors which can induce pluripotency. Loss of the miRNA processing enzyme, Dicer, leads to an inability of mouse embryonic fibroblasts to differentiate even in the presence of OCT3/4, KLF4, SOX2 and c-Myc, indicating the importance of post-transcriptional regulation in controlling stem cells (Kim, *et al*., 2012).

Alternatively, instead of relying entirely on internal factors, stem cells can depend on the surrounding environment for extrinsic signals, which control their behaviour. This environment is referred to as the 'niche' and can be made up of permanent somatic cells or the extracellular matrix (Decotto and Spradling, 2005; Nystul and Spradling, 2007; Morrison and Spradling, 2008). In this case, stem cells must express intrinsic factors that allow them to respond to these signals, while their neighbouring somatic cells do not. The structure of niches can vary greatly, with different stem cells being distributed through their corresponding tissue differently. For example, the germline stem cell (GSC) niche in the *Drosophila* ovary has specific locations for each stem cell population, which will be described later. Other niches, however, have stem cells scattered randomly within tissues. An example of this is seen in the *Drosophila* intestine where the intestinal stem cells (ISC) reside. ISCs are required to produce the cells that form the epithelium of the gut. These stem cells reside inside crypts where they receive signals which control their behaviour from the muscle cells lying below the basement membrane of the gut wall (Lin *et al*., 2008).

The use of a niche also allows stem cells to divide in response to the demands of the tissue they find themselves in. Examples of stem cells that are dependent on the niche for signals are seen in tissues which have to repair themselves after injury. Satellite cells in muscle tissue, for instance, are responsible for producing new muscle tissue. Normally they are quiescent, however, damage to muscle tissue triggers a local inflammatory response and subsequent changes to the niche structure, leading to satellite cell activation (Wozniak *et al*., 2005; Gopinath and Rando, 2008; Gopinath *et al*., 2010; Shavlakadze *et al*., 2010)**.**

Other niche dependent stem cells include intestinal stem cells in the Drosophila gut. In flies which are fed well, the midgut increases in size, while fasted animals have a shrunken midgut. O'Brien *et al* (2011), demonstrated that this increase in gut size is due to an increase in the number of symmetric divisions of the ISCs and this is reliant on insulin like peptides secreted from the underlying visceral muscle tissue. This shows the importance of the niche in modulating stem cell activity (O'Brien *et al*., 2011).

Mammalian GSCs, known as spermatogonia, are another niche dependent stem cell population. They divide continuously to produce sperm throughout a male's reproductive lifespan and reside in specialised structures called seminiferous tubules. In order to function, spermatogonia require glial cell line-derived neurotrophic factor (GDNF) to be produced by the surrounding somatic tissue known as Sertoli cells. (Meng *et al*., 2000; Tadokoro *et al*., 2002). Interestingly, Sertoli cells will rapidly boost the expression of GDNF in the absence of spermatogonia. It has been suggested this upregulation may be to ensure any remaining spermatogonia are kept in an undifferentiated state. In this case, the niche is responding to an absence of stem cells, suggesting that the signalling between the niche and resident stem cells functions in both directions (Zohni *et al*., 2012).

Finally, systemic signals are known to play a role in regulating stem cells. This allows for co-ordination of stem cells over much greater distances. Hormones such as insulin are important for regulating the behaviour of stem cells. In the *Drosophila* female, for example, insulin-like peptides synthesised in the brain are known to play a role in controlling egg production. Another example is the control of GSC maintenance in the ovary by Ecdysone (Hsu and Drummond-Barbosa, 2009; Konig *et al*., 2011). In mammals, it is known that a systemic factor called Insulin-like growth factor -1 is essential for maintaining muscle mass in mammals (Shavlakadze *et al*., 2010**).** In addition to this, old satellite cells which are exposed to a young systemic environment are able to upregulate Notch, as young satellite cells do, further supporting the idea that systemic factors play a role in regulating satellite cells (Conboy *et al*., 2005). It has also been shown that epithelial stem cells and progenitor cells found in human endometrial tissue rely on systemic estrogen to control their proliferation during the menstrual cycle (Cooke *et al*., 1997; Gargett *et al*., 2008).

2.4 Why study stem cells?

Since stem cells have the ability to self-renew and they are able to differentiate into multiple cell types, they may also have immense therapeutic potential. For example, transplantation of stem cells which have been forced to differentiate into particular cell types may be capable of alleviating the symptoms of diseases such as Parkinson's and diabetes (Ramiya *et al*., 2000; Kim *et al*., 2002; Soldner *et al*., 2009; Jian *et al*., 2012). In other instances, stem cells are providing useful models for understanding diseases such as autism spectrum disorders and hepatitis C infections (Kim *et al*., 2012; Schwartz *et al*., 2012). Induced pluripotent stem cells generated from patients with dystrophic epidermolysis bullosa (DEB), which is caused by mutation in collagen 7, are currently being investigated as a potential therapy for reducing the

severe blistering symptoms of DEB (Tolar *et al*., 2011a; Tolar *et al*., 2011b). Embryonic stem cells have also been shown to regenerate heart tissue in mice which have suffered myocardial infarctions (Orlic, 2003). Stem cells may also eventually be used in the treatment of Duchenne's muscular dystrophy (Bittner *et al*., 1999). In addition, embryonic stem cell research will enhance our understanding of early development (Bongso and Richards, 2004). Another example of stem cell based therapy is the use of bone marrow transplants. This involves the use of stem cells harvested from a donor and injected into a patient as treatment for disorders of the blood or bone marrow, for example, leukaemia and thalassemias. This therapy relies on the principle that stem cells are able to colonise bone marrow, divide, differentiate and self-renew (Sadelain *et al*., 2008). Understanding stem cell behaviour may also provide insights into how diseases such as cancer progress and how aging occurs.

2.4.1 Cancer

Misregulation of cell behaviour which leads to aberrant proliferation may lead to tumour formation in multicellular organisms (Campisi, 2004). Some malignant tumours contain different cell types, including a population of cells which retain the ability to proliferate, i.e. so-called cancer stem cells (Lapidot *et al*., 1994)

Only a small number of cells in an ovarian tumour are capable of forming colonies in agar, suggesting that few cells in a tumour may have the ability to divide (Hamburger and Salmon, 1977). Additionally, a small subset of cells in brain tumours seem to have stem cell like properties, including self renewal, multipotency and expression of stem cell associated markers, thus suggesting a hierarchy in tumours where stem cell-like cells proliferate and give rise to non-dividing, differentiated cells (Bapat *et al*., 2005; Singh *et al*., 2004).

A role for the microenvironment in controlling the behaviour of cancer stem cells has been demonstrated in a mouse model for brain tumours (Calabrese *et al*., 2007).

This is also true for endothelial cells which upregulate Notch, providing a niche for glioblastoma cancer stem cells, thus leading to cancer stem cell maintenance (Zhu *et al*., 2011). Another example which demonstrates the importance of the surrounding environment in cancer development is seen with breast cancer stem cells. In this instance, induction of hypoxia leads to an increase in the number of cancer stem cells present in the tumour (Conley *et al*., 2012). Thus a potential therapeutic aim in the treatment of cancer may be to target components of the microenvironment. In addition to specifically targeting the niche to kill cancerous cells, another approach may be to modulate niche signalling to protect the normal stem cell population during treatment with cytotoxic compounds. For example, Adams, *et al* (2006) found that, by using parathyroid hormone as a prophylactic, they could maintain the haematopoietic stem cell population in a mouse model for leukaemia during treatment with cyclophosphamide, a drug used to treat cancer which usually reduces the number of haematopoietic stem cells (Adams *et al*., 2007). Finally, Hansford, *et al* (2007) demonstrated that it was possible to identify a population of cancer stem cells in patients with neuroblastomas. In some cases, these cells were isolated from patients who were clinically in remission and later relapsed, suggesting that the ability to identify cancer stem cells may allow physicians to offer a more accurate prognosis for patients who are at a higher risk of relapse (Hansford *et al*., 2007). Other cancer stem cell markers have been identified for various types of cancer, such as Aldehyde dehydrogenase 1 (ALDH1), which may make assessing the efficacy of cancer therapies more accurate (Loebinger *et al*., 2008; Ma and Allan, 2011; Brunner *et al*., 2012).

All of the above require a better understanding of both the cancer stem cell niche and the behaviour of cancer stem cells themselves in order to make them viable options for treating different types of cancer.

2.4.2 Aging

Many diseases which are associated with old age are caused by the degeneration of specific tissues, for example, osteoporosis, sarcopenia and anaemia. A reduction in stem cell number or a decline in function may be partially responsible for the onset of these diseases (Rossi *et al*., 2008). If the rate of cell death is not modulated appropriately, the result is a decline in the differentiated cell population, leading to reduced tissue function.

Greying hair in humans is an example of how reduced stem cell numbers leads to changes in tissues; this appears to be a result of the loss of melanocyte stem cells (Nishimura *et al*., 2005). Another example is the reduction of mesenchymal stem cells in human bone marrow which may contribute to the reduction in bone density associated with age (D'Ippolito *et al*., 1999). A dramatic example of the importance of stem cells in maintaining tissues is seen in the genetic disorder, Hutchinson-Gilford progeria syndrome (HGPS), in which affected children develop many of the disorders associated with advanced age, including stiff joints and heart disease (DeBusk, 1972; Merideth *et al*., 2008). Mouse models of HGPS show decreased numbers of epidermal stem cells in the skin which leads to problems such as impaired wound healing (Rosengardten *et al*., 2011).

Aging in stem cells may be caused by intrinsic factors, including telomere attrition, generation of reactive oxygen species and the accumulation of DNA damage (Reviewed in Rossi *et al*., 2008). It has recently been demonstrated that restoring the expression of telomerase in adult mice which are telomerase-deficient rescues many of the degenerative phenotypes found in such mice, including atrophy of the testis and intestinal lining (Jaskelioff *et al*., 2011). Jaskelioff, *et al*. (2011) also found that restoring telomerase expression led to a reduction in the amount of apoptosis and DNA damage present in telomerase-deficient mice (Jaskelioff *et al*., 2011). Another example

of an intrinsic factor which has been associated with ageing is the aberrant form of LaminA, Progerin, which is responsible for the nuclear abnormalities seen in patients with HGPS (Glynn and Glover, 2005). Overexpression of Progerin in an immortalised human mesenchymal stem cell population leads to the misregulation of downstream targets of Notch signalling and abnormal differentiation which may explain the rapid aging seen in HGPS patients (Scaffidi and Misteli, 2008). Sporadic expression of Progerin has also been observed in wild type cells, suggesting that Progerin may play a role in the normal ageing process (Scaffidi and Misteli, 2006).

A reduction in extrinsic signalling, such as Notch signalling, has also been implicated in an age-dependent decline in muscle satellite cell function in mice (Conboy *et al*., 2005). Older satellite cells are less likely to upregulate the expression of Delta, the Notch ligand, when damaged than younger satellite cells (Conboy *et al*., 2005). Bone Morphogenetic Proteins (BMP) have also been implicated in the reduction of stem cell activity in the *Drosophila* germline (Pan *et al*., 2007). A third example of how extrinsic signals can influence stem cell behaviour is seen with Matrix metalloproteinase-1 in the Drosophila midgut. The expression of this secreted protein increases with age, concurrent with a reduction in the number of ISCs. Conversely, reducing Matrix metalloproteinase-1 leads to an increase in ISC proliferation (Lee *et al*., 2012), showing that age-related changes in the extracellular environment can contribute to a reduction in stem cell proliferation. Thus understanding stem cell regulation may lead to improved methods of managing age related diseases.

3 Drosophila ovaries

3.1 Why use Drosophila as a model system?

Drosophila melanogaster has many qualities which make it amenable to genetic manipulation. This includes their short life span, their ability to reach breeding age quickly and the fact that they are easy to maintain. In addition to this, there are several

genetic tools available which are useful for studying many biological processes. These include balancer chromosomes which inhibit recombination in females and contain a lethal dominant marker to allow for simple identification of desired mutants. Another powerful genetic tool is the P-element, which, in the presence of transposase, can be inserted into the genome at random points. Both of these features allow large scale screens for identifying genes to be constructed and carried out.

The *Drosophila* ovary is an ideal model for studying regulation of tissue renewal because the female constantly produces mature eggs. Each egg is composed of several different cell types which must be produced in the appropriate proportions in order to assemble a viable egg. Since these cell types are descended from two separate cell lineages, the ovary must be able to co-ordinate both the proliferation and differentiation of two different populations of stem cells. This co-ordination of stem cells is dependent on surrounding somatic cells. The cap cells are required for maintaining GSCs while escort cells (ECs) are essential for both maintaining follicle cells and moving cysts through the germarium (Fig. 1D). This means the *Drosophila* ovary is also a useful model for understanding niche-dependent stem cells.

The fact that the two ovarian stem cell lineages have invariant positions within the germarium is also a useful feature of a model for studying stem cell behaviour. Markers, such as α-Spectrin and Coracle, can be used to recognise key structures in the germarium, allowing for the easy identification of stem cells (Margolis and Spradling, 1995; Spradling *et al*., 1997; Decotto and Spradling, 2005) (Fig. 1E). In conjunction with this, given that eggs at various stages of development have key morphological features, determining an egg chamber's age within the ovarioles is relatively simple (King, 1957; Spradling *et al*., 1997). Taken together, this means that the phenotypes of genes linked with oogenesis can be analysed easily. Additionally, there are parallels between the stem cells found in the *Drosophila* ovary and stem cells in other tissues.

For example, follicle stem cells resemble human basal epithelial stem cells in that they both use Hedgehog (HH) signalling (Johnson *et al*., 1996; Spradling *et al*., 1997).

3.2 Drosophila ovary structure and oogenesis

Female *Drosophila melanogaster* are capable of producing large numbers of eggs throughout their lifetime. This efficient production of eggs is due to the organisation of the ovary. Adult females contain two ovaries which are surrounded by a peritoneal sheath and connected by a common oviduct. Inside each ovary are around fifteen to twenty ovarioles that function as independent egg producing chambers (Fig. 1A). The germarium, where egg production begins, can be found at the anterior tip of the ovariole. Egg chambers bud off from the germaria and move towards the posterior end of the ovariole, becoming progressively more mature until they reach the posterior tip of the ovariole. They then pass into the common oviduct where they are fertilised by sperm stored in the seminal receptacle (Fig. 1A). The outer sheath of each ovariole contains smooth muscle cells which help push the maturing egg chambers to a more posterior position (Spradling *et al*., 1997).

Each ovariole consists of several egg chambers at varying stages of maturity which can be identified as one of fourteen different developmental stages (Fig. 1B,C). Each egg chamber contains an oocyte and fifteen nurse cells which are surrounded by a layer of follicle cells. These follicle cells will go on to form the outer layer of the egg and the oocyte will form the embryo. At a late stage in oogenesis, the nurse cells will dump messenger ribonucleic acid (mRNA) into the oocyte, establishing the polarity of the oocyte (Foley and Cooley, 1998).

The germarium, which can be divided into regions 1, 2a, 2b and 3 along its anterior-posterior axis, houses the stem cell populations which are needed to produce the cell types found in egg chambers (Fig. 1D,E). Region 1 contains the GSCs, which

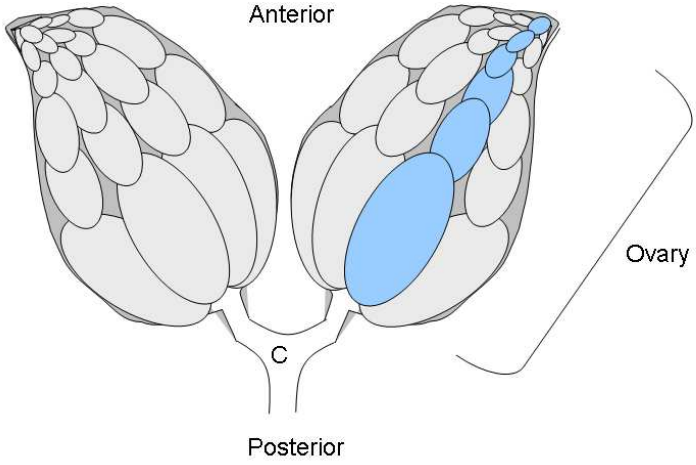
reside at the anterior most tip of the germarium, next to the cap cells and the terminal filaments, and cysts which are undergoing incomplete mitosis (Robinson *et al*., 1994). Typical division of a GSC generates a replacement GSC and a daughter cell, known as a cystoblast (CB), which will subsequently undergo four incomplete mitoses to produce a cluster of sixteen cells. This is referred to as a cyst. All the cells in a cyst are linked by a branched structure known as the fusome, which allows for the exchange of cytoplasmic components and may be needed for controlling the synchronicity of these mitotic divisions (Lin *et al*., 1994). In the developing cyst, only two of the cells will have four ring canals. These are actin-rich structures that form at the junction between two connected germline cells (Fig. 1F). One of these two cells will differentiate into the oocyte, the other will become a nurse cell (King, 1957; Gonzalez-Reyes, 2003). The fusome is formed from the spectrosome, which is a round structure seen in GSCs and is in contact with somatic cells at the anterior most tip of the germarium. Both the spectrosome and fusome contain specific proteins such as α-Spectrin, and Hu-Li Tai Shao which can both be identified by immunohistochemistry (Yue and Spradling, 1992; de Cuevas *et al*., 1996). The developing cyst moves through the germarium and is enveloped by the cytoplasmic processes of stationary ECs (Morris and Spradling, 2011).

Region 2a consists of sixteen-cell cysts which are beginning to be surrounded by follicle cells. By region 2b, the cyst will flatten into a distinctive lens shape (Robinson *et al*., 1994). The second population of stem cells, known as follicle stem cells (FSCs), are found within the germarium at the 2a/2b region boundary (Decotto and Spradling, 2005). These stem cells differentiate to form follicle cells that will surround the CB. Following this, these follicle cells will then go on to further differentiate into either epithelial cells that surround the egg chamber or stalk cells that connect each individual egg chamber, giving ovarioles the appearance of beads on a string (Torres *et al*., 2003; Assa-Kunik *et al*., 2007). After region 2b, the cyst moves into region 3 where it

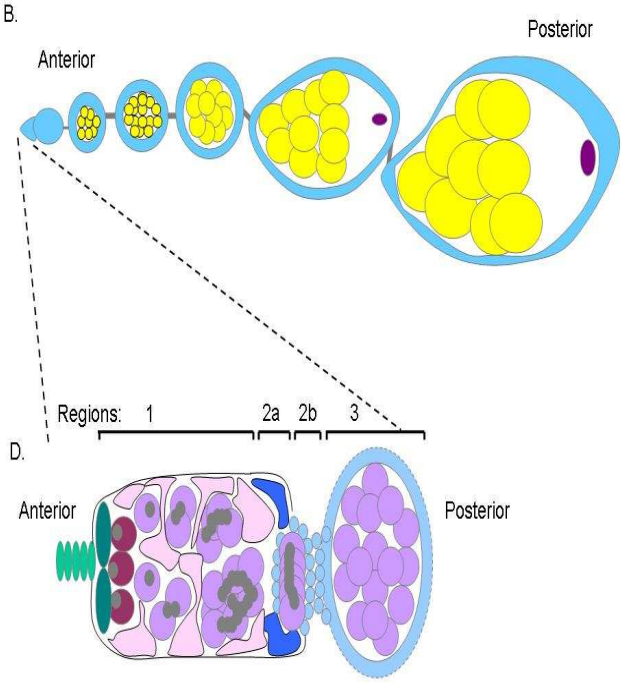
becomes more spherical and is ready to bud off from the germarium (Robinson *et al*., 1994).

Fig. 1. Drosophila ovaries and egg production in Drosophila melanogaster. **A.** The two ovaries, surrounded by a periotoneal sheath, share a common oviduct (C). Each ovary consists of around 15-20 ovarioles (example is illustrated in blue). **B.** The ovariole consists of several egg chambers of different maturitiy. The most mature egg chambers are found in the posterior region of the ovariole. (blue= follicle cells, yellow= nurse cells, purple oocyte. **C.** Microscopic image of an ovariole. 1st egg chamber stage and germarium (white line) are indicated. **D.** The germarium consists of terminal filament cells (pale green) and cap cells (dark green). Next to these are the germline stem cells (dark purple) (GSCs) which contain a spectrosome (grey circle). The GSCs divide and form the cystoblast (pale purple circles). The cystoblast is surrounded by escort cells (pale orange). The escort cells are replaced by follicle cells (pale blue) which are the progeny of somatic stem cells (dark blue). The regions of the germaria are indicated above the illustration. **E.** Microscopic image of a germarium. Blue=nuclei, Magenta=Coracle (a septate junction marker), Green=αSpectrin (a component of the cytoskeleton). EC= Escort cell, GSC= germline stem cell. Numbers indicate germarial regions. **F.** The germline stem cell (blue) undergoes four rounds of incomplete mitosis to form the cystoblast (magenta). The cells of the cystoblast are linked by ring canals and fusomes (black circles and red lines respectively). (Adapted from King, 1957; de Cuevas *et al*., 1996; Spradling *et al*., 1997).

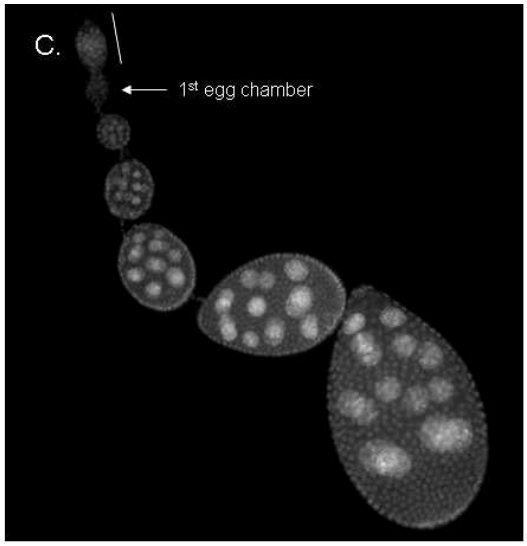
A.


Anterior
Ovary
C
Posterior

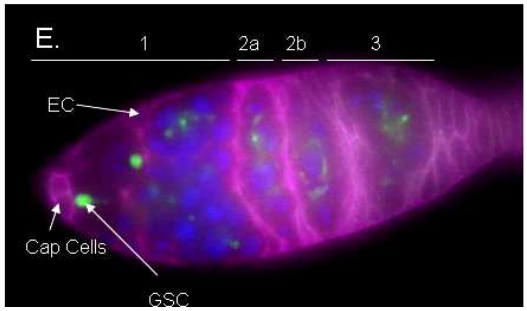
B.


Posterior
Anterior
Regions: 1 2a 2b 3
D.
Anterior
Posterior

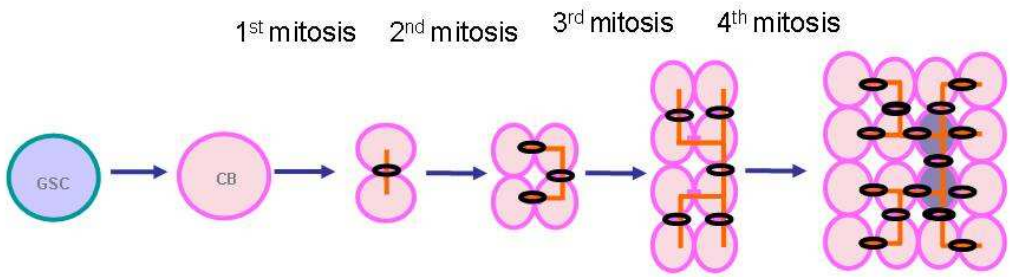
C.


1st egg chamber

E.


1 2a 2b 3
EC
Cap Cells
GSC

F.


1st mitosis
2nd mitosis
3rd mitosis
4th mitosis
GSC
CB

3.3 Germline stem cells

Of the two stem cell populations in the *Drosophila* ovary, the pathways controlling GSC behaviour are the best characterised. The niche at the most anterior tip of the germarium houses 2-3 GSCs (Wieschaus and Szabad, 1979). Ablation of GSCs leads to a loss of egg chambers, demonstrating their importance for maintaining the germline (Lin and Spradling, 1993; Morris and Spradling, 2011).

3.3.1 Self-renewal and differentiation

GSC division typically leads to the generation of a cell which will differentiate into a CB and a cell which will retain a stem cell-like identity. There are several mechanisms which contribute to this selection process. One is diffusion of morphogens secreted by niche cells. The orientation of the mitotic spindle is another example, which is also needed to promote differentiation by allowing the daughter cell to be pushed away from extrinsic signals. The extracellular matrix can further sequester these extrinsic factors which are required for differentiation, limiting their range of influence and ensuring that daughter cells which are leaving the niche start differenting (Xie and Spradling, 2000). Maintenance, differentiation and self-renewal are controlled by both intrinsic factors within the GSCs and CB and extrinsic factors secreted by the terminal filament and cap cells (King, 1957, Spradling *et al*., 1997). Adhesion complexes are also required for stem cell maintenance and selection. Despite ablation of GSCs, terminal filament and cap cells do not degenerate immediately, demonstrating that the GSC niche is an example of a stable niche (Kai and Spradling, 2003).

Two extrinsic factors needed for GSC maintenance are Decapentaplegic (DPP) and Glass bottom boat (GBB), which are both members of the BMP family (Zhu and Xie, 2003). They are secreted from terminal filaments cells, Cap cells and ECs (Xie and Spradling, 2000) (Fig. 2). An upregulation of Bag of Marbles (BAM) in the ovary as

well as a GSCs loss can be seen when DPP signaling is reduced, suggesting DPP functions by inhibiting *bam* expression and that BAM is a differentiation promoting factor. GBB is necessary but not sufficient for regulating GSCs (Song *et al*., 2004). DPP inhibits BAM by binding to the DPP receptor, Thick veins (TKV), and activating Mothers against Decapentaplegic (MAD) and Medea by phosphorylation (Fig. 3). By binding to a silencer element upstream of the *bam* gene, MAD and Medea then inhibit BAM expression (Chen and McKearin, 2003, Song *et al*., 2004). BAM functions by inhibiting Pumilios (PUM) and Nanos (NOS), who in turn function by inhibiting the translation of mRNAs required for differentiation (Szakmary *et al*., 2005). Once the GSC divides and pushes one daughter cell away from the GSC niche, BAM expression is upregulated which suppresses PUM and NOS, leading to translation of these mRNAs (Szakmary *et al*., 2005) (Fig. 2).

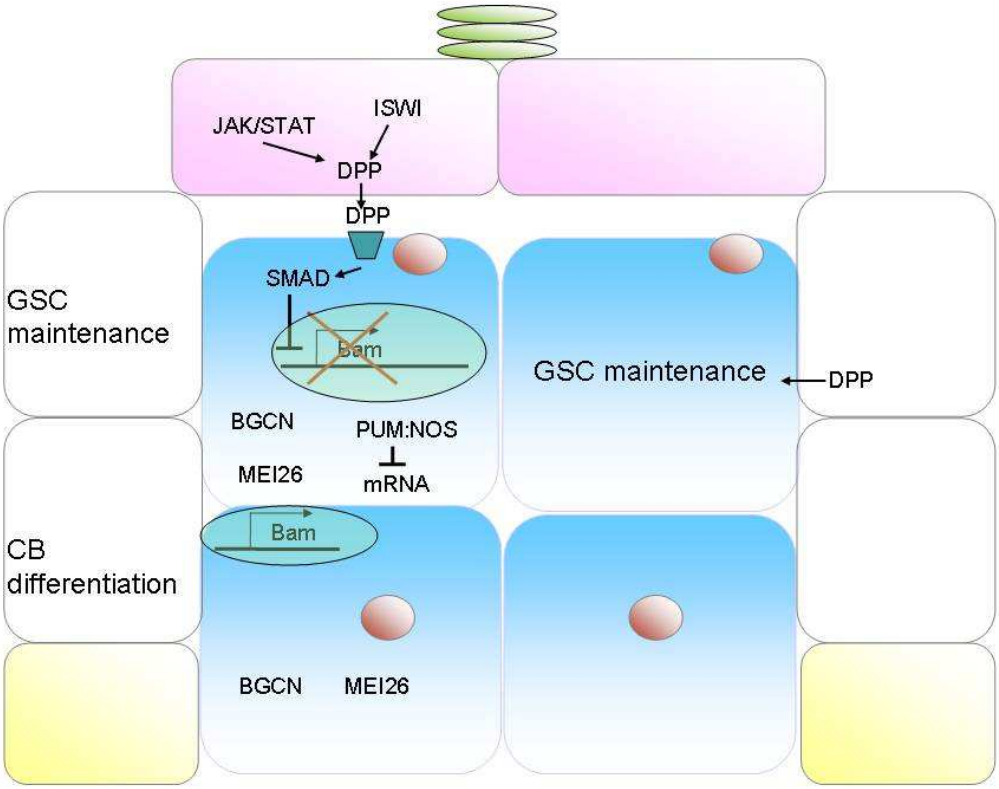


Fig. 2 Role of DPP and BAM in GSC maintenance. Inhibition of differentiation is regulated by the extrinsic factor, DPP. DPP downregulates the expression of BAM, allowing PUM:NOS-mediated translational inhibition of mRNAs required for differentiation (Left side of diagram). Additionally, DPP from escort cells is also able to promote a GSC like fate (Right side of diagram). DPP expression in the cap cells is dependent on the chromatin remodeling protein, ISWI, and JAK/STAT. In cystoblasts (lower row of blue cells), DPP signaling is switched off and *bam* expression us upregulated. BAM forms a complex with BGCN and MEI26, which are both present in the cytoplasm (Adapted from Yang *et al*., 2007; Shen and Xie, 2008).

Given the importance of DPP in regulating GSC maintenance, DPP activity must be tightly regulated. DPP activity in cap cells is thought to be induced by Janus Kinase/ Signal Transducers and Activators of Transcription (JAK/STAT) and Imitation SWI (ISWI), a chromatin remodelling factor (Szakmary *et al*., 2005, Wang *et al*., 2008) (Fig.2). Additionally, DPP activity is downregulated by several mechanisms, suggesting a functional redundancy which ensures that only one daughter cell will differentiate (Yamashita *et al*., 2005).

First, the diffusion of DPP is limited by a type IV collagen, Viking, found in the extra cellular matrix. Deletion of a small region of the C terminus of Viking leads to loss of interaction with DPP, demonstrating the Viking binds directly to DPP (Wasng, L. *et al.*, 2008). By sequestering DPP close to GSCs, this prevents activation of TKV in differentiating CBs, leading to enhanced GSC maintenance while promoting differentiation in CBs. Evidence which supports this is seen in *viking* mutants, which have an increased number of GSCs which can be located away from the anterior tip of the germarium, suggesting DPP in these mutants has a greater range of influence in the germarium (Wang *et al*., 2008). Another extracellular protein implicated in regulating DPP signalling is the cell surface glypican, Development abnormally delayed (DALLY). DALLY has been shown to function as a co-receptor for DPP in the developing wing. In contrast to Viking, which restricts DPP in the adult ovary, DALLY is required to enhance expression of the DPP ligand in cap cells. Over expression of DALLY leads to an expansion of the niche, producing ectopic GSCs. Conversely, loss-of-function *dally* mutants lose their GSCs over time (Hayashi *et al*., 2009).

Additionally, BAM itself is able to downregulate DPP signaling within the differentiating CB. This may be a concentration dependent mechanism whereby a lower concentration of DPP, secreted from ECs, is more readily inhibited by BAM than in regions where DPP signalling is high and BAM expression is low (i.e. near the cap cells) (Fig. 2). The result is a negative feedback loop which reinforces a CB fate (Xie

and Spradling, 2000). Recently, another protein which has been demonstrated to be a potent promoter of differentiation is BRAT. Germline mitotic clones which do not have functional BRAT are unable to differentiate. Conversely, ectopic BRAT expression leads to germline differentiation. BRAT is downregulated by PUM and NOS. Once NOS is removed by BAM, BRAT forms a complex with pum and inhibits mRNAs associated with self-renewal such as the DPP transducer, MAD, thus making developing cysts immune to DPP signalling (Harris *et al*., 2011).

A final mechanism which downregulates the activity of DPP is the degradation of MAD by Smad Ubiquitination Regulatory Factor (SMURF), an E3 ubiquitin ligase found in CBs. MAD loss in CBs would prevent the CB from reverting back to a GSC like state (Chen *et al*., 2005; Yamashita *et al*., 2005). SMURF also functions by forming a complex with Fused (FU) and driving the rapid turnover of the DPP receptor, TKV, thus further desensitizing developing cystoblasts to DPP (Xia *et al*., 2010). PIWI, the founding member of the Argonaute family, is also reported to downregulate SMURF. PIWI is expressed in niche cells in the germaria (Somatic PIWI), where its stability and localization is controlled by fs(1)Yb (YB), a component of the PIWI-interacting ribonucleic acid (piRNA) pathway (King *et al*., 2001, Qi *et al*., 2011) (Fig. 3). *Yb* mutants have both a loss of germline tissue and somatic PIWI suggesting the piRNA pathway in somatic tissue is important for regulating the germline (Qi *et al*., 2011). GSCs also express PIWI internally (Germline PIWI). This is not seen in SSCs or ECs, suggesting an exclusive role in germline stem cell function (Szakmary *et al*., 2005). Somatic PIWI antagonises BAM, while germline PIWI is repressed by BAM, producing another negative feedback loop which regulates GSC maintenance (Szakmary *et al*., 2005). The mechanism by which this may occur is unknown (Lin, 2002).

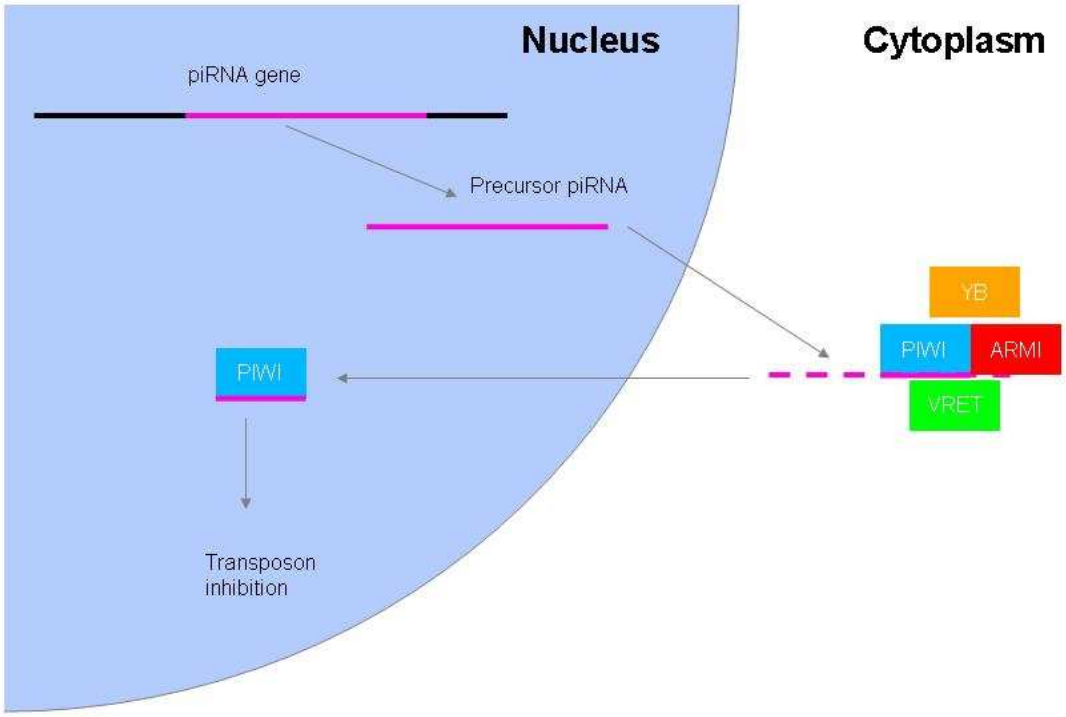


Fig. 3 PIWI-interacting RNA pathway. piRNA genes are transcribed and the resulting immature precursor piRNA is transported out of the nucleus. In the cytoplasm, the precursor piRNA associates with YB, Vreteno (VRET), Armitage (ARMI) and PIWI. This complex then processes the precursor piRNA into its mature form. VRET, YB and ARMI then dissociate and the mature piRNA and PIWI are translocated into the nucleus where they play a role in the inhibition of transposable elements (adapted from Pek *et al.* 2012).

3.3.2 Adhesion and Maintenance

In addition to DPP and PIWI signalling, GSC maintenance is also dependent on adhesion between GSCs and cap cells. *Drosophila* E-Cadherin, a member of the Ca^{2+} dependent Cadherin family, is present in high levels at the interface between cap cells and GSCs (Song *et al*., 2002). Without E-Cadherin, GSCs begin to differentiate (Song *et al*., 2002). Armadillo, the *Drosophila* homolog of β-Catenin, is another adhesion protein that is required for GSC maintenance (Song, 2002). In addition to proteins associated with adhesion junctions themselves, many other proteins may be implicated in maintaining adhesion complexes. For example, RAB11 GTPase, which is essential for the maintenance of adherens junctions, has been shown to be required for asymmetric division of GSCs (Bogard *et al*., 2007). RAB proteins are associated with trafficking of proteins (reviewed in Zerial and McBride, 2001). Loss of RAB11 leads to reduced levels of E-cadherin and armadillo on the surface of GSCs, suggesting RAB11 may function by trafficking adherens junction proteins to the cap cell-GSCs interface (Bogard *et al*., 2007). Another protein required for the accumulation of E-Cadherin as well as the stability of MAD, is Lissencephaly-1 (LIS-1). LIS-1 mutants lose their GSCs (Chen *et al*., 2010). Adhesion complexes allow for the recruitment of various signalling molecules within the cells expressing the adhesions proteins (Reviewed in Yap and Kovacs, 2003), thus, adhesion proteins such as E-Cadherin and Armadillo could allow GSCs to initiate internal signalling pathways needed for GSC maintenance in response to adhesion to cap cells.

Individual GSCs only survive for four or five weeks and are subsequently lost through differentiation (Margolis and Spradling, 1995, Xie and Spradling, 2000). Thus, to ensure the female is capable of laying eggs throughout her lifetime, the germarial niche must be able to replace a lost GSC. This is achieved through symmetric division, during which the plane of division of the remaining stem cell must be turned on a 90º

angle. In this instance, both daughter cells remain GSCs (Xie and Spradling, 2000). Evidence supporting this model is seen in *schnurri* (SCN) mutants, whose GSCs have a shortened lifespan. Recently divided GSCs in these mutants are connected by elongated fusomes which lie perpendicular to the anterior-posterior axis, suggesting after one GSC is lost, its neighbour undergoes a symmetric division to fill the niche (Deng and Lin, 1997, Xie and Spradling, 2000). The spectrosome, which lies at the cap cell-GSC interface, has been implicated in anchoring itself to the mitotic spindle in proliferating GSCs and thus may play a role in controlling the direction of GSC division (Deng and Lin, 1997).

E-Cadherin may also play a role in the selection of GSCs. Jin *et al*, (2008) introduced differentiation defective mutants (i.e. mutants that could not produce BAM) into wild type niches. These mutants were able to outcompete their wild type neighbours for niche occupancy. E-Cadherin is expressed at higher levels in *bam* mutants, thus Jin *et al* suggested that upregulation of BAM may lead to a direct reduction of E-Cadherin expression. This implies that germline stem cells may have a quality control mechanism for selecting GSCs which have less BAM expression (Jin, *et al*., 2008).

3.4 Cyst progression and germline differentiation.

After a decrease in DPP signalling and a subsequent rise in BAM protein production, the developing CB must undergo four rounds of incomplete mitosis to produce a sixteen cell cyst as it is pushed through the germarium to the FSCs. At the sixteen cell stage, the cyst stops dividing and begins the process of terminal differentiation. At this point, one cell in the cyst will become an oocyte while the other fifteen will differentiate into the polyploid nurse cells that will supply the embryo with maternal mRNA. Both the initial differentiation of the CB into a cyst and the

differentiation of the cyst into oocyte and nurse cells require the coordination of many intrinsic factors such as BAM and Sex lethal (SXL) (Fig. 4).

Contrary to a reduction in DPP, loss of BAM leads to an increase in number of GSC like cells (McKearin and Spradling, 1990). BAM is found in the cytoplasm of CBs and later localises to fusomes in sixteen cell cysts. After the sixteen cell stage, BAM is completely downregulated (McKearin and Spradling, 1990). Ectopic BAM promotes GSC differentiation, leading to GSC loss, suggesting BAM is essential for inducing CB formation (Lin and Spradling, 1993, Ohlstein and McKearin, 1997). The amino acid sequence of BAM contains a PEST sequence which is usually associated with proteins that have a high turnover rate and may be important for regulating the unusual incomplete cell cycles that the CB undergoes (McKearin and Ohlstein, 1995). It has been demonstrated that BAM is required for controlling the number of transit amplifying steps in the development of sperm in the Drosophila testis. If BAM is reduced, sperm cysts undergo extra rounds of mitosis, indicating that BAM may have to reach a threshold concentration before the next step of differentiation occurs (Insco *et al*., 2009). This has not yet been demonstrated to be true in the ovary, however, BAM is completely down-regulated in the sixteen cell cystoblast stage, when synchronicity of cell germ cell divisions is lost and specification of the nurse cells and oocyte begins (McKearin and Ohlstein, 1995). Interestingly, Tokusumi *et al*, (2011) have shown that, unlike in the ovary where BAM promotes differentiation, in the lymph gland, BAM maintains the haematopoietic progenitor cell population in an undifferentiated state. This suggests that BAM may have multiple targets which differ depending on the tissue in which it is expressed (Tokusumi *et al*., 2011).

BAM interacts with Benign Gonial Cell Neoplasm (BGCN); *bam* mutants are indistinguishable from *bgcn* mutants. The *bgcn* mutant phenotype is not rescued by the addition of BAM, suggesting these two proteins play a role in regulating differentiation together (Lavoie *et al*., 1999). BGCN is expressed in CBs and GSCs, unlike BAM

which is only expressed at low levels in GSCs, implying that GSCs are "primed" to become CBs (Ohlstein *et al*., 2000). BAM and BGCN are thought to control the expression of a pool of mRNAs required for cystoblast formation. It is also possible that they control the downregulation of mRNAs which are required for maintaining a GSC fate, such as NOS (Li *et al*., 2009). Another intrinsic protein which, when mutated, causes loss of GSCs is PUM (Lin and Spradling, 1997; Szakmary *et al*., 2005). PUM is responsible for repressing the translation of mRNAs in the *Drosophila* embryo in conjunction with NOS (Forbes and Lehmann, 1998). Pelota, another putative translational repressor, has also been implicated in GSC self-renewal (Xi *et al*., 2005). It has also recently been demonstrated that SXL, which is required for GSC differentiation (Chau *et al*., 2009), is also capable of translational repression in S2 cell culture (Medenbach *et al*., 2011). This suggests that translational regulation is a key mechanism for regulating GSC behaviour.

mei26 mutants have a phenotype similar to *bam* mutants. In this instance, BAM is not able to rescue the *mei26* mutant phenotype, suggesting that MEI26 and BAM are part of the same pathway. One model that explains how these proteins interact is that MEI26, once activated by BAM, possibly inhibits the activity of PUM and NOS. This would relieve the translational inhibition of mRNAs required for CB differentiation (Neumuller *et al*., 2008; Shen and Xie, 2008) (Fig. 4).

Components of the micro ribonucleic acids (miRNA) processing pathway have recently been implicated in the regulation of GSCs (Park *et al*., 2007). miRNAs are small RNA sequences which are required for the degradation or translational inhibition of specific mRNAs (Shen and Xie, 2008, Valencia-Sanchez *et al*., 2006). When Argonaute1 (AGO1), an enzymatic component of the miRNA pathway, is overexpressed, over-proliferation of GSCs is seen whereas loss of AGO1 causes GSCs to differentiate, implying that AGO1 inhibits differentiation (Jin and Xie, 2007, Yang *et al*., 2007a). Similarly, Dicer1 (DCR1) and Loquacious (LOQS) mutants also lead to a

loss of GSCs. These three proteins are thought to be involved with GSC maintenance by inhibiting the expression of factors required for differentiation, similar to PUM and NOS (Jin and Xie, 2007, Park *et al*., 2007).

In addition to BAM, another protein involved in regulating GSC behaviour is the sex-determination gene, SXL, which is normally found in the cytoplasm of GSCs and in the nuclei of cyst cells at the sixteen cell stage (Bopp *et al*., 1993) (Fig. 4). SXL mutants have large tumourous ovarioles similar to *bam* mutants. However, while *bam* tumours are filled with single cells that contain spectrosomes, *Sxl* mutants produce cysts, suggesting that the germline in *Sxl* mutants is able to partially differentiate (Chau *et al*., 2009). Loss of SXL in the germline leads to upregulation of *nanos*. It has been shown that *nanos* is required for continued growth of germline tumours that result from *Sxl* loss of function, although not for the undifferentiated cyst phenotype itself. (Chau *et al*., 2012).

A mutant in Sans-fille (SNF) which leads to a total loss of germline SXL has an overabundance of BAM but is unable to complete differentiation, demonstrating that SXL is essential for BAM-induced germline differentiation (Chau *et al*., 2009). Interestingly, in *snf* and *bam* double mutants the germline tumours are able to produce branched fusomes but cannot produce polyploid nurse cells. This implies that, in a *Sxl* deficient background, BAM is not essential for the early stages of development (Chau *et al*., 2009). Additionally, since these mutants produce branched cysts but not mature nurse cells, SXL may play a role in promoting the final stages of germline differentiation. As well as both producing tumours, *bam* and certain *Sxl* mutants have the same molecular signature; both types of mutants express male specific mRNAs suggesting that BAM and SXL are both involved in regulating the same downstream targets (Chau *et al*., 2009). In agreement with this, BAM mutants also produce the male splice form of SXL, indicating that both BAM and SXL are part of the same pathway (Chau *et al*., 2009).

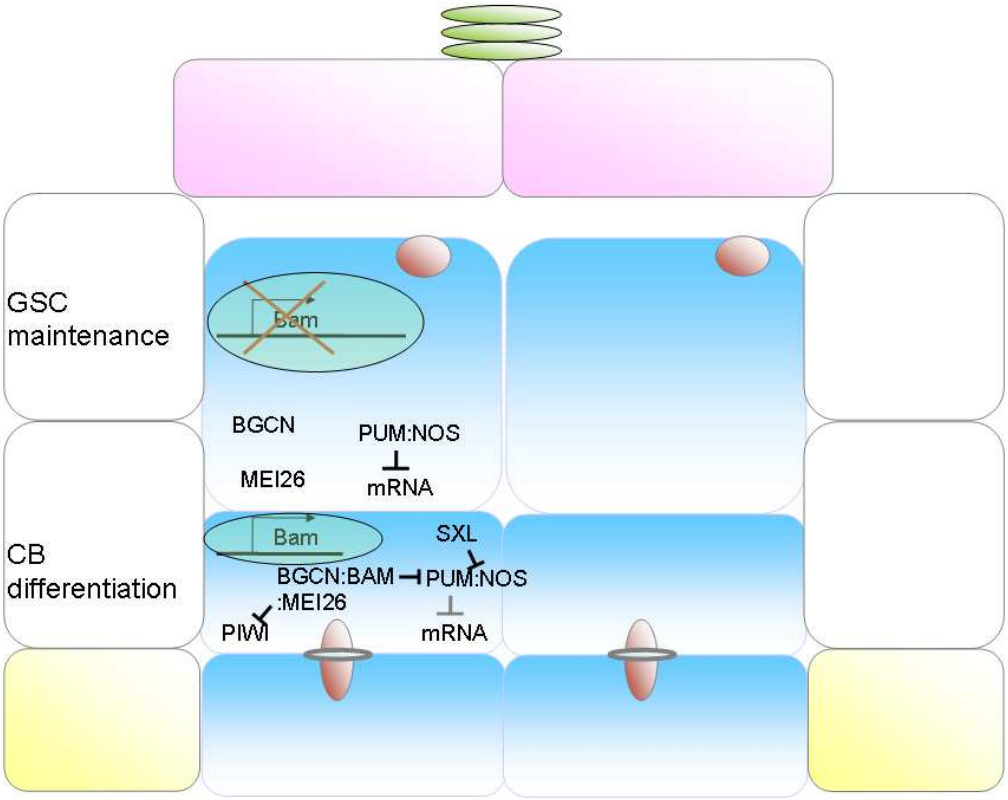


Fig. 4 BAM and SXL in germline differentiation. Low levels of BAM lead to GSC maintenance. In the cystoblast, reduced DPP signalling leads to an increase in BAM expression, allowing BAM to repress PIWI, PUM and NOS. Additionally, SXL is able to downregulate Nanos at this stage. This leads to cystoblast differentiation. After the cystoblast stage, BAM becomes localised to the fusome and SXL remains inhibited between the 2-8 cell stage. (Adapted from Chau *et al*., 2009; Chau *et al*., 2012).

3.5 Escort cells

While cysts are dividing in region 1, their movement through the germarium is driven by the activity of ECs. These cells contain of thin cytoplasmic projections that remain in close contact with the GSCs and developing germline cysts. It was initially thought that ECs were the progeny of "escort stem cells." After the escort stem cell had undergone division, the resulting EC was thought to move through the germarium with the developing cyst and undergo apoptosis at the region 2a/2b boundary, since ECs in this region are occasionally positive for apoptotic markers (Decotto and Spradling, 2005). However, recent live imaging data has demonstrated that ECs appear to be permanently anchored to the basement membrane of the germarium and it is the dynamic movement of the microtubule rich projections that push the cyst through the germarium (Morris and Spradling, 2011). Loss of GSCs leads to the gradual apoptosis of ECs, suggesting that ECs are able to sense and respond to changes in GSC activity (Kai and Spradling, 2003). Also, EC cytoplasmic projections do not surround the GSC-like cells in *bam* mutants, demonstrating that the differentiating germline plays a role in regulating EC behaviour (Kirilly *et al*., 2011).

In addition to their role in the development of the germline, it has also been proposed that escort cells may define part of the FSC niche through the formation of adherens junctions since loss of ECs lead to the degeneration of the FSC niche (Kai and Spradling, 2003, Morris and Spradling, 2011, Song and Xie, 2002). This illustrates the importance of ECs in the regulation of both the germline and the follicle cell population.

Little is known about the signalling pathways which regulate EC behaviour; however, Decotto and Spradling (2005) demonstrated that the escort cell population requires JAK/STAT signalling to function. Without JAK/STAT signalling, the germarium tip and muscle sheath is disorganised. An increase in JAK/STAT signalling in the germarium also leads to an increase in the number of ECs (Decotto and Spradling,

2005). In addition to JAK/STAT, Rhomboid (RHO) is known to influence the cytoplasmic projections of ECs. Loss of RHO in ECs leads to poor EC invasion and an accumulation of GSC like cells in the germarium. It has also been shown that expression of RHO, which functions through Epidermal Growth Factor (EGF) signalling, in ECs is important for restricting DPP signalling to the niche (Kirilly *et al*., 2011).

3.6 Follicle cell differentiation; epithelial cells, stalk cells and polar cells

The final part of egg production which takes place in the germarium occurs in region 3 where the developing cyst becomes enveloped by follicle cells (Fig. 5). The *Drosophila* ovary contains two FSCs which reside at either side of the germarium (Margolis and Spradling, 1995). These FSCs divide to produce immature follicle cells which express markers such as Fasciclin III (McGregor *et al*., 2002). Once an FSC divides, the daughter cell which will go on to differentiate has to migrate laterally across the germarium until it is at either the posterior or anterior side of the cyst. This decision appears to be dependent on Notch signalling since FSCs which have defective Notch signalling are unable to produce follicle cells which migrate across the cyst (Nystul and Spradling, 2010) (Fig. 5). Additionally, Nystul and Spradling (2010) demonstrated that Delta, the Notch ligand, is required in the cyst which the follicle cell will associate with for this migratory behaviour to occur.

When the follicle cell is in an appropriate position, it will divide and surround the sixteen cell cyst (Fig. 5). The generation of mitotic clones in older egg chambers has shown that all follicle cells have the potential to differentiate into either polar cells, stalk cells or epithelial cells (Nystul and Spradling, 2010). After this, the enveloped cyst will bud off from the germarium and begin to mature into an egg. Once out of the germarium, the follicle cell population continues dividing as the nurse cells and the oocyte begin to expand. A subgroup of follicle cells will stop dividing as the cyst buds

off from the germarium and will eventually differentiate into polar cells and stalk cells (Nystul and Spradling, 2010) (Fig. 5).

Polar cells are two pairs of cells which are found at opposite ends of the egg chamber. In the early stages of egg maturation, the polar cells are found at the end of the stalks and require Notch for their development (Nystul and Spradling, 2010). Expression of Unpaired, the JAK/STAT ligand, in polar cells is essential for proper stalk formation. A reduction of JAK/STAT signalling in the ovary leads to improper stalk cell specification and inappropriate fusion of adjacent egg chambers (McGregor *et al*., 2002) (Fig. 5).

JAK/STAT, along with EGF signalling, is also required for differentiation of follicle cells which cover the germline cysts (McGregor *et al*., 2002). During the final stages of follicle cell differentiation, JAK/STAT signalling in a subpopulation of epithelial follicle cells called border cells is required for their migration from the anterior end of the egg chamber to the oocyte (McGregor *et al*., 2002). This is mediated by a miRNA called mi-R279 which functions with Eyeless to ensure the border cells differentiate properly (Yoon *et al*., 2011) (Fig. 5).

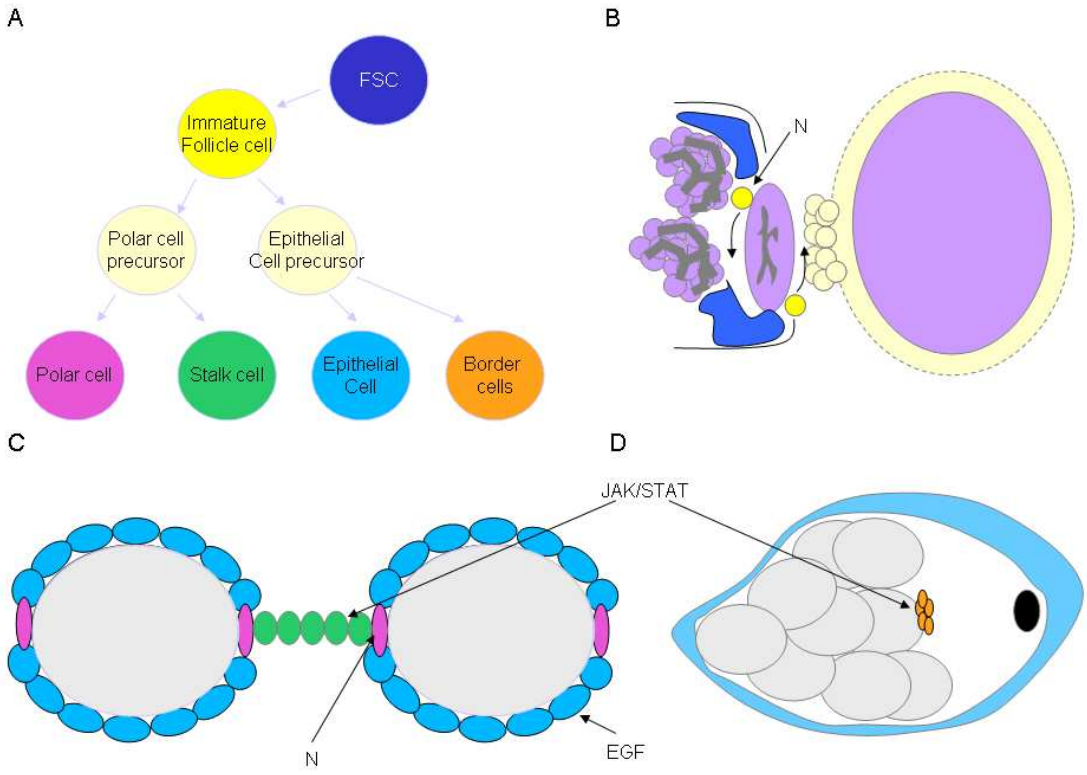


Fig. 5 Follicle cell lineages. **A.** Diagram showing follicle cell lineage. Colours match corresponding cell types in B-D. **B.** In the germarium, follicle stem cells (blue) divide to produce immature follicle (yellow) cells which will migrate laterally across the germline cyst (purple). These then divide and give rise to the follicle cell precursors (pale yellow). **C.** Follicle cells then further differentiate into polar cells (magenta) and stalk cells (green). This requires Notch and JAK/STAT, respectively. The remaining follicle cells will differentiate into epithelial cells. **D.** At around stage 10, a subset of the epithelial cells will differentiate into border cells (orange) at the anterior end of the egg chamber. These will then migrate up to the oocyte (black).

3.7 Follicle stem cells

It has been demonstrated that the maintenance, self-renewal and proliferation of FSCs is dependent on a variety of signals. These include the presence of adhesion junctions between FSCs and their neighbouring cells as well as a combination of signals from somatic cells and the extra cellular matrix.

3.7.1 Follicle stem cell maintenance

One interesting feature of the FSCs is that they exist in two distinct locations, one at either side of the germarium. These two separate niches are capable of repopulating each other, i.e. when one stem cell is lost, a FSC in the opposite niche can migrate laterally across the germarium to repopulate the empty niche (Nystul and Spradling, 2007). This means that FSCs undergo long-range stem cell replacement while GSCs only undergo short-range stem cell replacement (Nystul and Spradling, 2007). Since lateral migration of follicle cells depends on Notch signalling, this suggests Notch is important in the long-term maintenance of the FSC population, while not being required in the FSC itself. This type of stem cell replacement is analogous to the epithelial stem cell niches found in the *Drosophila* gut (Ohlstein and Spradling, 2006).

Another interesting feature of FSCs is that their behaviour does not appear to be dependent on locally secreted signals from nearby non-dividing somatic cells (Nystul and Spradling, 2007). Nystul and Spradling (2007) found that FSCs are attached to their daughter cells via ring canals which may be needed to stabilise FSCs in the correct position. FSC maintenance is also dependent on physical attachment to escort cells via E-Cadherin and β-catenin which may anchor the FSCs in place (Song and Xie, 2002). The loss of E-Cadherin leads to a loss of maintenance of FSCs (Song and Xie, 2002).

This adhesion is thought to be important because it ensures FSCs are held in the presence of signalling molecules such as HH and Wingless (WG) (Song and Xie, 2003).

Integrins have also recently been implicated in binding FSCs to the basement membrane of the germarium (O'Reilly *et al*., 2008). Integrins are transmembrane proteins found at the cell surface which regulate adhesion between the cell and its surrounding environment. This means that integrins are capable of transducing external signals, such as those required for survival or proliferation, into intracellular compartments (Reviewed in Hynes, 2002). It is possible that a patch of the basement membrane expresses the ligand which the FSCs integrin binds to which would explain why FSCs are always found at the 2a/2b region boundary in the germarium (O'Reilly *et al*., 2008). O'Reilly *et al* (2008) demonstrated that FSCs secrete Laminin A, a ligand for integrins, suggesting that FSCs may be capable of generating their own extracellular matrix. The implication is that FSCs control their own local environment, thus allowing them to function in exogenous positions in the germarium (O'Reilly *et al*., 2008).

FSCs are also dependent on intrinsic factors such as Cyclin E. Loss of Cyclin E leads to a reduction in Cyclin E- Cyclin dependent kinase 2 activity and results in a loss of FSCs. Overexpression of E-Cadherin is able to rescue this loss of FSCs, suggesting that adhesion to the niche is important for regulating both FSC division and maintenance (Wang and Kalderon, 2009).

Another intrinsic factor which is required for FSC maintenance is Domino (DOM) which is an ATP-dependent chromatin remodelling factor (Xi and Xie, 2005).The miRNA pathway, another intrinsic regulatory mechanism, has also been implicated in the regulation of FSCs (Jin and Xie, 2007). Jin *et al* (2007) found that loss of DCR1 led to a loss of FSCs and GSCs. However, it is uncertain how miRNA controls FSC behaviour. Identifying the target miRNAs associated with FSC self-renewal would greatly aid our understanding of how miRNAs function in both GSCs and FSCs (Jin and Xie, 2007).

3.7.2 Follicle stem cell self-renewal and proliferation

FSCs are capable of populating the GSC niche when GSCs are ablated, demonstrating that FSC behaviour can be controlled by the same signals as GSCs (Kai and Spradling, 2003). For example, GBB can control self-renewal and proliferation of FSCs through activation of the TKV receptor found at the cell membrane of FSCs (Kirilly *et al*., 2005). It is uncertain whether GBB functions in FSCs by repressing the expression of differentiation factors or by positively regulating factors that promote maintenance (Kirilly *et al*., 2005).

A second signalling pathway reported to be involved in FSC self-renewal and proliferation is HH, whose upregulation leads to the production of excess follicle cells. Conversely, disruption of HH signalling leads to a rapid loss of FSCs (Zhang and Kalderon, 2001). YB has been implicated in the proliferation of FSCs by controlling HH signalling in cap cells (King *et al*., 2001). This demonstrates that cap cells are also part of the niche which maintains FSCs, thus cap cells may coordinate the activity of two different stem cell populations in two different regions of the germarium (King *et al*., 2001). HH acts on FSCs through deactivation of the Patched receptor, allowing Smoothened to activate Cubitus interuptus. The result of this is the transcription of Cubitus interruptus' target genes (Lin, 2002). Mutation of these components leads to the misregulation of FSC proliferation (Lin, 2002).

A third pathway implicated in the regulation of FSC proliferation is WG (Song and Xie, 2003). The loss of downstream components of the WG signalling pathway, such as Frizzled and Dishevelled, leads to a loss of FSCs (Song and Xie, 2003). Mutation of proteins which negatively regulate the HH pathway, such as Patched, leads to the maintenance of FSCs (Zhang and Kalderon, 2001) while mutation of negative regulators of the WG pathway, such as Axin, leads to the destabilisation of FSCs (Song

and Xie, 2003). This implies that HH and WG may regulate the activity of FSCs in different ways (Song and Xie, 2003).

3.8 External factors which affect stem cell behaviour

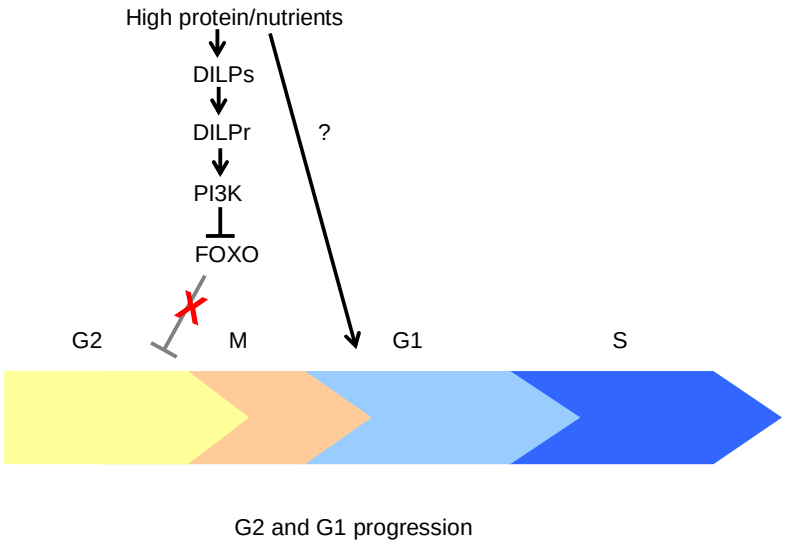
The molecular mechanisms that control egg production in the *Drosophila* ovary can also be regulated in response to a variety of extrinsic factors such as the presence of males, the abundance of food and overcrowding (Pearl, 1932; Partridge *et al*., 1986; Yang *et al*., 2008). The benefit of this regulation in response to the environment is that the *Drosophila* female can optimise the production of eggs. This means that the female is not expending more energy on oogenesis than can be afforded and she is not producing offspring in an environment that is unsuitable (i.e. producing less offspring reduces the competition for nutrients). Nutrition has a direct effect on the production of eggs by altering stem cell activity via Insulin signalling. In addition to Insulin signalling, Juvenile hormone regulation is known to play a part in regulating the behaviour of the ovarian niche (Konig *et al*., 2011). Additionally, there are two points in the egg production pathway where apoptosis can be induced in the *Drosophila* ovary. These are found at the start (cyst degradation) or mid-egg production (degradation of stage 8 egg chambers) (Cavaliere *et al*., 1998; Drummond-Barbosa and Spradling, 2001).

3.8.1 Nutrition

On a protein rich diet, the proliferation rates of GSCs and FSCs are raised. Conversely, protein deficiency leads to a reduction in the proliferation of GSCs and FSCs as well as inhibition of vitellogenesis, which is the stage when yolk accumulation begins (Drummond-Barbosa and Spradling, 2001). Drosophila Insulin-Like Peptides (DILPs) regulate this response to dietary protein. The influence of DILPs on GSCs is an example of how a signal generated far from the ovary can have an impact on stem cell

regulation (LaFever and Drummond-Barbosa, 2005). DILPs act directly on GSCs, demonstrated by the fact that loss of the DILP receptor on the surface of GSC leads to a reduction in GSC proliferation (LaFever and Drummond-Barbosa, 2005). FSC proliferation in response to nutrient deprivation, however, is not directly influenced by DILPs (LaFever and Drummond-Barbosa, 2005). It has been shown that Target of Rapamycin (TOR) signalling is involved in regulating follicle cell survival (LaFever *et al*., 2010). Additionally, TOR is able to regulate GSC proliferation at the G2 stage of mitosis. TOR is also important for regulating germline survival; TOR mutant cysts undergo apoptosis at the sixteen cell stage independently of insulin signalling (LaFever *et al*., 2010).

The signal generated by DILPs is thought to be regulated via the Phosphoinositide 3-kinases (PI3K) pathway. This pathway is required for the deactivation of Forkhead box, subgroup O (FOXO), a key forkhead transcription factor that regulates the cell cycle. Lack of FOXO leads to progression of the cell cycle (Hsu *et al*., 2008) (Fig. 6). This regulatory pathway is thought to act on the cell cycle at the G2/M transition; this point in mitosis requires a large amount of protein and energy to complete. GSCs have an unusually long G2 phase, perhaps to allow for synthesis of components which will be required in the fusome, a nutritionally demanding process (Hsu *et al*., 2008). Halting the cell cycle at the G2/M transition in response to nutrient deprivation may be a mechanism which ensures division does not occur before the fusome has had chance to develop. Diet may also influence the G1/S checkpoint (Hsu *et al*, 2008). Again, the synthesis of DNA is a process which requires a large amount of energy. Halting division here allows the cell to ensure there are enough resources to complete the process before entering synthesis.



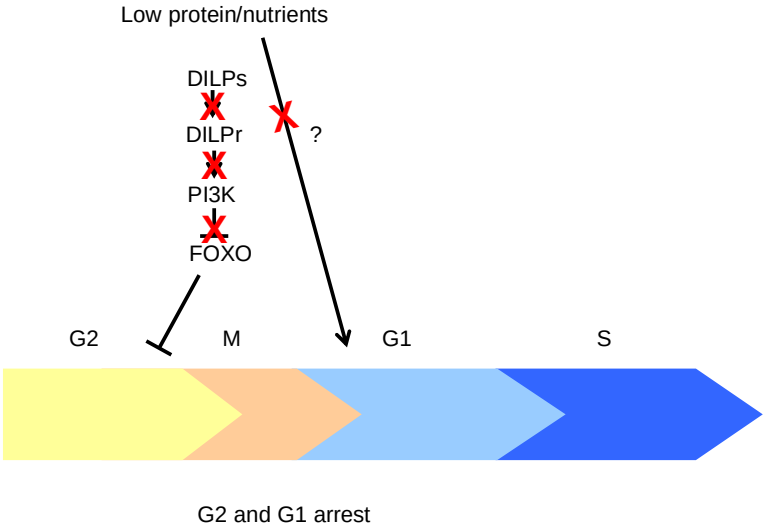


Fig. 6 Regulation of stem cell divisions in response to nutrient availability. **A.** On a high protein diet, DILPs are expressed in the brain. These interact with DILP receptor (DILPr) and then, through the PI3K pathway, allow G2 arrest by deactivating FOXO. **B.** Conversely, lack of DILPs leads to activation of FOXO and inhibition of the cell cycle. It is thought a secondary signal which acts on the G1 phase of the cell cycle is also mediated by DILPs (?). (Adapted from Hsu *et al.*, 2008).

3.8.2 Apoptosis

There are two points in the *Drosophila* ovary where apoptosis can be induced. The first is found in region 2 of the germarium where germline cysts undergo apoptosis when the fly is fed a diet lacking protein (Drummond-Barbosa and Spradling, 2001). One hypothesis for the presence of this checkpoint is that it allows the germarium to co-ordinate the rate of GSC production with FSCs as FSCs respond to a poor diet much more rapidly than GSCs (Drummond-Barbosa and Spradling, 2001). Evidence which supports this is that cysts only degenerate after they have reached the sixteen cell stage, at the point when follicle cells associate with the developing egg (Smith *et al*., 2002). One protein implicated in regulating this checkpoint thus far is Daughterless (DA) (Smith *et al*., 2002). Loss of DA leads to an increase in the number of nurse cells suggesting that germline cells which should have undergone apoptosis in the germarium survived (Smith *et al*., 2002). Another protein implicated in regulating cyst survival is TOR. TOR mutants have more apoptotic cysts (LaFever *et al*., 2010). Loki and p53 have also been implicated in apoptosis in the germline (Bakhrat *et al*., 2010).

Flies fed on a poor diet show degenerating egg chambers at stage 7-8. This is the second apoptotic checkpoint in the ovary (Drummond-Barbosa and Spradling, 2001). This checkpoint is also activated in response to apoptosis-inducing toxins such as staurosporin (Nezis *et al*., 2000). Accumulation of mature eggs in the ovary may also lead to apoptosis of egg chambers at stage 8 (Wyman, 1979). Thus the importance of this checkpoint is that it allows the ovary to modify egg production in response to environmental factors. Insulin receptor (INR), p70 ribosomal S6 kinase (S6K) and Chico mutants have been shown to produce egg chambers which have no follicle cells, but produce a normal germline. Closer analysis of the germline cells revealed that they produce more *Drosophila* Inhibitor of Apoptosis 1 (DIAP1), a potent inhibitor of apoptosis, than wild type egg chambers which suggests that insulin signalling is

required for controlling the apoptotic checkpoint. Since young egg chambers have raised levels of DIAP1, this suggests that younger egg chambers may be protected against the signal for apoptosis (Pritchett and McCall, 2012).

3.8.3 Hormones and the ovary

Hormones are known to play a role in regulating both the development and function of the *Drosophila* ovary. A steroid hormone known as Ecdysone, which is essential for morphological changes during larval development, is required for many different aspects of ovary function (Gancz *et al*., 2011). Initially, Ecdysone functions by maintaining primordial germ cells in an undifferentiated state in the larval ovary. These primordial cells will become the GSCs in the adult ovary. In the larval ovary, Ecdysone also influences the differentiation of the niche. Finally, Ecdysone is also required for initiating oogenesis (Gancz *et al*., 2011).

In adult ovaries, Ecdysone positively regulates DPP signalling in GSCs, encouraging their maintenance and promoting proliferation at the G2/M checkpoint. This is thought to occur independently to Insulin signalling (Ables and Drummond-Barbosa, 2010). Ecdysone receptor mutants have also been shown to contain less ISWI protein, suggesting Ecdysone functions by regulating transcription of factors required for GSC maintenance (Ables and Drummond-Barbosa, 2010).

3.9 Effects of ageing in the Drosophila ovary

As an organism ages, the level of niche signalling is reduced leading to the degeneration of tissues. This loss of signalling is seen in the *Drosophila* ovary and is thought to be the cause of reduced egg production. As *Drosophila* females age, the number of cap cells and GSCs decline. Additionally, the number of eggs declines with age, due to a reduction in the proliferation of stem cells and an increase in the death of egg chambers (Zhao *et al*., 2008).

In the ovary, the reduction of E-Cadherin and BMP expression leads to a loss of GSCs, thus lowering fecundity. Overexpression of both GBB and cadherin leads to preservation of GSCs in aged flies (Pan *et al*., 2007). A reduction in DPP signalling also contributes to a loss of GSCs. Conversely, overexpression of DPP in older flies transiently rescues this loss of GSCs (Zhao *et al*., 2008). Overexpression of DILP2 is able to rescue the age dependent loss of GSCs (Hsu *et al*., 2008). In addition to this, Notch signalling in GSCs has been implicated in the survival of cap cells (Ward *et al*., 2006). INR mutants have a reduced niche size as wells as a reduction of GSCs which is dependent on Notch signalling (Hsu *et al*., 2008)**.** Insulin signalling is able to control Notch expression though FOXO and PI3K (Hsu and Drummond-Barbosa, 2011). This shows that many signalling pathways are altered with age.

4 Genetic screen for identifying genes required for stem cell regulation

Despite what is already known about stem cell regulation in the *Drosophila* ovary, much remains to be understood about other components which are involved in stem cell regulation, for example, which motor proteins, trafficking proteins and transcription factors (e.g. downstream targets of BAM) are required to regulate stem cells. Additionally, little is understood about the mechanisms which govern the rate of proliferation of stem cells and developing cysts. In an attempt to address this, a fertility screen of *Drosophila* stocks listed as semi-lethal was carried out to identify genes associated with the maintenance, differentiation and self-renewal of stem cells (Ponting, personal communication). The stocks were listed as semi-lethal because they had to be maintained as a heterozygous stock. Some stocks which are listed as semi-lethal are actually sub-fertile (i.e. do not reproduce efficiently), rather than homozygous lethal. In these instances, the stocks contain a mixture of heterozygous and homozygous flies.

The ovaries of homozygous mutants which were found to be sub-fertile were dissected and categorised into one of three classes of phenotypes which were indicative

of specific defects in early oogenesis (Ponting, personal communication). These include:

1) The maturity of the egg chamber adjacent to the germarium was assessed using the scale described in King (1957) (Fig. 7). Normally, the first egg chamber is a stage 2. A high average first egg chamber stage with a large number of FasIII enclosed cysts in region 2b, indicating a follicle cell deficiency, possibly caused by a reduction in FSC activity. If FSCs do not produce enough follicle cells to encapsulate all of the cysts being produced, there may be a delay in the pinching off of cysts at the posterior end of the germarium. This assumes that the rate of egg chamber development, once pinched off from the germarium, is independent of an egg chamber's position in an ovariole.

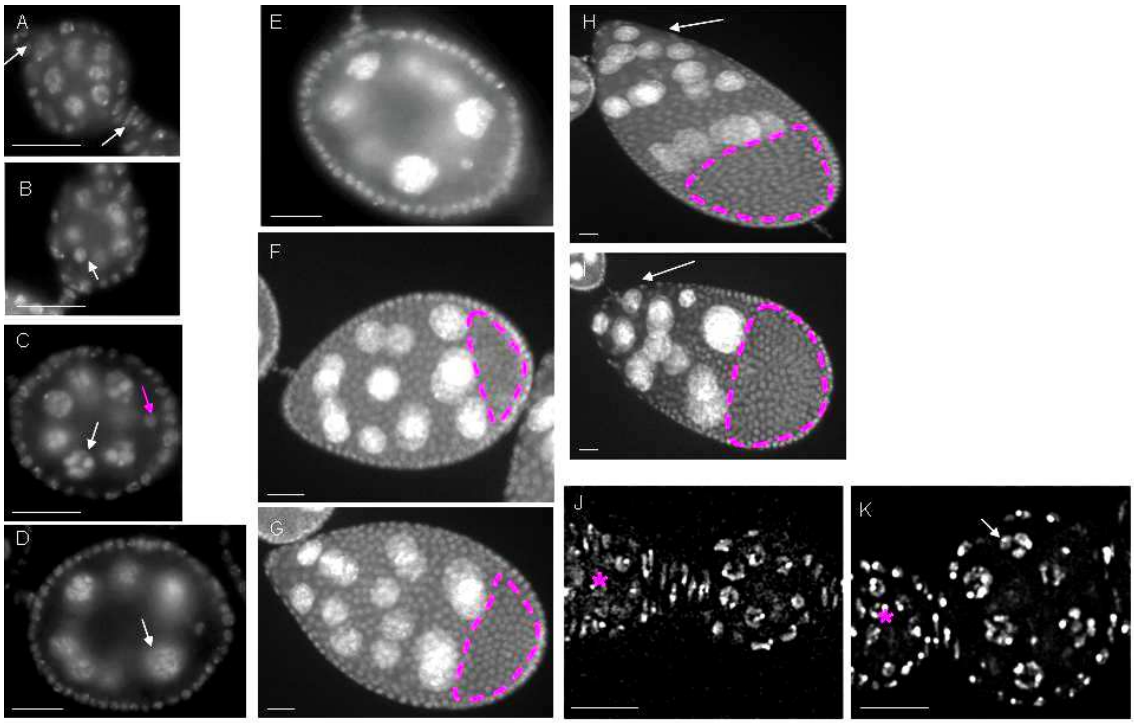


Fig. 7 Stages of egg chamber development. **A.** Stage 2 egg chambers have stalks which are made up of a cluster of cells (arrows). Oocyte is not distinguishable from nurse cells. **B.** The posterior stalk in a stage 3 egg chamber becomes straight, while the anterior remains clustered. The oocyte is visible (arrow). **C.** The oocyte is much smaller in stage 4 egg chambers (magenta arrow) and the nurse cells produce large, multi-lobed nuclei (white arrow). **D.** Stage 5 nurse cells become larger and take on a "speckled" appearance (white arrow). The anterior nurse cells are smaller. **E.** In stage 6, the nurse cells all become the same size. **F.** In stage 7, the anterior nurse cells are noticibly smaller than the posterior. The oocyte yolk starts to form (magenta dashed line). **G.** In stage 8, the yolk is larger (magenta dashed line) and the egg chamber becomes larger. **H.** The follicle cells from the anterior end of the oocyte begin to migrate towards the posterior end of stage 9 egg chambers. The yolk becomes larger. **I.** The follicle cell layer over the oocyte becomes columnar epithelial like and the yolk becomes larger. **J.** Example of a stage 2 egg chamber adjacent to a germarium (magenta asterisk). **K.** Example of a "high first egg chamber mutant." In this case, the egg chamber adjacent to the germarium (magenta asterisk) is stage 4, as identified by the characteristic blob like nurse cell nuclei (white arrow). Scale = 10 µm.

2) Conversely, a high first egg chamber stage and a reduction in the number of FasIII enclosed cysts is a possible indicator of a reduction in GSC activity. This category may be due to decreased numbers of GSCs or a reduction in frequency of GSC mitosis, or an increase in symmetric rather than asymmetric cell division. If GSCs do not produce enough cysts, there is nothing to push egg chambers further into the ovariole, thus the first egg chamber stage is higher than expected. Again, this assumes that the development of an egg chamber is independent of an egg chamber's position in an ovariole. For mutants in this category, the number of GSCs were counted to determine if the high first egg chamber/ low cyst phenotype was caused by GSC loss. This has not been carried out for all of the candidates as yet.

3) Another indicator of follicle cell deficiency is the presence of compound egg chambers which may be caused by several different mechanisms. The first is that the germarium is producing more cysts than FSCs can cope with, so multiple cysts become packaged into the same egg chamber. Conversely, compound egg chambers can also be caused by a deficiency in FSCs; if there are not enough follicle cells to ecapsulate a cyst, multiple cysts may be packaged into the same egg chamber. A final situation which may lead to the formation of compound egg chambers is an insufficiency of separating stalk cells which leads to egg chambers separated only by a bilayer of follicle cells, which subsequently degenerates to produce a compound egg chamber (Torres *et al*., 2003).

Of 391 stocks analysed in this screen, 107 were identified as semi-fertile. Twenty seven of these stocks had a phenotype associated with early oogenesis defects. These candidates are listed below in Table 1.

Candidate Gene	Stock number	First egg chamber stage	Cyst number	Compound egg chambers	GSCs
CycB	Kg08886	No egg chambers	No cysts	N/A	Lost
Deadlock	Kg10262	Increased	Reduced	N/A	Lost
Ncd	05884	No egg chambers	No cysts	N/A	Lost
Dpr9	l(3)04713	Increased	Reduced	Yes	Reduced
DLC90F	l(3)04091	Increased	Reduced	No	Reduced
E2F	Kg03332	Increased	Reduced	Yes	No effect
Mam	d02961	Increased	Reduced	Yes	No effect
GLCAT-1	f00247	Increased	Reduced	Yes	
CG8165/CG8176	KG06444	Increased	Reduced	Yes	No effect
L(2)K10411	l(2)k10411	Increased	Reduced		
Scribbled	C03872	WT	Reduced	Yes	
Eip63E	d02960	Increased	Reduced	No	Increased
No annotated gene	KG06463	Increased	Not scored	Yes	Present
	d05504	Increased	Reduced	Yes	No data
Met-tRNA synthetase	BG02730	Increased	Increased	Yes	No effect
Invalydolysin	C02816		Increased	Yes	
Tramtrak	d02388	Increased	WT	Yes	
Eagle	d04964	Increased	Reduced	Yes	
MAPKBP1	f05580	Increased	Reduced	Yes	
Not mapped	DO1157	Increased	Reduced	Yes	
Dally or l(3)87Df	06464	Increased	Increased		
CG31782	d03812	Increased	Reduced	Yes	No effect
GST-D1	d00284			Yes	
CG33298	D10678	Increased	Increased	Yes	
CycE	KG07848	Increased			
CG17574	l(2)k09328	Increased	No change		
Nhe2	KG03334	WT	Reduced	yes	
NLE or CG2807	k13714	Higher	Increased		

Table. 1. Blue cells = genes with published information linking them to stem cells. WT= wild type. Peach= genes examined in this thesis.

4.1 Aims of project

The aim of this project was to take candidate stocks identified in the screen and determine how they function in regulating stem cells in the Drosophila ovary. This included confirming that the insert was the cause of the phenotype by remobilising the transposable elements in these stocks, determining which genes were affected by the insert using complementation analysis with mutants carrying known molecular lesions, and trying to elucidate the function of these genes in regulating stem cells using in vivo RNAi and carrying out different genetic interactions. This thesis summarises the characterisation of three mutants identified in the original screen; *A2bp1*^{KG06463}, *GlcATI*^{F00247} and *l(3)04713*, and presents the work in the form of three research papers prepared for submission according to the alternative format thesis.

In the first paper the *A2bp1*^{KG06463} tumorous phenotype is described. I found that the insert affects two genes close to the insert; Ataxin 2 binding protein 1 and Gemin3. Since the original mutant produces branched cysts like mutants which affect SXL, we analysed SXL expression and found that *A2bp1*^{KG06463}mutants have an abundance of SXL. Genetic interactions suggested that A2BP1 and Gemin3 function through a gain of function of SXL and the SXL target Ornithine decarboxylase antizyme (ODA). ODA is an inhibitor of Ornithine decarboxylase enzyme, which is essential for the synthesis of polyamines. These small molecules are essential for growth and proliferation. Additionally, polyamines influence the translation of ODA by inducing a frameshift during the translation of ODA. This bypasses a non-sense mutation in the *Oda* mRNA, leading to the generation of a functional ODA molecule. Thus, ODA is part of a feedback loop which controls the synthesis of polyamines (Heby and Persson, 1990; Minois et al., 2011) (Fig. 8). I further identified the involvement of MAD, a known antizyme substrate.

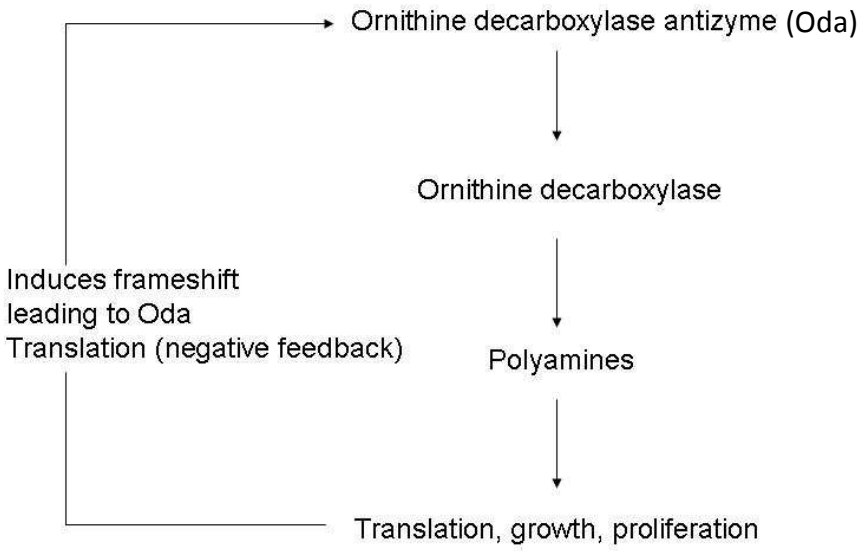


Fig. 8. Antizyme pathway. Ornithing decarboxylase is essential for the synthesis of polyamines, which are needed for growth and proliferation. Ornithine decarboxylase antizyme (Oda) targets Ornithine decarbozylase for proteosomal degradation. Polyamines are able to regulate the translation of Oda mRNA, thus generating a negative feedback loop which regulates the level of polyamines present in a cell.

The second paper describes a PiggyBac mutant known as *GlcATI*^{F00247}. This mutant had compound egg chambers, a reduction in cysts and a reduction in GSCs, suggesting a problem with both the germline and somatic tissue. Closer inspection of the germarium indicated that ECs were not invading properly, which may affect both GSCs and FSCs. Complementation and expression of a rescue construct confirmed that Glucuronyl transferase I (GlcATI) was causing the phenotype in *GlcATI*^{F00247}. RNAi indicated that GlcATI may be functioning in escort cells and FSCs. Genetic interactions indicated that multiple signalling pathways were affected in *GlcATI*^{F00247}, including JAK/STAT, HH, DPP and EGFR, showing that GlcATI is important for coordinating signalling in the germarium.

GlcATI is a putative glucuronyl transferase required for proteoglycan sytnehesis. Proteoglycans form a large and varied family of proteins which consist of a protein core coated in unbranched chains of polysaccharides. Their synthesis begins with the addition of xylose (Xyl) onto a serine residue in a protein. The next two residues added onto the Xyl are galactose (Gal) residues. Finally, a glucuronic acid (GlcA) residue is added onto the second Gal. This process is referred to as initiation and is common to a large group of proteoglycans. It is the last step in this process which is catalysed by GlcATI. The paper described in this thesis represents the phenotypic characterisation of a component of the GAG initiation process in Drosophila oogenesis. In addition to investigating the function of GlcATI in the germarium, we analysed mutants which affect the other two putative glucuronyl transferase genes in Drosophila melanogaster, GlcATS and GlcATP. While mutation of GlcATP only displayed a weak escort cell invasion phenotype, GlcATS mutation had similar consequences to *GlcATI*^{F00247}, suggesting a functional redundancy in proteoglycan synthesis. Additionally the GlcATS mutant also had cysts that had undergone less rounds of mitosis than a normal cyst, indicating that GlcATS may also have a function in the germline. Kim *et al* (2003) found that GlcATS is able to catalyse the transfer of glucuronic acids which are found

in glycolipids and other stages of proteoglycan synthesis (Kim *et al*., 2003). This raises the possibility that glycolipids could also be important for the regulation of tissue function in the germarium.

After the initiation step, GAG synthesis branches out into the different types of molecules that are generated by this process, including chondroitin sulphate, dermatan sulphate, heparin and heparan sulphate (Prydz and Dalen, 2000). While chondroitin and dermatan sulphate consist of glucuronic acid and N-acetyl galactosamine molecules, heparin and heparan sulphate GAGs consist of repeating N-acetyl galactosamine and glucuronic acid residues. GAGs are further modified by the addition of sulphate groups or removal of acetyl groups, leading to even greater diversity in this class of molecules. Thus, the generation of this large class of molecules requires many different enzymes, including sulphotransferases, epimerases, transferases and deacetylases (Reviewed in Prydz and Dalen, 2000) (Fig. 9). Additionally, mutation of the components of proteoglycan synthesis leads to the disruption of many different processes such as wing formation and embryonic patterning (Table. 2).

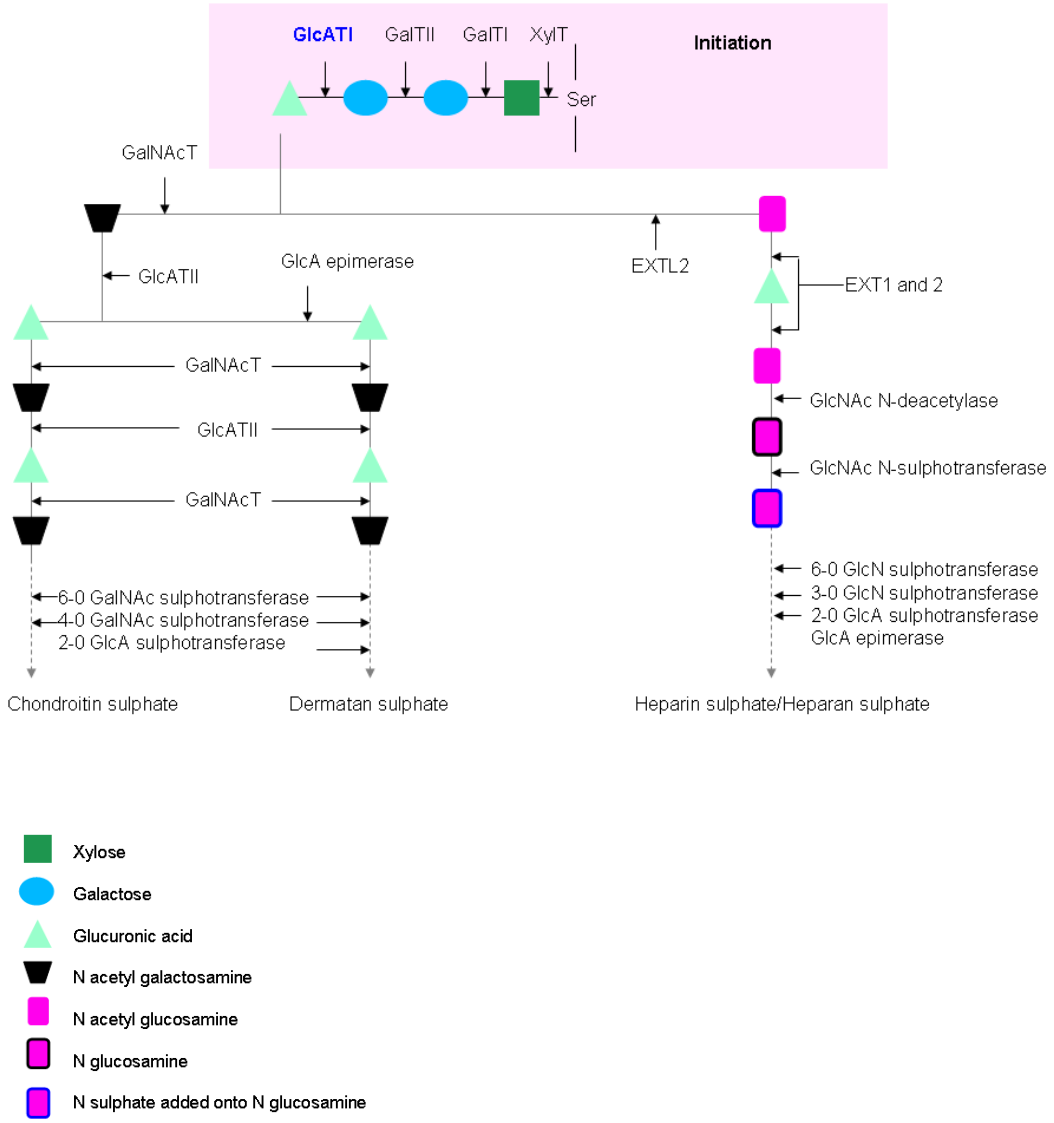


Fig. 9. Proteoglycan synthesis. Diagram showing the synthesis of chondroitin sulphate, dermatan sulphate, heparin and heparan sulphate. (Adapted from Prydz and Dalen, 2000).

Drosophila	Enzyme	Phenotype
GlcATI	GlcAT	Ovary; compound egg chambers, GSC loss, poor EC invasion
GlcATP	GlcAT	Reduced axon outgrowth (Pandey, *et al*, 2012)
GlcATS	GlcAT	Ovary; compound egg chambers, GSC loss, poor EC invasion, reduced germline mitosis
Dbeta3 GalT	GalT	RNAi in wing leads to wing curling alone anterior posterior axis. RNAi in eye causes rough eye phenotype (Ueyama *et al*., 2008)
Peptide O xylotransferase	XylT	Predicted to be lethal (Wilson, 2002)
	GalNAcT	Tracheal tube defect; poor apical basal polarity (Tian and Ten Hagen, 2007)
Heparan sulphate C5 epimerase	GlcA epimerase	Altered wg and hh signalling. Less viable if overexpressed (Kamimura *et al*., 2011)
Tout velu	EXT1	Mirror image dentical defects (Perrimon *et al*., 1996)
Sister of tout velu	EXT2	Wing vein loss, blistering, notches and narrowing. Segment polarity defects. (Han *et al*, 2004)
Brother of tout velu	EXTlike3	Similar to Sister of tout velu (Han *et al*., 2004)
Sulfateless	GalNAc deacetylase/ Sulphotransferase	Segment polarity defect (Zhu *et al*., 2005). Wing clones have wing margin "nicks" (Baeg *et al*., 2004). Mirror image duplication of dentical belt. Lethal. (Perrimon *et al*., 1996)

Table 2. Table listing the Drosophila homologs of different proteoglycan synthetic enzymes and their mutant phentoypes in Drosophila.

The final paper was analysing the phenotype of *l(3)04713* mutants. Removing the insert rescued the phenotype and complementation with a large deficiency indicated that there was at least one gene in the vicinity of the insert, which was responsible for the phenotype. RNAi suggested that Defective proboscis extension response 9 (DPR9) was affected in the *l(3)04713* mutants. Interestingly, *dpr9* is not expressed in the ovary, but in the brain. Finally, since *dpr9* is part of a large family of DPR-related brain expressed proteins, I analysed other mutations of other *dpr* genes and found two others which have similar phenotypes to *l(3)04713*, suggesting that this family of proteins may regulate stem cell behaviour in the ovary.

Chapter 2. *Results Part I*

The RNA binding proteins, Ataxin2 binding protein-1 and Gemin-3, cooperate to regulate somatic and germline cell differentiation during *Drosophila melanogaster* oogenesis.

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Abstract

In the *Drosophila* ovary, differentiation of the germline occurs through a series of defined intermediate stages and a tightly regulated number of mitotic divisions. Different mutations can result in a range of tumorous ovary phenotypes, from excessive self renewal of germline stem cells (GSCs) to over-proliferation of partially differentiated cysts. The latter category consists of a defective developmental lineage including GSCs and a hierarchy of partially differentiated cells. This resembles the heterogenous constitution now thought to comprise certain human tumours that are maintained by cancer stem cells. We identified the *A2bp1*^{KG06463} P-element insert in the 5' region of the splicing associated gene *A2bp1*, as having such a tumorous germline. Although disruption of *A2bp1* has previously been linked with similar outcomes, we find the strong *A2bp1*^{KG06463} phenotype results from disruption of both A2BP1 and the nearby Gemin3 protein, the latter also being involved in RNA processing. Using different allelic combinations and RNA interference (RNAi) we identified roles for the two proteins in various stages of germline development and a previously uncharacterised function to regulate the follicle cells in egg chamber formation. Additionally, we found that upregulated Sex lethal (SXL) protein associated with the *A2bp1*^{KG06463} phenotype is functional and reducing SXL function suppresses germline and follicle cell phenotypes of *Gemin3/ A2bp1* mutants. Following genetic interactions with known SXL targets, we suggest that Gemin3/A2BP1 normally act to repress SXL-dependent inhibition of Ornithine decarboxylase antizyme, and also demonstrate the involvement of Mother's against Decapentaplegic (MAD), a downstream target of Decapentaplegic (DPP) signalling and known antizyme substrate.

Introduction

The defining feature of a stem cell is its ability to divide and produce a differentiating daughter cell and a replacement stem cell. The ovaries of *Drosophila melanogaster* provide an excellent model system for analysing how the decision to differentiate occurs *in vivo*. The *Drosophila* ovary is composed of 15-20 ovarioles, each of which contains several egg chambers of varying maturity (King, 1957; Lin and Spradling, 1993). At the anterior region of the ovariole is the germarium, which houses the stem cells. The GSCs reside at the anterior end of this structure where they are anchored by E-Cadherin based junctions to the cap cells (Song *et al*., 2002). The GSCs divide to produce a GSC, which is retained in the niche and a cystoblast, which is the precursor to the sixteen differentiated cells of the germline lineage (Ong and Tan, 2010). Signals from nearby somatic cells in the microenvironment regulate self-renewal and differentiation of these two daughter cells of the GSC division. GSC maintenance requires DPP, which is secreted by the surrounding soma (Xie and Spradling, 1998). This leads to the repression of the differentiation promoting factor, Bag of marbles (BAM) (McKearin and Ohlstein, 1995). During GSC mitosis, the mitotic spindle is oriented such that one daughter cell is pushed away from the niche and each cell is exposed to small differences in a morphogen gradient of the DPP growth factor (Deng and Lin, 1997). Several mechanisms contribute to restricting the range of DPP activity to GSCs, including sequestering DPP to the extracellular matrix and degradation of downstream targets of DPP in cystoblasts (Chen and McKearin, 2005; Wang *et al*., 2008; Xia *et al*., 2010). Additionally, differences in DPP levels experienced by GSC and cystoblast cells are reinforced by numerous regulatory loops which ensure that the niche always contains an active GSC and that, once committed a daughter cell will not de-differentiate into a stem cell (Xie and Spradling, 2000; Chen and McKearin, 2005; Harris *et al*., 2011).

The GSCs can be identified by the presence of a spherical structure named the spectrosome, which, on GSC division, becomes partitioned into each daughter cell. In the differentiating cystoblast it becomes a fusome. The latter adopts an increasingly branched structure with each round of mitosis as it passes through the interconnecting ring canals of the cyst (Ong *et al*., 2010). After four mitotic divisions, germline mitosis is arrested and one cell in the cyst enters meiosis, beginning the process of differentiating into an oocyte (Barbosa *et al*., 2007). The sixteen cell cyst becomes surrounded by follicle cells, the progeny of the follicle stem cells (FSCs) to form an egg chamber and buds off into the ovariole (Morris and Spradling, 2011).

Disruption of the cystoblast differentiation through overexpression of DPP and loss of BAM function can produce germline tumours (Song *et al*., 2004). A number of other genes have been identified whose mutation disrupts germline differentiation at different stages, resulting in germ-line tumours. These include SXL and Sans fille (SNF) (Bopp *et al*., 1993; Chau *et al*., 2009). The phenotypes of *snf* and *Sxl* appear to be closely related since mutation of *snf* results in a loss of *Sxl* expression from the germline (Nagengast *et al*., 2003; Chau *et al*., 2009). Tumours arising from *snf/Sxl* loss of function differ from those resulting from loss of *bam* in that the frequent occurrence of branched fusomes in *snf/Sxl* mutants suggests that cyst development has been blocked at a later stage (Chau *et al* ., 2009). Mutations of the RNA binding factor, Ataxin 2-binding protein 1 (A2BP1), have also been reported to produce germline tumours (Tastan *et al*., 2010). Like *snf* mutants, these tumours appear to occur due to disrupted progression of cyst development since markers of late cyst development, such as RNA binding protein 9 (RBP9) and Oo18 RNA-binding protein (ORB) are not present in *A2bp1* mutants while early markers of cyst development, including BAM and MEI-P26 are still expressed (Tastan *et al*., 2010). Some alleles of *A2bp1* do not give rise to tumorous germaria but, instead, produce cysts which have undergone an extra round of mitosis (referred to as the 5n phenotype), thus doubling the normal number of germline

cells (Tastan *et al*., 2010). It is not clear whether this represents a weaker manifestation of the tumorous ovary phenotype, or the disruption of a different biological activity. The *A2bp1* mutant 5n phenotype is suppressed by the removal of one copy of *bam* but the mechanisms of action of A2PB1 and its other interacting partners and targets are not well understood (Tastan *et al*., 2010).

While screening a collection of viable P-element insertion lines for defects affecting early oogenesis, we identified $A2bp1^{KG06463}$ as an insert near the 5' end of *A2bp1* that produces germ line tumours when homozygous but did not disrupt the GSCs themselves. We show that the $A2bp1^{KG06463}$ phenotype results from disrupted function of both *A2bp1* gene and the nearby DEAD-box RNA helicase, *Gemin3*. Further complementation analysis and RNAi expression revealed that disruption of both genes contributes to regulation of germline differentiation and mitosis, and the supply of follicle cells. We also identify a role for Gemin3 in GSC maintenance. We investigate further the mechanisms of A2BP1/Gemin3 function and show their activity depends on SXL and Ornithine decarboxylase antizyme (ODA), a known SXL regulated gene (Vied *et al*., 2003). Antizyme is an inhibitor of the Ornithine decarboxylase (ODC) which is essential for polyamine synthesis (Heby and Persson, 1990). Further genetic interactions suggest the transcription factor MAD, a known substrate of ODA (Lin *et al*., 2002), is a downstream targets of A2BP1/Gemin 3 regulation in both germ line and somatic cells.

Methods.

Further details can be found in Appendix I.

Fly stocks and maintenance

The following alleles were obtained from Bloomington (Bloomington, Indiana, USA); *A2bp1*^{KG06463}, *A2bp1*^{EY00149}, *Gem3*^{rL562}, Df(3L)ED4457 Df(3L)Vin2, PTRiP. HMS0028attP2 (RNAi integration site), Nanos::VP16Gal4, *Oda*^{EY01073}, *Oda*^{lex47}, *Snf*^{148}, *Pum*^{1688}, Df(2R)BSC266, *Sxl*^{M1}, *sens*$^{Ly\text{-}1}$, Δ2-3,Sb (Transposase) and UAS-CD8-GFP. The following alleles were also aquired; c587Gal4 (T. Xie, Kansas city, KA, USA), *A2bp1*^{E03440} (Excelexis, Boston, MA,USA), *N*^{55ell} (S. Artivanis-Tsaksonas, Boston, MA, USA), *N*^{Axe2} (S. Artivanis-Tsaksonas, Boston, MA, USA), A2bp1VALIUM20, Gemin3VALIUM20 and NotchVALIUM20 were obtained from the Transgenic RNAi project (Boston, MA USA), Dad-LacZ (H. Ashe, Manchester, UK), E(spl)mβ$^{1.5\text{-}LacZ}$ (S.Bray, Oxford, UK) (Appendix I). All flies were maintained on standard cornmeal-agar media supplemented with live yeast. All crosses were carried out at 25^{o}C except RNAi crosses, which were carried out at 27^{o}C. All wild type controls were Oregon-R. Recombination crosses for generating *A2bp1*^{KG06463}, *Dad-LacZ* are described in Appendix I.

Dissections

Females were anaesthetised using ice and then pinned onto a SYLGARD® (Dow Corning, Barry, UK) containing plate using a 2 mm pin (Fine Science Tools, Heidelberg, Germany). The ovaries were removed using forceps (Fine Science Tools, Heidelberg, Germany) and ovarioles were separated using 0.1 mm pins (Fine Science Tools, Heidelberg, Germany) (more details in Appendix I). These were then placed in

phosphate buffered saline containing 0.1% Tween 20 (v/v) (PBS-Tw) and 4% formaldehyde for 20 mins at room temperature.

Antibody and Actin immunofluorescence.

Ovarioles were stained using either mouse IgG anti-Sex lethal 118 (1:50, Developmental Studies Hybridoma Bank, Iowa city, IA, USA), mouse IgG anti-Fasciclin III (1/20, Developmental Studies Hybridoma Bank, Iowa city, IA, USA), mouse IgG anti-βgal (1/1000, Promega, Madison, WI, USA) or mouse IgG anti-αSpectrin (1/20, Developmental Studies Hybridoma Bank, Iowa city, IA, USA). All were diluted in PBS-Tw. This was achieved by incubating samples with the primary antibodies overnight at 4°C. Samples were then washed with PBS-Tw. The appropriate secondary antibody was then added for 2 hours at room temperature (Listed in Appendix I). The secondaries used were donkey Cyanine5 anti-mouse IgG, donkey Cyanine3 anti-mouse IgG, donkey Rhodamine Red X anti-rat IgG (all purchased from Jackson ImmunoResearch, Suffolk, UK), diluted in PBS-Tw. Samples were then washed again and 4'-6-diamidino-2-phenylindole (DAPI) -containing mountant (H-1200, Vector Laboratories, Peterborough, UK) was added. Ovarioles were stained with rat anti-Bag of Marbles (1:500, gift from D. McKearin, Chicago, IL, USA) using a protocol described elsewhere (McKearin and Spradling, 1990, see Appendix I). Actin staining was achieved by incubating ovarioles with phalloidin- Fluorescein Isothiocyanate (1:100, Sigma-Aldritch, Dorset, UK) for an hour at room temperature. These were subsequently washed with PBS-Tw and mounted with DAPI containing mountant. Samples were left overnight at 4°C and were then mounted on a glass slide. All images were taken with a Hamamatsu digital camera mounted on a Zeiss Axioskop microscope. Subsequently, images were processed using Improvision Openlab or Velocity (PerkinElmer, Waltham, MA, USA). Where deconvolution was required, Z-section images were taken at 0.5 μm intervals and were then merged. Deconvolution of

Z-sections was achieved using Improvision Openlab software (PerkinElmer, Waltham, MA, USA).

Polymerase Chain Reaction, Reverse Transcription Polymerase Chain Reaction and electrophoresis.

30-50 male flies were mashed and incubated with ProteinaseK (Sigma-Aldritch, Dorset, UK) at a concentration of 20 µg/ml for 2 hours at 55°C. This was then incubated with RNase inhibitor for 30 mins at 37°C. DNA was then extracted using the phenol chloroform extraction method described elsewhere (Wilson, 2001, see Appendix I). DNA was precipitated using 100% ethanol and subsequently stored in distilled water. Primers against *A2bp1* were designed using primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). 5'-ACA ACT TGG CGC TCT TCT GT- 3' (15F1) and 5'- CGA ATT CAA CAG GCC AAT CT- 3' (15R1) were used to amplify part of intron 2 of *A2bp1*. PCRs and reverse transcription PCRs were carried out using a TGradient PCR machine (Biometra, Goettingen, Germany). The Taq polymerase and buffers used in the PCR were purchased from Roche Diagnostics (West Sussex, UK). RNA was extracted from 30 virgin female ovaries using the RNeasy Mini Kit and QIAshredder (QIAgen, West Sussex, UK) as per manufacturer's instructions. *Sxl* RT PCR primers were taken from Johnson *et al*., (2010). These primers are found in exons 2 and 4 of *Sxl*. The RT PCR was carried out using the SuperScript® III Reverse Transcriptase kit (Invitrogen, Life Technologies, Manchester, UK) as per manufacturer's instructions. All samples were run in a 1% agarose gel in Tris acetate ethylenediaminetetraacetic acid (EDTA) buffer. Control primers used were the following; 5' -AGA TGA CCA TCC GCC CAG CAT- 3' (RP49F) and 5' -CGA CCG TTG GGG TTG GTG AG- 3' (RP49RC). (*Tm* values used are listed in Appendix I).

X-Gal stain

Flies were aged to 3 or 6 day old. Ovaries were dissected in cold Grace's medium (Sigma-Aldritch, Dorset, UK) and fixed for 20 minutes at room temperature in 5 µl of 25% glutaraldehyde (Sigma-Aldritch, Dorset, UK) in 1 ml of PBS. This was rinsed with PBS and stained overnight with X-Gal (2nM MgCl2, 6mM $K4Fe^{II}CN6$, 6mM $K3Fe^{III}CN6$, 0.2% X-Gal). Ovarioles were subsequently washed with PBS and mounted in Vectamount™ (H-5000, Vector Laboratories, Peterborough, UK) or 70% glycerol.

Phenotypic analysis

The tumorous phenotype was identified by using DAPI and αSpectrin to stain nuclei and spectrosome/fusome structures respectively. Tumorous ovarioles contain a large number of non-polyploid germline cells which contain αSpectrin positive structures. Egg chambers which had undergone extra mitoses (5n) and egg chambers which consisted of multiple mispackaged cysts (compound) were identified by the use of phalloidin to stain ring canals (protocol described above). Chi^2 test was used to determine whether the number of abnormal ovarioles (tumorous+ 5n+compound) was significantly different from wild type. This was performed using the SPSS statistical software package.

Results

A2bp1^{KG06463} homozygotes do not undergo normal differentiation.

During a screen of transposon insertion mutants for disruptions to early oogenesis, we identified the recessive *A2bp1^{KG06463}* insertion line. The ovarioles of homozygous mutant flies contained an excess of germline cells (Fig. 1A,B). This phenotype was 100% penetrant and reverted by remobilisation of the P-element (Fig. 1C-E). Further analysis of these tumours showed that *A2bp1^{KG06463}* ovarioles have a large number of spectrosome-containing cells. In addition to this, the few developing cysts with branched fusomes present do not produce mature ring canals (Fig. 2A,B). To determine the developmental stage of the excess cells, we immunostained for the cystoblast differentiation marker BAM, which is normally present in early cystoblasts and cysts (McKearin and Spradling, 1990). Many of the spectrosome-containing cells in the posterior region of the tumorous ovarioles were not positive for BAM suggesting that germline differentiation had been disrupted, or the differentiating cysts had reverted to an early stage (Fig. 2C,D). Since DPP signalling in GSCs represses BAM expression we used a Daughters against DPP (DAD)-lacZ reporter to determine if the tumorous cells retained this stem cell characteristic. We found no difference in DAD-lacZ expression between wild type and *A2bp1^{KG06463}* (Fig. 2E,F). Indeed, the cap cell niche and associated GSC stem cells appeared normal in the *A2bp1^{KG06463}* ovarioles (Fig. 2G,H). The above results suggest that the germline in *A2bp1^{KG06463}* has stalled differentiation at an intermediate stage between GSCs and cystoblasts which is not dependent on DPP signalling from the cap cells. Alternatively, the phenotype may arise from breakdown and de-differentiation of early cysts, reflected in the immature ring canal interconnections observed in *A2bp1^{KG06463}*.

A2bp1 and Gemin3 are involved in the A2bp1^{KG06463} phenotype.

The insert in the *A2bp1*^{KG06463} line is located in a 5' intron of A2BP1, a protein that is known to regulate alternative splicing of pre-mRNA (Lasko, 2000; Nakahata and Kawamoto, 2005; Underwood *et al*., 2005; Fukumura *et al*., 2007; Lee *et al*., 2009; Gehman *et al*., 2011; Fogel *et al*., 2012) and which has recently been demonstrated to be involved in an intermediate step of female germline differentiation (Tastan *et al*., 2010). In order to confirm that disruption of *A2bp1* is involved in the *A2bp1*^{KG06463} phenotype, we carried out complementation at the insert site using several different alleles of *A2bp1* (Fig. 3A). When crossed to a deficiency that spans the insert site, all ovarioles appear tumorous. However, when *A2bp1*^{KG06463} was crossed to other alleles of *A2bp1*, the phenotype observed depended on the allelic combination used and tumorous ovarioles were only sporadically present (Fig. 3B).

The *A2bp1*^{E03440}/*A2bp1*^{KG06463} combination was a mixture of partially tumorous ovarioles and a 5n phenotype, displaying just one extra round of germ line mitosis. The latter was demonstrated by staining the actin ring canals since the single oocyte present possessed 5 ring canals instead of the usual four (Fig. 3C,E). Other allelic combinations of *A2bp1*^{KG06463} over *A2bp1*^{VK00039} or *A2bp1*^{EY00149} produced a compound egg chamber phenotype where multiple cysts were mis-packaged into one egg chamber (Fig. 3D), a phenotype not previously reported for A2bp1.

It was surprising that the *A2bp1*^{E03440}/*A2bp1*^{KG06463} combination produced only a weak tumorous ovary phenotype, since homozygous *A2bp1*^{E03440} flies have been reported to display a strong tumorous ovary phenotype similar to *A2bp1*^{KG06463} (Tastan *et al*., 2010). However, we were not able to confirm the *A2bp1*^{E03440} phenotype because, in our hands, the *A2bp1*^{E03440} line did not produce homozygous adults. Instead we considered whether the strong ovary phenotypes of *A2bp1*^{KG06463} allele might result from functional disruption of another neighbouring gene in addition to *A2bp1*. We

therefore carried out complementation with *Gemin3*, a DEAD box RNA helicase that is also involved in RNA processing and is situated close to *A2bp1*(Lasko, 2000; Cauchi *et al*., 2010). In $A2bp1^{KG06463}$/ $Gemin3^{rL562}$ flies, 66% of ovarioles contained at least one 5n egg chamber (Fig. 3E). Other *A2bp1* mutations in heterozygous combinations with $Gemin3^{rL562}$ did not interact (Fig. 3E. Note the dominant phenotype of $A2bp1^{E03440}$ heterozygotes). This suggests that only the $A2bp1^{KG06463}$ allele disrupts both *A2bp1* and *Gemin3* gene functions, possibly through disruption of a long range gene regulatory enhancer sequence or due to chromatin silencing induced by the *suppressor of Hairy wing* sequence found in the $A2bp1^{KG06463}$ insert. Other genes found in this region, *CG32063*, *CG32061*, *S-LAP4* and *CG6257*, are unlikely to be affected in the ovary of $A2bp1^{KG06463}$ mutants as they are expressed in the testis, but not the ovary (www.flybase.org).

A2bp1 and gemin3 regulate the supply of both germline and follicle cells

Since complementation had identified *A2bp1* and *Gemin3* as candidate genes involved in the $A2bp1^{KG06463}$ phenotype, we decided to further confirm this by carrying out RNAi. Furthermore, RNAi using the Gal4/ Upstream Activating Sequence (UAS) system would allow us to identify which tissues require functional A2BP1 and Gemin3. Removing A2BP1 from the germline using Nanos::VP16 Gal4 and the A2BP1VALIUM20 RNAi expressing line (Ni *et al*., 2011) results in an extra round of germline mitosis. This phenotype was highly penetrant, with 96% of ovarioles containing multiple 5n egg chambers (Fig. 4C). However, removing A2BP1 from follicle stem cells and escort cells, using c587Gal4 (Supplemental S1), also produces abnormal ovarioles which contain compound egg chambers, also at a high frequency of 87% (Fig. 4C). This supports the above described complementation data which defines roles for A2BP1 in both germline differentiation and follicle cell production. Expression of Gemin3 RNAi in the germline led to a complete loss of germline in most ovaries

(Fig. 4A). However around 6% of ovarioles retained the germline and these had tumorous ovarioles (Fig. 4B). Loss of Gemin3 in somatic cells, using c587Gal4, also produces a low frequency of ovarioles with compound egg chambers (Fig. 4C). These results suggest that Gemin3 plays a key role in the maintenance and differentiation in GSCs and, to a lesser extent, in maintaining the follicle cell lineage. The results further support the conclusion that the *A2bp1*^{KG06463} phenotype is due to disruption of both *Gemin3* and *A2bp1* gene functions.

A2bp1 and Gemin3 regulate Drosophila oogenesis through inhibition of Sxl function

It has been shown previously that *A2bp1* alleles affect SXL expression (Tastan *et al*., 2010). As *A2bp1*^{KG06463} produces tumours which contain branched fusomes, similar to mutants with altered SXL expression (Bopp *et al*., 1993; Chau *et al*., 2009; Vied and Kalderon, 2009), we investigated whether SXL expression was also affected in the *A2bp1*^{KG06463} mutant line. We found that in wild type ovarioles, SXL is present in the cytoplasm of GSCs and cystoblasts but is down-regulated during cystoblast mitosis (Fig. 5A). This staining pattern is consistent with published data (Bopp *et al* ., 1993, Chau *et al* ., 2009). In contrast, *A2bp1*^{KG06463} homozygotes do not downregulate SXL in cystoblasts. (Fig. 5B). Similarly, a combination of weaker alleles of *A2bp1* and *Gemin3*, which are able to produce polyploid nurse cells, also have persistent SXL expression that appears to be mostly localised to the cytoplasm (Fig. 5C,D). However, the germaria of these mutant combinations appear to be normal. Since Gemin3 and A2BP1 are RNA associated proteins, we carried out RT PCR to determine whether SXL splicing in *A2bp1*^{KG06463} ovaries is normal. RT PCR indicated that *Sxl* mRNA is correctly spliced to produce female SXL and thus the upregulated protein is likely to be functional (Supplemental S2). Note, this contrasts with cytoplasmic accumulation of SXL in germline cells that has been reported to result from Hedgehog loss of function and is

associated with increased expression of the non-functional male specific form of SXL (Vied and Horabin, 2001).

To investigate whether misregulation of SXL is likely to be involved the generation of the *A2bp1* mutant phenotype, we tested for genetic interactions with the heterozygous $A2bp1^{E03440}$ allele, which has a dominant 5n phenotype. A loss of function mutation, Sxl^{f4}, suppressed the 5n phenotype, with only 2% of ovarioles containing a 5n egg chamber. Similarly, a loss of function allele of *snf*, which is required for SXL expression in the germline (Chau *et al*., 2009), leads to rescue of the $A2bp1^{E03440}$/+ phenotype. These data indicate that the activity of SXL lies either downstream of, or parallel to, A2BP1 and is required for the 5n phenotype resulting from the latter's loss of function (Fig. 6).

We next tested known downstream targets of SXL regulation for genetic interactions with the $A2bp1^{E03440}$/+ mutant phenotype. One known downstream target of SXL is Notch (N). Expression of Notch is reduced in females through SXL activity and this contributes to sex specific developmental differences (Penn and Schedl, 2007). Notch has been shown to act in somatic cells of the niche to indirectly control GSC maintenance but no role in subsequent germline development has previously been identified (Song *et al*., 2007). While investigating oogenesis phenotypes of different Notch mutant alleles, we noticed that females that were heterozygous for the gain of function N^{Axe2} allele (Portin, 1975) sometimes produce egg chambers in which the germline had undergone only 3 rounds of mitosis, with 14% of ovarioles containing at least one of these “3n” egg chambers (Fig. 7A). We found that germline expression of E(spl)m$\beta^{1.5\text{-lacZ}}$, a Notch signal reporter (Furriols and Bray, 2001)**,** was increased in N^{Axe2} mutant ovarioles, consistent with a gain of Notch function in the germline. Curiously, flies that were heterozygous for the N^{55e11} deficiency of Notch also showed a 3n mitosis phenotype, with 12% of ovarioles containing a 3n egg chamber (Fig. 7B). Also surprisingly, removing one copy of *Notch* led to increased germline Notch

signalling (Fig. 7C,D). We wondered whether increased SXL expression in the *A2bp1* mutants might suppress a previously unknown function of Notch to limit the numbers of germline divisions. In flies that were heterozygous for $A2bp1^{E03440}$ and either N^{Axe2} or N^{55e11} there was mutual suppression of both phenotypes and a wild type number of germline cell divisions was restored demonstrating a functional interaction (Fig. 7B). We tested whether loss of Notch signalling could explain the 5n phenotype of *A2bp1* mutants by expressing Notch RNAi in the germline. However we observed no germline phenotypes despite strong loss of function phenotypes in the follicle lineage when the same RNAi was expressed in somatic tissues by the c587Gal4 driver (Supplemental S3). The most likely explanation is that the *Notch* mutations are neomorphic (i.e. showing novel gene function which is gain of function) for this phenotype leading to ectopic signalling in the germline rather than reflecting a normal function of Notch. This may be because wild type levels of Notch normally suppress, through cis-inhibition, the ability of the ligands in the somatic cells to signal to adjacent germline cells. Reducing the copy number of Notch might relieve this block on ligand function. *Abruptex (Ax)* mutant alleles have been reported to show defective cis-interactions with Notch ligands (de Celis and Bray, 2000). However it is still possible that down-regulation of Notch through SXL may normally play a role in ensuring such inappropriate Notch activation does not take place in the germline.

We next tested whether there was an interaction between $A2bp1^{E03440}$ and $CyclinB^{2}$ which has previously been associated with SXL regulation (Vied *et al* ., 2003). SXL has been reported to promote CyclinB stability and entry into the nucleus (Vied *et al* ., 2003). Thus, if A2bp1 mutation led to increased SXL function then this may increase germline mitosis through promoting cyclinB activity. However, we found that when flies were double heterozygous for $A2bp1^{E03440}$ and $CyclinB^{2}$ mutations, the small reduction in the frequency of 5n ovarioles observed was not significant. Interestingly however the double mutant produced a weak compound egg chamber phenotype.

An alternative target that is negatively regulated by SXL is Ornithine decarboxylase antizyme (ODA). (Vied *et al* ., 2003). In turn ODA is a negative regulator of ODC which catalyses the rate limiting step of polyamine synthesis (Heby and Persson, 1990). The latter has been implicated in cell survival and proliferation (Pohjanpelto *et al*., 1985; Auvinen *et al*., 1992; Packham and Cleveland, 1994). When combined with heterozygous Oda^{Lex47}, in which most of the Oda coding region has been removed (Salzberg *et al*., 1996), the $A2bp1^{E03440}$ phenotype is strongly enhanced with 96% of ovarioles containing 5n egg chambers (Fig. 6). Oda^{Lex47} was also able to produce compound egg chambers when combined with the recessive mutant, $Gemin3^{rL562}$/+, with 13% of ovarioles containing at least one compound egg chamber (Fig. 6). Thus ODA may function downstream of SXL and Gemin3/A2BP1 in both somatic and germline cells.

To see if the downstream targets of ODA are involved in the germline phenotype, we combined a deletion which removes both *Odc1* and *Odc2* (Df(3R)BSC266) with $A2bp1^{E03440}$. However, loss of *Odc1* and *Odc2* did not significantly affect the dominant 5n phenotype of $A2bp1^{E03440}$, suggesting that the ovary phenotype is not influenced by the polyamine synthesis pathway (Fig. 6). There have been few other targets of ODA identified, but in mammalian cells, ODA is able to bind to and promote the degradation of SMAD1, a downstream target of the Bone Morphogenetic Protein (BMP) signalling pathway (Lin *et al*., 2002). We found that Mad^{1-2}, a mutant with a small deletion in *Mad* (Wiersdorff *et al*., 1996), is able to significantly rescue the 5n phenotype of $A2bp1^{E03440}$, suggesting that ODA may be enhancing the germline phenotype by functioning through MAD (Fig. 6). However, as discussed above, we do not see up-regulation of DADLacZ expression in *A2bp1* mutants suggesting a non-canonical function of MAD might be involved. Together the results suggest a model in which A2BP1 and Gemin3 normally function through suppressing SXL activity. This may relieve repression of ODA expression leading to

down regulation of MAD to regulate a number of different downstream components that together ensure tissue renewal is properly coordinated across germline and somatic cell lineages of the ovary (Fig. 8).

Discussion

Here we demonstrate the involvement of two RNA binding proteins A2BP1 and Gemin3 in the regulation of germline and follicle cell proliferation and differentiation during *Drosophila* oogenesis. Different mutant combinations of *A2bp1* and *Gemin3* result in numerous germline and somatic cell phenotypes. We show that these phenotypes result from misregulation of SXL which in turn acts through its downstream target ODA. We also demonstrate the involvement of the BMP target, MAD, which has previously shown to be regulated by Antizyme. Finally we uncover a possible role of A2BP1 in ensuring Notch signalling is suppressed in the germline.

A2bp1 and Gemin3, two RNA associated proteins, function to control germline differentiation.

The $A2bp1^{KG06463}$ insertion mutant was able to produce tumours when in a transheterozygous combination with deficiencies that completely removed both *Gemin3* and *A2bp1*. However, complementation analysis between $A2bp1^{KG06463}$ and different *Gemin3* and *A2bp1* alleles had less severe phenotypes. Some allelic combinations resulted in an extra round of germline mitosis while others were able to produce egg chambers which contained multiple cysts. This suggests that the $A2bp1^{KG06463}$ tumorous phenotype results from the reduced activity of both A2BP1 and Gemin3. This was further confirmed by the use of RNAi in the ovary with tissue specific Gal4 drivers. Loss of A2BP1 in the germline produced many 5n egg chambers. Additionally, loss of A2BP1 in both escort cells and follicle stem cells led to the mispackaging of cysts to produce compound egg chambers. This suggests that A2BP1 may have an additional role in the regulation of follicle cells as well as in regulating germline differentiation. Interestingly, RNAi of Gemin3 in the germline produced a small percentage of tumorous ovarioles. The majority of ovarioles in the Gemin3 RNAi had completely lost

their germline. The fact that this was not observed during the complementation between *Gemin3*^{rL562} and *A2bp1*^{KG06463} may be due to the fact that *A2bp1*^{KG06463} is not a complete null, since loss of function Gemin3 is lethal (Shpargel *et al*., 2009). This result suggests that Gemin3 may be essential for both GSC maintenance and germline differentiation. Interestingly, the *C. elegans* homolog of Gemin3, Maternal Effect Lethal (MEL)-46, is required to produce mature eggs, suggesting some aspects of Gemin3 function may be conserved.

A2bp1 and Gemin3 function through regulation of Sxl and Oda.

We found that there are elevated levels of SXL protein present in *A2bp1* and *Gemin3* mutants, which is consistent with published data (Tastan *et al*., 2010). However it has not been shown previously whether the increase in SXL expression is functionally relevant to *A2bp1* mutant phenotypes. Indeed, previous published data has shown loss of function of SXL can lead to defects in germline differentiation that are similar to *A2bp1*^{KG06463} phenotypes (Chau *et al*., 2009). Several of our results suggest that the *A2bp1/Gemin3* loss of function phenotypes result in part from increased SXL activity. Firstly, we analysed the splicing pattern of *Sxl* in *A2bp1*^{KG06463} mutant ovarioles and found *Sxl* mRNA to be spliced into the functional female specific form. Secondly, we found that mutations which reduce the function of *Sxl* rescued the 5n phenotype of the dominant *A2bp1* allele, *A2bp1*^{E03440}. Tastan *et al.,* (2010) demonstrated that A2BP1 marks an intermediate stage in germline development between a GSC-like state and early cyst development. They found that late expressed markers, such as RBP9, ORB and Bruno, require the expression of A2BP1. Interestingly, loss of function mutations of Bruno cause germline tumour phenotypes which, like *A2bp1*^{KG06463} mutants, do not form proper ring canals (Parisi *et al*., 2001). Bruno has been found promote germline differentiation by repressing SXL in the germline, and SXL over expression only significantly affects germline differentiation if the Bruno binding sites in its mRNA are

removed (Wang and Lin, 2007). One possibility, therefore, is that A2BP1 and Gemin3 may indirectly repress SXL levels through Bruno.

Since ODA is a known downstream target of SXL, we looked for interactions between an *Oda* mutant and the dominant *A2bp1*^{E03440} mutant (Vied *et al*., 2003). The reduction of *Oda* led to a strong enhancement of the 5n phenotype. The most well known function of ODA is the inhibition of ODC, which catalyses the synthesis of polyamines (Heby and Persson, 1990). Polyamines are known to be important for cell division and survival (McCann *et al*., 1977; Minois *et al*., 2011). However, a reduction of ODC did not affect the dominant phenotype of *A2bp1*^{E03440}. It is known that polyamines are able to upregulate the amount of ODA by controlling translation of *Oda* mRNA, thus leading to a feedback loop where an excess of polyamines will lead to a reduction in the amount of ODC (McCann *et al*., 1977). One possible explanation for the lack of interaction between *Odc* and *A2bp1*^{E03440} is that this feedback loop is able to compensate for a reduction in the amount of ODC by reducing the amount of the ODC inhibitor, ODA. Another possibility is that the germline function of A2BP1 and ODA is dependent on alternative ODA targets. Antizyme1, the mammalian homolog of ODA, is able to target SMAD1 for proteosomal degradation (Gruendler *et al*., 2001). SMAD1 is a homolog of MAD, which is phosphorylated by the DPP receptor, Thick veins (TKV) (Newfeld *et al*., 1997). We found that reducing MAD was able to substantially rescue the 5n phenotype of *A2bp*^{A2bp1E03440}. Thus ODA and A2BP1 may be able to downregulate MAD, subsequently promoting differentiation of GSCs. This interaction was unexpected since expression of DADLacZ, a reporter for DPP signalling, did not seem to be perturbed in *A2bp1*^{KG0646}. One possible explanation is that MAD is functioning in a non-canonical manner. Some SMAD proteins are able to influence the processing of miRNAs which will then go on to promote the degradation of specific mRNAs, thus the interaction we observed may be due to MAD activating different downstream targets independently of DAD (Oh and Irvine, 2011).

A2bp1^{E03440} also had a genetic interaction with a *CyclinB* mutant. While the resulting reduction in 5n phenotype was not significant, the double mutation unexpectedly produced a compound egg chamber phenotype. This was interesting because it suggests there may be an involvement of CyclinB in conjunction with A2BP1 to control follicle cell prolifration. Nevertheless the *CyclinB* mutation is known to inhibit the 5n phenotype of a mutant allele of *effete*, an E2 ubiquitin conjugating enzyme (Lilly *et al*., 2000), so a role controlling germline mitosis downstream of A2BP1 and SXL remains plausible.

Ectopic Notch signalling in the germline suppresses germline mitosis.

Whilst investigating a possible involvement of possible targets of SXL for germline mitosis defects, we found an unexpected germline phenotype associated with different *Notch* alleles. We found that both an *Abruptex* mutation of Notch and loss of one copy of the Notch gene led to a premature exit from mitosis in the germline, producing egg chambers which only have seven nurse cells and an oocyte. Both *Notch* alleles suppressed the *A2BP1* mutant 5n phenotype. Notch is already known to be important for regulating the exit from mitosis into the endocycle in follicle cells (Deng *et al*., 2001) but no function of Notch in the germline has previously been identified. We were unable to detect any germline phenotype when Notch was knocked down in the germline using RNAi. Using a Notch reporter, we found that, in both the *Abruptex* mutant and the heterozygous *Notch* deficiency, Notch signalling is inappropriately active in the germline. The 3n *Notch* phenotypes may, therefore, reflect a neomorphic activity. Notch is a transmembrane receptor protein whose activation is normally dependent on neighbouring cells presenting the Notch ligands, Delta or Serrate (Simpson, 1990). This leads to cleavage of Notch and translocation of the intracellular domain to the nucleus where it activates transcription of Notch targets (Okochi *et al*., 2002). Normally, in the ovary, Notch signalling is unidirectional with ligands present in

the germline signalling to somatically expressed Notch (Deng *et al*., 2001). However, Notch signalling can also be regulated through a process known as cis-inhibition, by which Delta, expressed in the same cell as the Notch receptor, is able to downregulate Notch and vice versa (de Celis and Bray, 2000). Cis-inhibition helps to reinforce unidirectional signalling so that signal-receiving cells become incompetent to signal back. It is possible that reduction of Notch expression in the null mutation relieves cis-inhibition of ligand function in the somatic cells enabling them to signal back to Notch in the germline. Similarly the N^{Axe2} mutation used may be incompetent to cis-inhibit the ligand, again leading to inappropriate activation of Notch in the germline. Given that SXL is known to down regulate Notch activity in somatic tissue (Penn and Schedl, 2007) it will be interesting to determine whether SXL also functions in the germline to keep Notch from being active.

Functional links between of A2bp1 and Gemin3.

To our knowledge, this is the first report of the close functional association between A2BP1 and Gemin3 and it is interesting, therefore, that both Gemin3 and A2BP1 are important in regulating RNA processing. Gemin3 is an RNA helicase that interacts with Survival Motor Neuron and other Gemin proteins in a complex which has a critical role in assembly of small nuclear ribonucleoproteins (snRNP) (Supplemental S4). The latter are constituents of ribonucleoprotein (RNP) particles known as U bodies, Cajal bodies and Gemini bodies, which are involved in regulation of pre-mRNA splicing (Cauchi *et al*., 2010). Interestingly, the snRBP complex protein U1C has been found to bind in a two-hybrid assay to mammalian homolog of A2BP1, Forkhead box 1 (FOX1) protein (Ohkura *et al*., 2005). Thus, there may be a direct mechanistic link between these proteins that underlies the functional interaction reported in this study. A2BP1 has itself been linked to regulation of splicing; it contains a conserved domain called an RNA Recognition Motif (RRM) domain and is required for alternative

splicing during development. Primary human neural progenitor cells, which have been treated with A2BP1 RNAi show altered splicing of mRNAs required for neuronal development (Fogel *et al*., 2012). Additionally, mice that have a deletion of the *FOX1* gene show altered neuronal excitation and changes in the splice pattern of genes associated with synaptic function (Gehman *et al*., 2012). The importance of post-transcriptional control is already well known in the *Drosophila* germline; the initial differentiation of the cystoblast requires the activity of Pumilios (PUM) and Nanos (NOS) which are thought to repress the translation of mRNAs required for differentiation (Forbes and Lehmann, 1998). It is the recruitment of BAM which inhibits the activity of PUM and NOS and promotes the differentiation of the germline (Li *et al*., 2009; Kim *et al*., 2010). In addition to this, it has recently been shown that BRAT is able to bind to NOS and inhibits the translation of mRNAs required for GSC maintenance (Harris *et al*., 2011).

It is interesting that both *Gemin3* and *A2bp1* genes have links to inherited neurodegenerative disorders. The human homologue of A2BP1 is a binding partner of Ataxin2 which is the gene affected in patients suffering from spinocerebellar ataxia type 2 (Huynh *et al*., 1999). A2BP1 has also recently been implicated in other neurological disorders such as autism spectrum disorder, bipolar disorder and schizophrenia (Martin *et al*., 2007; Le-Niculescu *et al*., 2009; Elia *et al*., 2010). Gemin3 is known to interact directly in a complex with Survival Motor Neuron (SMN) to promote proper neuromuscular function in Drosophila (Shpargel and Matera, 2005). Human homologues of SMN have been linked with the neurodegenerative disorder, spinal muscular atrophy (Shpargel and Matera, 2005). It will be interesting therefore to determine whether a conserved functional interaction between Gemin3 and A2BP1 is involved in maintaining normal neuronal function in humans.

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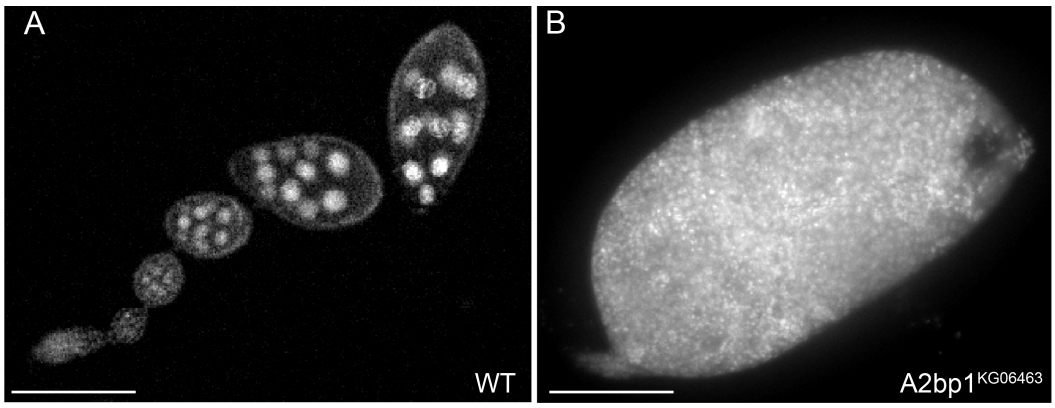
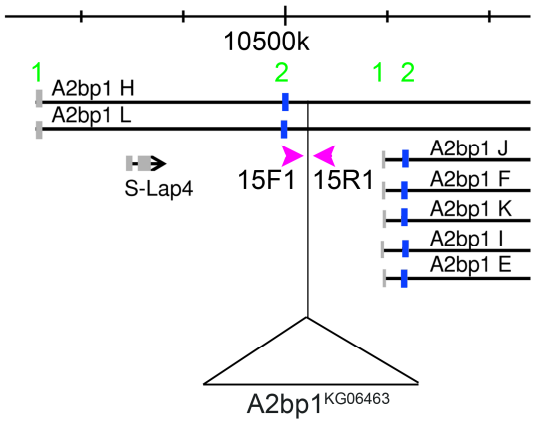
Figures

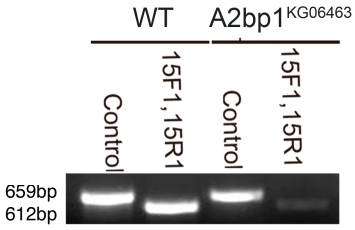
Fig. 1 Excision of the *A2bp1*^{KG06463} insert rescues ovariole phenotypes. **A.** WT ovariole showing egg chambers with normal polyploid nurse cells. **B.** *A2bp1*^{KG06463} ovariole showing complete lack of germline differentiation. A and B are stained with DAPI. Scale bars= 100 µm. **C.** Map showing location of *A2bp1*^{KG06463} insert. Arrows indicate the site of the 15F1/R1 primers. Green = Exon numbers, blue = translated exons, grey = untranslated regions. **D.** PCR shows amplification of the region between 15F1/R1 in wild type (WT) but not in *A2bp1*^{KG06463}. **E.** Frequency of tumorous ovaries for each P element excision line. W-1,-3 and -4 were completely rescued while W-2 retained fully penetrant phenotype. W+1 was a control line established from the same crosses which fully retained the P-insertion. **F.** PCR analysis of the P-element excision stocks. W-1 and W-4 are clean excisions, confirmed by sequencing. W-2 and W-3 retain 1.5kb and 39bp P-element sequence respectively. Scale bar = 65 µm.



C



D



E

Genotype	Tumorous ovaries
A2bp1$^{KG06463[-1]}$	0% (p=>0.9, n=8)
A2bp1$^{KG06463[-2]}$	100% (p=<0.005, n=20)
A2bp1$^{KG06463[-3]}$	0% (p=>0.9, n=8)
A2bp1$^{KG06463[-4]}$	0% (p=>0.9, n=14)
A2bp1$^{KG06463[+1]}$	100% (p=<0.005, n=16)

F

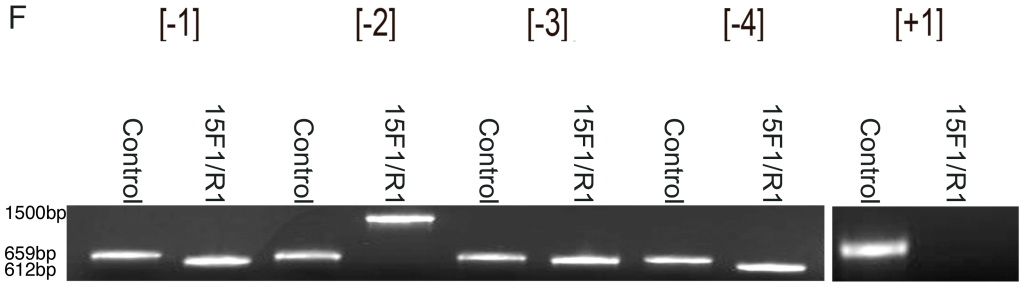


Fig. 2 GSCs in *A2bp1*^{KG06463} germline tumours are regulated normally. A. WT ovariole showing normal cyst development. Inset; ring canals (green) can be seen in older cysts surrounding the fusome (magenta). **B.** *A2bp1*^{KG06463} ovariole showing incomplete differentiation. Inset; ring canals are not present in branched cysts in *A2bp1*^{KG06463} ovarioles **C.** Bam can be seen in 2-4 cell cysts (arrowhead) of WT ovarioles but not in older cysts which are towards the posterior end of the germarium (inset). **D.** *A2bp1*^{KG06463} ovarioles have Bam staining (green) at the anterior end of the germarium (arrowhead), as in WT. Numerous spectrosome (magenta) containing cells towards the posterior end of the ovariole are not expressing Bam (inset). **E.** DADLacZ expression (magenta) in a WT background is confined to the proximity of the GSC niche (arrowhead). **F.** DADLacZ expression in a *A2bp1*^{KG06463} is similar to WT (arrowhead). **G.** Cap-cell niche of a WT ovariole with 2 GSCs present (Arrow heads). **H.** Cap cell niche of a *A2bp1*^{KG06463} ovariole has a wild type number of GSCs. Scale bar A-F= 15µm (scale in inset A=10 µm and inset in C=6 µm). Scale bar G-H 5 µm. WT= wild type.

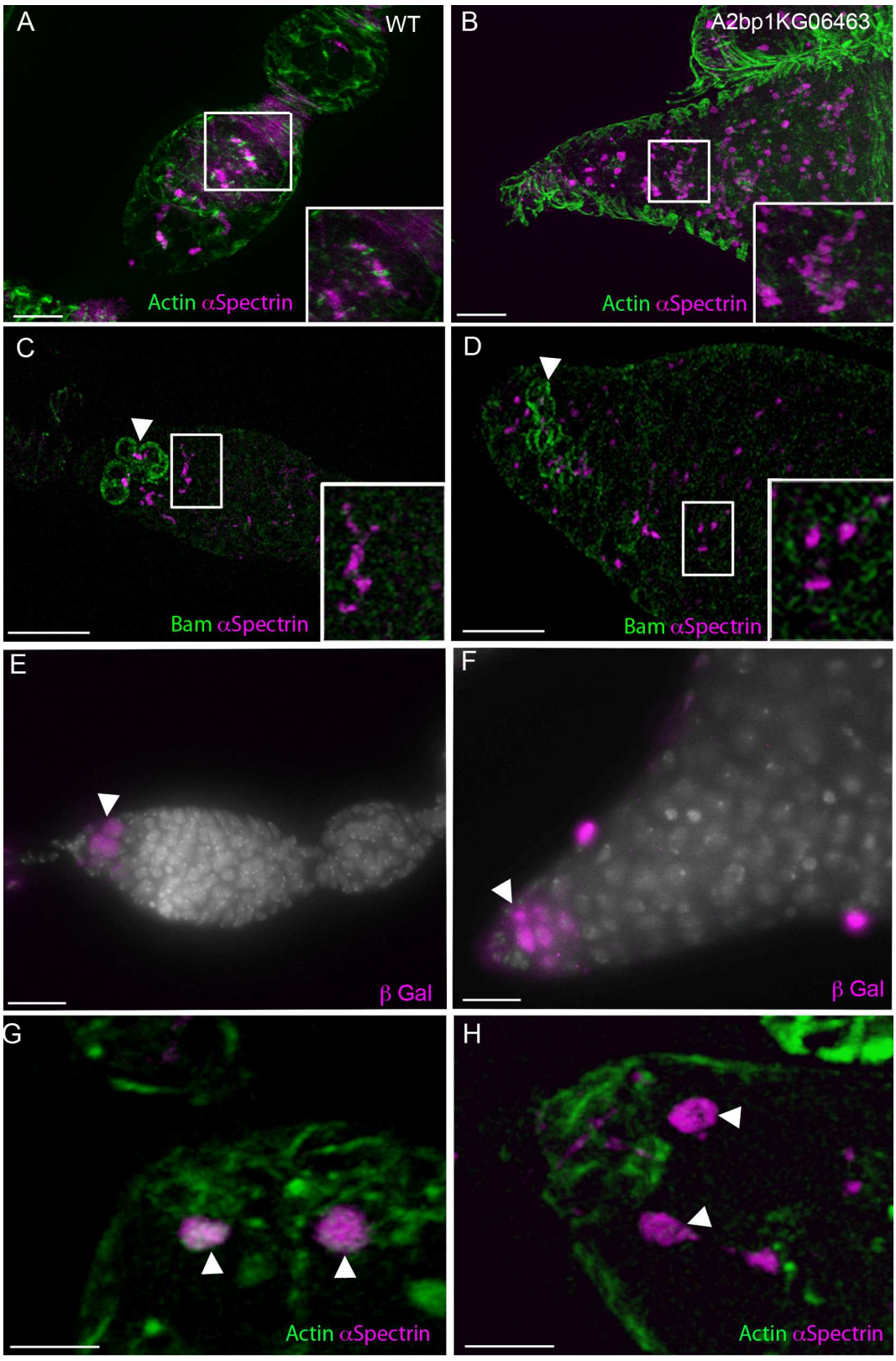

A
WT
Actin αSpectrin
B
A2bp1KG06463
Actin αSpectrin
C
Bam αSpectrin
D
Bam αSpectrin
E
β Gal
F
β Gal
G
Actin αSpectrin
H
Actin αSpectrin

Fig. 3 Complementation analysis implies that disruption of both Gemin3 and A2bp1 contributes to the *A2bp1*^{KG06463} phenotype. A. Genome map of the *A2bp1* and *Gemin3* loci. A2bp1 spliceforms E-L, CG32061, CG32063, SLap-4 and CG6527 are shown on the diagram. Blue = exons, grey = untranslated regions. **B.** Partial germline tumour phenotype of *A2bp1*^{E03440}/*A2bp1*^{KG06463} Phalloidin staining (magenta) reveals lack of actin-rich ring canals. **C.** 5 ring canals are clearly visible surrounding single oocyte in *A2bp1*^{KG06463}/*Gemin3*^{rL562}, indicating a 5n phenotype. **D.** Compound egg chamber phenotype in *A2bp1*^{KG06463}/*A2bp1*^{VK00039}. Four ring canals surrounding oocyte indicate germline cyst has undergone the normal number of mitotic divisions. **E.** Complementation analysis of the *A2bp1* and *Gemin3* alleles, phenotypes were scored as % abnormal ovarioles with proportions of germline tumour, 5n and compound egg chamber phenotypes indicated on graphs. Scale bar = 15 μm in A-D, n= >30. All samples were compared to wild type. * indicates $P \leq 0.05$ as determined by Chi^2 test. Images in C,D represent a merged stack of 15 consecutive deconvolved Z-sections sampled at 0.5 μm intervals.

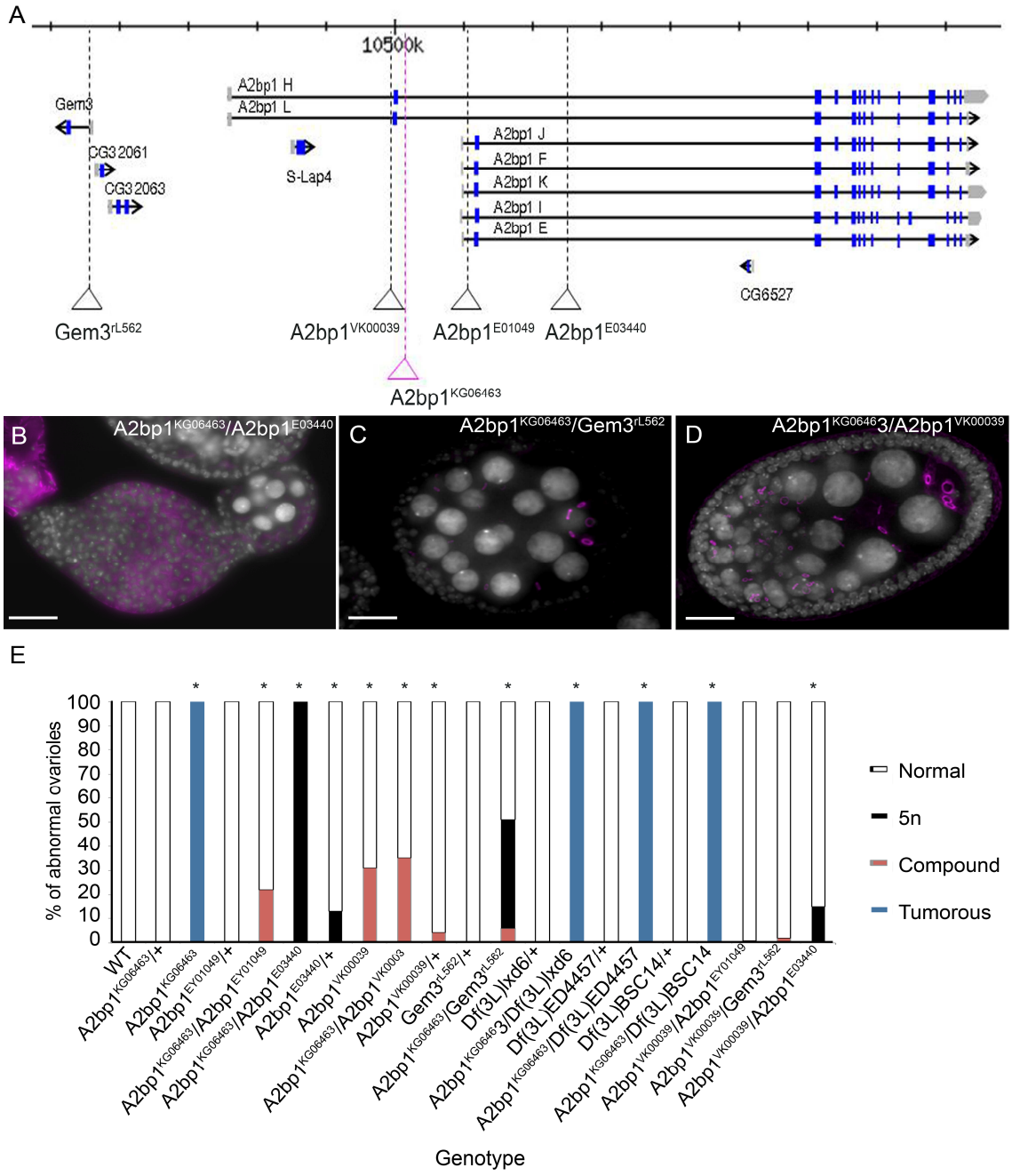
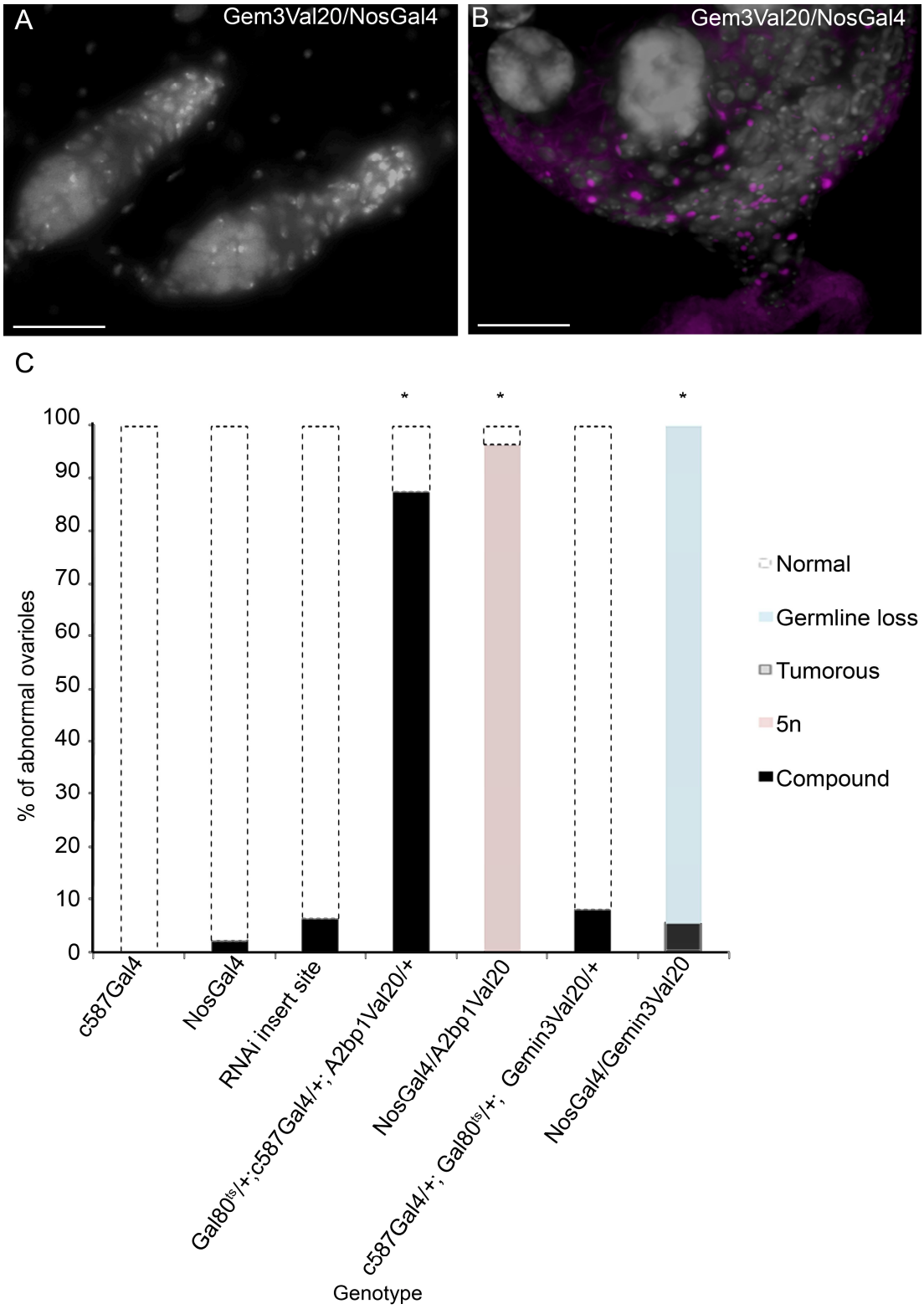

A
10500k
Gem3
CG32061
CG32063
A2bp1 H
A2bp1 L
S-Lap4
A2bp1 J
A2bp1 F
A2bp1 K
A2bp1 I
A2bp1 E
CG6527
Gem3rL562
A2bp1VK00039
A2bp1E01049
A2bp1E03440
A2bp1KG06463
B
A2bp1KG06463/A2bp1E03440
C
A2bp1KG06463/Gem3rL562
D
A2bp1KG06463/A2bp1VK00039
E
% of abnormal ovarioles
100
90
80
70
60
50
40
30
20
10
0
WT
A2bp1KG06463/+
A2bp1KG06463
A2bp1EY01049/+
A2bp1KG06463/A2bp1EY01049
A2bp1KG06463/A2bp1E03440
A2bp1E03440/+
A2bp1VK00039
A2bp1KG06463/A2bp1VK00039
A2bp1VK00039/+
Gem3rL562/+
A2bp1KG06463/Gem3rL562
Df(3L)Ixd6/+
A2bp1KG06463/Df(3L)Ixd6
Df(3L)ED4457/+
A2bp1KG06463/Df(3L)ED4457
Df(3L)BSC14/+
A2bp1KG06463/Df(3L)BSC14
A2bp1VK00039/A2bp1EY01049
A2bp1VK00039/Gem3rL562
A2bp1VK00039/A2bp1E03440
Genotype
Normal
5n
Compound
Tumorous

Fig. 4 RNAi of A2BP1 and Gemin3 indicates that they are required in germline and somatic cells for oogenesis. A. Example of a tumorous germarium in which *Gemin3* is knocked down in the germline. (αSpectrin, purple; DAPI, grey). **B.** Example of an ovariole which has lost its germline after *Gemin3* was knocked down with nanos-Gal4. **C.** % abnormal ovarioles scored after driving A2BP1 and Gemin3 RNAi expression in germline and somatic cells using nanos-gal4 or c587-gal4. Proportions of germline tumour, 5n and compound egg chamber phenotypes are indicated on graphs. All samples were compared to GlcATIVal20/+ (vacant RNAi chromosomal insert site line) and their respective Gal4 driver controls. * indicates $P \leq 0.05$ as determined by Chi^2 test. (Scale bar = 10 µm).


A
Gem3Val20/NosGal4
B
Gem3Val20/NosGal4
C
% of abnormal ovarioles
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Normal
Germline loss
Tumorous
5n
Compound
c587Gal4
NosGal4
RNAi insert site
Gal80ts/+;c587Gal4/+; A2bp1Val20/+
NosGal4/A2bp1Val20
c587Gal4/+; Gal80ts/+; Gemin3Val20/+
NosGal4/Gemin3Val20
Genotype

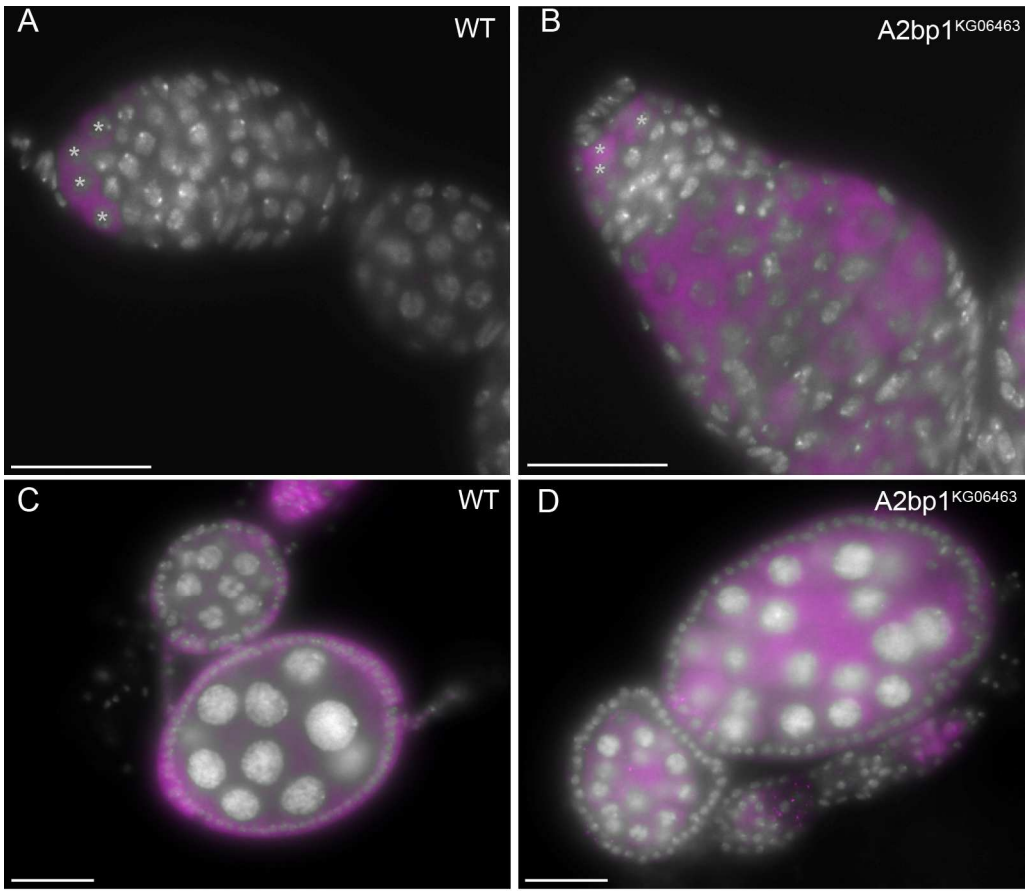


Fig. 5 SXL is upregulated in A2bp1^{KG06463}. A. In wild type ovarioles, SXL (magenta) is present in GSCs and cystoblasts (asterisks). **B.** SXL in *A2bp1^{KG06463}* is present throughout the germarium as well as in GSCs (asterisks). **C.** In WT egg chambers, SXL is not present in germline cells, demonstrated by a lack of magenta staining in the cytoplasm of nurse cells. **D.** In *A2bp1^{KG06463}*, SXL (magenta) is present in the cytoplasm of nurse cells of *A2bp1^{KG06463}/Gemin3^{rL562}*. Scale bar = 30 µm.

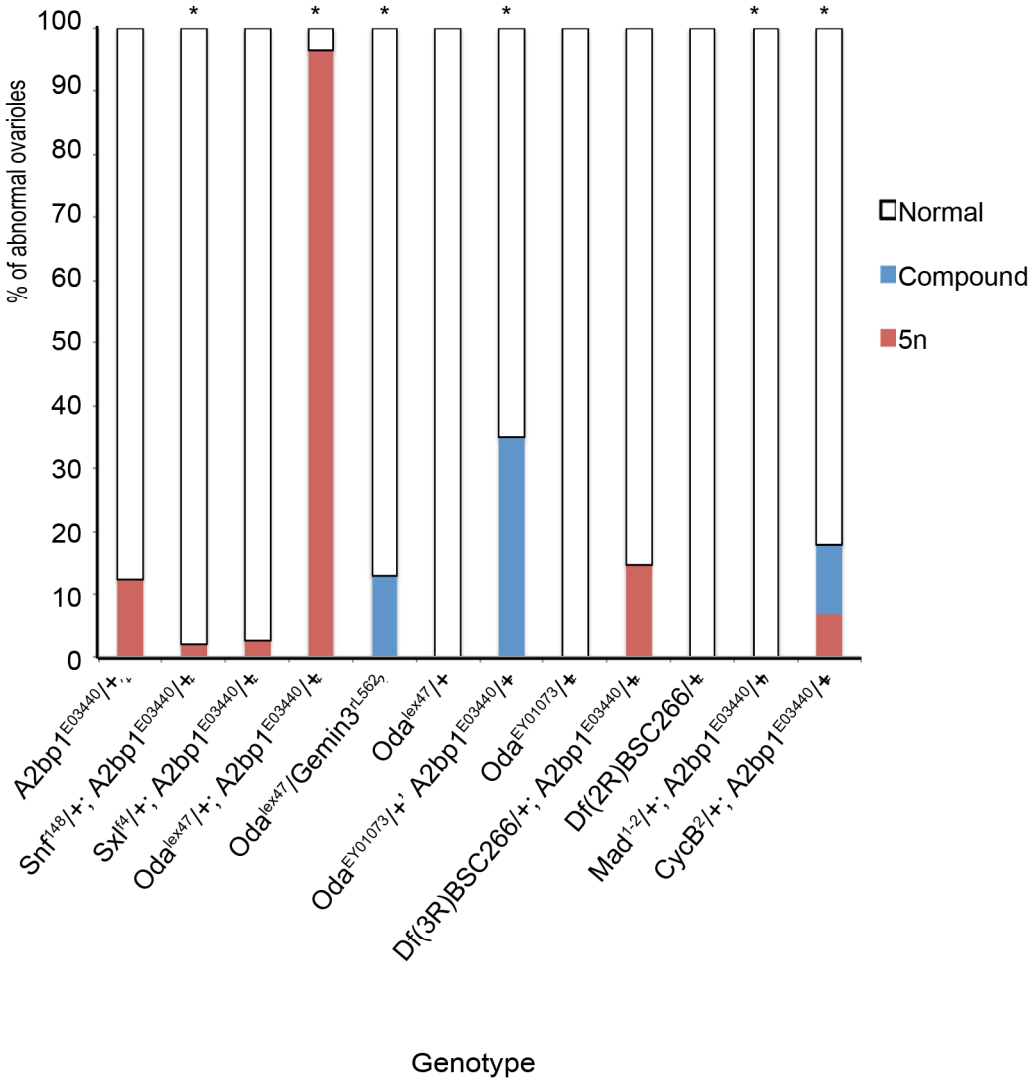


Fig. 6 Genetic interactions with *A2bp1*^{E03440} and *Gemin3*^{rL562}. % abnormal ovarioles were scored and compared to *A2bp1*^{E03440}/+. * indicates P≤0.05 as determined by Chi^2 test. Proportions of germline tumour, 5n and compound egg chamber phenotypes indicated on graphs.

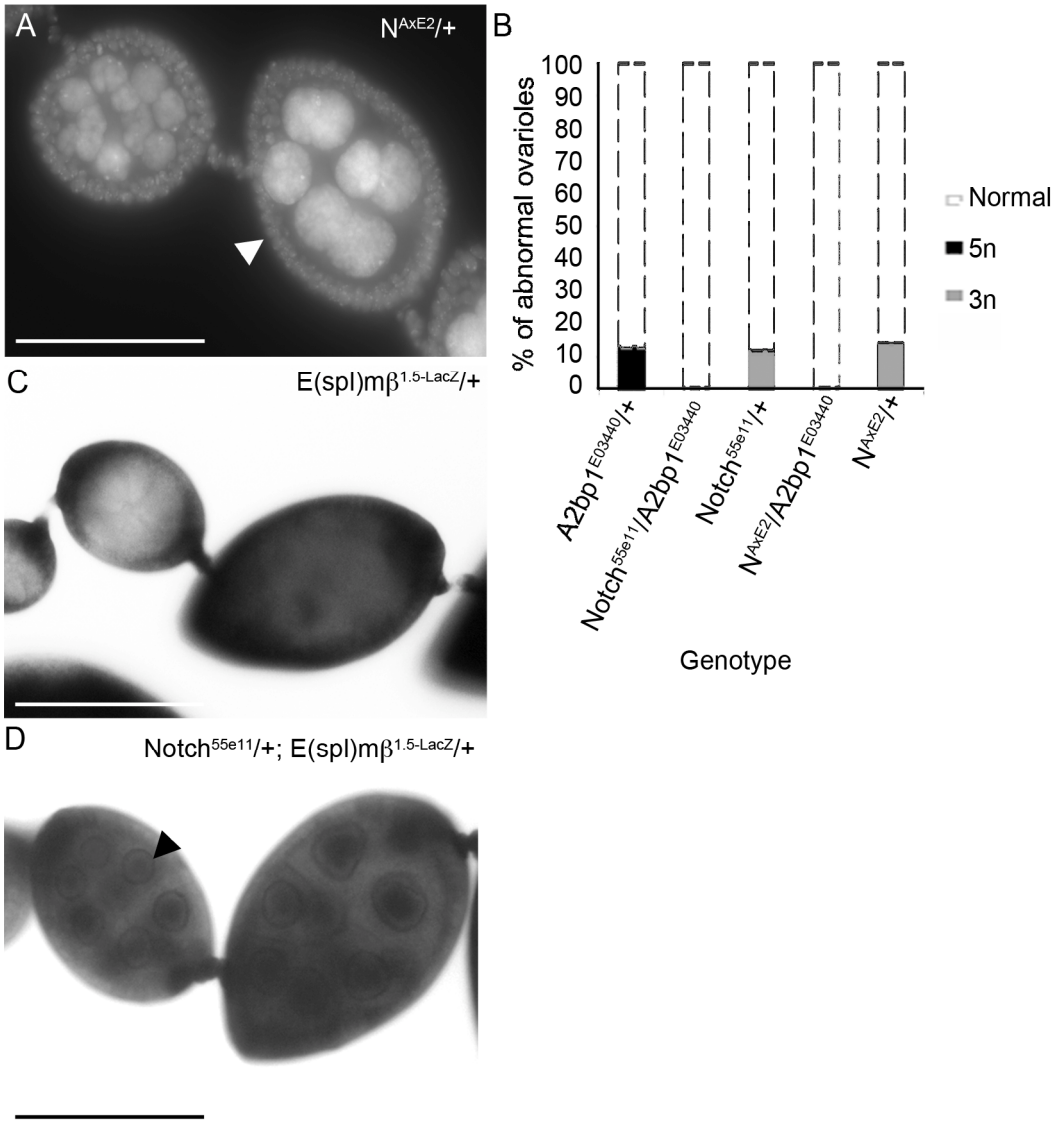


Fig. 7 Deregulated Notch signalling suppresses germline mitosis. A. Example of a 3n egg chamber (arrowhead) in N^{Axe2}/+ ovariole stained with DAPI. **B.** Mutual suppression of *Notch* 3n and *A2bp1* 5n phenotypes in double mutant combinations. Penetrance was determined by analysing a group of ovarioles from each genotype, determining the number of abnormal ovarioles per genotype and dividing this by the total number of ovarioles analysed for that genotype. This was then expressed as a percentage. N^{55e11}/+, N^{Axe2}/+ and $A2bp1^{E03440}$/+ phenotypes were significantly different when compared to wild type and double mutant combinations. * indicates P≤0.05 as determined by Chi^2 test. **C.** E(spl)m$\beta^{1.5}$/+ has little germline lacZ, indicated by a lack of lacZ accumulation near the nucleus. **D.** Example of inappropriate Notch activation in the germline. XGal was seen accumulating near the nucleus (arrowhead). Scale bar= 60 µm. C and D is stained with XGal.

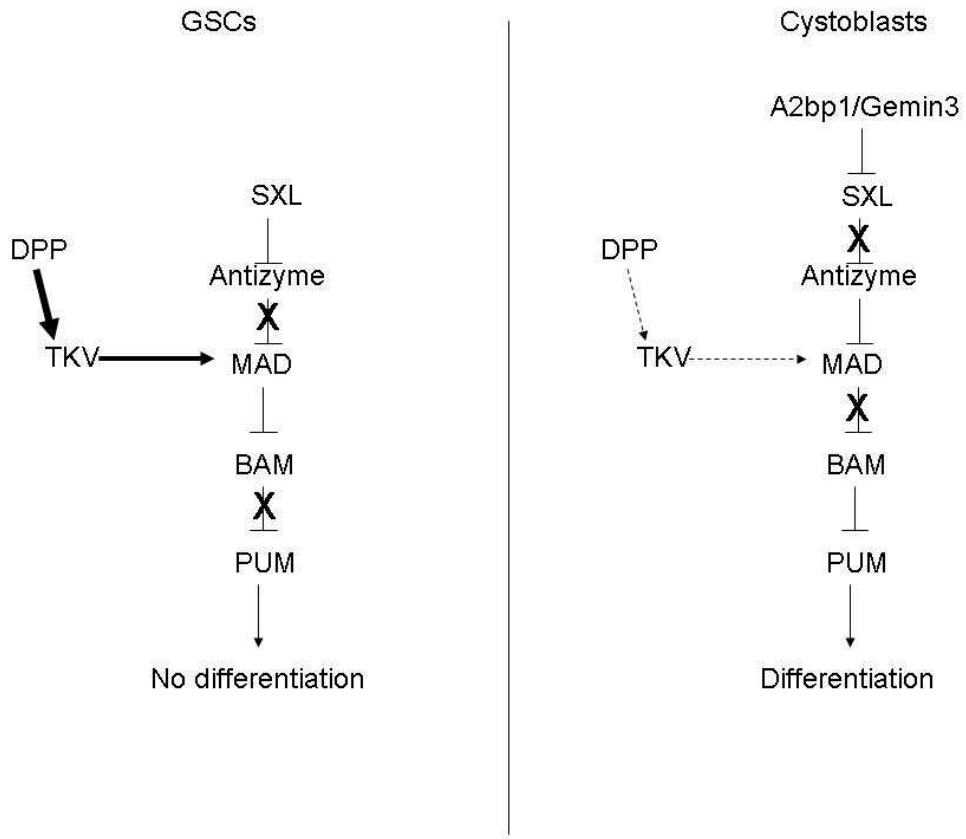
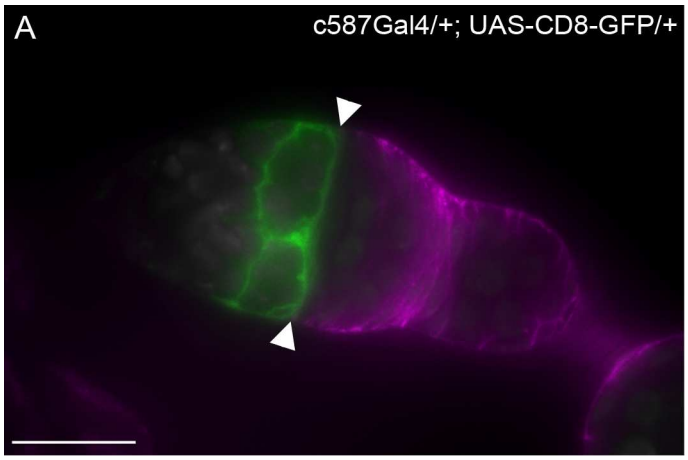
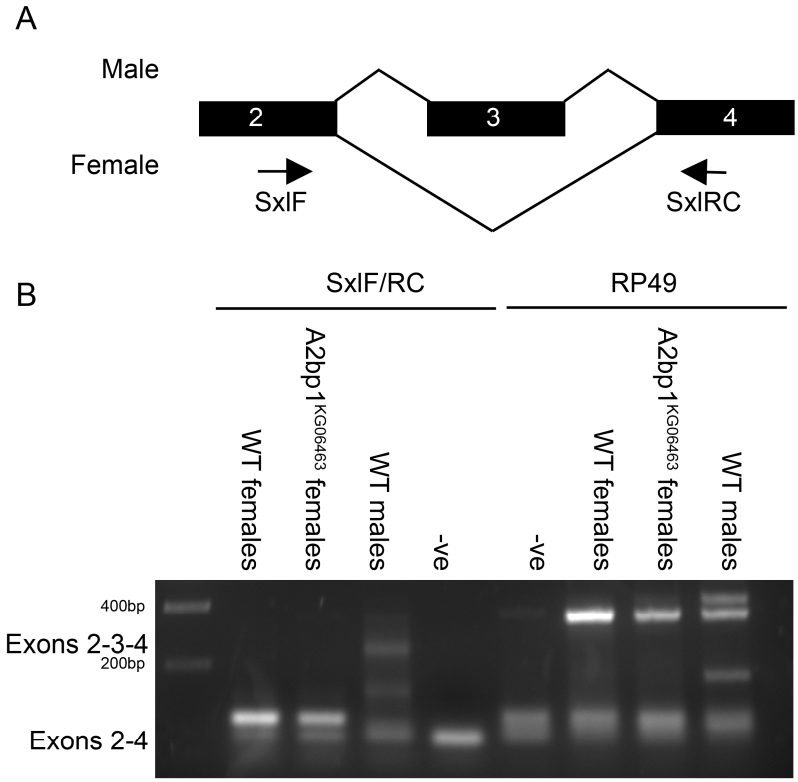


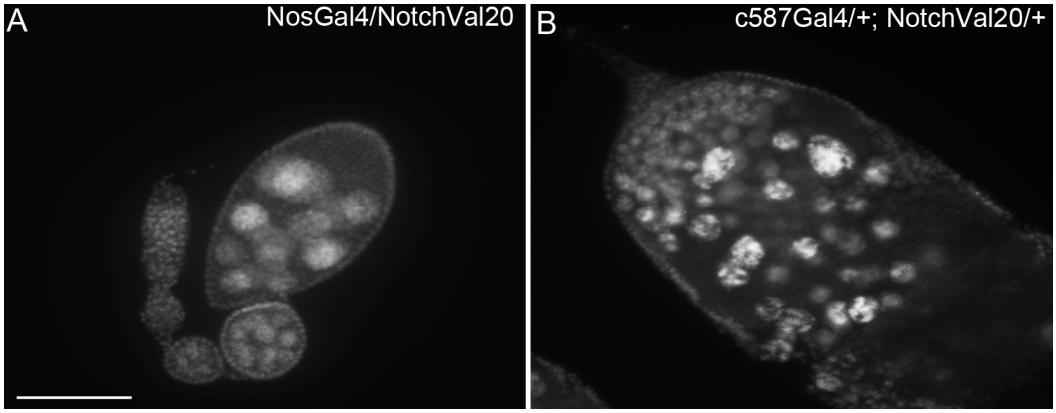
Fig. 8. Model explaining A2bp1/Gemin3 possible function in the germline. In the GSCs, Sxl inhibits Antizyme activity which leads to upregultion of MAD. Together with DPP, MAD promotes a GSC phenotype by repressing the expression of BAM. In cystoblasts, A2bp1 and Gemin3 would reduce SXL which leads to upregulation of Antizyme, which in turn targets MAD for proteosomal degradation. This would lead to upregulation of BAM, thus driving germline differentiation.



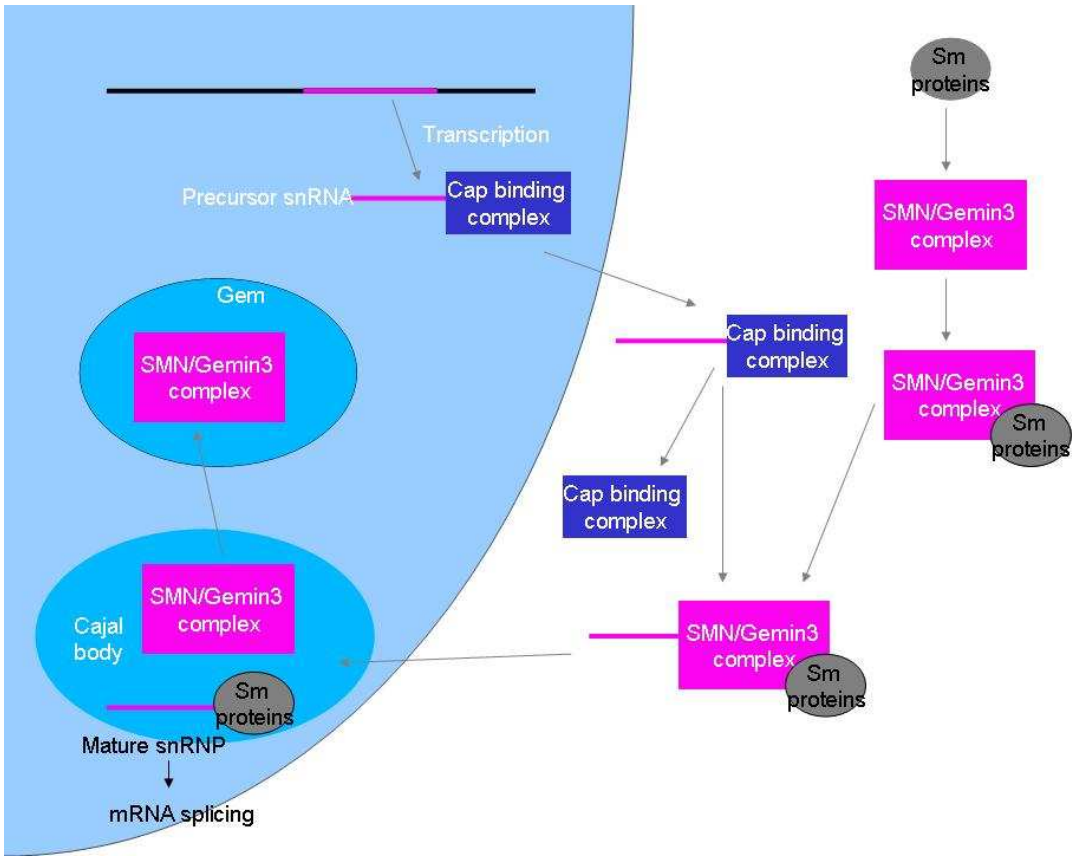
Supplemental S1. Expression pattern of c587 Gal4 in the adult germarium. A. In order to determine the expression pattern of c587Gal4, we crossed flies containing the c587Gal4 to flies containing a UAS-CD8-GFP construct. We chose to costain with FasIII (magenta), a marker for immature follicle cells. Follicle stem cells are located immediately anterior to FasIII expression domain and do not express FasIII, themselves. c587Gal4 drives the expression of a UAS-CD8-GFP (green) construct in both escort cells and follicle stem cells, indicated by a lack of space (arrowheads) between the expression of CD8-GFP and FasIII. Scale bar = 15 µm.



Supplemental S2. Sxl mRNA is normally spliced in *A2bp1*^{KG06463}. A. Exon 3 is spliced out of the female form of *Sxl*. Arrows indicate SxlF and SxlRC primer location. **B.** RT PCR showing the female splice form of *Sxl* is produced normally (left panels). Right panels show control PCR amplification of Rp49 cDNA. Lanes marked "-ve" are respective no template controls.



Supplemental S3. Loss of Notch in the germline does not produce a 3n phenotype. **A** NotchVal20 RNAi expressed with nanosGal4, a germline Gal4 driver, does not result in a germline phenotype (N=50), demonstrating loss of Notch in the germline is not the cause of the germline phenotype seen in Notch mutants. **B.** NotchVal20 RNAi is able to produce a strong compound egg chamber phenotype when driven in somatic cells with c587Gal4. This shows that the NotchVal20 RNAi construct is functional, suggesting Notch is not present in the germline. Scale bar= 65 µm.



Supplemental S4. Gemin3 plays a role in snRNP biogenesis. SMN/Gemin proteins associate with the Sm proteins required for splicing in the cytoplasm. In the nucleus, precursor snRNAs are transcribed and then protected by the cap binding complex. This complex is then exported out of the nucleus. In the cytoplasm these snRNAs associate with SMN/Gemin3. This complex is imported back into the nucleus and localised to Cajal bodies. Here, the SMN/Gemin proteins dissociate and are localised to Gem particles. The mature snRNP then goes on to control mRNA splicing. (Adapted from Pellizzoni, 2007).

Chapter 3. *Results Part II*

Disruption of Glucuronyl transferase I activity impairs escort and follicle cell contributions to *Drosophila* oogenesis.

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Abstract

The extracellular matrix (ECM) plays important roles in tissue formation, providing mechanical support and a substrate for cell migration. Additionally, the ECM facilitates and regulates pattern formation by interacting with components of different signalling pathways. An important constituent of the ECM are the extensively glycosylated proteins known as proteoglycans. The synthesis of this class of molecules is initiated by addition of an O-linked tetrasaccharide, which is subsequently modified by the addition of further sugar and sulphate residues. We identified a mutant in *Drosophila* which had reduced germline stem cells, defective escort cell invasion and defective packaging of cysts into egg chambers. P-element excision, complementation analysis and expression of a rescue construct identified the disrupted gene to be Glucuronyl transferase I (GlcATI), which is required for the final step in the synthesis of the linker tetrasaccaride which is added onto the serine of -Serine-Glycine-X-Glycine- sequences found in proteoglycans. Additionally, RNA interference indicated that GlcATI functions primarily in escort cells and follicle stem cells. We found that GlcATI is able to influence the activity of several different signalling pathways, including Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT), Decapentaplegic (DPP), Hedgehog (HH) and Epidermal Growth Factor (EGF) signalling, suggesting that proteoglycans produced as a result of GlcATI activity essential for modulating tissue function in the *Drosophila* ovary. GlcATI is one of three *Drosophila* Glucuronyl transferases. We also found that GlcATS and GlcATP, which catalyse the same and additional reactions during glycosaminoglycan synthesis, were also able to influence different aspects of oogenesis.

Introduction

Maintenance of a tissue throughout a multicellular organism's lifespan requires balancing the production of cells with cell loss. If this balance is not maintained and the tissue is unable to renew itself properly, then its function will be compromised. In diseases such as cancer, over-proliferation and poor differentiation of cells leads to disruption of the surrounding tissue, while under proliferation may contribute to tissue decline in certain age-related conditions. Both instances highlight the importance of understanding how healthy tissues are maintained and how tissue renewal occurs. The *Drosophila* ovary is a useful model for understanding the process of tissue renewal as the production and differentiation of numerous cell types from different stem cell lineages must be coordinated and maintained throughout the lifespan of an adult fly.

The *Drosophila* female has two ovaries, each of which is split into 15-20 independent egg producing structures called ovarioles. At the anterior tip of each ovariole is the germarium, where the two populations of stem cells that are required to produce eggs are housed. The first population are the germline stem cells (GSCs), which divide to produce a replacement GSC and a daughter cell known as a cystoblast (CB) (Lin and Spradling, 1993). The CB will undergo four rounds of incomplete mitosis to become a cyst, with each cell being connected through an actin-rich structure known as a ring canal and a branched organelle called the fusome (Lin *et al*., 1994; Ong and Tan, 2010). At this 16 cell stage, one of the interlinked cells undergoes meiosis, beginning the process of becoming an oocyte while the other 15 cells will differentiate into nurse cells, whose function is to supply the oocyte with maternal mRNAs (Barbosa *et al*., 2007). GSC maintenance is dependent on DPP signalling from cap cells and the terminal filament while the developing cyst is enveloped by a set of cells with long, thin projections known as escort cells (EC) (Fig. 1 in Chapter 1). Disruption of EC invasion

through loss of JAK/STAT results in a disorganised germarium (Kai and Spradling, 2003; Decotto and Spradling, 2005). Furthermore, germaria with disrupted EC invasion have a reduction in the number of GSCs, demonstrating that ECs are important in maintaining the GSC niche (Kirilly *et al*., 2011). Interestingly ECs appear to be able to sense the presence of differentiating cysts, since in ovaries where the germline is unable to differentiate, EC invasion in the germarium is defective (Kirilly *et al*., 2011); it is also known that ECs gradually undergo apoptosis when germline cells are absent (Kai and Spradling, 2003). It has recently been shown that Epidermal Growth Factor (EGF) is required both to regulate the ability of ECs to surround germline cysts and to restrict DPP signalling to the niche, which is essential for controlling proper differentiation of the germline. The long projections of ECs drive the movement of cysts through the germarium towards the second population of stem cells, the follicle stem cells (FSC). At this stage, the FSCs produce follicle cells that surround the developing cyst, replacing the EC cells. This developing cyst then buds off from the germarium and begins the process of differentiating into an egg as it is pushed through the ovariole. FSCs are anchored to the most posterior ECs through E-Cadherin and loss of this adhesion leads to FSC loss (Decotto and Spradling, 2005). Thus ECs are an important part of the niches of both GSCs and FSCs.

While screening P-element insertion lines for early oogenesis phenotypes we identified recessive mutants of the *GlcATI* gene which displayed compound egg chambers, reduced numbers of GSCs and mature cysts in the germarium, and disruption of EC invasion. Mutant phenotypes were phenocopied by expression of GlcATI specific ribonucleic acid interference (RNAi) and rescued by expression of wild type *GlcATI* complementary deoxyribonucleic acid (cDNA). GlcATI is so named because of the homology it shares with the catalytic domain of human β1-3 glucuronosyl transferase and its ability to catalyse the addition of a glucuronic acid residue onto a galactose

residue, which is an essential step in the early stages of proteoglycan synthesis (Kim *et al*., 2003). We also found similar oogenesis phenotypes for mutations of GlcATS which has similar biochemical function (Kim *et al*., 2003). Proteoglycans in the extracellular matrix and at the cell surface are known to be essential for regulating signalling. We tested genetic interactions of *GlcATI* mutants with a number of developmental signalling pathways to show phenotype-specific functional interactions of GlcATI with JAK/STAT, DPP, EGF and HH signalling in oogenesis, and the development of other adult tissues. These results extend the known contributions of the extracellular matrix and its modifications in regulating adult development and tissue renewal.

Methods

Further information can be found in Appendix I.

Fly stocks and maintenance

All stocks were maintained on yeast-molasses agar at 25°C. The following alleles were obtained from Bloomington (Bloomington, Indiana, USA); *GlcAT-I*^{E04384}, PTRiP.HMS0028attP2 (RNAi integration site), *GlcAT-I*^{F00247}, Df(1)BSC580, Df(1)ED6716, Df(1)ED6720, Nanos::VP16Gal4, Actin5CGal4, *hh*^{AC} , *hh*^{MRT} , *Egfr*^{t1} , *tkv*$^{Sz-1}$, *Stat92e*^{F}, Df(1)BSC352, P(Tub-PBac\T)2, CyO (Transposase), ptcGal4, *GlcATP*^{PL00294}, *GlcATS*^{EY01481}and UAS-CD8-GFP. The following alleles were also obtained; c587Gal4 (T. Xie, Kansas city, KA, USA), *hop*^{Tuml} (S. Brown, Sheffield, UK) and GlcATI VALIUM20 (Transgenic RNAi project, Boston, MA USA) (Full genotypes are listed in Appendix I). Crossing schemes for remobilising *GlcAT-I*^{F00247}, recombination of *GlcAT-I*^{F00247} with *hop*^{Tuml}, the rescue construct experiments and the genetic interaction experiment are listed in Appendix I. All experimental crosses were carried out at 25°C, except the RNAi experiments which were carried out at 27°C. All wild type controls were Oregon-R.

Dissection

Three day old or nine day old female flies were pinned to a SYLGARD® (Dow Corning, Barry, UK) plate containing phosphate buffered saline with 0.1% Tween (v/v) (PBS-Tw) and ovaries were removed with forceps (Fine Science Tools, Heidelberg, Germany). Ovarioles were separated and the sheath removed using 0.1 mm fine pins (Fine Science Tools, Heidelberg, Germany) (More details in Appendix I). Ovarioles were transferred to an eppendorf and fixed with 4% formaldehyde (v/v) in PBS-Tw for 20 mins.

Wings were dissected using forceps (Fine Science Tools, Heidelberg, Germany). These were arranged on a slide containing isopropanol and then allowed to air dry. Legs were also removed using forceps (Fine Science Tools, Heidelberg, Germany) and were arranged on the slide without isopropanol. Gary's magic mountant (1.5 g Canada balsam and l ml methylsalicylate) was added to the slide, which was then covered with a coverslip and sealed.

Coracle, αSpectrin and FasIII immunofluoresence.

Ovarioles were incubated overnight in either guinea pig anti-Coracle IgG (1/10 000, gift from R. Fehon, Chicago, IL, USA), mouse anti-αSpectrin IgG (1/20, Developmental Studies Hybridoma Bank, Iowa city, IA, USA) or mouse anti-FasciclinIII IgG (1/20, Developmental Studies Hybridoma Bank, Iowa city, IA, USA) diluted in PBS Tw. After washing the samples with PBS Tw, the secondary antibodies were added at a 1/800 dilution and left overnight (Appendix I). The secondary antibodies used were Donkey anti-mouse Alexa 488 fluorophore or donkey anti-guinea pig Rhodamine Red X IgG (Jackson ImmunoResearch, Suffolk, UK). Samples were again washed with PBS Tw and mounted in glycerol containing 4'-6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Peterborough, UK) was added. Images were acquired using a Ziess Axioskop microscope mounted with a Hammamatsu camera. Images were processed using Openlab (PerkinElmer, Waltham, MA, USA). Where deconvolution was required, stacks were produced by taking images at 0.5 µm intervals along the z axis. Deconvolution was also carried out in Openlab using 3 nearest neighbours.

Polymerase Chain Reaction

Primers were designed against GlcATI using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The following primers were used; 5'-GAC AGC TCG CCG ATT TGT TTG- 3' (GlcATIF), 5' -GCC TGC GGA TTC CTG ATG AAG- 3' (GlcATIRC), 5' -GAA AAG GTC CAA AGT CGC AA- 3' (PBac3F) and 5' -TCC AAG CGG CGA CTG AGA TG- 3' (5R2) (Thibault *et al*., 2004). PCR was carried out using DNA Taq polymerase (Roche Diagnostics, West Sussex, UK) in a TGradient PCR machine (Biometra, Goettingen, Germany). Control primers used were the following; 5' -AGA TGA CCA TCC GCC CAG CAT- 3' (RP49F) and 5' -CGA CCG TTG GGG TTG GTG AG- 3' (RP49RC). DNA was extracted from 30-50 male flies by mashing them and incubating with ProteinaseK (Sigma-Aldritch, Dorset, UK) at a concentration of 20 µg/ml for 2 h at 55°C. This was then treated with RNase inhibitor for 30 mins at 37°C. A phenol chloroform extraction method described elsewhere (Wilson, 2001, see Appendix I) was used to extract DNA which was then precipitated using 100% ethanol (v/v) and subsequently stored in distilled water. All PCRs were run in a 1% agarose gel in a Tris-acetate ethylenediaminetetraacetic acid buffer (EDTA). *Tm* values are listed in Appendix I.

Reverse Transcription Polymerase Chain Reaction

Primers which span the exon-exon boundary in GlcATI mRNA were designed for use in RT PCR experiments. The primers used in the RT PCR experiments were 5' -GTT ATT TAG GCA CAC AGC TCG C- 3' (F2) and 5' -GCC TGC GGA TTC CTG ATG AAG- 3' (R2). RNA was extracted from 30-50 ovaries using the QIAgen RNA extraction kit (QIAgen, West Sussex, UK) and the RT PCR was carried out for 18 or 40 cycles using the Superscript® III RT PCR kit (Invitrogen, Life Technologies,

Manchester, UK), following the manufacturer's instructions. *Tm* values are listed in Appendix I.

Rescue construct generation

GH05057 (Drosophila Genomics Resource Centre, Bloomington, IN, USA) was PCR amplified using 5' -GGG GAT CCG TTT TTA CCA ACT GCC GCA G- 3' (forward 5' primer) and 5' -GGT CTA GA G GAA CAC ATT AAG TAA ATT CAC TA- 3' (reverse 3' primer). The 5' primer and 3' primer contained BamHI and XbaI restriction sites, respectively (both enzymes were purchased from New England Biolabs, Ipswitch, MA, USA). The BamHI/XbaI digested PCR product was purified using a QIAquick gel extraction kit (QIAgen, West Sussex, UK), as per manufacturer's instructions and cloned into BamHI/XbaI cut pUASp plasmid (Rorth, 1998) using a T4 DNA ligase kit (Invitrogen, Life Technologies, Manchester, UK) as per manufacturer's instructions. The resulting plasmid was purified by QIAgen Midi Kit (QIAgen, West Sussex, UK) and was injected into y^-,w^- embryos by Bestgene (Bestgene Inc, Chino Hills, CA, USA). See Appendix I for more details.

In situ probe generation and in situ hybridisation

pOT2 GH05057 (Drosophila Genomics Resource Centre, Bloomington, IN, USA) was digested using either EcoRI or XhoI and purified using phenol; chloroform and ethanol precipitation (Wilson, 2001, see Appendix I). The linearised plasmid was then labelled with Digoxygenin using the DIG RNA labelling kit (Roche Diagnostics, West Sussex, UK) and GlcATI mRNA was transcribed using either SP6 (Roche Diagnostics, West Sussex, UK) or T7 (Roche Diagnostics, West Sussex, UK) for the sense or anti-sense probes respectively, as per manufacturer's instructions. The polymerase reaction was stopped using 0.2 M EDTA (pH 8.0). The resulting probe was

precipitated using 4M LiCl, 0.5 µl *Saccharomyces cerevisiae* tRNA (Sigma-Aldritch, Dorset, UK) and ethanol at -20^{o}C for 24 h. The RNA yield was estimated using agarose gel electrophoresis. See Appendix I for more details.

Wing discs were dissected from inverted larvae and were fixed in 4% formaldehyde for 30 mins. The tissue was washed several times in PBS Tw and prehybridised for 1 h at 70^{o}C in hybridisation solution (HSW) which consisted of 50% formamide, 5x saline-sodium citrate buffer, 0.1% Tween (v/v), 30 mM citric acid. 2 µl of the appropriate probe was added to 30 µl of HSW and was then denatured at 85^{o}C. The probe was added to the prehybridised tissue and incubated overnight. Following this, samples were washed in HSW heated to 68^{o}C and 500 µl of anti-DIG antibody (1/1000, Roche Diagnostics, West Sussex, UK) was added for 2 hours. This was rinsed and samples were then incubated with NMTT which consisted of 0.1 M NaCl, 50 mM $MgCl^{2}$, 0.1 M Tris pH 9.5, 0.1% Tween (v/v) and water. This was then removed and replaced with 20 µl of stain solution (Roche Diagnostics, West Sussex, UK) and 1ml of NMTT. Wing discs were then allowed to develop in the dark, after which, the reaction was stopped using 0.1% Tw (v/v) and 20 mM EDTA. Samples were precipitated using ethanol and were washed in PBS Tw before being mounted onto glass slides in glycerol.

Phenotypic analysis

Germaria were stained with FasciclinIII, which is a marker of immature follicle cells, to enable to counting of cysts. Only germline cysts which were completely enwrapped by follicle cells were counted. Cap cells and GSCs were counted after staining for using coracle and α-spectrin to identify cap cells and GSCs respectively. Only spectrosome like structures which were touching coracle positive cap cells were counted as GSCs. After confirming that the distribution was normal using a Kolgomov-Smirnov test, a t-test was used to determine whether phenotypes were significantly

different from wild type for both assays described above. Compound egg chambers were identified using DAPI to allow counting of nurse cells. Escort cell invasion was assessed following staining for coracle and αSpectrin to identify escort cells and fusomes, respectively. The number of enwrapped cysts were counted and compared to the total number of cysts present per germaria. If less than 50% of cysts in a germarium were surrounded by coracle, this was counted as having poor escort cell invasion. For the compound and the escort cell phenotype, a Chi^2 test was carried out. All statistical tests were carried out using the SPSS statistical software package.

Results

Loss of function of GlcATI disrupts Drosophila oogenesis

While screening transposon insertion lines for defects in early oogenesis, we identified *GlcATI*^{F00247} as a candidate mutant. This mutant line was semi-viable and adult homozygous escapers displayed weak leg and wing phenotypes which included a slight bend in the tibia of the leg and a narrowing of the space between wing veins L3 and L4 (Supplemental S1). We immmunostained stained homozygous *GlcATI*^{F00247} with Fasciclin III (FasIII), a marker used to identify immature follicle cells which surround the developing cysts in region 2b and 3 of the germarium. We found that this mutant has a reduction in the number of FasIII-surrounded cysts (Fig. 1A,B,G). Additionally, we found that *GlcATI*^{F00247} ovarioles often contain abnormal egg chambers, with 54% of ovarioles containing at least one compound egg chamber (Fig. 1B,H).

Closer inspection of the germarium was carried out using an anti-αSpectrin antibody, which stains a spherical structure found in GSCs known as the spectrosome. We found that there was a significant reduction in the number of GSCs from 2.42, found in the wild type, to 1.71 in *GlcATI*^{F00247} (Fig. 1I). In order to determine if this reduction was due to a reduction in niche size, we immunostained cap cells using an antibody against the septate junction marker, Coracle. The number of cap cells was slightly higher in *GlcATI*^{F00247} than in wild type ovarioles suggesting that the observed GSC loss is not caused by degeneration of the niche itself (Fig. 1E,F,I,J). An additional phenotype, which was seen in *GlcATI*^{F00247} was a loss of EC invasion into the germarium. Germline cysts are normally enveloped by one of the long, thin projections of the ECs. This was observed in 100% of the wild type germaria analysed, but only 60% of the time in *GlcATI*^{F00247} homozygotes (Fig. 1C,D,K). All of the identified phenotypes mentioned were recessive (data not shown).

The insert in *GlcATI*^{F00247} is reported to be in an exon in Glucuronyl transferase I (Thibault *et al*., 2004) and this location was confirmed using PCR (Fig. 2A,B,C). RT PCR was carried out on homozygous *GlcATI*^{F00247} ovaries to determine the nature of the lesion in these mutants. Using primers against the insert itself and *GlcATI*, we showed that the insert is retained in the messenger RNA (Fig. 2D). Additionally, RT PCR using primers against *GlcATI* alone suggests that there is a reduction in the amount of *GlcATI* mRNA being produced (Fig. 2E). In order to confirm that the *GlcATI*^{F00247} insertion gene is responsible for the observed phenotypes, we first remobilised the element using a PiggyBac transposase (Thibault *et al*., 2004). Loss of the insert from *GlcATI*^{F00247} led to a restoration of wild type phenotypes (Fig. 3). Next, we carried out complementation analysis for compound egg chamber phenotypes using mutants around the *GlcATI* locus. There was a compound egg chamber phenotype in flies which were transheterozygous for *GlcATI*^{F00247} and two deletions, Df(1)ED6716 or Df(1)BSC580 that each remove the *GlcATI* gene (Fig. 4A,B, Supplemental S3), confirming that the mutant phenotype is loss of function. Both deletions also produced a strong reduction in the number of FasIII surrounded cysts (Fig. 4C). A nearby deficiency, Df(1)ED6720, which does not delete *GlcATI*, complemented *GlcATI*^{F00247}*for* the observed phenotypes. (Fig. 4C). *GlcATI*^{E04384}, a transposon insertion 2bp upstream of *GlcATI*^{F00247}, failed to complement *GlcATI*^{F00247} and *GlcATI*^{E04384} homozygotes also displayed similar phenotypes (Fig. 4C). Taken together, these data suggest that *GlcATI*^{F00247} is causing a loss of GlcATI function.

Replacing GlcATI function in a GlcATIF00247 mutant background is able to rescue the mutant phenotype.

To confirm that GlcATI is required in the ovary, ActinGal4 was used to drive the expression of an Upstream Activating Sequence (UAS) *GlcATI* cDNA rescue construct in a *GlcATI*^{F00247} homozygous mutant background. We found that this was

able to significantly rescue both the compound egg chamber phenotype and the EC invasion phenotype (Fig. 5). The construct also rescued the leg and wing phenotypes (Supplemental S1). However, we found that outcrossing *GlcATI*^{F00247} produced other phenotypes in the wing, seen in the control flies (*GlcATI*^{F00247} *; ActinGal4/+* and *GlcATI*^{F00247} *; UAS-GlcATI/+).* This included loss of cross veins between L3 and L4 and slight expansion of the wing vein tissue at L2 and the cross vein between L4 and L5 (Supplemental S1E). It is possible that there is something in the genetic background of *GlcATI*^{F00247} which leads to the suppresion of these phentoypes. We were not able to test for rescue of the GSC loss phenotype, as crossing *GlcATI*^{F00247} into the ActinGal4 driver line alone recovered the GSC number to wild type levels (data not shown). The stem cell loss phenotype may be sensitive to genetic background. Interestingly other *GlcATI*^{F00247} mutant phenotypes were also less penetrant in this background, but remained significant compared to wild type (Fig. 5). The UAS *GlcATI* cDNA rescue construct was tested using a Patched Gal4 driver in the wing discs of 3rd instar larvae, to demonstrate that the construct would express *GlcATI* mRNA appropriately (Fig. 5A,B).

Loss of GlcATI in escort cells and follicle stem cells is sufficient to cause the GlcATI^{F00247} phenotype.

To further confirm that GlcATI is affected in *GlcATI*^{F00247} and to identify which tissues it functions in, we carried out RNAi using flies which contain a GlcATI hairpin in the VALIUM21 vector. This vector has been reported to function well in both the germline and somatic tissue and, using the yeast Gal4/UAS system, allows for tissue specific knock down of target genes (Ni *et al*., 2011). NanosGal4::VP16, which drives the expression of UAS constructs in the germline and in the embryo, did not produce a phenotype with the GlcATI hairpin (Fig. 6A,C). As a positive control, we found that this Gal4 driver was able to induce a tumorous ovary phenotype with a control *bam*

RNAi (Supplemental S2). However, driving the hairpin in the escort cells and progenitor cells of the follicle cell lineage using c587Gal4 (Supplemental S2) produced a strong compound egg chamber phenotype (Fig. 6B). We additionally observed a reduction in the number of FasIII encapsulated cysts, GSCs and EC invasion (Fig. 6E-H). All the observed mutant oogenesis phenotypes of *GlcATI* mutants are thus recapitulated by RNAi knockdown of GlcATI expression in the escort cells and follicle stem cell population.

Functional overlap of GlcAT genes in oogenesis.

GlcATI catalyses the addition of a glucuronic acid residue onto galactose during the synthesis of the tetrasaccharide linkage region which is essential for all proteoglycans (Kim *et al*., 2003). Two other GlcAT gene products, GlcATS and GlcATP, are thought to be capable of catalysing the same step. These latter proteins share sequence similarity with GlcATI and also play a role in the synthesis of glycolipids and other reactions, which include the transfer of glucuronic acid during proteoglycan synthesis (Kim *et al*., 2003) (Fig. 7). We tested to see if the other two genes also play a role in oogenesis. We found that $GlcATS^{EY01481}$, which is recessive viable, has similar phenotypes to $GlcATI^{F00247}$, exhibiting GSC loss with germaria having on average 0.47 GSCs. Many ovarioles were devoid of germline cells (Fig. 8C,D). The GlcATS mutations also resulted in 31% of ovarioles containing a compound egg chamber and only 43% of germaria had normal EC invasion (Fig. 8A,C). Interestingly, this mutant also produced a novel germline phenotype. We found that 6% of ovarioles contained germline cysts that had undergone less than the normal four rounds of germline mitosis, which was indicated by the reduced number of ring canals connected to the oocyte. (Fig. 8B,C). In contrast the mutation of $GlcATP^{PL00294}$ did not cause any germ line or follicle cell phenotypes although there was a slight reduction in

EC cell invasion with 20% of ovarioles showing defects in this process (Fig. 8C). These results demonstrate overlapping contributions of *GlcAT* genes to numerous processes in oogenesis.

GlcATI functionally interacts with several signalling pathways in early oogenesis

In order to determine how GlcATI functions in the ovary, we combined $GlcATI^{F00247}$ with mutations in components of several different signalling pathways that have been implicated in regulating egg production in *Drosophila*. Since the $GlcATI^{F00247}$ phenotype indicated a reduced escort cell invasion, compound egg chambers which may indicate reduced follicle cell production and reduced GSC numbers, we chose to investigate interactions with mutants from JAK/STAT, HH, DPP and EGF signalling. JAK/STAT and EGF signalling have been implicated in escort cell function (Kirilly, *et al.* 2011), while HH signalling is known to be important for follicle cell production (King, *et al.* 2001) and DPP is essential for GSC maintenance (Zhu and Xie, 2003). To account for any genetic background effects we similarly outcrossed $GlcATI^{F00247}$ flies with wild type chromosomes as a control. The wild type outcross had a mean of 1.72 GSCs, 12% germaria with failure of EC invasion, and 21% ovarioles with a compound egg chamber phenotype. All phenotypes were significant compared to wild type ($P \leq 0.05$).

First we analysed the EC phenotype. Since EGF signalling is required for EC function (Kirilly *et al*., 2011), we looked at the effect of $GlcATI^{F00247}$ on a loss of function EGF receptor mutant, $EGFR^{t1}$ (Clifford and Schupbach, 1994). We found that $EGFR^{t1}$/+ alone showed a weak EC phenotype (5% germaria defective), but there was no significant genetic interaction with homozygous $GlcATI^{F00247}$, with 9% of germaria showing the EC phenotype in the double mutant combination (Fig. 9H). JAK/STAT signalling is also known to be required for escort cell morphology. Loss of JAK/STAT

leads to poor EC invasion (Decotto and Spradling, 2005; Kirilly *et al*., 2011). We used a loss of function STAT allele known as $Stat92E^{F}$ (Decotto and Spradling, 2005) in combination with homozygous $GlcATI^{F00247}$. While the *Stat92E* mutant alone showed no EC defects, its addition to $GlcATI^{F00247}$ led to an increase in the number of germaria with poor EC invasion up to 56% which was significantly enhanced compared with the wild type outcrossed $GlcATI^{F00247}$ ovarioles (Fig. 9H). A mutant with a deletion spanning the three JAK/STAT ligands, *outstretched*, *unpaired (UPD) 2* and *upd3*, also showed a dominant EC invasion phenotype (9% abnormal) and this was increased slightly when in transheterozygous combination with $GlcATI^{F00247}$ (17%), while $GlcATI^{F00247}$/+ alone showed no phenotype. These data suggest that *GlcATI* mutation may reduce JAK/STAT activity in the EC cells. Consistent with the above conclusion, the gain of function mutant in the JAK/STAT signal transducer, *hopscotch* (HOP), known as hop^{tuml} (Luo *et al*., 1995), slightly reduced the $GlcATI^{F00247}$ phenotype (12% to 7%), although this was not statistically significant (P=0.45).

HH is expressed in the niche and diffuses through the EC region to regulate the follicle cells (Forbes *et al*., 1996). We investigated the effect of altering hh signalling in a $GlcATI^{F00247}$ mutant background using a gain of function mutant, hh^{MRT} (Felsenfeld and Kennison, 1995), and a loss of function allele, hh^{AC} (Park *et al*., 2003). We found that hh^{MRT}/+ alone had a phenotype, with 15% of germaria having poor EC invasion, suggesting a possible function for HH in regulating ECs (Fig. 9H). There was no significant interaction with $GlcATI^{F00247}$ however (Fig. 9H). The hh^{AC}/+ mutant had no germaria with abnormal EC invasion, however, it produced a phenotype stronger than that seen in homozygous $GlcATI^{F00247}$, although this was not statistically significant (32%, P=0.069). We next analysed the effect of GlcATI on DPP signalling using a gain of function mutant in the DPP receptor, Thick veins (TKV), known as $tkv^{Sz\text{-}1}$ (Terracol and Lengyel, 1994). The *tkv* mutant alone had a dominant phenotype in the ECs (7%

abnormal). The addition of *GlcATI*^{F00247} produced a significantly enhanced phenotype with 47% of ovarioles showing poor EC invasion indicating that GlcATI interacts with DPP signalling to control EC invasion (Fig. 9H). This may be an indirect effect, however, through the effects of enhanced DPP signalling on germline differentiation, which is discussed below.

Next we analysed the consequence of mutating GlcATI on GSC number when combined with signalling pathway mutants. Reduction of EGFR signalling with *Egfr*^{t1}/+ was able to rescue the loss of GSCs associated with *GlcATI*^{F00247}, with an average of 3 GSCs (Fig. 9G). Similarly the addition of *Stat92E*^{F}/+ into a homozygous *GlcATI*^{F00247} background was sufficient to increase the GSC number from 1.7 in the *GlcATI*^{F00247} control, up to 2.32. The number of GSCs was not significantly affected, however, by the addition of the gain of function *hop*^{tuml} mutant. The *hh*^{AC} mutant was also unable to rescue the GSC phenotype of *GlcATI*^{F00247}, with an average of 1.89 GSCs, although the gain of function mutant was able to significantly raise the number of GSCs from 1.7, seen in the control, to 3.15. The addition of the gain of function *tkv*$^{Sz\text{-}1}$ into a homozygous *GlcATI*^{F00247} background also raised the number of GSCs to 2.25, although this was not significantly different to the *GlcATI*^{F00247} mutant (P=0.86). However in the latter case we observed that the *tkv*$^{Sz\text{-}1}$ mutant alone had defective germline differentiation expected from a gain of DPP signalling (Xie and Spradling, 1998); 29% of germaria showed a slight accumulation in region 1 of cells containing the spectrosome structure that is characteristic of GSCs. This is consistent with the known role of DPP signalling, i.e. to oppose GSC differentiation (Fig. 9A,B). The addition of homozygous *GlcATI*^{F00247} enhanced both the severity (Fig. 9C) and the frequency of this phenotype, with 63% of germaria showing a large accumulation of spectrosome containing cells in the germarium. Thus, GlcATI may act to limit DPP signalling to the germline.

In the follicle cells, the *Egfr*^{t1} allele was able to fully rescue the compound phenotype of *GlcATI*^{F00247} to wild type suggesting that EGF signalling may be altered in *GlcATI*^{F00247} (Fig. 9I). JAK-STAT signalling is involved in egg chamber development and separates adjacent egg chambers by promoting stalk formation (Assa-Kunik *et al*., 2007). Unexpectedly, and in contrast to the EC phenotype, the *Stat92E*^{F} mutant was also able to significantly rescue the compound egg chamber phenotype of *GlcATI*^{F00247} (to 1.7%) (Fig. 9I). However, a new phenotype was observed in the double mutant flies. We found that 28% of ovarioles contained egg chambers that showed inappropriate accumulations of follicle cells inside maturing egg chambers (Fig. 9D). This suggests that JAK/STAT signalling may play a direct or indirect role in regulating egg chamber formation in combination with *GlcATI*. However, the gain of function, *hop*^{tuml}, did not significantly alter the EC phenotype (Fig. 9I).

HH signalling is known to play an important role in regulating FSCs (Forbes *et al*., 1996). The gain of function *hh*^{MRT} significantly rescues the compound phenotype of *GlcATI*^{F00247}, with only 5% of ovarioles containing a compound egg chamber. The *hh*^{MRT} allele may compensate for reduced HH signalling in the FSCs in the *GlcATI*^{F00247} mutation. It is also possible that an increase in HH activity may act in parallel to compensate for other defects resulting from loss of *GlcATI*^{F00247}. Consistent with this explanation, the loss of function *hh*^{AC} mutant did not significantly affect the *GlcATI*^{F00247} phenotype. Interestingly, we found a contrary result in the wing of the adult fly. The *hh*^{MRT} mutant caused partial duplication of wing territories, which was enhanced in combination with *GlcATI*^{F00247}. The latter combination also led to an increase in the number of ectopic wing margin bristles (Fig. 9E,F). This suggests that, in the wing, the gain of Hedgehog signal is more effective when proteoglycan sysnthesis is reduced. Finally the gain of function *tkv*$^{Sz\text{-}1}$ mutant was also able to rescue the compound egg chamber phenotype of *GlcATI*^{F00247}, to 5% (Fig. 9I).

Discussion

In order to function properly, cells must be able to interpret different environmental cues from both the ECM and their neighbouring cells. The extracellular matrix is comprised of secreted and cell-associated proteins, many of which are extensively post-translationally modified with a considerable variety of polysaccharide molecules, known as glycosaminoglycans (GAG)s, which contribute to its physical and functional properties (Oxlund and Andreassen, 1980; Karus *et al*., 2011). The ECM provides structural integrity and support to tissues and cells, affecting their three dimensional form and mechanical stiffness, which in turn regulates cell polarity, behaviour and differentiation (Wang and Ingber, 1994). As well as its structural properties, ECM components affect cell fate by providing a reservoir of growth factors, affecting their diffusion and presentation to signalling receptors found on the cell surface, and by binding to cell adhesion molecules (Ayers *et al*., 2010). Thus ECM function must be taken into account when trying to uncover mechanisms of developmental patterning. The ECM also contributes considerably to providing appropriate microenvironments that are populated by stem cells and their progeny. This includes providing anchorage for niche cells or making direct contact to stem cells, and regulating the range and activity of key signalling molecules that control stem cell fate (Fujise *et al*., 2003; Lin *et al*., 2008; O'Reilly *et al*., 2008). *Drosophila melanogaster* provides an ideal model organism to explore the role of the extracellular matrix in development. The *Drosophila* ovary in particular has many features that make it amenable to investigation of the many roles of the ECM. For example GSC maintenance and differentiation are dependent on the fine control of DPP diffusion in the extracellular space. Disturbing this gradient produces extra GSCs as seen when the collagen IV protein, Viking, is mutated (Wang *et al*., 2008). Cell adhesion to the ECM

is also important for hub cells which are anchored to the basement membrane and are essential for maintaining GSCs of the *Drosophila* testis (Tanentzapf *et al*., 2007)**.** Additionally, FSC maintenance is dependent on Laminin A which is a component of the basement membrane (O'Reilly *et al*., 2008). Cysts also need to be able to migrate through the germarium, a process which might involve the ECM.

Here we show that loss of function of key enzymes in the proteoglycan synthesis pathway, a key component of the ECM, results in defects in several important processes in different cell types required for the coordinated tissue renewal that maintains egg production in adult *Drosophila*.

GlcATI is required for Drosophila oogenesis

Several lines of evidence allowed us to conclude that GlcATI is required for normal oogenesis. The ovarioles of a transposon insert line, $GlcATI^{F00247}$, located within the *GlcATI* locus had numerous defects in oogenesis including compound egg chambers, reduction in the number of germ line cysts, fewer GSCs and defective EC invasion. The insertion was incorporated into the *GlcATI* mRNA and was associated with reduced expression levels of the gene. Complementation analysis, GlcATI targeted RNAi and phenotypic rescue by overexpression of a wild type cDNA together confirmed *GlcATI* loss of function caused the oogenesis defects. Furthermore, RNAi expression in ECs and FSCs was able to replicate all the mutant phenotypes, whereas its expression in the germline had no effect, suggesting a requirement for the gene's expression in the somatic cells of the germarium.

GlcATI is a protein required for one of the early steps in proteoglycan synthesis. The synthesis of proteoglycans begins with the addition of four different sugar residues, the last of which is the addition of glucuronic acid onto a galactose residue. It is this last step which is catalysed by GlcATI (Kim *et al*., 2003). After initiation, proteoglycan

synthesis branches into different groups, leading to the synthesis of Heparin/Heparan sulphates, Chondroitin and Dermatan sulphate, and the synthesis of the glypican family of proteins (Prydz and Dalen, 2000), thus GlcATI is essential for the post-transcriptional modification of a large group of proteins. There are three *Drosophila* GlcAT genes that have been identified (Kim *et al*., 2003). These are GlcATI, GlcATS and GlcATP. All three are able to catalyse the same step in initiation. GlcATI is only able to catalyse this one reaction, while GlcATS and GlcATP are broad specificity transferases, which are able to catalyse other reactions involved in the synthesis of glycolipids and proteoglycans (Kim *et al*., 2003). In agreement with the conclusion that defective proteoglycan synthesis results in the observed phenotypes we found that GlcATS mutants displayed similar phenotypes to GlcATI mutants. GlcATP mutants revealed only a minor role for this enzyme although its contribution to oogenesis may be masked by redundancy. An additional role for GlcATS in the germline was shown by the fact that the GlcATS mutant produced egg chambers in which germline cells had only undergone three incomplete rounds of mitosis rather than four.

Proteoglycans are known to play an important role in regulating signalling in many different processes that affect cell fate and behaviour. One function of proteoglycans is to sequester morphogens and thus control morphogen gradient formation. In the zebrafish embryo, reducing the production of heparan sulphate GAGs by using Heparinise I leads to an expansion of Fibroblast Growth Gactor 8 (FGF8) protein diffusion (Yu *et al*., 2009). Another example is the role of the glypican, Development abnormally delayed (DALLY), which acts as a co-receptor for DPP both in the wing disc and in S2 cell culture. In this instance, DALLY is able to increase DPP signalling by delaying DPP endocytosis and degradation (Akiyama *et al*., 2008; Dejima *et al*., 2011). Similarly, DALLY is able to restrict the diffusion of Wingless (WG) during wing development. The WG morphogen is unable to diffuse across cells that are

incapable of synthesising heparan sulphate proteoglycans (HSPGs), suggesting that WG does not diffuse freely in the ECM (Han *et al*., 2005).

In the Drosophila ovary, the ECM is known to play an important role in regulating oogenesis. Firstly, DALLY is known to be required as a co-receptor for DPP signalling in cap cells (Guo and Wang, 2009). Secondly, mutants in *sulfateless*, a gene required for heparan sulphate synthesis, is also important for ensuring BAM expression is repressed during germline development in the larval ovary (Hayashi *et al*., 2009). In developing eggs, two proteoglycans, Perlecan and Dystroglycan, are essential for defining apical/basal polarity of follicle cells (Schneider *et al*., 2006). Thus glycosaminoglycans are important for regulating many aspects of tissue function in the ovary.

GlcATI shows phenotype specific functional interactions with signalling pathways involved in oogenesis.

In order to determine how GlcATI may be functioning in regulating egg production in the ovary, we looked at the effect of genetically altering different signalling pathways in a $GlcATI^{F00247}$ mutant background. We analysed the effect these mutants had on the compound egg chamber phenotype, the GSC number and the poor EC invasion of the $GlcATI^{F00247}$ mutant. While it is not possible to determine whether the consequences are direct or indirect the results showed GlcATI loss interacted with several signalling pathways involved in oogenesis. Reduction of JAK/STAT activity using the $Stat92E^{F}$ mutation enhanced defective EC invasion phenotype of the GlcATI allele. This is consistent with published data demonstrating that JAK/STAT is essential for EC function (Decotto and Spradling, 2005). The $GlcATI^{F00247}$ phenotype may therefore reflect a reduction of JAK-STAT signalling. The genetic interactions of $GlcATI^{F00247}$ with a deficiency of the *upd* ligands or a gain of function *hopscotch* allele,

enhancing and moderating *GlcATI*^{F00247} respectively, were also consistent with this conclusion although these interactions were not statistically significant. UPD1 and UPD3 are secreted ligands which are thought to bind to heparan sulphate proteoglycans since treatment of Kc167 cells or S2 cells with heparin leads to an increase in JAK/STAT signalling mediated by UPD and attenuation of JAK/STAT signalling initiated by UPD3 (Harrison *et al*., 1998; Wright *et al*., 2011). It is possible that GlcATI is involved in the synthesises of a proteoglycan which is essential for restricting UPD or enhancing UPD3 function.

The functional interaction of GlcATI with JAK/STAT signalling during egg chamber formation was more complex to interpret. The *GlcATI*^{F00247} mutant compound egg chamber phenotype is consistent with a reduction of JAK/STAT signalling because the latter is known to be involved in stalk cell differentiation and loss of stalks, which separate adjacent egg chambers, result in compound egg chamber phenotypes (Assa-Kunik *et al*., 2007). However, combination with the *Stat92E*^{F} allele, which would be expected to further decrease JAK/STAT signaling, instead suppressed the compound egg chamber phenotype of *GlcATI*^{F00247}. It is possible that precise levels of JAK/STAT signalling are critical for the differentiation of follicle cells, particularly because the mutual antagonism seen with Notch signalling in stalk cell differentiation makes phenotypes based on incomplete loss of JAK/STAT activity difficult to interpret (Assa-Kunik *et al*., 2007). A further complication was that the combination of mutations resulted in a novel phenotype consisting of egg chambers that contained an excess of follicle cells that invaded the egg chamber and surrounded the nurse cell nuclei. JAK/STAT is known to function in regulating different aspects of follicle cell differentiation, both specifying the stalk cells (McGregor *et al*., 2002) and controlling border cell invasion (Silver and Montell, 2001). Thus JAK/STAT plays a role in both differentiation and migratory behaviour. Follicle cells continue dividing until stage 6,

and express FasIII until this point when they exit mitosis and become terminally differentiated. JAK/STAT is known to be important for the latter step and follicle cells in JAK/STAT mutant clones continue to express FasIII (McGregor *et al*., 2002). One explanation for the excess follicle cell phenotype is that follicle cells are not differentiating and continue dividing inappropriately.

Another surprising result was the ability of the loss of function STAT mutants to rescue the GSC phenotype, since loss of JAK/STAT is reported to lead to a loss of GSCs (Decotto and Spradling, 2005). However the $GlcATI^{F00247}$; $Stat92E^{F}$/+ combination also had a more severe defect in EC function. Since ECs are known to be important for promoting cystoblast differentiation by restricting DPP signalling in region 1 of the germarium (Kirilly *et al*., 2011) then it is possible that their loss may explain the observed increase in the number of GSCs.

The hh^{MRT} gain of function mutant was also able to rescue the GSC phenotype of $GlcATI^{F00247}$. This was unlikely to be an indirect effect of changes to ECs, because the $GlcATI^{F00247}$ EC phenotype was neither enhanced nor rescued by hh^{MRT}. Overexpression of HH is known to rescue the GSC loss observed in *female sterile 1 Yb (YB)* mutants (King *et al*., 2001), which suggests that HH can play a role in maintaining GSCs. GlcATI modified proteins in the niche may act to restrict HH signalling to the location where it is needed, thus increasing HH signalling locally. However, the loss of function hh^{AC} mutant did not cause a further reduction of GSCs of the $GlcATI^{F00247}$*phenotype*, and at normal expression levels the impact of HH on GSCs may be insignificant. The observed GSC rescue by hh^{MRT} may therefore reflect a compensatory function in parallel to GlcATI rather than directly restoring a loss of HH signalling in the $GlcATI^{F00247}$ mutant background. Similarly the gain of function hh^{MRT} was able to rescue the compound phenotype of $GlcATI^{F00247}$ which may reflect an increase in FSC activity, which is known to be a HH target. Expression of GlcATI targeted RNAi with the c587

Gal4 driver reproduced the compound egg chamber phenotype and this expression pattern included the FSC but was switched off in follicle progeny. It is likely therefore that GlcATI has a required function to modify key molecules expressed in the FSC and this may have an effect in boosting HH signalling, although again we were unable to demonstrate a genetic interaction with the loss of function hh^{AC} allele. One possible regulator is Development abnormally delayed which is important for the production of HH in the cap cells (Guo and Wang, 2009). Interestingly we noted a converse interaction with hh^{MRT} in the adult wing, which displayed duplication of certain areas. These phenotypes were enhanced in the $GlcATI^{F00247}$ background suggesting that a protein modified by glycosaminoglycans, which are synthesised by GlcATI, can act to limit HH signalling during wing development. The impact of ECM proteins on HH function in the wing has been previously noted. The DSulphatase 1 protein functions by removing sulphate groups from heparan sulphate molecules found in the ECM, allowing the diffusion of the HH morphogen, thus ensuring that HH signals appropriately in the wing (Wojcinski *et al*., 2011).

We also saw genetic interactions of EGFR with $GlcATI^{F00247}$ mutants carrying a loss of function *Egfr* mutant. There has been no direct role for EGF signalling in GSC maintenance reported although EGF from the germline regulates EC function and this may have reciprocal consequences on GSC regulation (Kirilly *et al*., 2011). However, changes in the ECs seem unlikely to explain the GSC rescue in this case as $GlcATI^{F00247}$ EC phenotype was unchanged by $Egfr^{t1}$. This is despite a known function of EGF in the EC cells (Kirilly *et al*., 2011) and a weak phenotype of reduced EC invasion in $Egfr^{t1}$/+ flies alone. The loss of function $Egfr^{t1}$ mutation did however suppress the compound egg chamber of $GlcATI^{F00247}$. EGF signalling is known to be required for the differentiation of epithelial follicle cells which cover the oocyte during stage 6

(McGregor *et al*., 2002) but to our knowledge, a role in earlier stages of egg chamber formation has not been described.

The *tkv* mutant also rescued the compound egg chamber phenotype. This result is consistent with a report that Glass bottom boat (GBB), a Bone Morphogenetic Protein-like ligand for TKV, is essential for FSC function (Kirilly *et al*., 2005). If GBB signalling in the FSCs were reduced in $GlcATI^{F00247}$, this might lead to poor FSC maintenance. Thus increasing signalling with a gain of function *tkv* mutant would be expected to restore FSC function. As with other interactions that suppressed the compound egg chamber phenotype we observed an increase in GSC number compared to $GlcATI^{F00247}$ alone, although in this case it did not reach statistical significance. However, as increased DPP signalling would be expected to increase GSC numbers (Xie and Spradling, 1998) then a direct effect may also be involved. One candidate proteoglycan that might provide this direct effect is Development abnormally delayed (DALLY), which acts as a co-receptor for DPP. Mutations of *dally* lead to GSC loss, similar to that seen in $GlcATI^{F00247}$, (Hayashi *et al*., 2009) and a gain of function mutation of the *tkv* receptor would be expected to suppress this. Interestingly, we also saw the *tkv* gain of function mutation resulted in a weakly expanded population of GSC-like cells that were not adhered to the niche. This phenotype was significantly enhanced by the $GlcATI^{F00247}$ mutant background. The latter result suggests that, in region 1 of the germarium, another GlcATI modified protein may normally act to restrict the range of DPP signalling, thus preventing GSCs from losing dependence on niche adherence. Such a role for the ECM has previously been proposed following similar phenotypes being observed with mutations of *Drosophila* collagen IV (Wang *et al*., 2008). The *tkv* mutation alone also displayed reduced EC invasion, but unlike with $Egfr^{t1}$ allele, this phenotype was strongly enhanced by $GlcATI^{F00247}$. However, the latter effect may

reflect the disrupted germline progression resulting from the overproliferation of GSCs throughout region 1 of the germarium in the combination mutation.

Conclusions

Here we describe the identification and characterisation of mutant phenotypes of genes associated with a key biochemical step in synthesis of the core structure of proteoglycans. These molecules comprise a major component of the ECM with essential roles in cell signalling, cellular migration, developmental patterning and mechanical properties of tissues. Loss of GlcATI highlighted the importance of the ECM in regulating egg development, with multiple phenotypes affecting most of the cellular components of the ovariole including the activity of the germline and somatic stem cells. We found that GlcATI activity underpins the function of several different signalling pathways, including DPP, EGF, JAK/STAT and HH, demonstrating the importance of the ECM in cellular communication, particularly between somatic tissue and the germline. Further characterisation will be required to determine which of these interactions reflect direct molecular interactions of signals with GlcATI modified proteins and the identity of those GlcATI targets.

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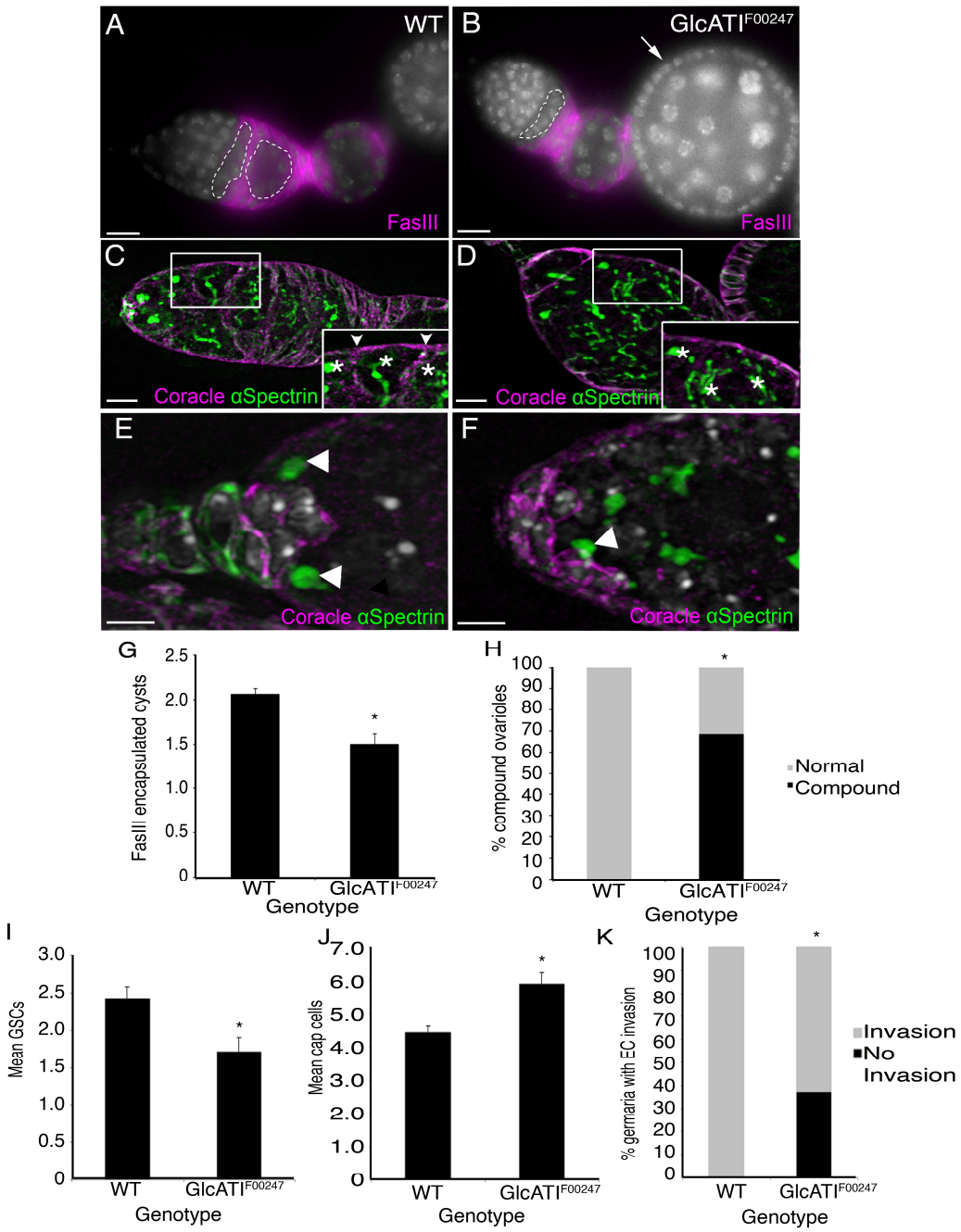
Figures

Fig. 1 GlcATI^{F00247} homozygotes have early oogenesis phenotypes. **A.** WT germaria often have two cysts which are encapsulated by FasIII (Arrow heads). **B.** *GlcATI^{F00247}* germaria have less FasIII positive cysts. There are also compound egg chambers in *GlcATI^{F00247}*ovarioles. **C.** Wild type (WT) germaria showing normal escort cell invasion. Inset shows three cysts (Asterisks) which are separated by escort cells (Arrow heads). **D.** *GlcATI^{F00247}* homozygotes produce germaria in which the escort cells are unable to invade. Inset shows three cysts (asterisks) which are not separated by escort cells. **E.** WT germaria usually have two GSCs, identified by the presence of a spectrosome which is in contact with cap cells (Arrow heads) while **F.** *GlcATI^{F00247}* homozygotes lose GSCs (Arrow heads). If not contacting the cap cells, structures which contain αSpectrin were not counted as stem cells. A and B are stained for FasIII (Magenta). C-F are stained for Coracle (Purple) and αSpectrin (green). Scale in A-D = 5 µm. Scale bar in insets, C-D=3.6 µm. Scale bar in E-F = 2.5 µm. **G.** Graph showing reduction in number of cysts. **H**. Graph showing the number of ovarioles containing compound egg chambers. **I.** Graph showing reduction in GSCs in *GlcATI^{F00247}*. **J.** The reduction in GSCs is not due to a loss of cap cells. **K.** Graph showing the number of ovarioles which have poor escort cell invasion. For both genotypes in G-K, n=45. Error bars represent standard error. * indicates $P \leq 0.05$ as determined by t test (panels G, I and J) Chi^2 test (panels H and K).


A
WT
FasIII
B
GlcATI F00247
FasIII
C
Coracle αSpectrin
D
Coracle αSpectrin
E
Coracle αSpectrin
F
Coracle αSpectrin
G
FasIII encapsulated cysts
2.5
2.0
1.5
1.0
0.5
0
WT
GlcATI F00247
Genotype
H
% compound ovarioles
100
90
80
70
60
50
40
30
20
10
0
WT
GlcATI F00247
Genotype
Normal
Compound
I
Mean GSCs
3.0
2.5
2.0
1.5
1.0
0.5
0
WT
GlcATI F00247
Genotype
J
Mean cap cells
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
WT
GlcATI F00247
Genotype
K
% germaria with EC invasion
100
90
80
70
60
50
40
30
20
10
0
WT
GlcATI F00247
Genotype
Invasion
No Invasion

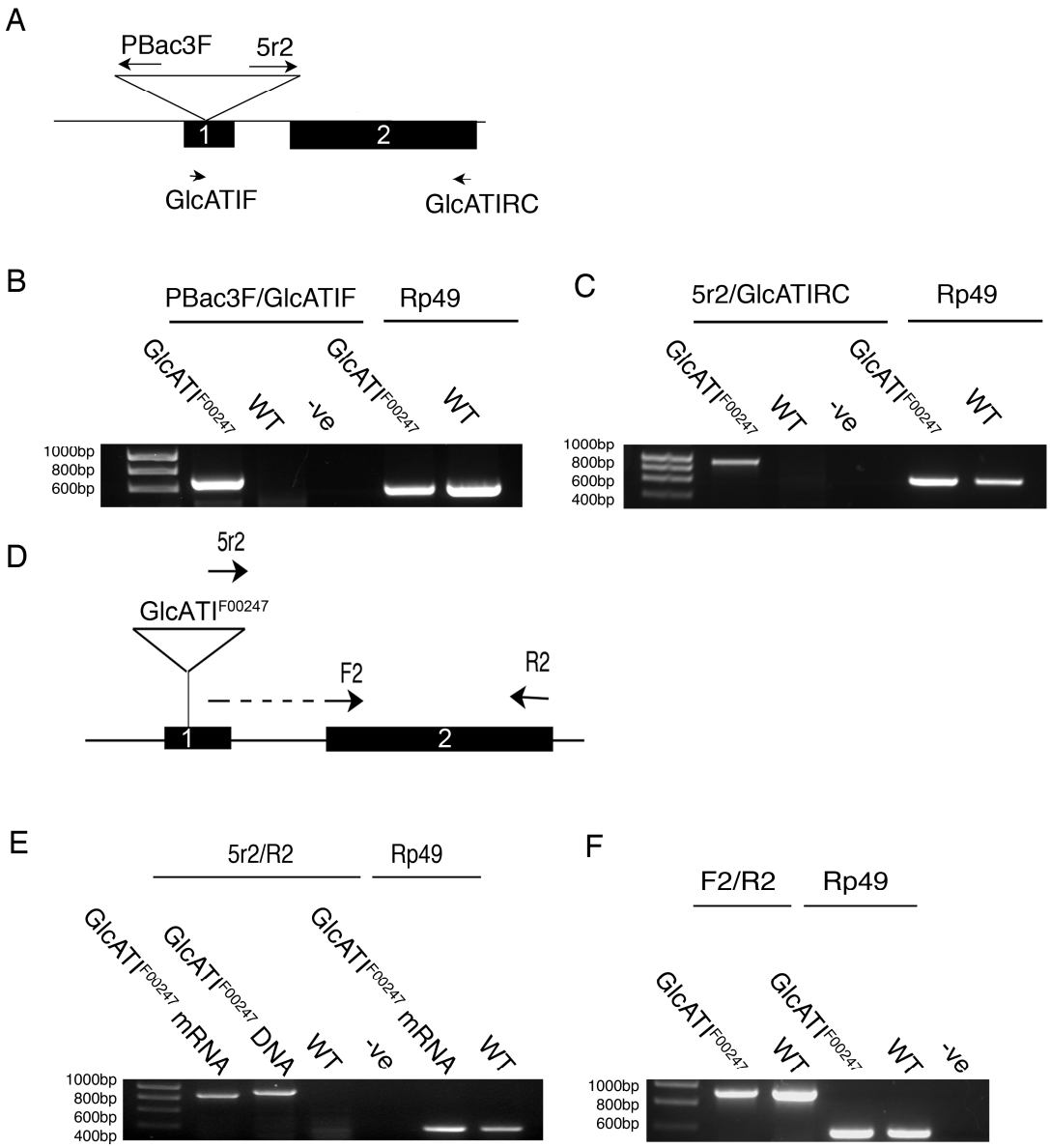


***Fig. 2 GlcATI^{F00247} is located in the GlcATI gene and alters GlcATI mRNA*. A.** Primers used to confirm the insertion site of F00247. **B.** It was possible to amplify the 3' region of the PiggyBac insert using primers in GlcATI and the insert itself, while this same band was not present in wild type (WT). **C.** It was possible to amplify the 5' region of the PiggyBac insert using primers in GlcATI and the insert itself, while this same band was not present in wild type (WT). **D.** Diagram illustrating the primers used in determining the effects of the insert on GlcATI mRNA. **E.** The insert is retained in the mRNA transcript as it was possible to amplify part of the GlcATI mRNA using primers in GlcATI and the PiggyBac insert. It was not possible to amplify the same region in WT. **F.** The presence of the insert leads to a reduction in the amount of GlcATI mRNA in comparison to WT. Control Rp49 mRNA expression was not changed.

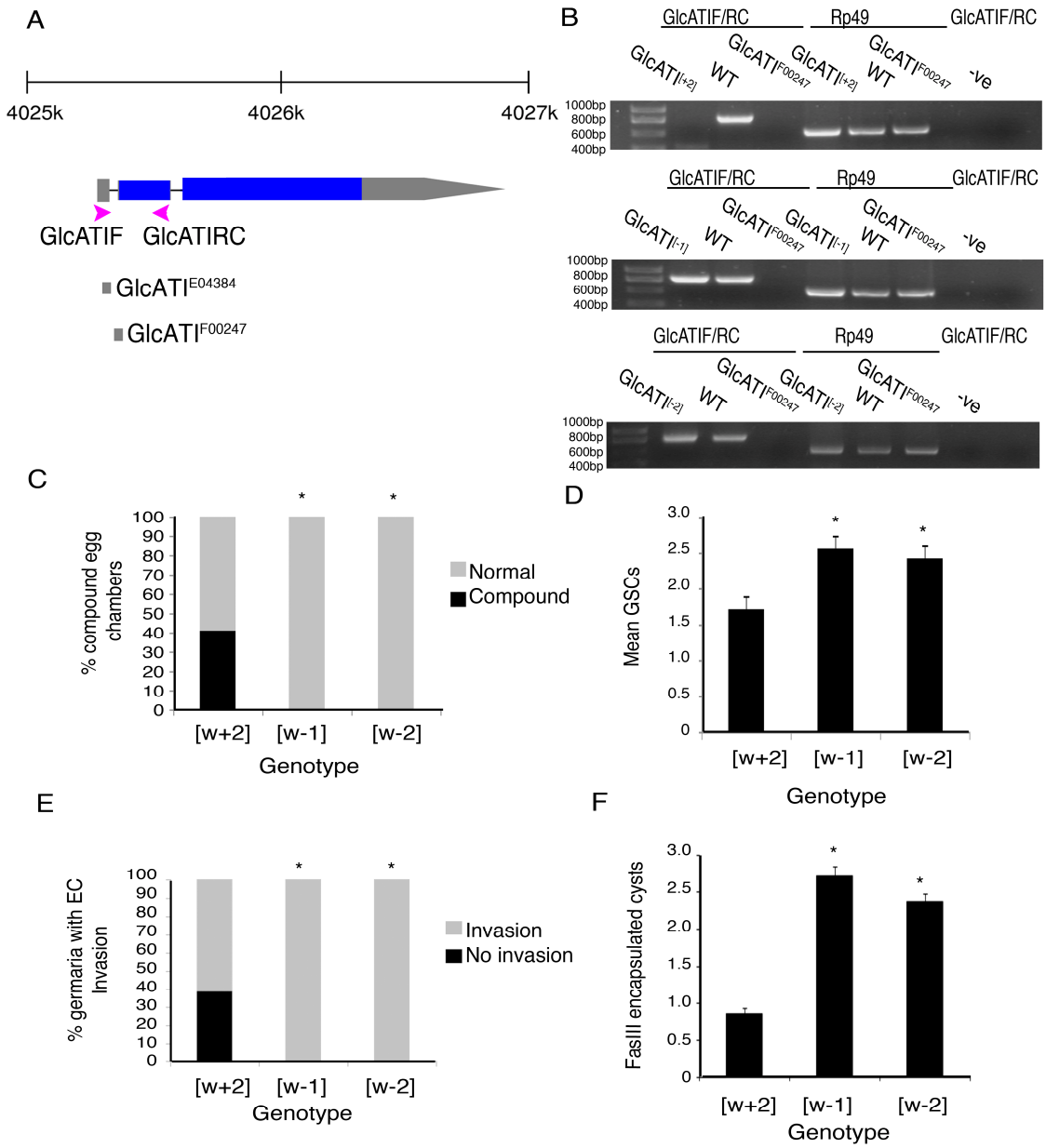


***Fig. 3 Remobilising the PiggyBac element in GlcATI^{F00247} rescues oogenesis phenotypes*. A.** Map of the GlcATI locus, showing the insert site of both *GlcATI^{F00247}* and a second insert used in the complementation, *GlcATI^{E04384}*. Locations of primers used to confirm P-excision are indicated (magenta). Blue = translated exons, grey = untranslated exons. **B.** PCR showing that the insert has been lost in the two jump-out stocks, [w-1] and [w-2]. Presence of insert is indicated by absence of PCR band. Control RP49 amplified band is present in all samples **C** The compound phenotype is rescued in [w-1] and [w-2] but not in the control (w+2), a line which retained the P-element insert. N≥50. **D.** The GSC phenotype is rescued in [w-1] and [w-2] but not in the w+2 control. N≥25. **E.** The escort cell invasion phenotype is rescued in [w-1] and [w-2] but not in the w+2 control. N≥25. **F.** The number of FasIII positive cysts is rescued in w-1 and w-2 but not in the w+2 control. N≥50. Error bars represent standard error. * indicates P≤0.05 as determined by t test (panels D and F) Chi^2 test (panels C and E).

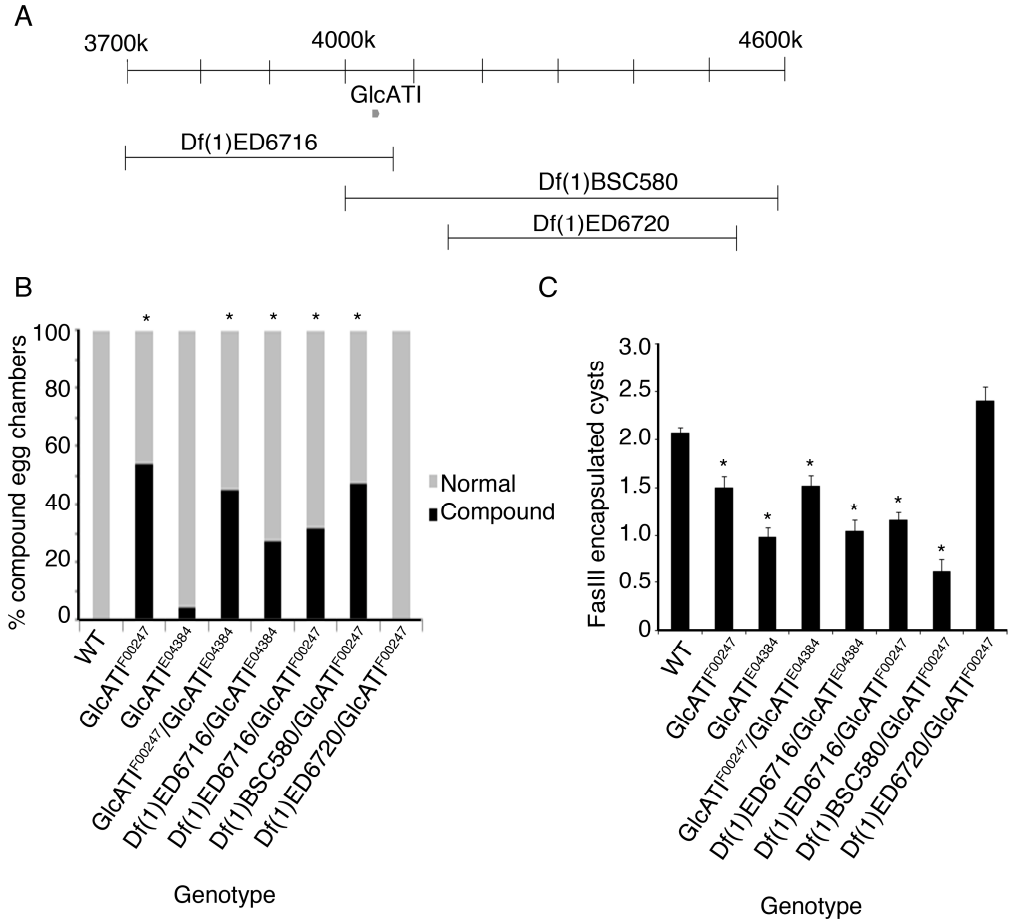


Fig. 4 Complementation analysis of GlcATI alleles **A.** Location of deficiencies used in the complementation analysis. See Supplemental S3 for more details on genes which map to these regions. **B.** Complementation analysis scored for % ovarioles with compound egg chamber phenotypes. N=26-60. **C.** Complementation analysis scored for numbers of FasIII encapsulated cysts. N=26-60. Error bars represent standard error. * indicates $P \leq 0.05$ as determined by Chi^2 test (panel B) and t test (panel C).

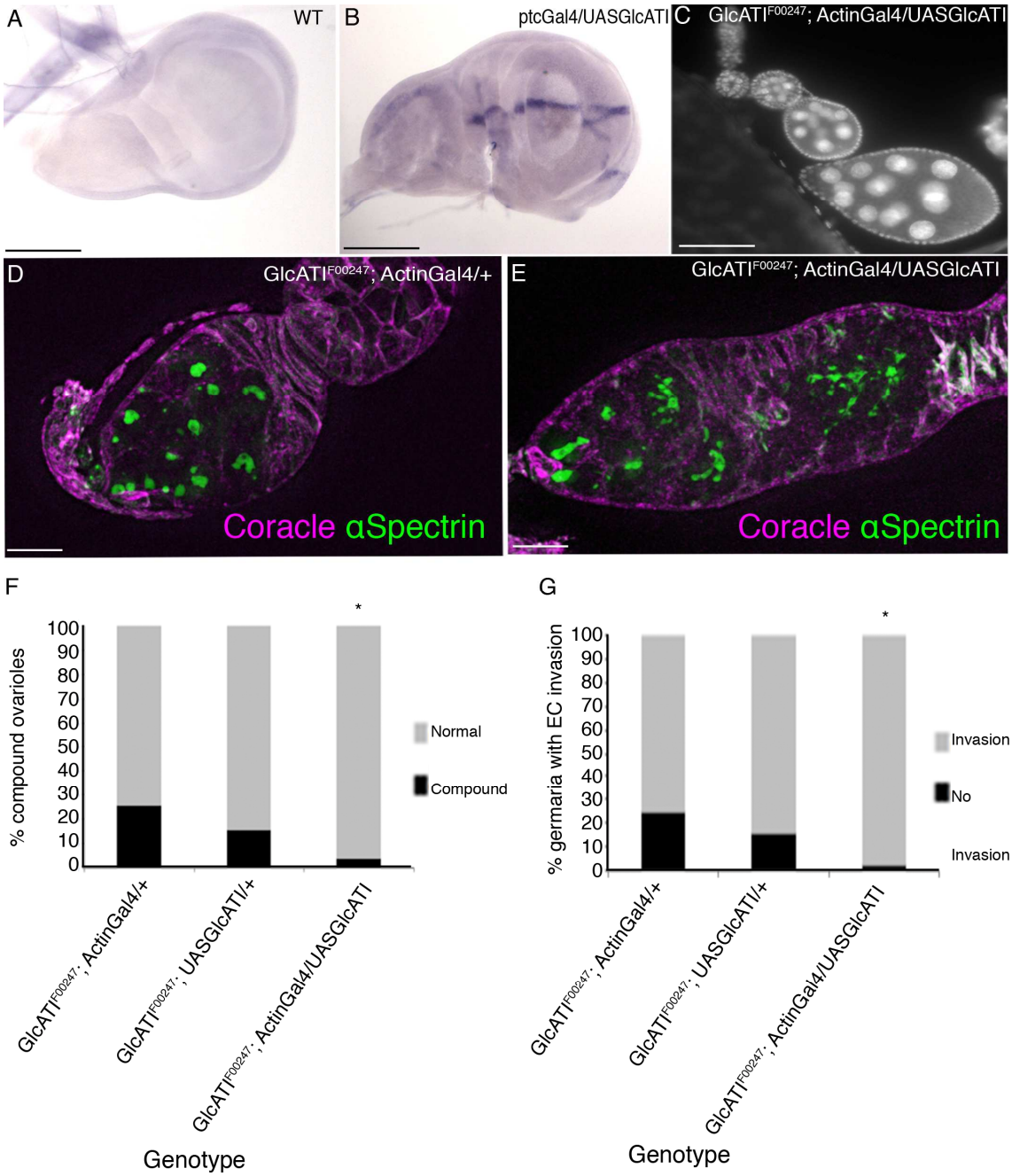
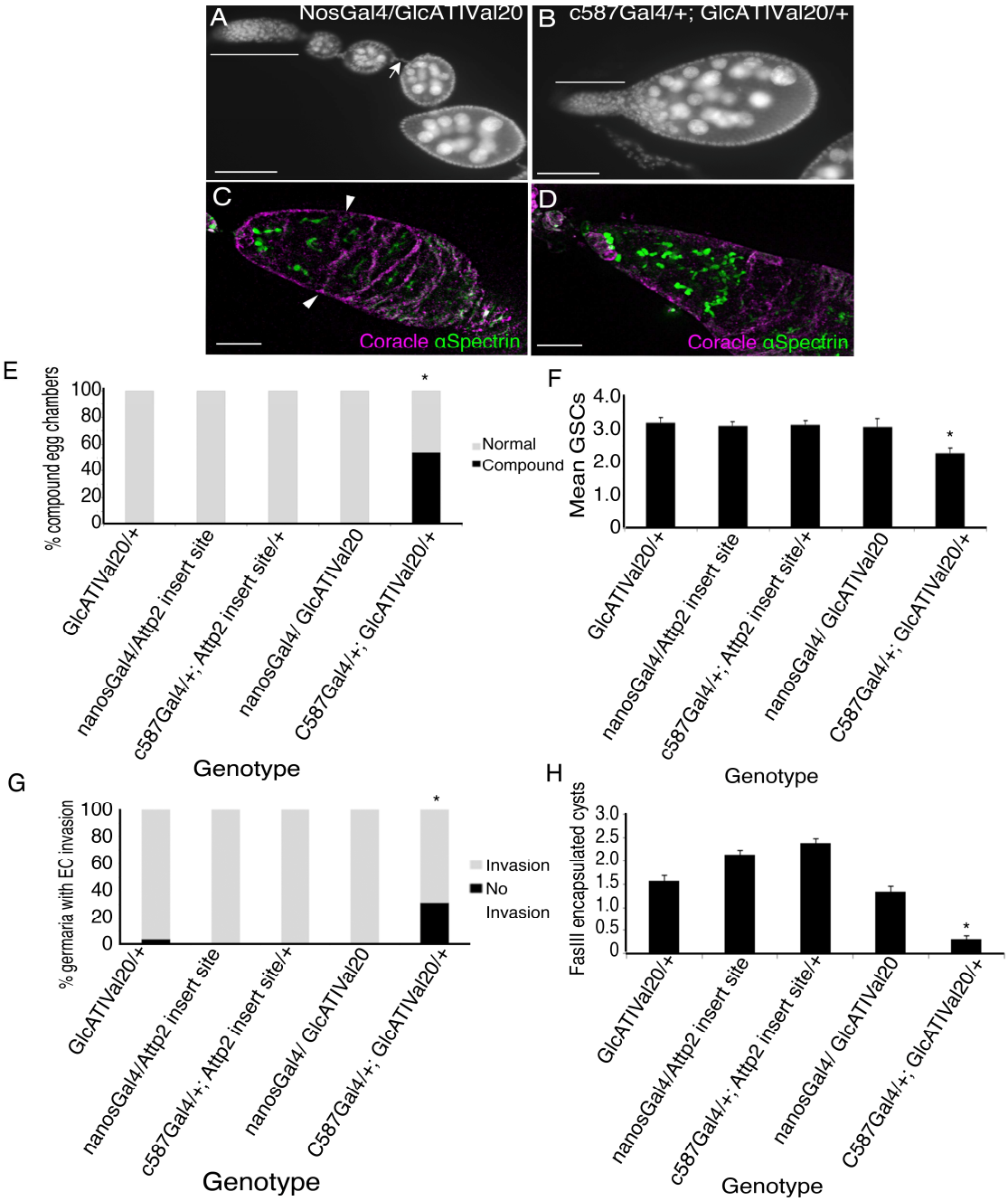


Fig. 5 Expression of GlcATI cDNA rescues the phenotypes of GlcATI^{F00247}. **A.** Wild type wing disc treated with *in situ* probe against *GlcATI* cDNA. **B.** *In situ* showing expression of *GlcATI* cDNA construct using a ptcGal4 driver. This experiment demonstrates that it is possible to express the *GlcATI* cDNA construct using a Gal4 driver. **C.** *GlcATI^{F00247}* ovariole expressing *GlcATI* cDNA with Actin-Gal4 driver appears wild type. (Scale bar in A-C= 60 µm.) **D.** *GlcATI^{F00247}* germarium displays defective escort cell invasion when only Actin Gal4 driver is present. **E.** Actin-Gal4 driven expression of the *GlcATI* cDNA rescues escort cell phenotype. (Scale bar in D-E = 5 µm) **F.** Actin-Gal4 driven expression of the *GlcATI* cDNA rescues the *GlcATI^{F00247}* compound egg chamber phenotype. Control *GlcATI^{F00247}* with either Actin-Gal4 or UAS-GlcATI alone showed no rescue. N=35-50. **G.** Actin-Gal4 driven expression of the *GlcATI* cDNA rescues the *GlcATI^{F00247}* compound egg chamber phenotype. Control *GlcATI^{F00247}* with either Actin-Gal4 or UAS-GlcATI alone showed no rescue. N=35-50. Grey=DAPI, Green=αSpectrin, Magenta=Coracle. * indicates P≤0.05 as determined by Chi^2 test.

Fig. 6. Expression of GlcATI RNAi in escort cells and follicle stem cells replicates the GlcATI^{F00247} phenotypes. **A.** Expression of GlcATI RNAi in the germline using a nanosGal4 produces wild type ovarioles. Line = germarium, arrow = stalk. **B.** Driving *GlcATI* RNAi in the escort cells and follicle stem cells with c587-Gal4 produces a strong compound egg chamber phenotype. Note the lack of stalks. Line = germarium. Scale in A-B = 60 µm. **C.** Expression of GlcATI RNAi in the germline using nanosGal4 does not affect escort cell invasion. **D.** Driving GlcATI RNAi in the escort cells and follicle stem cells with c587-Gal4 reduces the ability of escort cells to invade in the germarium. Scale in C-D = 15 µm. **E.** Scoring of % ovarioles with a compound egg chamber phenotype following expression of GlcATI RNAi with c587 Gal4. Controls with either Gal4 or UAS construct alone, or the Attp2 insert site alone displayed no phenotype. N=19-52. **F.** Reduction in the number of GSCs following expression of c587-Gal4 driven of GlcATI RNAi**.** N=19-52. Error bars represent standard error. **G.** Scoring of Escort cell invasion phenotype following expression of GlcATI RNAi with c587 Gal4. Controls with either Gal4 or UAS construct alone, or the Attp2 insert site alone displayed no phenotype. N=19-52. **H**. Reduction in the number of FasIII enclosed cysts following expression of c587-Gal4 driven of GlcATI RNAi. N=19-52. Error bars represent standard error. * indicates $P \leq 0.05$ as determined by t test (panel F and H) and Chi^2 test (panel E and G).


A
NosGal4/GlcATIVal20
B
c587Gal4/+; GlcATIVal20/+
C
Coracle αSpectrin
D
Coracle αSpectrin
E
% compound egg chambers
100
80
60
40
20
0
*
Normal
Compound
GlcATIVal20/+
nanosGal4/Attp2 insert site
c587Gal4/+; Attp2 insert site/+
nanosGal4/ GlcATIVal20
C587Gal4/+; GlcATIVal20/+
Genotype
F
Mean GSCs
4.0
3.0
2.0
1.0
0
*
GlcATIVal20/+
nanosGal4/Attp2 insert site
c587Gal4/+; Attp2 insert site/+
nanosGal4/ GlcATIVal20
C587Gal4/+; GlcATIVal20/+
Genotype
G
% germaria with EC invasion
100
80
60
40
20
0
*
Invasion
No Invasion
GlcATIVal20/+
nanosGal4/Attp2 insert site
c587Gal4/+; Attp2 insert site/+
nanosGal4/ GlcATIVal20
C587Gal4/+; GlcATIVal20/+
Genotype
H
FasIII encapsulated cysts
3.0
2.5
2.0
1.5
1.0
0.5
0
*
GlcATIVal20/+
nanosGal4/Attp2 insert site
c587Gal4/+; Attp2 insert site/+
nanosGal4/ GlcATIVal20
C587Gal4/+; GlcATIVal20/+
Genotype

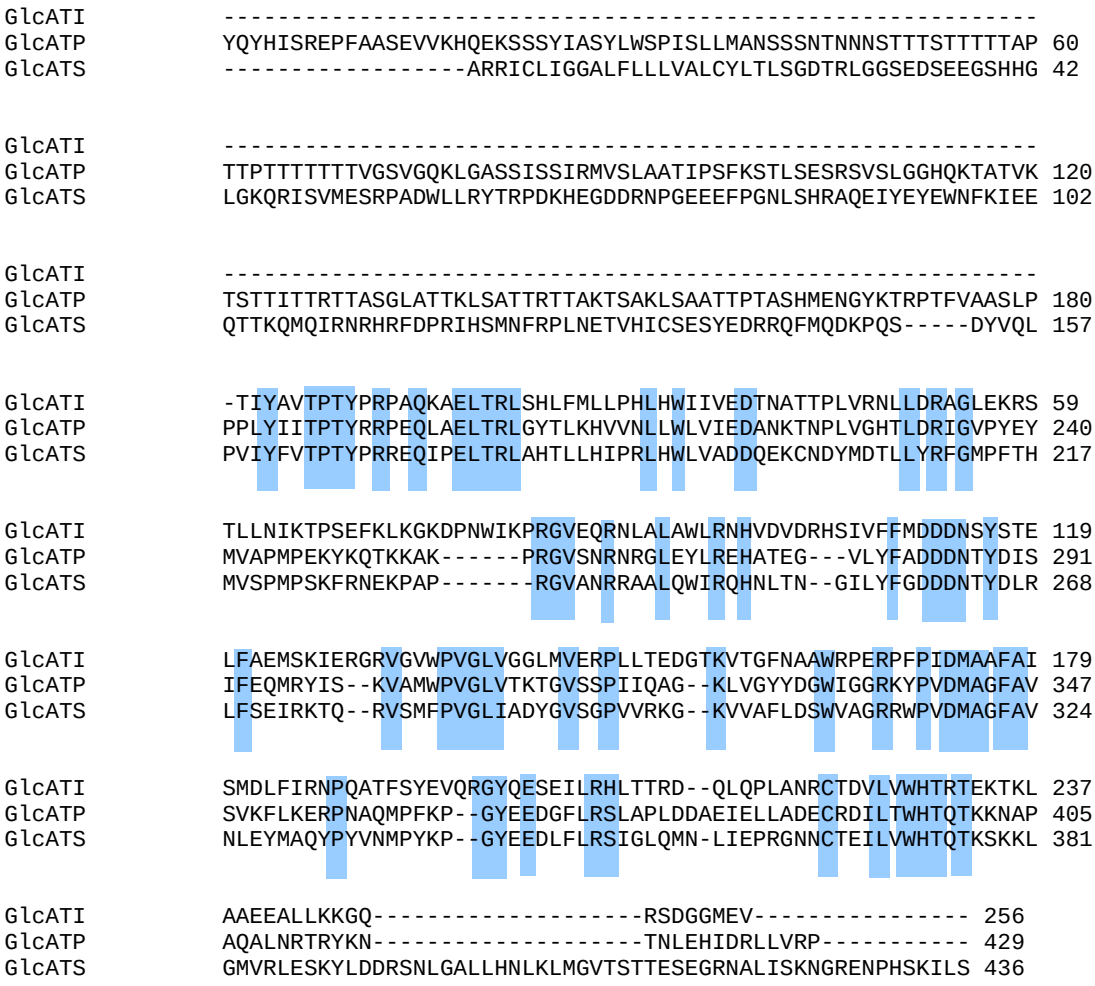


Fig. 7. GlcATI, GlcATS and GlcATP share sequence similarities. Regions which are identical between the three putative GlcAT genes are highlighted in blue. Sequences aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

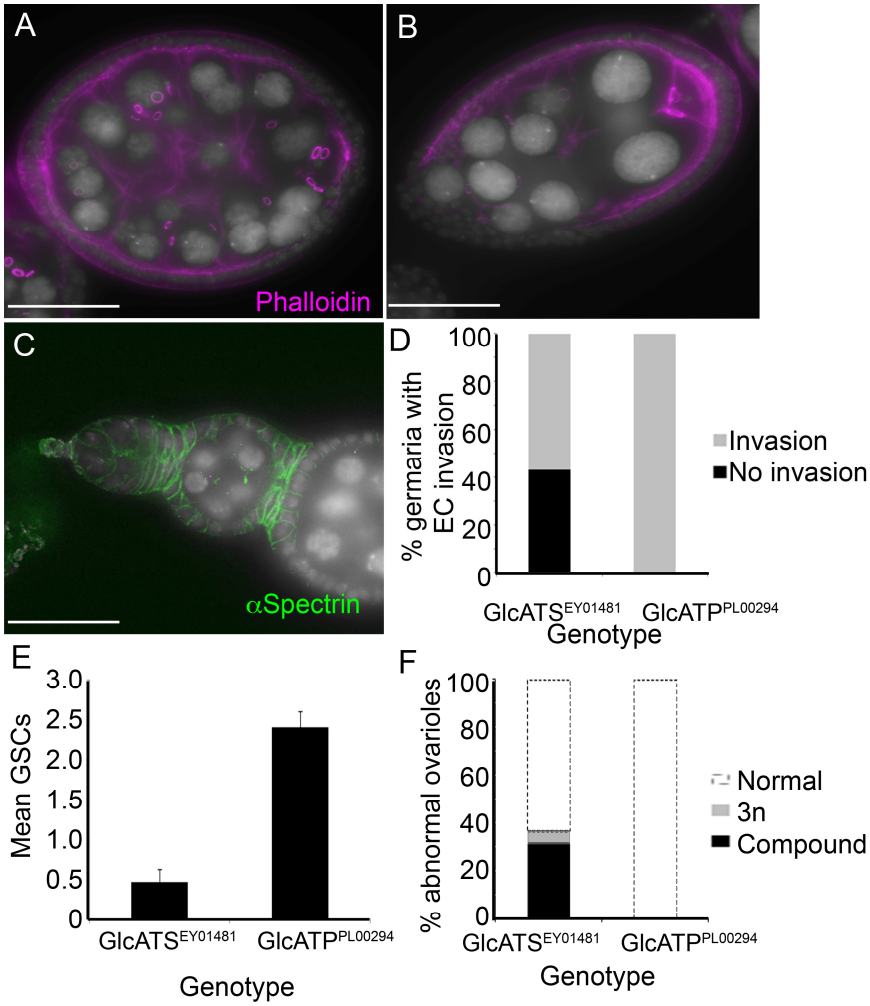
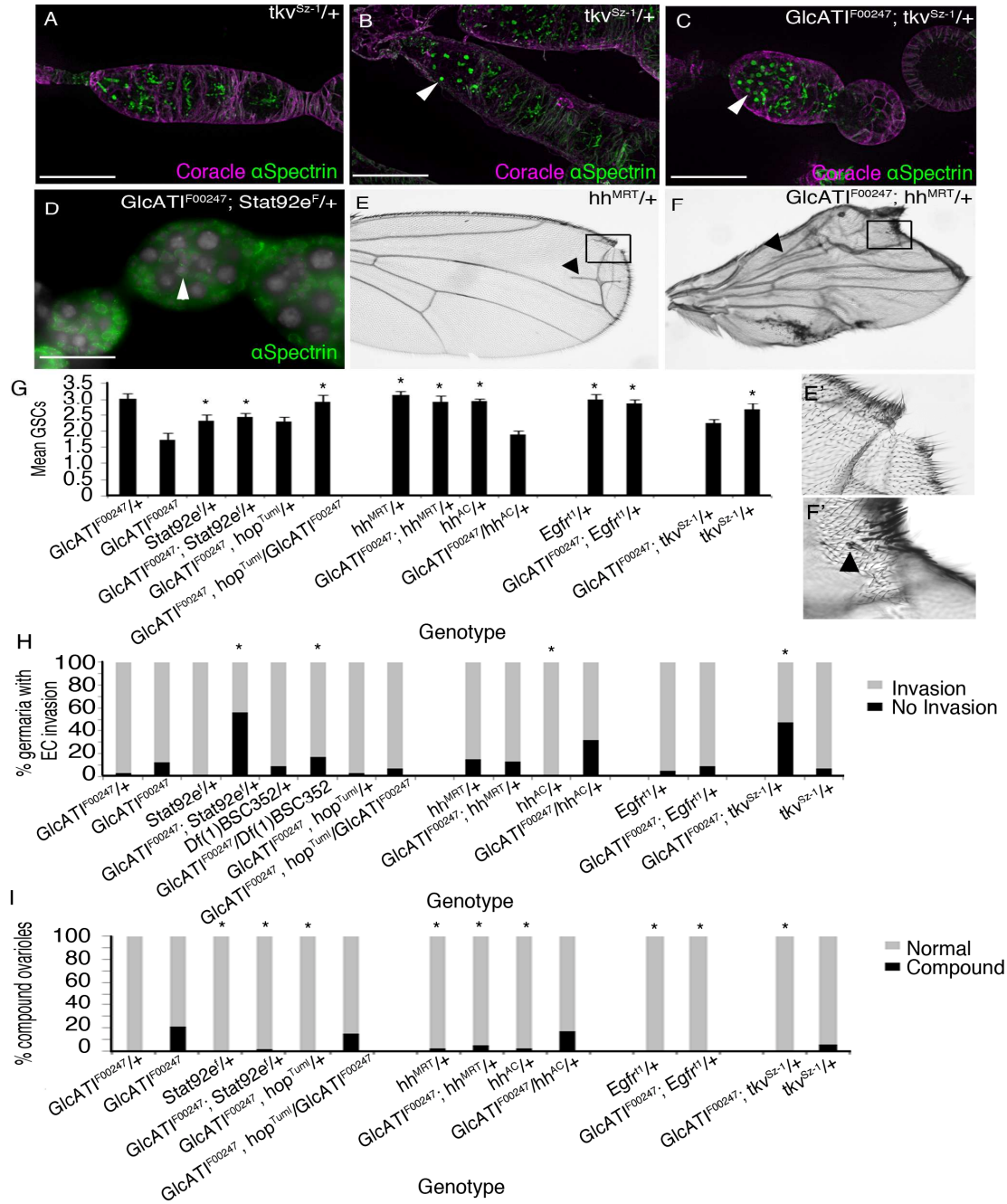
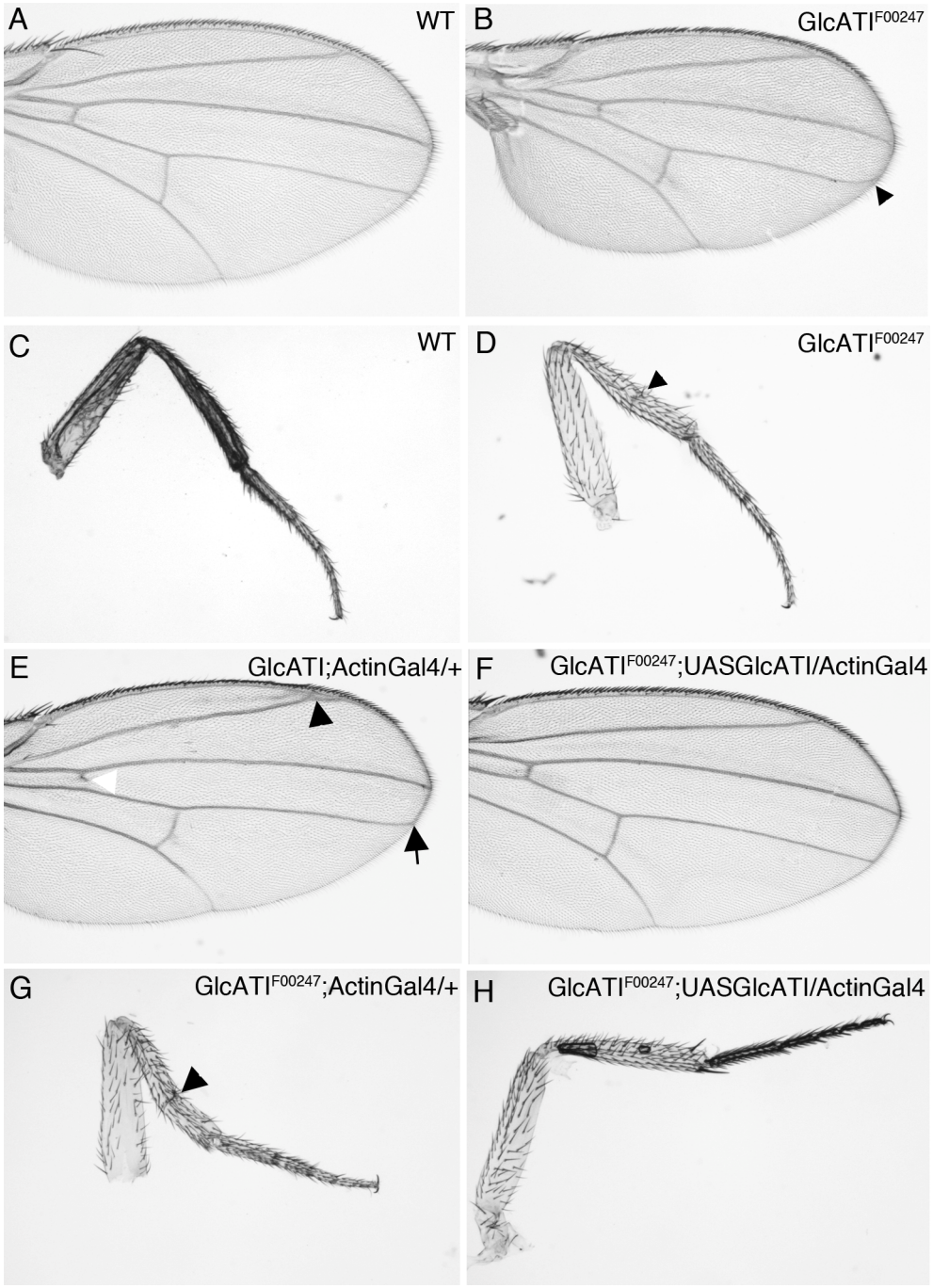


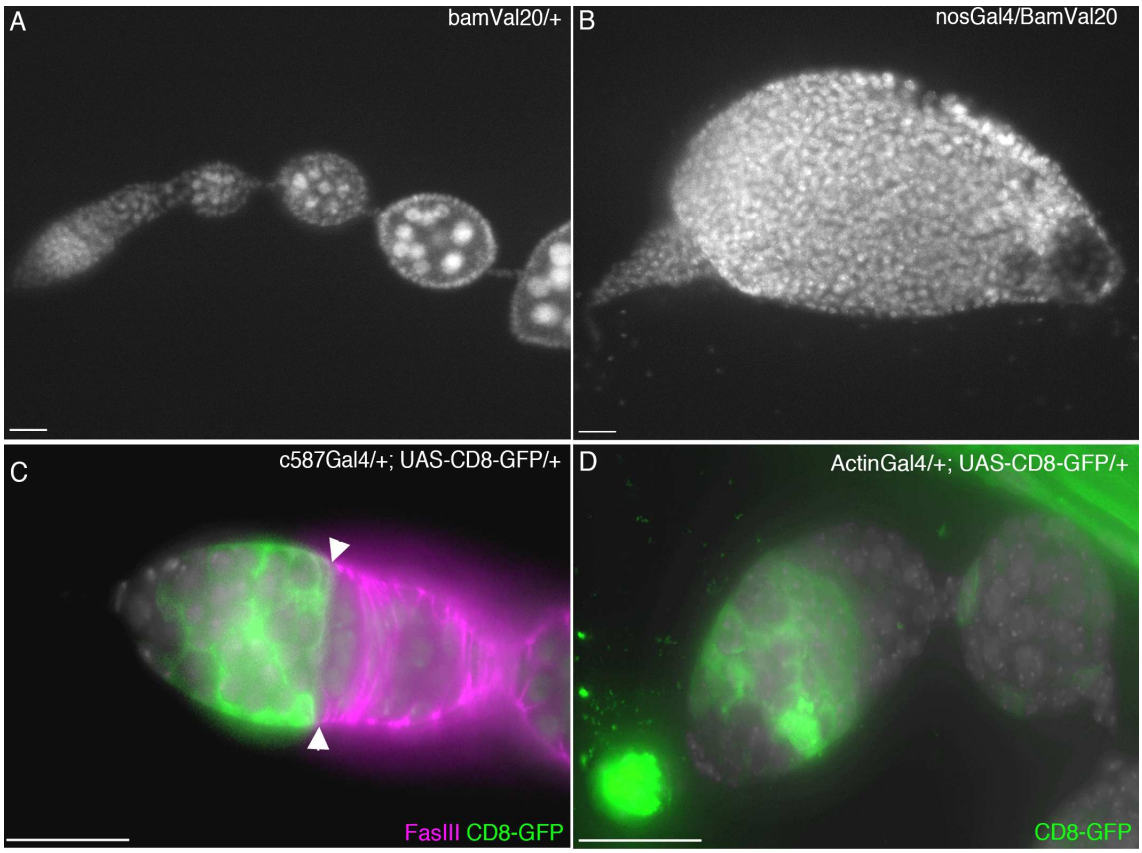
Fig. 8 GlcATS mutant has germline and follicle cell phenotypes similar to GlcATI^{F00247}. **A.** Compound egg chamber in *GlcATS^{EY01481}*. Phalloidin staining of Actin (magenta) indicates there are only four ring canals near the oocyte, despite the egg chamber having more than fifteen nurse cells. **B.** 3n egg chamber in *GlcATS^{EY01481}* has only 3 ring canals connected to the oocyte, despite having 10 nurse cells. **C.** *GlcATS^{EY01481}* homozygotes often lose their germline cells (demonstrated by the lack of fusome/spectrosome structures which contain αSpectrin). Stained with anti-αSpectrin (green). Scale bar in A-C = 15 µm. **D.** Reduced escort cell invasion in *GlcATS^{EY01481}* and *GlcATP^{PL00294}* germaria **E.** Numbers of GSCs scored for *GlcATS^{EY01481}* and *GlcATP^{PL00294}*. Loss of GSCs for *GlcATS^{EY01481}* compared to wild type is significant ($P \leq 0.05$, T Test) **F.** Scoring of % abnormal ovarioles in *GlcATS^{EY01481}*. Proportions showing 3n or compound egg chamber phenotypes are indicated on the graph. (N=15-32). Error bars represent standard error.

Fig. 9 GlcATI^{F00247} interacts genetically with JAK/STAT, HH, EGF and DPP signalling components. **A.** *tkv^{Sz-1}*/+ has wild type germaria, and **B.** germaria with a slight increase in the number of cystoblasts. **C.** The accumulation of cystoblasts is enhanced in *GlcATI^{F00247}*; *tkv^{Sz-1}*/+ germaria. **D.** Example of follicle cells invading egg chambers (arrowhead) in *GlcATI^{F00247}*; *Stat92E^{F}*/+. Scale in A-D = 30 µm. Stainings in A-D are Coracle (magenta), αSpectrin (green). **E.** The wing phenotype in *hh^{MRT}*/+. **E'.** a magnified image of boxed region in E. **F.** In *GlcATI^{F00247}*; *hh^{MRT}*/+, the wing phenotype is enhanced. **F'.** a magnified image of boxed region in F. **G.** GSC numbers for *GlcATI^{F00247}* interactions with *Stat92E^{F}* , *hop^{tuml}*, *hh^{MRT}*, *Egfr^{t1}* and *tkv^{Sz-1}*. N=21-60, error bars represent standard error. **H.** Scoring of escort cell invasion phenotype for *GlcATI^{F00247}* interactions with *Stat92E^{F}* , *hop^{tuml}*, *hh^{MRT}*, *Egfr^{t1}* and *tkv^{Sz-1}*. N=21-60. **I.** Scoring of compound egg chamber phenotype for *GlcATI^{F00247}* interactions with *Stat92E^{F}* , *hop^{tuml}*, *hh^{MRT}*, *Egfr^{t1}* and *tkv^{Sz-1}*. * indicates significant rescue of the *GlcATI^{F00247}* phenotype. N=21-60. * indicates P≤0.05 as determined by t test (panel G) and Chi^2 test (panels H and I).


A
tkv^Sz-1/+
Coracle αSpectrin
B
tkv^Sz-1/+
Coracle αSpectrin
C
GlcATI^F00247; tkv^Sz-1/+
Coracle αSpectrin
D
GlcATI^F00247; Stat92e^F/+
αSpectrin
E
hh^MRT/+
F
GlcATI^F00247; hh^MRT/+
G
Mean GSCs
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
GlcATI^F00247/+
GlcATI^F00247
Stat92e^1/+
GlcATI^F00247; Stat92e^1/+
GlcATI^F00247, hop^Tuml/+
GlcATI^F00247, hop^Tuml/GlcATI^F00247
hh^MRT/+
GlcATI^F00247; hh^MRT/+
hh^AC/+
GlcATI^F00247/hh^AC/+
Egfr^1/+
GlcATI^F00247; Egfr^1/+
GlcATI^F00247; tkv^Sz-1/+
tkv^Sz-1/+
Genotype
E'
F'
H
% germaria with EC invasion
100
80
60
40
20
0
Df(1)BSC352/+
Df(1)BSC352/Df(1)BSC352
Invasion
No Invasion
I
% compound ovarioles
Normal
Compound
Genotype



Supplemental S1. GlcATI^{F00247} has wing and leg defects which are rescued by a GlcATI cDNA construct. **A.** Wild type wing showing normal wing vein morphology. **B.** *GlcATI^{F00247}* wing showing narrowing of gap between wing vein L3 and L4 (arrowhead). **C.** Wild type leg, showing normal leg morphology. **D.** *GlcATI^{F00247}* legs had slight bend in tibia (arrowhead). **E.** *GlcATI^{F00247}* wing phenotypes are not affected by crossing in Actin-Gal4 (black arrow). These wings also show loss of cross veins (white arrowhead) and extra wingvein tissue (black arrow head). **F.** Actin-Gal4 driven expression of *GlcATI* cDNA construct rescues wing phenotypes of *GlcATI^{F00247}*. **G.** *GlcATI^{F00247}* leg phenotype is not affected by crossing in Actin-Gal4. (arrowhead) **H** Actin-Gal4 driven expression of *GlcATI* cDNA construct rescues leg phenotype of *GlcATI^{F00247}*.



Supplemental S2. Expression controls for c587 Gal4, nanos Gal4 and Actin-Gal4 in the ovary. **A.** bamVal20/+ RNAi construct alone produces normal egg chambers. **B.** Driving bamVal20 RNAi with nanos-Gal4 produces germline tumours. A and B are stained with DAPI. **C.** Expression pattern of c587Gal4. C587 drives UASCd8GFP (Green) in escort cells and follicle stem cells. Stained with FasIII (Purple). **D.** The ActinGal4 drives in escort cells in the germarium. The escort cells can be identified by their distinctive morphology in the germarium; they have long cytoplasmic processes which surround the developing cyst. These can be identified by the expression of the UAS-CD8-GP construct (green). Scale bars = 15 µm.

Supplemental S3. Genes deleted by deficiencies used in complementation. Blue= Df(3R)BSC580, black= Df(3R)ED6716, magenta= common to both Df(3R)BSC580 and Df(3R)ED6716, green=Df(3)ED6720.

Gene Symbol	Gene Name	Any known functions
bi	Bifid	
brn	Brainiac	Acetylglucosaminyltransferase (Wilson, 2002)
Cbp80	p binding protein 80	
CG11436	-	
CG11444	-	
CG12179	-	
CG12184	-	
CG12684	-	
CG12688	-	
CG12691	-	
CG12692	-	
CG12693	-	
CG15239	-	
CG15375	-	No published information
CG15473	-	
CG15570	-	
CG15571	-	
CG15572	-	
CG15576	-	
CG15577	-	
CG15578	-	
CG15579	-	
CG15912	-	
CG2901	-	
CG2930	-	
CG2938	-	
CG2941	-	
CG2982	-	
CG3009	-	
CG3062	-	
CG3081	-	
CG32773	-	
CG32783	-	
CG32786	-	
CG34336	-	
CG3527	-	
CG3546	-	

CG3556	-	
CG3568	-	
CG3626	-	
CG42541	-	
CG43134	-	
CG43135	-	
CG43288	-	
CG43689	-	
CG6379	-	
CG6414	-	
CG6428	-	
CHOp24	CHOp25	
cib	Ciboulot	cytoskeleton organisation (Boquet *et al*., 2000)
CTP	cut up	
dgt4	dim γ-tubulin 4	mitotic spindle organisation (Hughes *et al*., 2008)
ec	Echinus	
FasII	Fasciclin 2	neural development (Hebbar and Fernandes, 2005)
Fd3	forkhead domain 59A	
Femcoat	Femcoat	
GlcATI	Glucuronyl transferase I	glucuronyl transferase (Kim *et al*., 2003)
HIP	Hsc/Hsp70-interacting protein	
HLH4c	helix loop helix 4c	
Hsf	Heat shock factor	
lva	lava lamp	
mei-9	meiosis 9	
mRpL33	Mitochondrial ribosomal protein L33	
muc4B	Mucin 4B	
norpA	no receptor potential A	
Nsun2	NOP2-Sun domain family, member 2 ortholog	RNA methylation (Abbasi-Moheb *et al*., 2012)
peb	Pebbled	
pon	partner of numb	
Pp2c1	Protein phosphatase 2C	
rap	retina aberrant in pattern	
rb	ruby	
rox1	RNA on the X 1	
TiP60	Tip60	histone acetylation (Kusch *et*

		al., 2004)
Torsin	Torsin	
tyf	twenty-four	positive regulation of translation (Lim *et al*., 2011)
Vap33-1	Vap-33-1	
VhaAC38-1	VhaAC38-2	
Xpac	Xeroderma pigmentosum group A-like	
yin	Yin	

Chapter 4. *Results Part III*

Dpr9, a brain expressed IgG domain protein, regulates oogenesis in Drosophila melanogaster.

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Abstract

In the *Drosophila* ovary, the rate of oogenesis and egg laying behaviour are strongly linked to the quality of food source. The production of Insulin in the *Drosophila* brain allows the female to adjust the rate of egg production to match the availability of sugar and protein in the environment. This systemic signal acts in addition to local niche-derived signalling to control germline stem cell proliferation. The mechanisms that link behavioural and physiological responses to food quality are, however, poorly understood. Here we describe the identification of a mutant that affects *defective proboscis extension response-9* (*dpr9*), a gene expressed in the brain. Loss of *dpr9* in the nervous system leads to germline stem cell loss and poor follicle cell production. DPR9 belongs to a large family of membrane bound Ig domain DPR proteins, related to the Neurotrimin family of neuronal cell adhesion proteins. To date, the only known functions of these proteins is to mediate behavioural responses to food source constituents. DPR has been implicated in the behavioural response to salt and DPR9 in the response to alcohol. In addition to *dpr9,* mutations of several brain expressed members of the *dpr* family, including *dpr,* displayed similar oogenesis phenotypes. The results suggest a possible pathway which links the detection of food quality and the regulation of oogenesis. The functional overlap of several members of this family suggests that there may be other dietary cues to be identified which are important for controlling stem cell behaviour and oogenesis at a systemic level.

Introduction

The role of adult stem cells is to produce enough cells to ensure the maintenance of tissue homeostasis. In order to do this, stem cells require environmental cues to ensure that they do not over- or under-proliferate. Such cues are often provided by the surrounding microenvironment, known as the stem cell "niche." Examples of niche-dependent stem cells are seen in the *Drosophila* lymph gland, where haemocytes require Janus kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT) mediated signalling for their maintenance (Gao *et al*., 2009). The mammalian hair follicle is another site where signals produced from the niche control stem cell fate. In this case, WNT signalling drives the differentiation of bulge stem cells (Andl *et al*., 2002). In addition to local signals produced by the niche, systemic signals, such as Insulin, are able to control the activity of adult stem cells to ensure that they only proliferate under the appropriate conditions (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009).

The *Drosophila* ovary is a useful model for understanding both the interactions of stem cells interact with their niche and for understanding the role of systemic factors in controlling stem cell behaviour. Each female has two ovaries, which are split into independent egg-producing structures known as ovarioles. At the anterior tip of each ovariole is the germarium, which maintains the two populations of stem cells that are required to produce a viable egg. These are the germline stem cells (GSCs) and the follicle stem cells (FSCs). GSC maintenance is dependent on Decapentaplegic (DPP) produced by somatic cells known as cap cells (Xie and Spradling, 1998). Once a GSC divides, one of the daughter cells is pushed out of the range of DPP signalling and begins to differentiate into a cystoblast. At this stage, the cystoblast will divide four times to produce a cyst, in which each cell is linked by an organelle known as the fusome. This branched structure passes through each cyst at actin-rich junctions called

ring canals (McKearin, 1997; Ong and Tan, 2010). Each cyst is enveloped by an escort cell, which pushes the developing cyst through the germarium to the region where the FSCs are found (Morris and Spradling, 2011). Each germarium has two FSCs that reside in separate niches and rely on signals from both escort cells and the underlying basement membrane to control their maintenance (Song and Xie, 2002; O'Reilly *et al*., 2008). The progeny of FSCs give rise to the follicle cells which will surround the developing cyst. At this stage, the cyst and associated follicle cells begin to bud off from the germarium, prior to the final stages of differentiation. The GSC will differentiate into one oocyte and fifteen nurse cells while the follicle cells will differentiate into polar cells, stalk cells and follicle cells which cover the germline cells (King, 1957; Lin and Spradling, 1993; McGregor *et al*., 2002; Barbosa *et al*., 2007). If any part of this process is disrupted, the female's fertility is reduced. In addition to local signals from the niche, the maintenance of GSCs is known to require Insulin produced in the brain. Loss of Insulin signalling leads to a reduction in the number of GSCs (Hsu and Drummond-Barbosa, 2009). Insulin appears to play a role in maintaining GSC number as flies age by acting to increase Notch signalling levels in the cap cell niche (Hsu and Drummond-Barbosa, 2011). The latter is thought to maintain the levels of DPP from the niche to the GSCs. Additionally, in conditions of poor nutrition, egg production slows markedly due to loss of Insulin signalling. The Insulin receptor is essential for GSC proliferation, where lack of Insulin halts GSC proliferation which indirectly results in a matching reduction in follicle cell division (Drummond-Barbosa and Spradling, 2001). Insulin deficiency also causes developing egg chambers at stage 8 to undergo apoptosis (Drummond-Barbosa and Spradling, 2001). These findings demonstrate the importance of signals produced outside the niche in co-ordinating egg production.

In order to identify other genes that play a role in regulating stem cell behaviour, we carried out a screen of transposon insertion lines. From these stocks, we identified several mutants which had defects in early oogenesis. Adult homozygous escapers of the *l(3)04713* line showed a reduction in the number of maturing cysts associated with a loss of GSCs. The production of egg chambers with multiple cysts inside them, known as compound egg chambers, suggested this mutant also has an impaired ability to regulate the supply of follicle cells. Inverse polymerase chain reaction (PCR) showed that the P-element found in this mutant was present in a brain-expressed gene called *defective proboscis extension response 9* (*dpr9*). We did not detect *dpr9* expression in the ovary but we confirmed expression in head derived mRNA, which was strongly reduced in *l(3)0413* homozygotes. Remobilising this P-element restored *dpr9* mRNA expression and rescued all of the mutant phenotypes. To confirm that disruption of *dpr9* caused the observed mutant phenotypes, we expressed *dpr9* targeted RNAi using the Gal4/Upstream Activating Sequence (UAS) system to knock down *dpr9* in different tissues in the adult fly. We found that loss of *dpr9* in the brain reproduced the compound egg chamber phenotype, but found no consequences of *dpr9* knockdown in follicle cells or the germline. DPR9 belongs to a large family of brain-expressed Immuoglobulin (Ig) domain proteins whose functions to date have been only linked to behavioural responses to the constituents of food sources. We found that mutants in other members of the DPR family have similar phenotypes to *l(3)04713*, suggesting that they have overlapping roles in regulating tissue renewal in the *Drosophila* ovary.

Methods

Further information can be found in Appendix I.

Fly stocks and maintenance

All stocks were maintained on standard yeast-cornmeal agar media. The following alleles were obtained from Bloomington (Bloomington, Indiana, USA); *l(3)04713*, Df(3R)ED5660, PTRiP.HMS0028attP2 (RNAi integration site), GAL4::VP16-nos, TM3, delta2-3, Sb, dpr^{1}, $dpr4^{MB03978}$, $dpr8^{KG01318}$, $dpr8^{MB07155}$, $dpr11^{EY06824}$, $dpr13^{MB08759}$ and elav-Gal4. We also obtained c587Gal4 (T. Xie, Kansas city, KA, USA) and *dpr9* VALIUM20 (Transgenic RNAi project, Boston, MA USA) (See Appendix I for genotypes). All experiments were carried out at 25^{o}C except the RNAi expression which was performed at 27^{o}C. All wild type controls were Oregon-R.

Dissection

Nine day old female flies were pinned to a sylgard plate containing phosphate buffered saline with 0.1% Tween (v/v) (PBS-Tw) and ovaries were extracted with forceps (Fine Science Tools, Heidelberg, Germany). Ovarioles were separated and the sheath removed using 0.1 mm fine pins (Fine Science Tools, Heidelberg, Germany) (See Appendix I for more details). Ovarioles were transferred to an eppendorf and fixed with 4% formaldehyde (v/v) in PBS Tw for 20 mins.

Coracle, αSpectrin, FasIII, Actin immunofluorescence.

Ovarioles were incubated overnight with PBS-Tw. The following day, either Guinea pig anti-Coracle IgG (1/10 000, gift from R. Fehon, Chicago, IL, USA) and mouse anti-αSpectrin IgG (1/20, Developmental Studies Hybridoma Bank, Iowa city, IA) or mouse anti-FasciclinIII IgG (1/20, Developmental Studies Hybridoma Bank,

Iowa city, IA) was diluted in PBS-Tw and applied to the ovarioles which were then incubated overnight. Samples were subsequently washed three times for 20 mins each using PBS-Tw. Alexa488 donkey anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK) and Rhodamine Red X donkey anti-guineapig IgG (Jackson ImmunoResearch, Suffolk, UK) were diluted in PBS-Tw and added to the ovarioles overnight. Samples were washed again and mountant containing 4'-6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Peterborough, UK) was added to the ovarioles. This was, again, incubated overnight. Actin was immunostained using phalloidin- Fluorescein Isothiocyanate (Sigma-Aldritch, Dorset, UK) at a 1/100 dilution for 1 h at room temperature in PBS-Tw. Samples were thoroughly washed with PBS-Tw before being stained overnight with DAPI (H-1200, Vector Laboratories, Peterborough, UK). In both protocols, ovarioles were mounted onto plain microscope slides. See Appendix I for more details.

All images were captured on a Hammamatsu digital camera and an Axioscope microscope. Images were captured in Velocity and processed using Openlab (PerkinElmer, Waltham, MA, USA). Where deconvolution was required, Z section images were collected at 5 µm intervals.

Inverse polymerase chain reaction

30-50 male flies were digested with ProteinaseK (Sigma-Aldritch, Dorset, UK) at a concentration of 20 µg/ml for 2 h at 55°C and then incubated with RNase inhibitor for 30 mins at 37°C. DNA was then extracted using phenol/chloroform as described elsewhere (Wilson, 2001, see Appendix I). Genomic DNA was precipitated using 100% ethanol and subsequently stored in distilled water. DNA was digested using HinPI (New England Biolabs, Ipswitch, MA, USA) for 2.5 h at 37°C followed by 20 minutes at

65°C to inactivate the enzyme. Ligation was carried out on digested DNA (10 µl) at 4°C overnight using 0.5 µl T4 ligase, 40 µl ligation buffer with adenosine triphosphate (ATP) and 348 µl of distilled water.

PCR was carried out on the ligated mixture using the following primers; 5' -CAC CCA AGG CTC TGC TCC CAC AAT- 3' (Plac1) and 5' -ACT GTG CGT TAG GTC CTG TTC ATT GTT- 3' (Plac4) or 5' -CCT TAG CAT GTC CGT GGG GTT TGA AT- 3' (Pry1) and 5' -CAA TCA TAT CGC TGT CTC ACT CA- 3' (Pry4) and the resulting PCR samples were analysed using agarose gel electrophoresis. Reactions containing a single band were sequenced and analysed using BLAST (Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Tm* values are listed in Appendix I.

PCR and reverse transcription PCR

Primers were designed using Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). PCR was carried out using the Roche PCR kit at the concentrations recommended by the manufacturer in a TGradient PCR machine (Biometra, Goettingen, Germany). The following primers were used to amplify *dpr9*; 5' -CAG CAC GCG AAG ATG AAT AA- 3' (38F1) and 5' -TTT TGG CCC ACT GTT CTA GG- 3' (38R1). The annealing temperature was 51°C. RT PCR was carried out using the Superscript® III RT PCR kit (Invitrogen, Life Technologies, Manchester, UK) as per the manufacturer's instructions using the following primers; 5' -CGC CGT TGG GGT TGG TGA GA- 3' (LP20) and 5' -GCG GCT CCG GTG AGT TTT GTA- 3' (LP21). The annealing temperature was 55°C. All PCR reaction products were run in 1% agarose gel in a Tris-acetate ethylenediaminetetraacetic acid buffer. Control primers used were the following; 5' -AGA TGA CCA TCC GCC CAG CAT- 3' (RP49F) and 5'

-CGA CCG TTG GGG TTG GTG AG- 3' (RP49RC). *Tm* values are listed in Appendix I.

Phenotypic analysis

The first egg chamber stage for each ovariole was assessed using DAPI staining and the staging criteria outlined elsewhere (King, 1957). The average first egg chamber stage of mutants was compared to wild type using a Mann-Whitney U test. The number of cysts per germaria was assessed using FasIII which is a marker of immature follicle cells. Only germline cysts which were completely enwrapped by follicle cells were counted. The number of GSCs was determined using coracle and α-spectrin which identify cap cells and GSCs, respectively. Only spectrosome-like structures which were touching coracle positive cap cells were scored as GSCs. These two assays were compared to wild type using a t-test; normal distribution was confirmed using a Kolgomov-Smirnov test. Compound egg chambers were assessed by DAPI staining and counting the number of nurse cells. This was compared to wild type using a Chi^2 test. All statistical tests were carried out using the SPSS statistical software package.

Results

l(3)04713 affects dpr9.

We identified a p-element insertion line *l(3)04713* which was recessive semi-viable with homozygous escapers displaying early oogenesis defects. These included egg chambers with supernumerary germline cells, a reduction in the number of Fasciclin III encapsulated cysts and a raised first egg chamber stage that indicated a delay in egg chamber assembly (Fig. 1). We found that around 50% of ovarioles analysed had lost all their GSCs (Fig. 1). We examined the egg chamber phenotype by staining the actin-rich ring canals with phalloidin and showed oocytes were linked to nurse cells through four ring canals. This confirmed that the phenotype resulted from mispackaging of multiple cysts rather than additional rounds of germ line mitosis (Fig. 1G).

We used inverse PCR to confirm the insertion site of the P-insert mutation in the 5' end of *dpr9* gene (CG12601) (Fig. 2A). We mobilised the P-element to generate an imprecise excision and restore the wild type sequence. We generated two lines which had lost the *white*$^{+}$ eye colour marker indicating p-element excision lines, [-1] and [-2]. The [-1] line contained an imprecise excision, which retained 91 bp of non coding sequence. However, despite this, the line fully rescued the oogenesis phenotypes (Fig. 2F,G,H). The [-2] did not give a detectable genomic band either, indicating a large part of the P-element was retained or that primer sites were deleted. This line displayed an incomplete rescue of the phenotypes (Fig. 2B,F,G,H).

We investigated the consequences of the P-element insertion on the expression of *dpr9*. Expression of *dpr9* has been detected in brain but not other tissues (www.flybase.org). Consistent with this we detected expression of *dpr9* in adult head extracted mRNA but not from *Drosophila* ovaries (Fig. 2C,D). This *dpr9* expression was strongly reduced in *l(3)04713* homozygotes but appeared fully restored in the [-1] P-excision line. Interestingly the [-2] line, which retained weak oogenesis phenotypes,

showed an incomplete restoration of *dpr9* mRNA expression (Fig. 2E). Additionally, the P-insertion failed to complement a deficiency that removed the *dpr9* locus (Fig. 3A,B,C,D).

To confirm that loss of *dpr9* function is associated with the observed phenotypes we utilised a Gal4 dependent RNAi expression construct which targets *dpr9*. Expression in somatic and germline tissues of the germarium produced no phenotypes consistent with the lack of any observed ovary expression for *dpr9*. In contrast, expression of RNAi in the nervous system with elavGal4, a pan-neuronal Gal4 driver, phenocopied the *dpr9* mutant phenotype, producing 27% of ovarioles which contained compound egg chambers and a reduction in the number of cysts, with 23% containing no cysts (Fig. 4). There was also a significant increase in the average first egg chamber stage up to 3.44. However, there was no significant reduction in the number of GSCs (Not shown). Separately, the elav-Gal4 and UAS RNAi controls alone produced no phenotype.

Other Dpr mutants share similar phenotypes

DPR9 is a member of a large family of Ig-domain proteins (Fig. 6). To determine if other members of the family mediate similar functions we analysed mutants in other *dpr* family genes for the number of GSCs and the presence of compound egg chambers. We chose to analyse *dpr* genes which have the same expression profile as *dpr9*, according to Flybase (www.flybase.org). We found that dpr^{1}, $dpr8^{MB03631}$ *and* $dpr13^{MB08759}$ have significantly less GSCs than wild type, while dpr^{1}, $dpr4^{MB03978}$ and $dpr11^{EY06824}$ have compound egg chambers (Fig. 5C,D). In addition to the loss of GSCs, some of the ovarioles analysed appeared very short, with fewer egg chambers than wild type, suggesting a reduction in GSC output (Fig. 5A,B).

Therefore, several brain-expressed *dpr* genes appear to have overlapping functions to regulate egg formation.

Discussion

We identified the *1(3)04137* P-element insertion line as part of a screen to find genes which are involved in regulating tissue renewal in the *Drosophila* ovary. Analysis of the *l(3)04137* phenotype revealed a reduction in the number of mature cysts and an increase in the average first egg chamber stage, suggesting a defect in germline production. Coracle and α-Spectrin staining showed that *l(3)04137* was losing GSCs. In addition to this, *l(3)04137* also produced compound egg chambers, suggesting follicle cell production was also abnormal. Remobilising the insert rescued the phenotype while complementation with a deficiency at the *l(3)04137* locus suggested the region spanned by the deficiency was affected by the insert. Interestingly, RT PCR demonstrated that *dpr9* is not expressed in the ovary, but in the brain. Since inverse PCR demonstrated that the insert was in the *dpr9* gene, we knocked this gene down with brain expressed RNAi and found that this replicated the compound phenotype and the reduction in cysts. However, there was no reduction in the number of GSCs. Either the RNAi knockdown was producing a partial loss of function which left the GSCs intact or an untested tissue is involved in generating this phenotype. Despite this, the results indicate that *dpr9* is required in the *Drosophila* brain for proper regulation of oogenesis. Previous work has shown that systemic signals are able to control stem cells in the ovary. For example, both follicle and germline stem cells respond to changes in nutrition; flies which are raised on a poor diet show germline and follicle cells which divide at a much slower rate than flies fed on a rich diet (Drummond-Barbosa and Spradling, 2001). This fluctuation is dependent on insulin signalling since insulin receptor (InR) mutants lose their GSCs rapidly over time (Barbosa, 2001, Barbosa, 2008). Mutation of the *InR* in GSCs leads to reduced GSC proliferation, suggesting that Insulin is able to act directly on GSCs (LaFever and Drummond-Barbosa, 2005) and this may account for the response to poor nutrition. Poor nutrition also leads to reduced egg laying which is caused by the

apoptosis of both stage 8 egg chambers and germline cysts in region 2a/2b of the germarium in response to starvation (Drummond-Barbosa and Spradling, 2001). Additionally, mutations in *InR* and *chico*, another component of the Insulin signalling pathway, lead to a reduction in the number of GSCs (Hsu *et al*., 2008). Reduced Insulin also leads to a loss of Notch signalling in cap cells which is essential for the maintenance of the cells, demonstrating that Insulin has an impact on the niche itself as well as on stem cells (Hsu *et al*., 2008). Thus there is good evidence that Insulin has an important role in maintaining an active niche as flies age.

While the GSC loss in *l(3)04713* is reminiscent of *InR* mutants, no compound egg chamber phenotype has been described for *InR* mutants. Proliferation of follicle cell clones that contain mutant *InR* is unaffected, suggesting Insulin does not directly affect follicle cells and that the reduction in follicle cell proliferation is due to a signal from the germline (LaFever and Drummond-Barbosa, 2005). This suggests that *dpr9* may function via alternative or additional mechanisms. Besides insulin signalling, another process under systemic control is the regulation of Target of Rapamycin (TOR), which is essential for cell survival, growth and proliferation. TOR responds to the presence of amino acids and growth factors, and is also controlled by the energy status of the cell (Wang and Proud, 2009). In the ovary, TOR is essential for controlling GSC proliferation and maintenance, independently of Insulin (LaFever *et al*., 2010). Unlike Insulin signaling, TOR is also able to control FSC proliferation, but not FSC maintenance or the proliferation of progeny follicle cells. Loss of TOR signalling was found to significantly reduce FSC proliferation (LaFever *et al*., 2010). This might therefore account for follicle cell phenotypes observed in *dpr9* mutants. Further candidate signals required for follicle cell proliferation and differentiation that may be affected by loss of *dpr9* include Notch and Hedgehog signalling. Delta, the ligand for Notch, is expressed in the germline cells and controls follicle cell differentiation and

proliferation and also the switch between the mitotic phase of immature follicle cells and the endocycle phase of differented follicle cells (Forbes *et al*., 1996; Zhang and Kalderon, 2000; Deng *et al*., 2001; Lopez-Schier and St Johnston, 2001). Loss of *hedgehog* function also causes the generation of compound egg chambers while its upregulation results in excess follicle cells which continue dividing inappropriately up to stage 10 (Zhang and Kalderon, 2001). It will be interesting to determine whether DPR9 could be controlling the expression of a systemic factor, which is important for regulating Notch or HH in the ovary.

Other members of the Dpr gene family play a role in regulating Drosophila oogenesis

DPR9 is member of a family of twenty related membrane bound proteins containing at least one Ig domain in their extracellular domain (Nakamura *et al*., 2002). A BLAST search of the DPR9 amino acid sequence (Fig. 6) suggested that, apart from other DPR family genes, it is most closely related to the neural adhesion protein, Neurotrimin. DPR9 and related DPR family proteins may therefore function as neural adhesion molecules. The first *dpr* gene identified has been linked to the gustatory response to salt. Flies which lose *dpr* will eat salty solutions while their wild type counterparts will not (Nakamura *et al*., 2002). We found that loss of *dpr* also produces compound egg chambers and a reduction in the number of GSCs, similar to *l(3)04713*. Several other brain expressed *dpr* family genes produced similar phenotypes suggesting there is functional overlap between them. Interestingly *dpr9* has recently been implicated in the behaviour response to alcohol (Kong *et al*., 2010). Under normal circumstances, flies show increased locomotor activity upon exposure to alcohol vapours, while *dpr9* mutants do not. Additionally, alcohol exposure leads to an upregulation of *dpr9* expression in the adult fly, suggesting that *dpr9* may play a role in the adult rather than during development (Kong *et al*., 2010). Thus, it is possible that the *dpr* family of genes

may be involved in detecting appropriate food sources, as well as food with a high calorific content. The different DPR family proteins may therefore be involved in detecting the presence of suitable environments for egg laying and for boosting egg production in these appropriate environments. For example, *Drosophila melanogaster* will preferentially lay their eggs on food containing a small percentage of alcohol (McKenzie and McKechnie, 1978; McKenzie and Parsons, 1972). Given the large number of DPR family proteins which have been identified, it is possible that other food cues besides sugar and protein may be able to influence the production of viable eggs.

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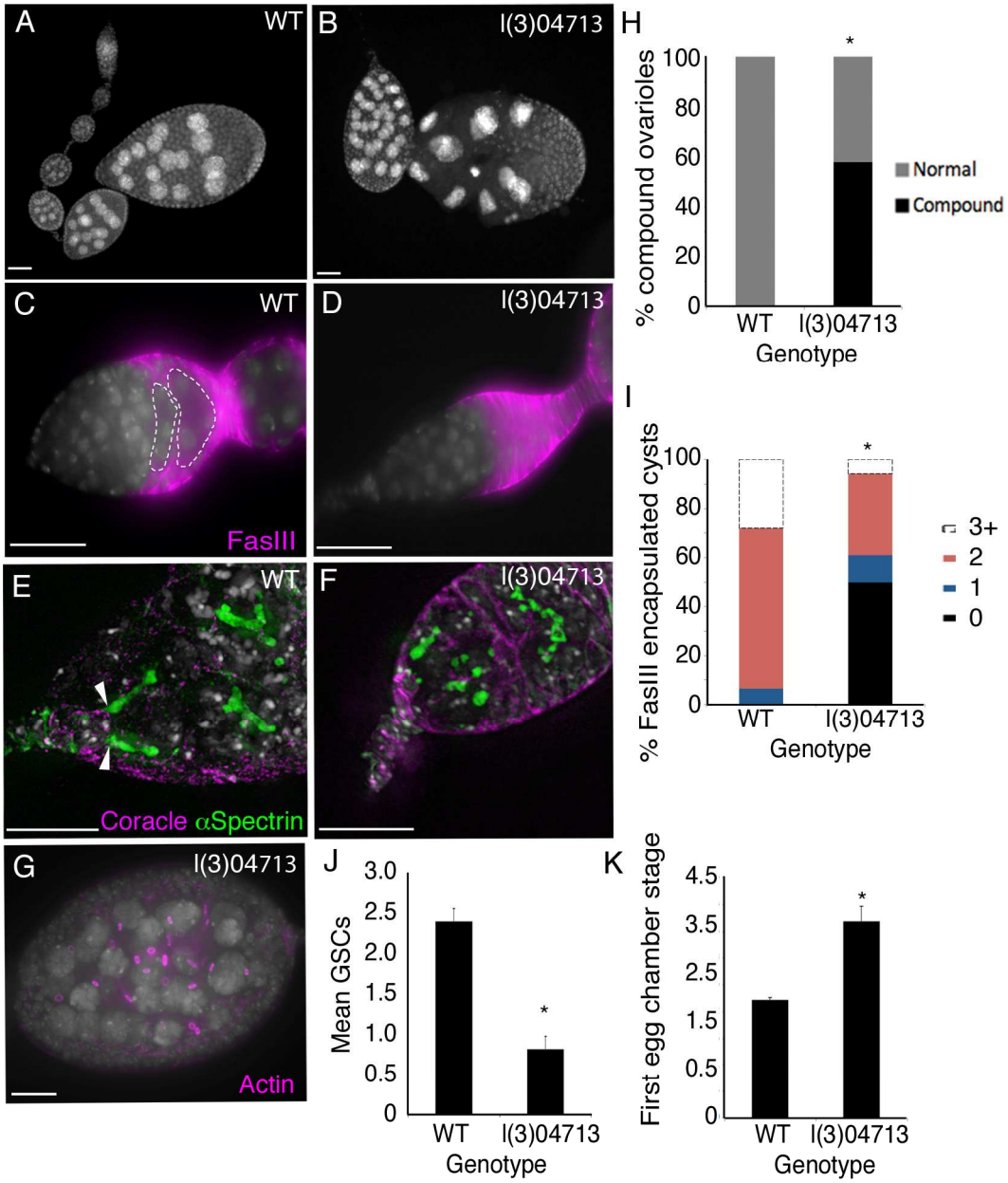
Figures

Fig. 1 l(3)04713 has a follicle and germline phenotype. **A.** Normal wild type (WT) ovarioles have several egg chambers. **B.** *l(3)04713* has reduced numbers of egg chambers, and frequently have compound egg chambers. A and B are stained with DAPI. Scale bar in A = 30 µm. **C.** WT ovarioles also have two or more FasIII encapsulated cysts (arrowhead) **D.** *l(3)04713* has reduced numbers of FasIII enclosed cysts. C and D are stained for FasIII (magenta). Proportions of germaria with 0, 1, 2, 3+ FasIII cysts are indicated. **E.** WT germaria have two or more GSCs (arrowheads) **F.** *l(3)04713* loses GSCs. E and F are stained for coracle (magenta) and α-Spectrin (green). **G.** Actin staining indicated that the extra nurse cell containing egg chambers are compound egg chambers since the oocytes only have four ring canals around the oocyte. Actin is stained with phalloidin (magenta) Scale bar in C, E and G= 15 µm. **H.** The % of ovarioles containing compound egg chambers compared to WT. N≥50. **I** The number of FasIII encapsulated cysts compared to WT. N≥50. **J** The number of GSCs compared to WT. N≥50. **K** The first egg chamber stage compared to WT. N≥50. Error bars represent standard error. * indicates P≤0.05 as determined by t test (panel J and I), Man Whitney U test (panel K) and Chi^2 test (panel H).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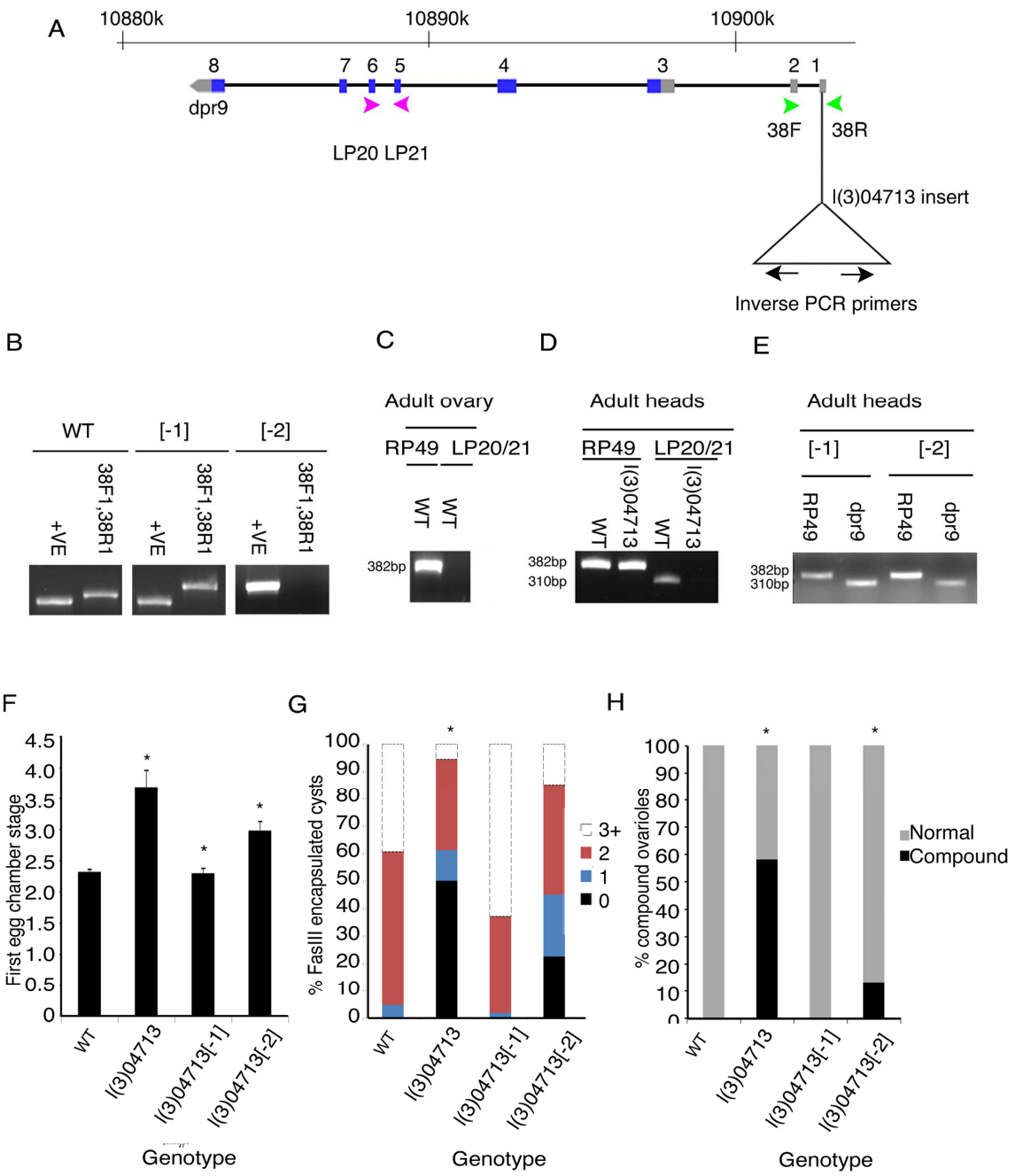
WT
B
l(3)04713
H
% compound ovarioles
100
80
60
40
20
0
*
Normal
Compound
WT
l(3)04713
Genotype
C
WT
D
l(3)04713
FasIII
I
% FasIII encapsulated cysts
100
80
60
40
20
0
*
3+
2
1
0
WT
l(3)04713
Genotype
E
WT
F
l(3)04713
Coracle αSpectrin
G
l(3)04713
Actin
J
Mean GSCs
3.0
2.5
2.0
1.5
1.0
0.5
0
*
WT
l(3)04713
Genotype
K
First egg chamber stage
4.5
3.5
2.5
1.5
0.5
0
*
WT
l(3)04713
Genotype

Fig. 2 l(3)04713 phenotype is rescued by P-element mobilisation. **A.** The *dpr9* locus and primer sites used in the PCR experiments presented in this figure. **B.** PCR shows that [-1] has retained part of the insert while [-2], due to the absence of a band, either has a deletion or has retained a large portion of the insert. **C.** It was not possible to amplify *dpr9* mRNA in adult ovaries. Control RP49 mRNA was successfully amplified. **D.** It was possible to amplify *dpr9* in adult heads. *l(3)04713* shows reduced *dpr9* mRNA expression. There is no effect on expression of control RP49 mRNA. **E.** *dpr9* mRNA expression is restored in [-1] and [-2] in adult heads. **F.** The first egg chamber phenotype in [-1] is suppressed compared to *l(3)04713* and weakly suppressed in [-2]). N≥50. Error bars represent standard error. **G.** The number of FasIII encapsulated cysts is reduced to WT in [-1] and [-2]. N≥50 **H.** The *l(3)04713* compound egg chamber phenotype is rescued [-1] but only partly suppressed in [-2]. N≥50. * indicates P≤0.05 as determined by Man Whitney U test (panel F) and Chi^2 test (panels G and H).


A
10880k
10890k
10900k
8
7 6 5
4
3
2 1
dpr9
LP20 LP21
38F
38R
I(3)04713 insert
Inverse PCR primers
B
WT
[-1]
[-2]
+VE
38F1,38R1
C
Adult ovary
RP49 LP20/21
WT
382bp
D
Adult heads
RP49 LP20/21
I(3)04713
382bp
310bp
E
Adult heads
[-1]
[-2]
RP49
dpr9
F
First egg chamber stage
Genotype
WT
I(3)04713
I(3)04713[-1]
I(3)04713[-2]
G
% FasIII encapsulated cysts
3+
2
1
0
H
% compound ovarioles
Normal
Compound

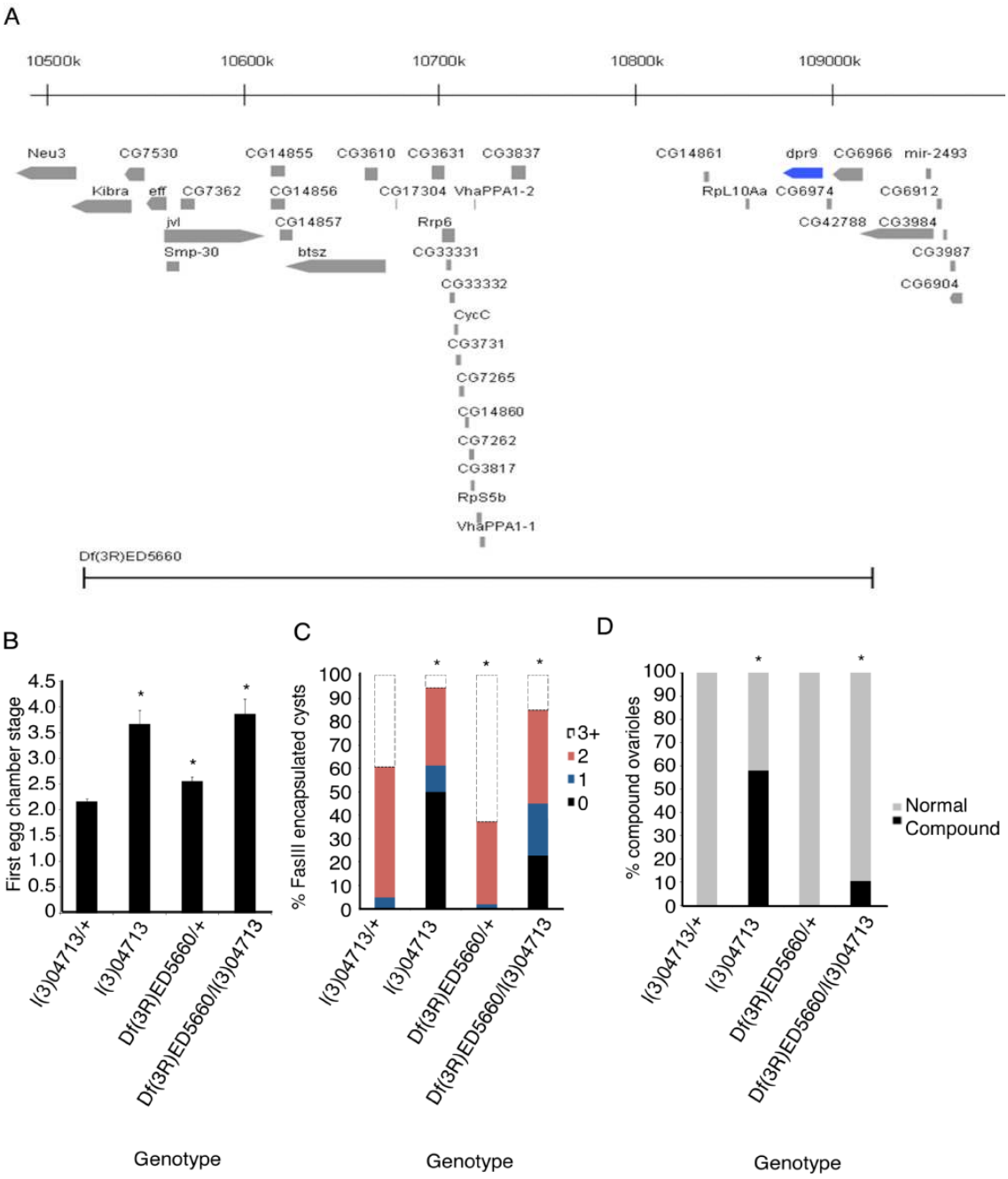


Fig. 3 l(3)04713 fails to complement a deficiency spanning the dpr9 locus. **A** Diagram illustrating the region of the genome deleted by Df(3R)ED5660 and all the genes deleted by this deficiency. **B** Df(3R)ED5660/+ has a slight dominant phenotype of a raised first egg chamber stage when compared to WT. This is significantly enhanced when placed over *l(3)04713*. N≥50. Error bars represent standard error. **C** Df(3R)ED5660/+ shows a slight raised number of FasIII cysts when compared to WT. However, this is enhanced when placed over *l(3)04713*. N≥50. Proportions of germaria with 0, 1, 2, 3+ FasIII cysts are indicated. **D** l*(3)04713* fails to complement Df(3R)ED5660 for the compound egg chamber phenotype when compared to *l(3)04713/+* which has no dominant phenotype. N≥50. * indicates P≤0.05 as determined by Man Whitney U test (panel B), t test (panel C) and Chi^2 test (panels D).

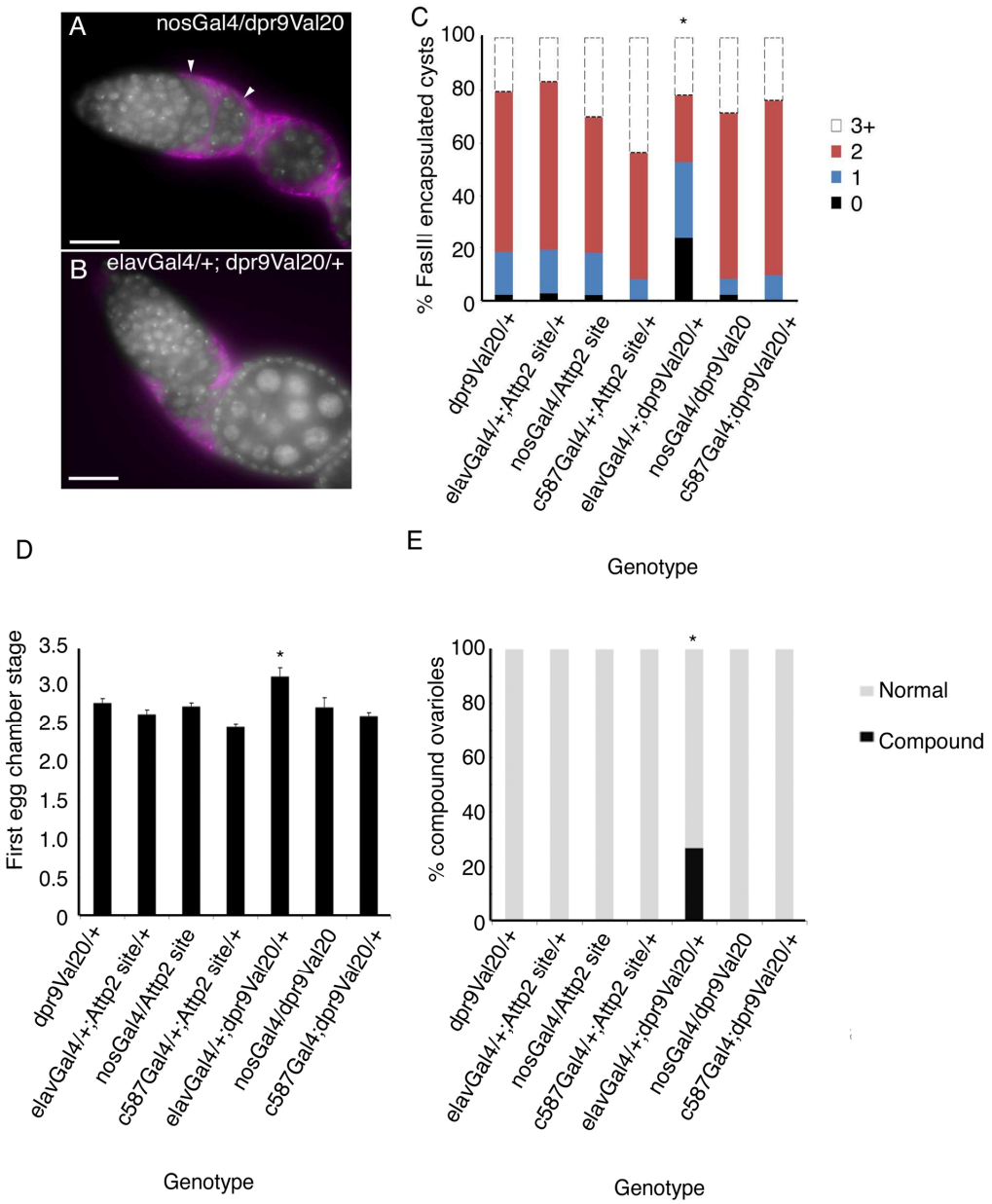


Fig. 4 Expression of dpr9 RNAi in the central nervous system mimics the phenotype observed in l(3)04713. **A.** Driving the *dpr9* Val20 RNAi with the nanosGal4 gave rise to a normal number of FasIII encapsulated cysts (arrowheads) **B.** Driving the RNAi with elavGal4 produced a strong reduction in the number of cysts. A and B are stained with FasIII (magenta). Scale bar in A = 10 µm. **C.** The number of FasIII encapsulated cysts is only reduced when the dpr9 RNAi was driven with elavGal4 when compared to *dpr9* Val20/+ and respective driver controls. N≥50. **D.** The first egg chamber stage is only increased when the *dpr9* RNAi was driven with elavGal4 when compared to *dpr9* Val20/+ and respective driver controls. N≥50. Error bars represent standard error. **E.** Ovarioles containing compound egg chambers are only present when the *dpr9* RNAi was driven with elavGal4 when compared to *dpr9* Val20/+ and respective driver controls. N≥50. * indicates P≤0.05 as determined by Man Whitney U test (panel D), t test (panel C) and Chi^2 test (panel E).

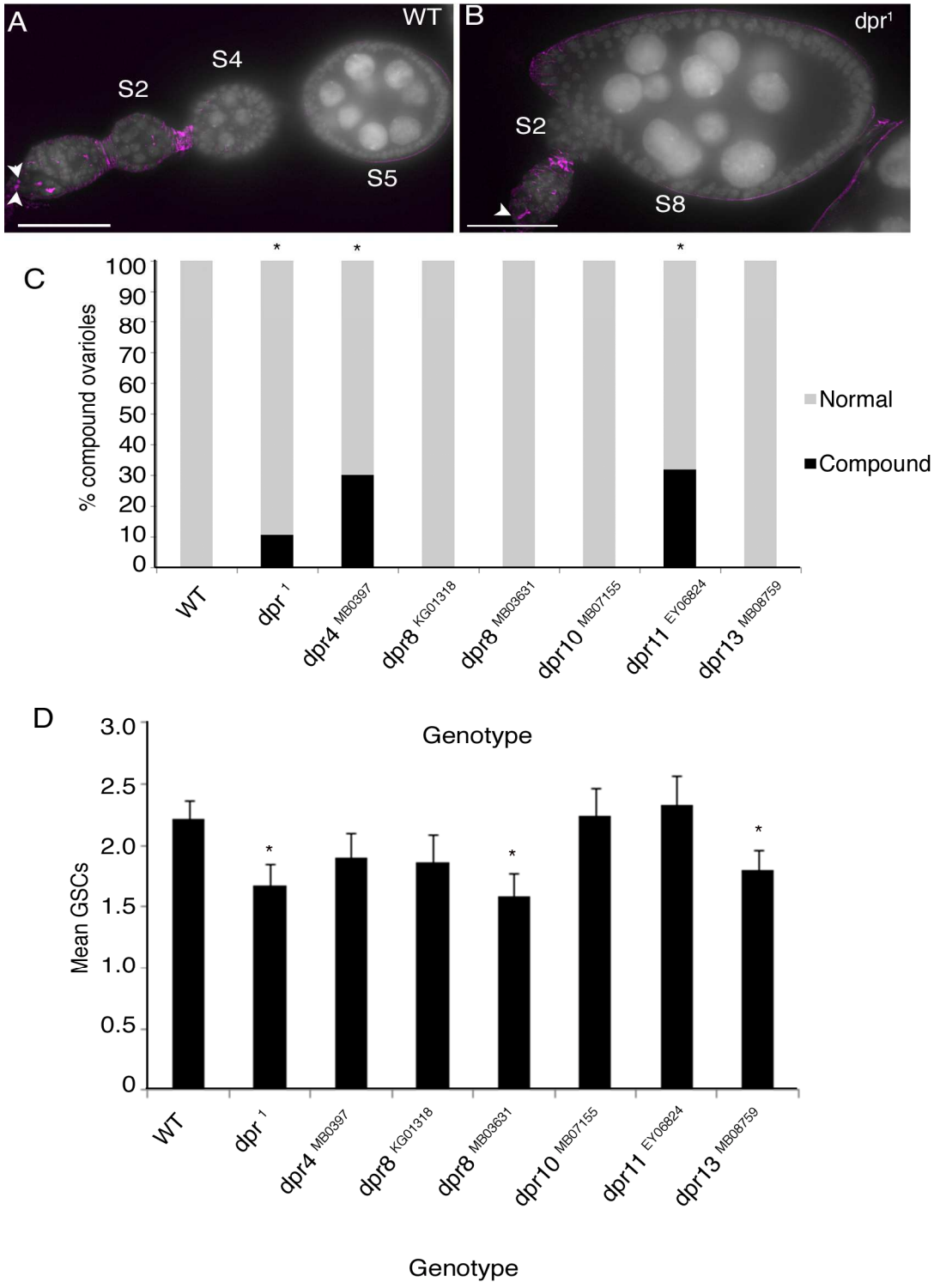
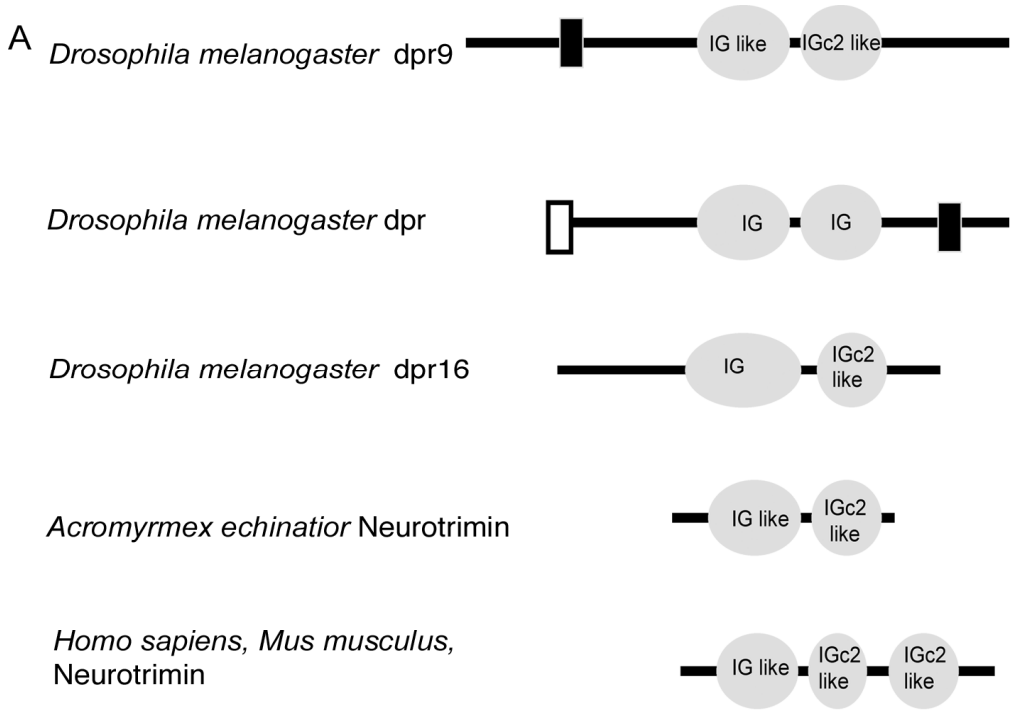


Fig. 5 Several members of the dpr gene family have phenotypes similar to l(3)04713. **A.** Wild type (WT) ovarioles have several egg chambers at relatively close stages of development (S=stage). Additionally, they have around two GSCs (arrowheads). **B.** dpr1 has less developmental stages represented in each ovariole (S= stage). This mutant also has less GSCs (arrowhead). Staining for Spectrin (magenta). Scale bar in A = 30 µm. **C.** *dpr*^{1}, *dpr4*^{MB03978} and *dpr11*^{EY06824} have a significantly higher percentage of ovarioles with compound egg chambers than WT. N≥50. **D.** *dpr*^{1}, *dpr8*^{MB03631} and *dpr13*^{MB08759} show have significantly less GSCs than WT. N≥50. Error bars represent standard error. * indicates P≤0.05 as determined by t test (panel D) and Chi^{2} test (panel C).

Fig. 6 DPR9 is similar to the Neurotrimin family of neural cell adhesion molecules. **A** Predicted protein domains of DPR9, DPR16 and DPR (Nakamura *et al*., 2002), compared to mammalian and *Acromyrmex echinatior* Neurotrimin. (Grey circles= Immunoglobulin domains, black rectangle= predicted transmembrane domain, white rectangle= putative signal sequence). Proteins are oriented N terminal on the left to C terminal on the right. **B** A BLAST search indicated that *Acromyrmex echinatior* Neurotrimin was the nearest relative to DPR9 outside of DPR family. A reciprocal BLAST search of *Acromyrmex echinatior* Neurotrimin against *Drosophila* proteins identified several of the DPR family members as being most closely related. Diagram shows part of the amino acid alignment between DPR family members and Neurotrimin (Ntm) (Blue = exact match, Alignment obtained using ClustalW software).

A



B

```
A.echinatior Ntm ------ALEEATRSGPYFDKSASKNVTALLGKTTYLNCRVKNLGNKTMTLQVSWVRHRDV 84
D.mel dpr9       FHRNSIDLEEARNAGPYFDKAFSKNVTALLGKTAYLNCRVKNLGNKTMLLQVSWVRHRDI 300
D.mel dpr6       ---FLQDLPTPGTGGPTFDTTIGTNITGLVGKTVKLTCRVKNLGNRT----VSWVRHRDI 82
D.mel dpr10      ----TAAYTHPKWMEPYFDPSTPRNVTALMGKSAYLSCRVRNLANKT----VSWIRHRDI 113
D.mel dpr4       ----SHYPHGHKWNEPYFDLTMPRNITSLVGKSAYLGCRVKHLGNKT----VAWIRHRDL 93
H.sapiens Ntm    ------HYWETPYSQPYFDNSSRREVTATVGQAALLHCRVRNLGDRA----VSWIRKRDL 85
M.musculus Ntm   ------TGVPVRSGDATFPKAMD-NVTVRQGESATLRCTIDNRVTRV-----AWLNRS-- 72
                 ------TGVPVRSGDATFPKAMD-NVTVRQGESATLRCTIDNRVTRV-----AWLNRS-- 72

A.echinatior Ntm HLLTIGRYTYTNDQRFRAIHNAHSDDWTLQIKYPQHRDSGIYECQVSTTPHMSHLVHLNV 144
D.mel dpr9       HLLTVGRYTYTSDQRFRAIHQPQTEDWMLQIKYPQHRDSGIYECQVSTTPHMSHYIHLNV 360
D.mel dpr6       HLLTVGRYTYTSDQRFEAMHSPHAEDWTLRIRYAQRKDSGIYECQISTTPPIGHSVYLNI 142
D.mel dpr10      HILTVGSYTYTSDQRFQATHHQDTEDWTLQIKWAQKRDAGMYECQISTQPVRSYFVRLNV 173
D.mel dpr4       HILTVGTYTYTTDQRFQTSYHRDIDEWTLQIKWAQQRDAGVYECQISTQPVRSYSVNLNI 153
H.sapiens Ntm    HILTVGILTYTNDQRFQSLHSEGSDEWTLRISSPQPRDSGTYECQVSTEPKISQGFRLNV 145
M.musculus Ntm   TILYAGNDKWCLDPRVVLLSNTQT-QYSIEIQNVDVYDEGPYTCSVQTDNHPKTSRVHLI 131
                 TIPYAGNDKWCLDPRVVLLGNTQT-QYSIEIQNVDVYDEGPYTCSVQIDNHPKTSRVHLI 131

A.echinatior Ntm I-----------------------------------------------EPKTEILGAPELF 158
D.mel dpr9       V-----------------------------------------------EPSTEIIGAPDLY 374
D.mel dpr6       V-----------------------------------------------EPVTDIIGGPELH 156
D.mel dpr10      V-----------------------------------------------VPTATILGGPDLH 187
D.mel dpr4       VDLIDAETSDIMQQYYNDDAFYIAENRVYQSSNDEFAGMFGPIQTVAVPTATILGGPDLY 213
H.sapiens Ntm    V-----------------------------------------------VSRAKILGNAELF 159
M.musculus Ntm   VQ----------------------------------------------VSPKIVEISSDIS 146
                 VQ----------------------------------------------VSPKIVEISSDIS 146

A.echinatior Ntm INRGSTINLTCVVLQSPEPPAYIFWNHN------DAIISYDSSRGGVSVVTEKGDSTTSF 212
D.mel dpr9       IESGSTINLTCIIQNSPEPPAYIFWNHNNAFPSHPQIINYDSPRGGVSVVTNKGDTTTSF 434
D.mel dpr6       INRGSTINLTCIVKFAPEPPPTVIWSHN------REIINFDSPRGGISLVTEKGVLTTSR 210
D.mel dpr10      VDKGSTINLTCTVKFSPEPPAYIFWYHH------EEVINYDSSRGGVSVITEKGDVTTSF 241
D.mel dpr4       VDKGSTINLTCIIKFSPEPPTHIFWYHQ------DKVLSEETSGGRLKFKTIKSEETKSI 267
H.sapiens Ntm    IKSGSDINLTCLAMQSPVPPSFIYWYKG------KRVMNY-SQRGGINVITERS-TRTSK 211
M.musculus Ntm   INEGNNISLTCIATGRPEP--TVTWRHIS-----PKAVGFVSEDEYLEIQGITREQSGDY 199
                 INEGNNISLTCIATGRPEP--TVTWRHIS-----PKAVGFVSEDEYLQIQGITREQSGEY 199

A.echinatior Ntm
D.mel dpr9       LLVQEAKPSDSGRYTCNPSNAQPKSITVHVLNGE------------------------- 246
D.mel dpr6       LLIKSARPSDSGHYQCNPSNAKPKSVTVHVLNGVSHSVSRGVPSSNAARGTSASSPLA-- 492
D.mel dpr10      LLVQKAITQDSGLYTCTPSNANPTSVRVHIVDGEHPAAMHTGNNGNST-ASQPPVLLP-- 267
D.mel dpr4       LLIQNADLADSGKYSCAPSNADVASVRVHVLNGEHPEAMQTGSSGCQYNWLTIVLLLG-- 299
H.sapiens Ntm    LLIYDADLLHSGKYSCYPSNTEIASIRVHVLQGERPEAMQTNAAPAAVALACWSCHFGQA 327
M.musculus Ntm   LLIAKATPADSGNYTCSPSSSDSASVVVHVINGEHPAAMQHGNSSATCLRPLSSTSVP-- 269
                 ECSASNDVAAPVVRRVKVTVNYPPYISEAKGTGVPVGQKGTLQCEASAVPSAEFQWYKD- 258
                 ECSASNDVAAPVVPRVKVTVNYPPYFSEAKGTGVPVGQKGTLQCEASAVPSAEFQWFKD- 258
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Chapter 5. *General discussion*

General discussion

The aim of this project was to study genes associated with stem cell regulation and tissue homeostasis, using the *Drosophila melanogaster* ovary as a model system. In order to do this, a screen designed to identify mutants with defects in early oogenesis was carried out in the Baron lab using publicly available stocks containing transposable elements which were annotated as being semi-lethal (Ponting, personal communication). These were stocks which, while balanced, were still able to produce homozygotes with reduced fertility. The goal of this screen was to identify genes involved in adult tissue renewal, which are not otherwise required for the development of wild type adults. The ovaries of these mutants were analysed for a high first egg chamber stage, which would suggest a delay in egg chamber production. This delay, in association with compound egg chambers or an increase in mature but unpackaged cysts in the germarium, would reflect a decreased supply of follicle cells. In contrast a high first egg chamber stage in association with a decrease in maturing cysts might suggest a reduction in germline stem cell (GSC) activity. Having identified a set of candidate mutants, our next goal was to determine which genes were affected in these mutants and understand how these genes contributed to the maintenance of the ovary throughout the fly's lifetime.

Three candidate mutants were chosen for further analysis, which are described in this thesis; $A2bp1^{KG06463}$, $GlcATI^{F00247}$ and *l(3)04317*. The first paper shows that two gene encoding RNA binding proteins, *Ataxin 2 binding protein 1* (*A2bp1)* and *Gemin3,* are affected in $A2bp1^{KG0646}$. The second paper identified *Glucuronyl transferase I* (*GlcATI)*, a gene involved in proteoglycan synthesis, to be affected by the $GlcATI^{F00247}$ allele. The final paper suggests that brain expressed *defective proboscis extension response 9 (dpr9)* may be affected in *l(3)04713*. In addition to identifying candidate genes affected in these mutants, the papers presented in this thesis identify potential

signalling pathways by which these gene products regulate tissue function either cell autonomously, non-autonomously in the stem cell niche or at a systemic level. This general discussion describes possible caveats to the work carried out and suggests future experimental directions that could be taken.

Paper 1. The RNA binding proteins, Ataxin2 binding protein-1 and Gemin-3, cooperate to regulate somatic and germline cell differentiation during Drosophila melanogaster oogenesis.

The first paper describes the tumorous germline phenotype seen in the *A2bp1*^{KG06463} line, which was rescued following mobilisation of the P-element insert (Chapter 2, Fig.1). Complementation with mutants in surrounding regions identified *A2bp1* and *Gemin3* as candidate genes that were involved in the phenotype (Chapter 2, Fig. 3). The role of both genes in the *A2bp1*^{KG06463} phenotype was further confirmed using expression of ribonucleic acid interference (RNAi) against each gene in different tissues in the ovary (Chapter 2, Fig. 4). Complementation analysis revealed additional phenotypes depending on the allelic combination involved. Weaker phenotypes had egg chambers that had undergone five instead of four rounds of mitosis, or egg chambers that consisted of multiple cysts which had been inappropriately packaged together (Chapter 2, Fig. 3).

Since differentiation appeared to be affected in *A2bp1*^{KG06463}, we tested to see whether Bag of marbles (BAM), a marker of differentiation, or Decapentaplegic (DPP), a key signalling molecule associated with differentiation, were altered (Chapter 2, Fig. 2). As has been reported for other alleles of *A2bp1* there was no change in the expression of Daughters against decapentaplegic (DAD) LacZ, a reporter for DPP signalling indicating the GSCs in the niche were being regulated normally by intrinsic signals. However we found that most of the sprectrosome-containing GSC-like cells were negative for BAM expression, a marker for cystoblast (CB) differentiation that is

normally expressed when DPP signalling is reduced. This result was unexpected because Tastan, *et al*. (2010) have shown that another allele of *A2bp1*, $A2bp1^{E03440}$, has an excess number of GSC like cells which express BAM. Although different staining conditions might contribute to such a discrepancy another possible explanation is that the different expression pattern reflects different alleles used. A2BP1 is a marker for an intermediate step of GSC differentiation. Its expression begins in the cyst after cytoplasmic BAM expression is reduced, and continues through to the sixteen cell stage (Tastan *et al*., 2010). It is possible that A2BP1 could function at different stages in cyst development and that more severely affected alleles show arrested development at an earlier stage, i.e. at the CB stage. Thus, more severely affected alleles would show an increase in BAM expression. Tastan *et al* (2010) used the $A2bp1^{E03440}$ allele in their study. Since $A2bp1^{E03440}$ has a dominant phenotype, we might expect this to be more severe than other alleles of *A2bp1*. However in our hands the $A2bp1^{E03440}$ allele did not produce viable adults so we were unable to test this possibility. Another explanation could be that $A2bp1^{KG06463}$ affects *Gemin3* as well as *A2bp1*, while $A2bp1^{E03440}$only affects *A2bp1*. This may alter the phenotypic consequences, and influence the observed the *bam* expression pattern.

The germline tumours in $A2bp1^{KG06463}$ were reminiscent of those seen in *Sxl* mutants; while most of the germline consisted of GSC-like cells, there were a few cysts which were able to develop beyond the CB stage. However, these cysts were unable to complete the differentiation process and produce polyploid nurse cells. Similar phenotypes have been observed following loss of *Sxl* function (Chau *et al*., 2009). $A2bp1^{KG06463}$ showed an excess of SXL protein in the cytoplasm which was not observed in the wild type (Chapter 2, Fig. 5). SXL was shown to be correctly spliced into the female splice form, that is, the *Sxl* mRNA in $A2bp1^{KG06463}$ mutants do not contain exon 3, which is only retained in wild type males (Johnson *et al*., 2010). This is

consistent with the misexpressed SXL that I observed being functional and genetic data suggested that SXL activity was required for at least some of the *A2bp1/Gemin3* mutant phenotypes. However it remains possible that misplicing is involved in generating the observed increase in Sxl accumulation. There are 25 splice forms of *Sxl* (http://flybase.org/reports/FBgn0264270.html), so it is possible that Gemin3 and A2BP1 are affecting the splicing of a different isoform of *Sxl* which results in altered localisation or stability of *Sxl* mRNA. I have not however determined if Gemin3 and A2BP1 have a direct effect on SXL itself or if the abundance of SXL is caused by the effect of Gemin3 and A2BP1 on another target. SXL has recently been shown to be required in the ovary for GSC differentiation. It functions by reducing Nanos protein in BAM-expressing cells (Chau *et al*., 2012). SXL is expressed in the cytoplasm of GSCs whereas it is downregulated in CBs (Chau *et al*., 2012). It is possible that A2BP1 and Gemin3 control the localisation of SXL, thus leading to inappropriate activation of SXL targets. Alternatively, SXL may function with other binding partners, which are affected by Gemin3 and A2BP1 (Fig. 1). Interestingly, a *Saccharomyces cerevisiae* DExD-box helicase, Dhh1, is able to regulate mRNA decay. In the absence of other components that control mRNA decay, Dhh1 is still able to reduce the amount of protein produced from mRNAs which it targets, suggesting that Dhh1 is able to inhibit translation (Carroll *et al*., 2011). It is possible that Gemin3 functions by repressing the translation of Sxl mRNA. If this were the case, reducing Gemin3 activity might be expected to produce more SXL protein.

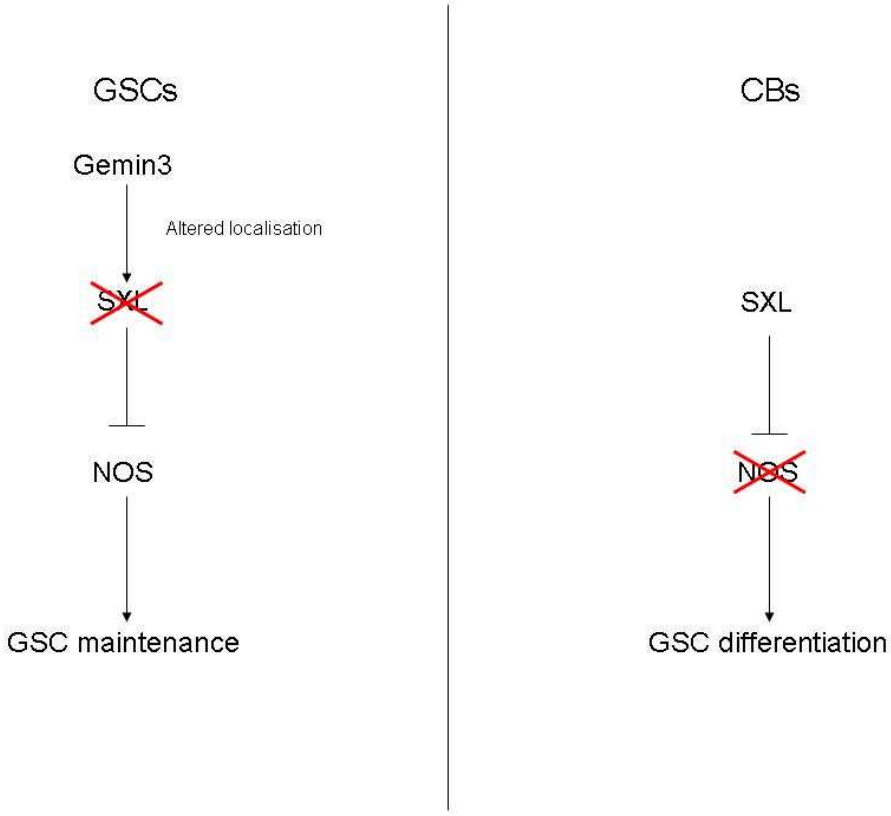

Fig.1 Possible function of Gemin3 in germline stem cells. SXL is essential for downregulating Nanos (NOS) in cystoblasts (CBs), which is required to allow differentiation of the germline. Gemin3 may alter SXL function by controlling its localisation. If SXL is downregulated, then NOS would be able to promote GSC maintenance.

SXL is essential for regulating sex determination in *Drosophila* (Gonzalez *et al*., 2008). In the ovary, as well as a function in regulating GSC differentiation, SXL controls the development of the female germline in the embryo. SXL is sufficient to change transplanted XY pole cells into female germline. Additionally, transplantation of XY pole cells without ectopic SXL expression leads to an accumulation of undifferentiated germline in female ovaries, demonstrating that male germline cells are unable to differentiate in females and that SXL expression is essential for ensuring that the germline retains a female identity (Hashiyama *et al*., 2011). Interestingly, the *Caenorhabditis elegans* homolog of Gemin3, Maternal effect lethal (MEL)-46, is required to produce mature eggs. Loss of MEL-46 in *C. elegans* hermaphrodites leads to an overproduction of immature female germline cells, known as ooids, and an increase in the production of sperm, which suggests a masculinisation of the germline (Minasaki *et al*., 2009). Similarly, Drosophila $bam^{\Delta 86}$ and snf^{148} mutations are known to express mRNAs associated with males (Chau *et al*., 2009). As SXL expression is altered in *A2bp1* and *Gemin3* mutants, one of the functions of A2BP1 and Gemin3 may be to ensure the female germline is suppressing expression of male specific mRNAs, possibly through SXL.

During complementation, only deletions which removed both the *Gemin3* and *A2bp1* loci were able to produce the tumorous phenotype. Complementation with transposable element mutations mostly produced 5n egg chambers or compound egg chambers. This suggested that both *A2bp1* and *Gemin3* mutations contribute to the tumorous phenotype. Since the null allele of *Gemin3*, $Gemin3^{rL562}$, is lethal (Shpargel *et al*., 2009), the fact that we can obtain homozygotes from $A2bp1^{KG06463}$ suggests it is not a null allele. It is possible that the 5n and compound phenotypes are weaker than the tumorous phenotype because they were only produced in combinations where either *A2bp1* or *Gemin3* alone was affected. A2BP1 and Gemin3 may regulate factors required

to promote differentiation at multiple stages of cyst development. In males, BAM is required to control the number of transit amplifying steps that developing cysts undergo. If BAM is reduced, the cysts will undergo an extra round of mitosis because the BAM threshold required to stop mitosis takes longer to reach (Insco *et al*., 2009). One possible explanation for the 5n phenotype seen in the *A2bp1*^{KG06463} complementation is that factors required for the switch between mitosis and cyst progression take longer to reach an appropriate threshold, thus the germline undergoes an extra round of mitosis. Conversely, it is also possible that factors which are required for maintaining an undifferentiated state are not turned over quickly enough in the absence of Gemin3 and A2BP1, leading to germline cysts which differentiate much more slowly and may undergo extra rounds of mitosis.

The presence of compound egg chambers was interesting because it suggested a function for A2BP1 in the follicle tissue which has not been identified previously (Tastan *et al*., 2010). Compound egg chambers can arise when stalk cells fail to differentiate appropriately, leading to egg chamber fusion. This may be one mechanism by which A2BP1 functions in the follicle cells. Additionally, A2BP1 may be important for regulating FSC proliferation. A reduction in follicle cell production might be expected to lead to packaging defects in the germarium. One way of differentiating between the two would be to carry out a Fasciclin III (FasIII) stain on the weaker alleles of *A2bp1*. This would mark the polar cells; if the compound phenotype resulted from egg chamber fusion, there should be an extra set of these cells. *A2bp1* RNAi using the c587Gal4 driver produced compound egg chambers which suggests follicle stem cells (FSCs) may be affected by loss of *A2bp1*, since I found that this driver expresses in the FSCs (Chapter 2, Fig. 4). The use of flippase (FLP)/FRT induced mitotic clones (Golic and Lindquist, 1989) would determine whether A2BP1 had a function in FSCs. Using recombination induced by FLP under the control of a heatshock promoter, this

experiment would allow the generation of FSC clones which do not have functional A2BP1 which would be identified by their lack of a marker such as GFP. By comparing the number of unmarked FSC clones between ovarioles with mutant *A2bp1* and a control which contains unmarked wild type clones, it would be possible to determine whether A2BP1 functions in FSCs. If A2BP1 did function in FSCs, we would expect the number of unmarked FSC *A2bp1* mutant clones to be less than the number of unmarked wild type clones, since any mutant FSCs would be replaced by FSCs containing GFP. It is also possible that, rather than being lost, FSCs may just not divide frequently enough in $A2bp1^{KG06463}$. It might be possible to determine if this were the case, again, by using mitotic clones. Since it has been shown that two FSCs are responsible for the generation of all follicle cells and that making a mitotic clone in one leads to half of the follicle cells being unlabelled (Margolis and Spradling, 1995), we could generate germaria with single mutant FSCs and count the number of unmarked mutant follicle cell clones and compare them to those which are marked. If there are significantly less than 50% which are unmarked, this might suggest a problem with FSC proliferation rather than maintenance.

While the ribonucleic acid interference (RNAi) of *A2bp1* produced many compound egg chambers when driven in the somatic tissue and many 5n egg chambers when driven in the germline, further demonstrating that A2BP1 is required for germline and follicle cell function, there were no tumours observed. This is possibly because the RNAi was not strong enough. Alternatively, A2BP1 might need to be reduced in both the germline and somatic tissue, or Gemin3 function may also need to be reduced. Consistent with a role for Gemin3 in oogenesis, RNAi against the latter produced a strong phenotype when driven in the germline. There were few ovarioles which produced a tumorous phenotype. The rest were devoid of any germline. This latter phenotype is reminiscent of *nanos* mutants (Chau *et al*., 2012). Since SXL is required to

inhibit the expression of Nanos in the germline (Chau *et al*., 2012), it follows that the loss of a protein which normally functions to inhibit SXL, for example, Gemin3, might produce a similar phenotype to *nanos* mutants (Fig. 2). Given that A2BP1 does not seem to be expressed in GSCs (Tastan *et al*., 2010), the fact that Gemin3 is able to cause GSC loss suggests that A2BP1 and Gemin3 may have different targets depending on the cellular context they find themselves in. It would be useful to determine the expression pattern of Gemin3 in the germarium, to confirm whether Gemin3 is present in GSCs.

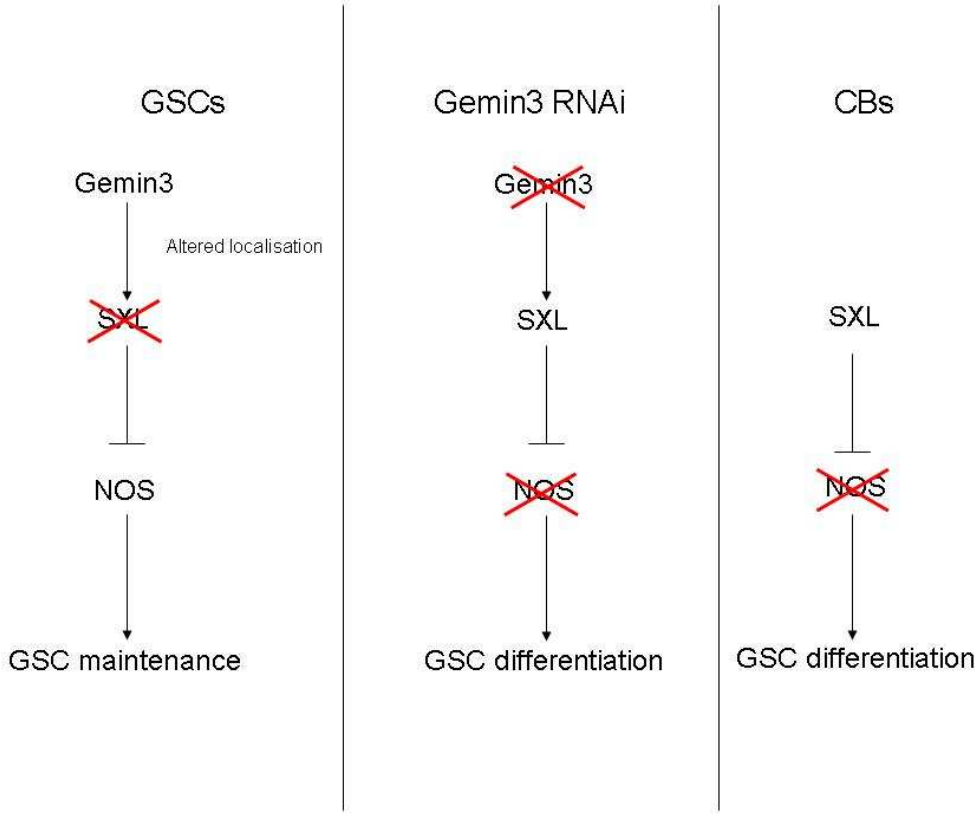

Fig. 2. Loss of Gemin3 in germline may lead to germline differentiation. In GSCs, Gemin3 may inhibit SXL which leads to upregulationg of Nanos (NOS) and subsequent GSC maintenance. In cystoblasts (CBs), SXL is able to downregulate NOS, leading to differentiation. In the Gemin3 RNAi, loss of Gemin3 leads to upregulation of SXL and loss of NOS, driving GSC differentiation.

One function of Gemin3 that has been published recently is to induce the separation of polytene chromosomes in nurse cells (Cauchi, 2012). This phenotype is similar to that seen in mutant clones of *survival motor neuron* (*smn*) in the germline (Lee *et al*., 2009). This was not a phenotype observed in any of the experiments carried out using $A2bp1^{KG06463}$. One possible explanation for this is that we used different combinations of mutants, as mentioned previously; the phenotypes seen in our study might require the disruption of both A2BP1 and Gemin3. It is also possible that the clones described by Cauchi (2012) are actually ovo^{D1} egg chambers rather than $Gemin3^{rL562}$ clones. ovo^{D1} mutant egg chambers are able to survive until stage 4, which has nurse cells with a characteristic "blob" like appearance, when germline development becomes arrested and the egg chambers later degenerate (Wang and Riechmann, 2007; Cauchi, 2012). Since the Cauchi (2012) paper describes the $Gemin3^{rL562}$ germline clones as having the characteristic "blob" like nuclei of a stage 4 egg chamber, it is difficult to determine the difference between an $ovo^{D1}/Gemin3^{rL562}$ egg chamber and a $Gemin3^{rL562}$ clone without confirming that the egg chambers being analysed are, in fact, $Gemin3^{rL562}$ clones. This could be done using a Gemin3 antibody to show that the Gemin3 clones do not contain any Gemin3 (Cauchi *et al*., 2010). If the egg chambers being analysed were not Gemin3 clones, this may explain why a Gemin3 rescue construct was unable to rescue the phenotypes which they described (Cauchi, 2012). Additionally, eggs which were laid were described as having fused dorsal appendages, which are characteristic of some *ovo* alleles when placed in combination with *snf* or *Sxl* mutants (Oliver *et al*., 1990). Since we know from our work that SXL is affected by Gemin3, it is possible that, although ovo^{D1}mutants do not normally lay eggs, by adding a Gemin3 allele into this background, this may have allowed some egg chambers to progress beyond stage 4.

In order to determine how A2BP1 might be functioning in $A2bp1^{KG06463}$, I tested to see if the dominant allele, $A2bp1^{E03440}$ had an interaction with mutants known to

affect SXL (Chapter 2, Fig. 6). First, *A2bp1*^{E03440} was rescued by an allele which abolishes SXL in the germline, *Snf*^{148}, suggesting that SXL function is increased in *A2bp1* mutants. Additionally, another *Sxl* mutant that leads to an increased accumulation of SXL in the germline also rescued the 5n phenotype of *A2bp1*^{E03440}. It is unconfirmed whether SXL in this mutant is functional or not (Bopp *et al*., 1993). A known downstream target of SXL is Ornithine decarboxylase antizyme (ODA) (Vied *et al*., 2003). *A2bp1*^{E03440} produced 5n egg chambers with a deletion in ODA. Additionally, a P-element mutant in *Oda* was able to produce compound egg chambers with *A2bp1*^{E03440}, suggesting that A2BP1 may be affecting ODA in both follicle cells and in the germline. ODA is a negative regulator of Ornithine decarboxylase (ODC), a key enzyme involved in polyamine synthesis (Heby *et al*., 1990; Heby and Persson, 1990). ODA binds to ODC and inactivates it, eventually targeting it for ubiquitin-independent degradation in the proteosome (Murakami *et al*., 1992). I had expected that reducing ODC would rescue the dominant phenotype. However, reducing ODC had no effect on the phenotype of *A2bp1*^{E03440}. In addition to its role in regulating ODC, ODA is able to target other proteins for degradation, including CyclinD1 (Newman *et al*., 2004) and Aurora A, which are essential for cell cycle progression (Lim and Gopalan, 2007). In addition to this, upregulation of antizyme is able to promote deoxyribonucleic acid (DNA) repair in human oral cancer cells by upregulating DNA dependent kinase and Ku70 (Tsuji *et al*., 2007). Another function for antizyme is the targeted destruction of Smad1, which is a downstream component in Bone Morphogenic Proteins (BMP) signalling (Lin *et al*., 2002). A mutant in Mothers against Decapentaplegic (MAD), the Drosophila homolog of SMAD1, was able to rescue the 5n phenotype in *A2bp1*^{E03440}. This suggests that DPP signalling is upregulated in *A2bp1*^{E03440}. However, this was unexpected given there was no increase in the amount of DADLacZ produced in *A2bp1*^{KG06463}. It is possible that the effect of altering DPP signalling is indirect. If the

components which are required for differentiation are reduced, then the developing cyst might be expected to take longer to differentiate. In reducing the level of DPP signalling by reducing MAD, this restores the balance between factors responsible for differentiation and GSC maintenance. Another explanation for this interaction could be that Mad is functioning through other downstream targets. For example, mad is known to interact with a non-DNA binding transcription factor, Yorkie. This is able to upregulate the expression of a micro ribonucleic acid (miRNA) gene known as bantam, which is required for growth (Oh and Irvine, 2011). It would be interesting to see if Yorkie or bantam and other components of the DPP signalling pathway interact with *A2bp1*^{E03440}.

Another target which is negatively regulated by SXL is Notch. Paradoxically, we found that both loss of function and gain of function alleles of Notch produced egg chambers which had only undergone three rounds of mitosis as opposed to four (Chapter 2, Fig. 7). Notch is able to regulate the switch from mitosis to endocycle in the follicle cells (Shcherbata *et al*., 2004). Perhaps inappropriate Notch activity in the germline has a similar effect. Notch is not known to have a function in the germline itself. This was confirmed using Notch RNAi in the germline; this had no effect on the number of mitoses the germline underwent. The inappropriate activation of Notch in the germline could be explained by the activity of cis-inhibition of Notch (de Celis and Bray, 2000). The E(spl)mβ$^{1.5}$ reporter in wild type ovarioles showed that normally Notch activity is present in follicle cells, but not in the germline. A Notch loss of function allele unexpectedly led to an increase in Notch signalling in the germline. It is possible that reduced amounts of Notch are unable to cis-inhibit ligands expressed in the somatic cells, allowing them to mis-activate Notch in the germline (Fig. 3).

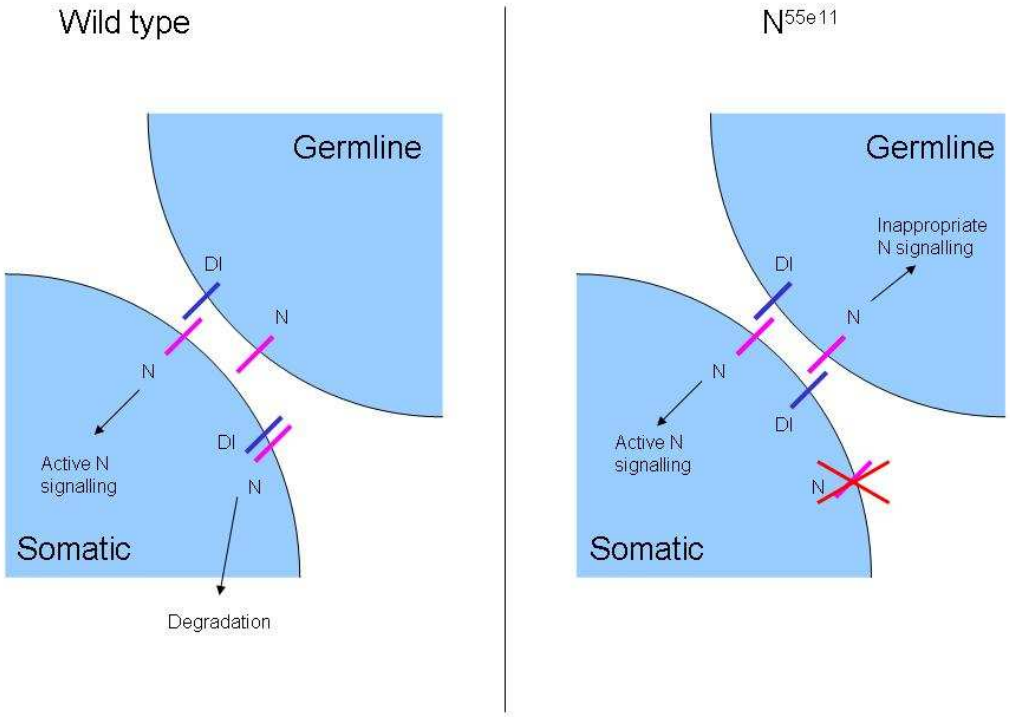

Fig. 3. Cis-inhibition of Notch in the ovary. In the wild type situation, Notch signaling is active in the somatic tissue but not the germline as Delta (Blue) expressed in the somatic tissue is targeted for degradation by Notch (Magenta). In N^{55e11}, a reduction in the amount of Notch leads to loss of cis-inhibition, leading to inappropriate Notch activation in the germline.

It would be interesting to see if there is a functional interaction between Gemin3 and A2BP1 homologs in other organisms. Both are involved in processing mRNAs and both have been implicated in regulating neuronal function in mammals (Bhalla *et al*., 2004; Martin *et al*., 2007; Sun *et al*., 2010). *smn*, which is known to be mutated in patients with spinal muscular atrophy (SMA), is part of the small nuclear ribonucleoprotein (snRNP) biogenesis pathway, which is essential for RNA storage and processing (Praveen *et al*., 2012). Gemin3 is known to interact directly with SMN via its C terminus and addition of a human mutant found in SMA patients is able to reduce this interaction, suggesting that Gemin3 and SMN function together (Charroux *et al*., 1999). Recently it has been shown that replacing SMN in a *smn* mutant background is able to rescue the lethality of *smn* in Drosophila larvae without restoring snRNA biogenesis, suggesting loss of snRNP biogenesis is not the cause of the SMA phenotype and that SMN may have other functions besides snRNP biogenesis (Praveen *et al*., 2012). Gemin3 is a putative DEAD-box RNA helicase. These proteins also form part of the spliceosome and have been implicated in mRNA processing. For example, the yeast homolog of Gemin3, Dhh1, is able to prevent translation by decapping mRNAs, thus promoting mRNA degradation (Sweet *et al*., 2012).

There is, as yet, no known interaction between A2BP1 and Gemin3. However, A2BP1 is able to bind to Ataxin 2 which is known to be mutated in patients with spinocerebellar ataxia type 2, thus A2BP1 is also linked to human neurodegenerative disorders (Ross *et al*., 2011). A2BP1 belongs to a family of proteins known as the Forkhead Box 1 (FOX1) family, which regulate alternative splicing by binding to a specific sequence, (U)GCAUG, in different unprocessed mRNA molecules. FOX-1 proteins are able to induce exon skipping by binding to upstream introns and preventing the spliceosome from forming at that junction and thus leading to inclusion of the downstream exon. Alternatively, they are able to induce exon inclusion by binding to

downstream exons and, again, preventing components of the spliceosome from binding to the intron (Reviewed in Kuroyanagi, 2009).

Paper 2. Disruption of Glucuronyl transferase I activity impairs escort and follicle cell contributions to Drosophila oogenesis.

The second paper describes the identification of GlcATI as a gene involved in regulating egg production. In this mutant, there was a loss of GSCs and the presence of compound egg chambers which suggested that both the germline and the follicle cell lineage were affected (Chapter 3, Fig. 1). Furthermore the escort cells were unable to invade and separate adjacent cysts. Viable adults also had defective wing and leg morphology (Chapter 3, Fig. 1). Remobilisation of the transposable element in this stock demonstrated that the phenotypes were not caused by a secondary mutation (Chapter 3, Fig. 3). We carried out complementation analysis, which suggested GlcATI was the affected gene (Chapter 3, Fig. 4). This was confirmed by RNAi knockdown of GlcATI, which replicated the phenotypes (Chapter 3, Fig. 6). Furthermore the mutant phenotypes were rescued by expression of a GlcATI complementary DNA (cDNA) rescue construct in a *GlcATI* ^{F00247} mutant background (Chapter 3, Fig. 5). The RNAi experiment indicated that GlcATI exerts its function either in the ECs or the FSCs. GlcATI is predicted to be a homolog of the human gene, β1-3 glucuronosyl transferase which catalyses an essential step in glycosaminoglycan (GAG) synthesis which form part of proteoglycans (Bai *et al*., 1999; Kim *et al*., 2003).

Proteoglycans are known to play important roles in many different signalling pathways and thus have an impact on many aspects of tissue function. For example, chondroitin sulphate proteoglycans (CSPG)s are able to act as a repellent to neurons (Wang, H. *et al*., 2008). After a brain injury, neurocan and NG2, two different CSPGs, are upregulated and deposited at the site of the injury, potentially contributing to the

formation of scar tissue in the brain (Yi *et al*., 2012). During the neuronal development of *Drosophila* embryos, heparan sulphate proteoglycans (HSPG)s are also able to control the gradient of a repellent known as Slit, preventing axons from inappropriately crossing the midline (Johnson *et al*., 2004). Additionally, GAGs are known to be important for regulating stem cell fates. In a culture plate containing different GAG molecules, human mesenchymal stem cells will begin expressing markers associated with differentiating osteoblasts, suggesting that the composition of the ECM is important for controlling differentiation (Mathews *et al*., 2011; Murphy *et al.,* 2012). Another example is seen in the ability of GAG molecules to influence the differentiation of heparan sulphate mouse embryonic stem cells into neural tissue (Maede *et al.,* 2012).

Since proteoglycans play key roles in regulating signalling, the activities of enzymes which modify proteoglycans are also important in tissue function. Loss of components of the proteoglycan synthesis pathway such as *tout velu, brother of tout velu* and *sister of tout velu* in *Drosophila* all have an impact on heparan sulphate synthesis which in turn results in altered Wingless (WG), DPP and HH signalling (Takei *et al*., 2004). One enzyme required for modifying heparan sulphate is Sulphated1 (SULF1) which functions as an endosulphatase. Butchar, *et al*, 2012 demonstrated that Epidermal Growth Factor (EGF) signalling is able to upregulate the activity of SULF1 which in turn acts as a negative regulator for EGF signalling in the wing, potentially by removing active sites from heparan sulphate. Thus, SULF1 contributes to a negative feedback loop which regulates the levels of EGF signalling in the wing (Butchar *et al*., 2012). Similarly, SULF1 is also able to act as a negative regulator of WG, thus suggesting that multiple signalling pathways may utilise the same mechanisms of controlling the output of a given pathway (You *et al*., 2011). Additionally, control of proteoglycan synthesis allows for simultaneous regulation of the multiple signals required for organogenesis. For example, Fat and Dachsous, both members of the Hippo

signalling pathway, are able to negatively regulate Development abnormally delayed (DALLY) and Dally-like protein (DLP), which are required for WG, HH and DPP signalling in the wing (Baena-Lopez *et al*., 2008). Finally, internalisation of morphogens which are bound by proteoglycans may play a role in enhancing cell signalling. This is seen in the wing, where DLP is endocytosed with patched and HH, which ensures proper activation of both WG and HH signalling (Gallet *et al*., 2008). It would be interesting to determine whether or not other enzymes associated with GAG synthesis are also able to influence ovary function and, if so, whether these had an interaction with $GlcATI^{F00247}$. It is possible that the phenotype of mutations which affect the protein core of a particular proteoglycan might be enhanced by affecting the activity of GlcATI, or vice versa, thus indicating which proteoglycans are important for ovary function.

After identifying GlcATI as a potential candidate gene, I tested to see if $GlcATI^{F00247}$ had a genetic interaction with components of different signalling pathways in an attempt to identify potential mechanisms by which GlcATI may function (Chapter 3, Fig. 8). $GlcATI^{F00247}$ was found to interact with several mutants that affect either HH, DPP, EGF or Janus kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT). Since GlcATI catalyses a step which is common to the synthesis of all proteoglycans, GlcATI may be influencing multiple signalling processes. Proteoglycans are able to both enhance and suppress signalling by sequestering secreted morphogens. An example of the former is seen when proteoglycans function as coreceptors for signalling molecules. Heparan sulphate is able to facilitate BMP and fibroblast growth factor (FGF) mediated signalling. The loss of heparan sulphate leads to attenuation of both signalling pathways and poor differentiation of mouse mesoderm in culture (Kraushaar *et al*., 2012). Another heparan sulphate proteoglycan which is essential for BMP signalling in the *Drosophila* wing is the glypican, DALLY. This protein functions as a coreceptor for

DPP, the *Drosophila* homolog of BMP, thus enhancing DPP signalling (Fujise *et al*., 2003). Additionally, the expression of a secreted form of DALLY is able to induce overgrowth in different tissues in Drosophila due to expansion of the range of HH signalling in these flies. Under normal circumstances, tethering DALLY to cell membranes functions to restrict the range of HH signalling (Takeo *et al*., 2005). In the ovary, DALLY also increases HH signalling from the cap cells (Guo and Wang, 2009). Since $GlcATI^{F00247}$ led to the enhancement of a dominant HH wing phenotype, suggesting an expansion of HH signalling, one possible explanation for this result might be that without proper glycosylation, DALLY is no longer able to sequester HH in $GlcATI^{F00247}$. Also, by sequestering morphogens, GAGs are able to sharpen the boundary between cells which respond to high levels of signalling and cells which respond to low levels. This is seen in the role of DLP in the regulation of HH signalling in the wing. In this instance, DLP, secreted from cells exposed to the highest levels of HH, is able to sequester HH**.** This is then internalised, thus preventing other cells being activated by HH signalling (Ayers *et al*., 2012). Thus proteoglycans are able to "fine tune" the expression of morphogens to ensure tissue develops properly during embryogenesis.

We found that a number of signalling pathway components showed genetic interactions with the GSC loss phenotype of $GlcATI^{F00247}$, but the results were not straightforward to interpret. A gain of function *thickveins* (*tkv*) mutant had excess GSC-like cells in the germarium which was expected as over activation of DPP signalling is known to increase GSC self renewal. This phenotype was enhanced when the *tkv* mutant was placed in a homozygous $GlcATI^{F00247}$ mutant background, suggesting that one of the products of GlcATI is able to restrain DPP signalling in the germarium (Fig. 3). If this were the main function of GlcATI in the ovary then it is difficult to reconcile this activity with its mutant phenotype which leads to loss of GSCs. Similarly, loss of

function JAK/STAT mutants were able to rescue the *GlcATI*^{F00247} GSC loss despite the known role of JAK/STAT signalling to promote DPP signalling from the niche. One possibility is that the GSC loss phenotype of *GlcATI*^{F00247} reflects consequences of this mutation on other cell types, with subsequent indirect consequences on GSC number that mask any enhancement of DPP signalling in the GSCs. I considered whether loss of Escort cell invasion might be linked to GSC loss. However there was no correlation between the strength of the escort cell phenotype and GSC number in the genetic interactions studied. There did seem to be a correlation between the presence of the compound egg chamber phenotype and GSC number. All the genetic interactions that rescued the follicle cell phenotypes also recovered GSC numbers. It is possible therefore that there is a form of feedback between proper egg chamber formation and GSC population. Recent unpublished work in our group shows that inducing egg chamber phenotypes by RNAi knockdown of Notch only in the follicle cells does reduce GSC number (Alessandro Bonfini, unpublished).

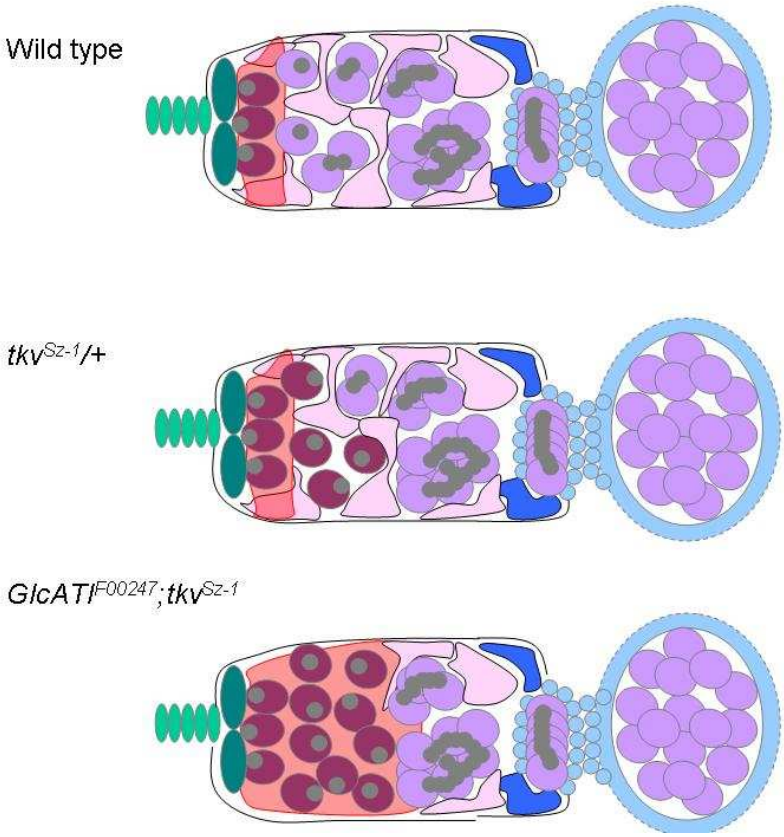

Fig. 3. Possible function of GlcATI in DPP signaling. In wild type germaria, the range of DPP (Red) signalling is limited. This means the number of GSC cells (dark purple) remains restricted to those which are in contact with cap cells (Dark green). In the *thick veins* gain of function mutant, there are more cells which become GSC like, possibly because they are more senstised to DPP (ie. Lower levels of DPP are required to convert germline cells into GSCs). With the addition of *GlcATI*^{F00247}, the number of GSC like cells is increased, possibly because one of the products of GlcATI is involved in controlling the diffusion of DPP.

Another interesting phenotype which we observed was the inappropriate invasion of follicle cells in the egg chambers of flies which were mutant for both *GlcATI*^{F00247} and heterozygous loss of function *Stat92E*. It would be interesting to determine if the invasion is due to poor differentiation of the follicle cells, which could be investigated using a marker such as FasIII which marks undifferentiated follicle cells. Another mutant which has been associated with invasive follicle cell behaviour is Discs large, which is a marker expressed on the lateral side of follicle cells. This phenotype is caused by an inability to halt proliferation or control polarity of follicle cells (Goode and Perrimon, 1997). Follicle cell over proliferation is also seen in *posterior sex comb* and *su(z)2* mutants which show follicle cells lacking apical/basal polarity. In this case, differentiation of follicle cells is blocked (Li *et al*., 2010) Also, blocking mitochondrial fission, by mutating *dynamin related protein-1*, leads to over proliferation of follicle cells (Mitra *et al*., 2012). Another possible explanation for this phenotype is that GlcATI is responsible for the synthesis of a molecule which acts as a repellent for follicle cells, inhibiting their ability to proliferate and invade the egg chamber. The basement membrane of tissues is known to be important for regulating tissue integrity and this is also a site where proteoglycans are known to accumulate and influence cell behaviour (Reviewed in Iozzo, 2005). Indeed, a *Drosophila* proteoglycan, Perlecan is needed to define the apical/basal axis of the follicular epithelium of older egg chambers (Schneider *et al*., 2006). If the synthesis of basement membrane were altered, the cells which rely on positional cues from the ECM would be unable to function.

We found that loss of GlcATI also had an impact on the ability of the germarium to appropriately package cysts. One pathway associated with follicle stem cell function is HH signalling (Nystul and Spradling, 2007). We found that a gain of function HH mutant was able to rescue the cyst packaging defect in *GlcATI*^{F00247}, suggesting a possible function of GlcATI is to control the diffusion of the HH morphogen secreted

from the cap cells. Again this could be through a proteoglycan, which functions as a coreceptor on the FSC surface, or a component of the basement membrane that is required to sequester HH at the surface of FSCs (Fig. 4). However we found no genetic interaction with a loss of function mutation of HH and further genetic analysis with other alleles and components of the HH signalling pathway will be required to confirm a role of GlcATI in HH signalling.

Thus, these results demonstrate that GlcATI has a critical role in many aspects of tissue regulation in the germarium and identify functional interactions with a number of signalling pathways. Further work will now be required to determine the relevant proteoglycans proteins involved in each case and to determine which interactions reflect direct molecular regulatory mechanisms.

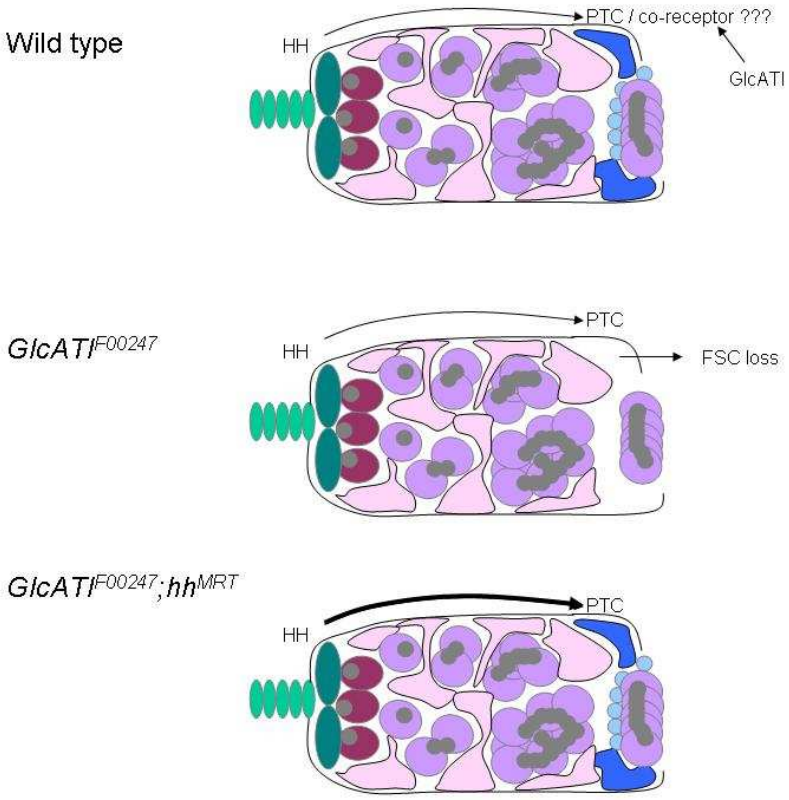

Fig. 4. Influence of GlcATI on HH signaling in FSC maintenance. In wild type germaria, HH produced by cap cells (Dark green) is essential for FSC maintenance. It is possible that GlcATI is involved in the production of a co-receptor for HH which promotes activation of HH signaling in FSCs (Dark blue). In *GlcATI*^{F00247}, the loss of the co-receptor would make FSCs less sensitive to the HH morphogen, thus leading to FSC loss. The addition of the gain of function *hh* mutant might lead to a boost in HH signaling which is enough to compensate for the loss of a putative co-receptor produced by *GlcATI*^{F00247}. This would prevent the loss of FSCs, thus reducing the number of compound egg chambers seen in *GlcATI*^{F00247}.

Paper 3. Dpr9, a brain expressed IgG domain containing protein, is required for egg production.

The final paper shows the characterisation of a third mutant identified in the screen, *l(3)04713*. This mutant produced compound egg chambers, and had a high first egg chamber stage caused by the premature loss of GSCs (Chapter 4, Fig. 1). Remobilising the insert rescued the phenotype, indicating that the phenotype was not due to a secondary mutation (Chapter 4, Fig. 2). Complementation with deficiencies showed that *dpr9* was a possible candidate for the phenotype in *l(3)04713* (Chapter 4, Fig. 3). It was found that *dpr9* was expressed in the ovary, and RNAi expressed in ovarian tissues had no phenotype. We therefore carried out RNAi using a pan-neuronal Gal4 and found that this was able to partially replicate the *l(3)04713* phenotype, suggesting that dpr9 does not function in the ovary, but in the brain (Chapter 4, Fig. 4). It would be useful to generate other mutants in *dpr9*, possibly using imprecise P-element excision events in the original mutant, in order to completely understand the role which *dpr9* plays in *Drosophila*. Additionally, confirmation of the role of *dpr9* in the *l(3)04713* mutant would be further strengthened by the rescue of the phenotype by a *dpr9* cDNA construct.

I also found that other members of this family of immunoglobulin containing proteins demonstrated the same phenotype. A BLAST search for the DPR9 protein sequence suggested that DPR proteins share homology with neurotrimin, a member of a class of neural cell adhesion molecules known as IgLONs (Chapter 4, Fig. 6). These proteins are able to influence neural adhesion and neurite outgrowth (Akeel *et al*., 2011; McNamee *et al*., 2011). They are also able to function as both homodimers or heterodimers with other members of the IgLON family (Reed *et al*., 2007). One possible explanation for the observation that other *dpr* genes have an ovary function is that there is functional redundancy in this family of proteins. But another possibility is

that they are involved in heterodimer based cell adhesion similar to the IgLON proteins. This could be tested in DPR-expressing cell culture using a cell-cell aggregation assay similar to that used to demonstrate Notch receptor/ligand interactions (Cordle *et al*., 2008). It would also be interesting to combine mutants in different *dpr* genes with each other to see if there is a phenotypic enhancement.

One possible function of the DPR proteins is that they are involved in the production of a systemic factor important in regulating the fly's responses to food. For example brain produced Insulin-like peptides are known to play a role in regulating GSC proliferation and maintenance (Hsu and Drummond-Barbosa, 2009; Hsu and Drummond-Barbosa, 2011). Additionally, Insulin is able to regulate the apoptosis of older egg chambers under conditions of starvation (Drummond-Barbosa and Spradling, 2001). A high protein diet is also known to have an effect on egg production (LaFever *et al*., 2010). The consequences of diet and loss of Insulin on the ovary do not directly phenocopy the consequences of disruption of DPR9 genes, however, and other possible sources need to be considered.

It is possible that the normal function of DPR9 is to influence the fly's egg production and egg laying behaviour in response to different components in their food. The first member of this family of genes to be identified was *dpr*, which is known to be important for controlling the response to the presence of salt in food. Under wild type conditions, flies will reject solutions which contain too much salt, while *dpr* mutants will consume salty solutions (Nakamura *et al*., 2002). Recently, *dpr9* has been implicated in the response to alcohol. Flies will normally exhibit increased locomotion when initially exposed to alcohol. *dpr9* mutants do not show this increased locomotion, suggesting that *dpr9* may, like *dpr*, be involved in detecting the presence of a particular food cue (Kong *et al*., 2010). This is interesting because it suggests that the systemic regulation of ovary function is dependent on more than just a high sugar or high protein

diet. It would be informative, therefore, to observe the effects of removing defined components of the diet on the *dpr9* mutant phenotype.

If the *dpr* family of genes are involved in regulating responses to food, it would be necessary to determine how they contribute to this process and how this influences the ovary. One possibility could be that *dpr9* is needed for neuronal development during embryogenesis, rather than affecting neuronal function in the adult. To assess this, *dpr9* could be selectively downregulated in the adult using an elavGal4 stock which contains a temperature sensitive Gal80 (McGuire *et al*., 2004). In this instance, if the embryos are allowed to develop at the permissive temperature which allows Gal80 to function, the brain should form normally. After eclosure, the flies would be shifted to a non-permissive temperature, and thus *dpr9* would be knocked down in the adult. If *dpr9* has a function in the adult, this would recreate the *dpr9* phenotype in the ovary.

It would also be necessary to determine which neurons are affected in the fly. Candidates might include neurons which are involved in the gustatory or olfactory response. It would be useful to determine the expression pattern of *dpr9* in the brain. This could be done using *in situ* hybridisation or by generating Dpr9 specific antibodies for immunostaining. Another possible experiment could be to use Gal4 drivers which affect subsets of neurons to selectively knock down *dpr9* in these neurons to see if the *dpr9* phenotype can be replicated (Pfeiffer *et al*., 2008). Additionally, *dpr9* may function in Insulin producing cells in the brain. It would be interesting to determine if *dpr9* exerts its function through Insulin. One key experiment to test this would be to drive Upstream Activating Sequence (UAS) Drosophila Insulin like peptide constructs in the ovary in a *dpr9* mutant background to see if this would rescue the *dpr9* phenotype (Hsu *et al*., 2008). It would also be interesting to see if *l(3)04713* interacts with other components in Insulin signalling, or mutants involved in Tor signalling.

General Conclusions

We found that stocks containing transposable elements were useful in allowing the identification of genes which are required for maintaining tissue function. One advantage of this strategy over an RNAi-based one is that there is no need to decide which tissue to knock the gene down in. Another advantage of this method is that it may reveal functional clusters of genes. For example, the *A2bp1*^{KG06463} allele affected two different genes which are both required for ovary function and when both genes were knocked out together, this produced a much stronger phenotype than if one locus alone had been affected. This suggests that transposable elements have the ability to reveal phenotypes which might not be identified in other screens.

However, a disadvantage of using transposable elements is that identifying the affected gene with certainty is problematic. Some transposable elements have the ability to influence multiple loci, as seen with *A2bp1*^{KG06463}. Additionally, confirmation of which loci are affected requires that there are enough characterised mutants which affect that particular locus for complementation. A further problem with this latter point is that narrowing down which genes are affected can be difficult if the mutants for complementation are not available. This is where the use of RNAi in conjunction with complementation is useful as this allows selective knock down of genes to see if the phenotype can be replicated. Employing this methodology, the screen was able to identify different categories of mutant phenotypes involving a wide range of protein functions: extracellular matrix, RNA binding and cell-cell adhesion. Furthermore, the screen uncovered genes which are required in several different somatic and germline cell types, and it identified both locally acting and systemically acting factors that, together, regulate the constant turnover of cells needed to maintain egg production in *Drosophila*.

Through the use of the screen, we found several proteins which play a role in this regulation of tissue turnover. First, we show that A2bp1 and Gemin3 may function together to control differentiation in the germline. Understanding this interaction is important since both have been linked to poor prognosis of certain human cancers (Tada *et al*., 2009; Yang *et al*. 2008; Cai *et al*., 2011). Secondly, we identified GlcATI as a protein which is important for egg production, demonstrating the importance of the ECM in regulating tissue turnover. Finally, we identified DPR9 as a protein produced in the brain which is able to influence egg production. A better understanding of how DPR proteins influence egg production may provide insights into the role of nutrition in human fertility.

Chapter 6. References

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Appendix I. Supplementary materials and methods.

Genotype list

Mutant	Full *Genotype*	Order Number	Obtained from
A2bp1^{KG06463}	*y^{1}; P{SUPor-P}A2bp1^{KG06463} ry^{506}/TM3, Sb^{1} Ser^{1}*	15104	Bloomington
A2bp1^{EY00149}	*y^{1} w^{67c23}; P{EPgy2}eg^{EY00149}/TM3, Sb^{1} Ser^{1}*	15285	Bloomington
Gem3^{rL562}	*P{PZ}Gem3^{rL562} ry^{506}/TM3, Sb^{1}*	12079	Bloomington
Df(3L)ED4457	*w[1118]; Df(3L)ED4457 / TM6C, Sb[1]*	150428	Bloomington
Df(3L)Vin2	*Df(3L)vin2, ru[1] h[1] gl[2] e[4] ca[1]/TM3, Sb[1]*	2547	Bloomington
PTRiP. HMS0028attP2 (RNAi integration site)	*y[1] sc[1] v[1] P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP2*	25710	Bloomington
Nanos::VP16Gal4	*w[*]; P{w[+mC]=UAS-eGFP-huLC3}1; P{w[+mC]=GAL4::VP16-nos.UTR}CG6325[MVD1]*	8730	Bloomington
Oda^{EY01073}	*y^{1} w^{67c23}; P{EPgy2}Oda^{EY01073}*	15831	Bloomington
Oda^{lex47}	*y^{1} w^{67c23}; Oda^{lex47}/CyO, P{en1}wg^{en11}*	4373	Bloomington
Snf^{148}	*y^{1} w^{1118} snf^{148} P{neoFRT}19A/FM6*	7398	Bloomington
Pum^{1688}	*P{PZ}pum^{01688} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}*	11544	Bloomington
Df(2R)BSC266	*w^{1118}; Df(2R)BSC266/CyO*	26500	Bloomington
Sxl^{M1}	*y[1] cm[1] Sxl[M1] v[1] f[1]/FM7a/Dp(1;2;Y)w[+]*	3719	Bloomington
sens$^{Ly\text{-}1}$	*sens$^{Ly\text{-}1}$ / TM3, Sb*		M. Baron
Δ2-3,Sb (Transposase)	*Δ2-3,Sb/TM6, Hu*	-	M. Baron
UAS-CD8-GFP	*UAS-CD8-GFP*	-	M. Baron
c587Gal4	*c587Gal4*	-	T. Xie, Kansas city, KA, USA
A2bp1^{E03440}	*W1118; PBac{RB}A2bp1^{e03440} / TM6, Hu*	-	Excelexis
N^{55ell}	*N^{55e11} P{neoFRT}19A/FM7c*	-	S. Artivanis-Tsaksonas, Boston, MA, USA
N^{Axe2}	*N^{Axe2} / FM7c*	-	S. Artivanis-Tsaksonas, Boston, MA, USA
A2bp1VALIUM 20,	*y^{1} sc* v^{1}; P{TRiP.HMS00478}attP2*	-	Transgenic RNAi project (Boston, MA USA)

Gemin3VALIUM20	*y^1 sc* v^1; P{TRiP.HMS00287}attP2*	-	Transgenic RNAi project (Boston, MA USA)
NotchVALIUM20	*y^1 v^1; P{TRiP.HMS00001}attP2*	-	Transgenic RNAi project (Boston, MA USA)
Dad-LacZ	*Dad-LacZ, r*/ TM3, Sb*	-	H. Ashe, Manchester, UK
E(spl)mβ$^{1.5\text{-LacZ}}$	*E(spl)mβ$^{1.5\text{-LacZ}}$*	-	S.Bray, Oxford, UK
baz^{106}/FM7c ; TM3, Sb/TM6, Hu	*baz^{106}/FM7c ; TM3, Sb/TM6, Hu*	-	University of Manchester communal fly facility
baz^{106}/FM7c ; noc^{Sco}/CyO	*baz^{106}/FM7c ; noc^{Sco}/CyO*	-	University of Manchester communal fly facility
GlcAT-I^{E04384}	*W1118, GlcAT-I^{E04384} / TM6, Hu*	-	Excelexis
GlcAT-I^{F00247}	*W1118, GlcAT-I^{F00247} / FM7c*	-	Excelexis
Df(1)BSC580	*Df(1)BSC580, w^{1118}/Binsinscy*	25414	Bloomington
Df(1)ED6716	*Df(1)ED6716, w[1118] P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6716/FM7h*	24145	Bloomington
Df(1)ED6720	*Df(1)ED6720, w[1118] P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6720/FM7h*	9055	Bloomington
Actin5CGal4	*y^1 w*; P{Act5C-GAL4}17bFO1/TM6B, Tb^1*	3954	Bloomington
hh^{AC}	*ry^{506} hh^{AC}/TM3, Sb^1*	1749	Bloomington
hh^{MRT}	*hh^{Mrt}/TM3, Sb^1*	26166	Bloomington
Egfr^{t1}	*Egfr[t1] bw[1]/CyO*	2079	Bloomington
tkv$^{Sz\text{-}1}$	*In(2L)tkv[Sz-1], al[1] tkv[Sz-1] b[1]/SM1*	860	Bloomington
Stat92e^F	*w*; e^1 Stat92E^F/TM6C, cu^1 Sb^1*	24757	Bloomington
Df(1)BSC352	*Df(1)BSC352, w^{1118}/FM7h/Dp(2;Y)G, P{hs-hid}Y*	24376	Bloomington
P(Tub-PBac\T)2, CyO (Transposase)	*w[1118]; CyO, P{Tub-PBac\T}2/wg[Sp-1]*	8285	Bloomington
ptcGal4	*ptcGal4*	-	M. Baron
GlcATP^{PL00294}	*w*; PBac{GAL4D,EYFP}GlcAT-P^{PL00294} P{FRT(w^{hs})}2A P{neoFRT}82B*	19444	Bloomington
GlcATS^{EY01481}	*y^1 w^{67c23}; P{EPgy2}GlcAT-S^{EY01481}*	20120	Bloomington
hop^{Tuml}	*y^1 v^1 hop^{Tum}/FM7c*	-	S. Brown, Sheffield, UK
GlcATI VALIUM20	*y^1 sc* v^1; P{TRiP.HMS00289}attP2*	-	Transgenic RNAi project, Boston, MA

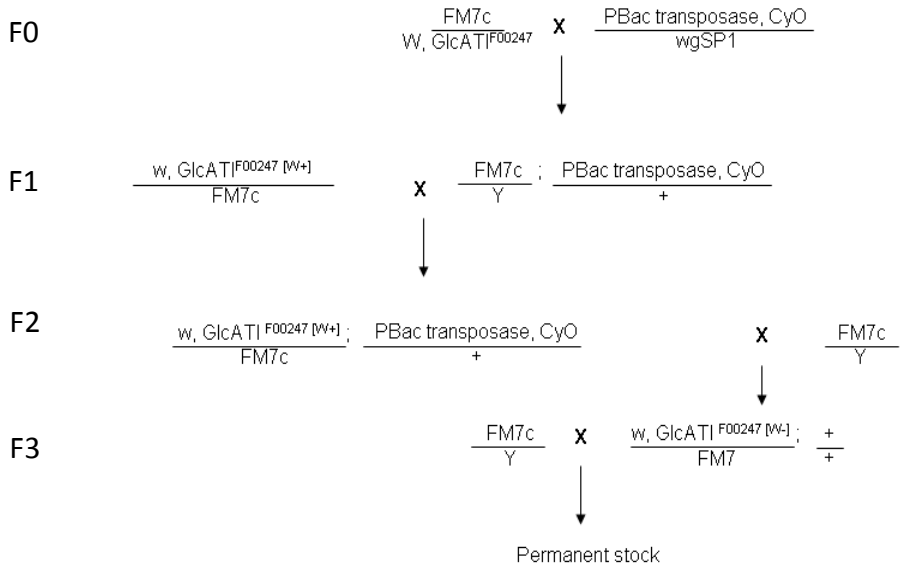
			USA
nmo^{adk1}/TM6, Hu	*nmo^{adk1}/TM6, Hu*	-	M. Baron
l(3)04713	*ry^{506} P{PZ}l(3)04713^{04713}/TM3, ry^{RK} Sb^{1} Ser^{1}*	11638	Bloomington
Df(3R)ED5660	*w^{1118}; Df(3R)ED5660 / TM6C, Sb^{1}*	150331	
dpr^{1}	*P{PZ}dpr^{1}; ry^{506}*	25079	Bloomington
dpr4^{MB03978}	*w^{1118}; Mi{ET1}dpr4^{MB03978}*	24553	Bloomington
dpr8^{KG01318}	*w^{1118} ; Mi{ET1}dpr8^{KG01318}*		Bloomington
dpr8^{MB07155}	*w^{1118}; Mi{ET1}dpr8^{MB07155}*		Bloomington
dpr11^{EY06824}	*y^{1} w^{67c23}; P{EPgy2}dpr11^{EY06824}*	16760	Bloomington
dpr13^{MB08759}	*w^{1118}; Mi{ET1}dpr13^{MB08759}*	26401	Bloomington
elav-Gal4	*elav-Gal4/CyO*	-	University of Manchester communal fly facility
dpr9 VALIUM20	*y^{1} sc* v^{1}; P{TRiP.HMS00288}attP2*	-	Transgenic RNAi Project

Fly husbandry and experimental crosses. Standard fly food consisted of the following; 7.9% (w/v) glucose, 7.2% (w/v) maize, 5% (w/v) yeast, 0.85% (w/v) agar, 0.3% (v/v) proprionic acid and yeast powder (Sigma-Aldritch). All crosses were carried out at 25˚C, unless otherwise stated. Any experiment requiring multiple crosses had an appropriate control which was similarly out-crossed to account for changes in genetic background. All females for dissection were aged with Oregon-R males in un-crowded conditions (~20 flies per vial) and were tipped every 2-3 days to provide new egg laying sites.

Ovary dissection Flies were anaesthetised by incubating on ice for 20 mins. Females were pinned by the thorax to SYGARD plates filled with phosphate buffered saline containing 0.1% Tween 20 (v/v). Ovaries were removed from the abdomen using a pair of forceps (size 55, Fine Science Tools). The ovarioles were separated by using a pair of forceps with 0.1 mm pins taped to the tips. This separation was achieved by holding the ovary steady with one pin through the broad posterior end of the ovary (mature egg containing end which appears opaque under the microscope) and brushing the narrow anterior tip of the germarium gently until ovarioles break free of their smooth muscle

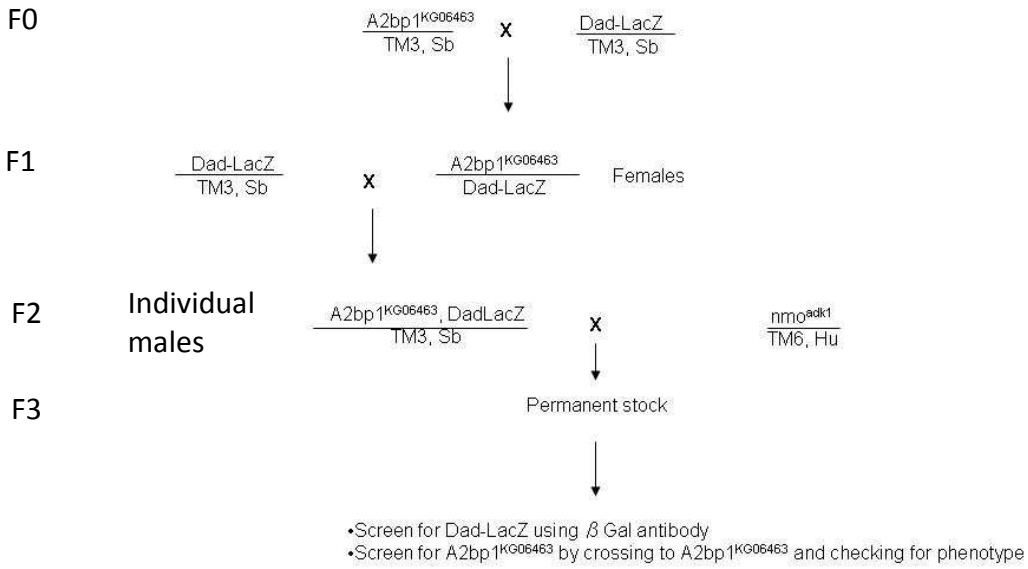
sheath (strings of individual egg chambers can be identified when this is accomplished. If the ovariole is not removed from its sheath, individual egg chambers cannot be identified). These ovarioles are pipetted into an eppendorf and immunohistochemistry is carried out as described.

P- element remobilisation The following crossing scheme was used for the remobilisation of the PiggyBac element in *GlcATI*^{F00247};

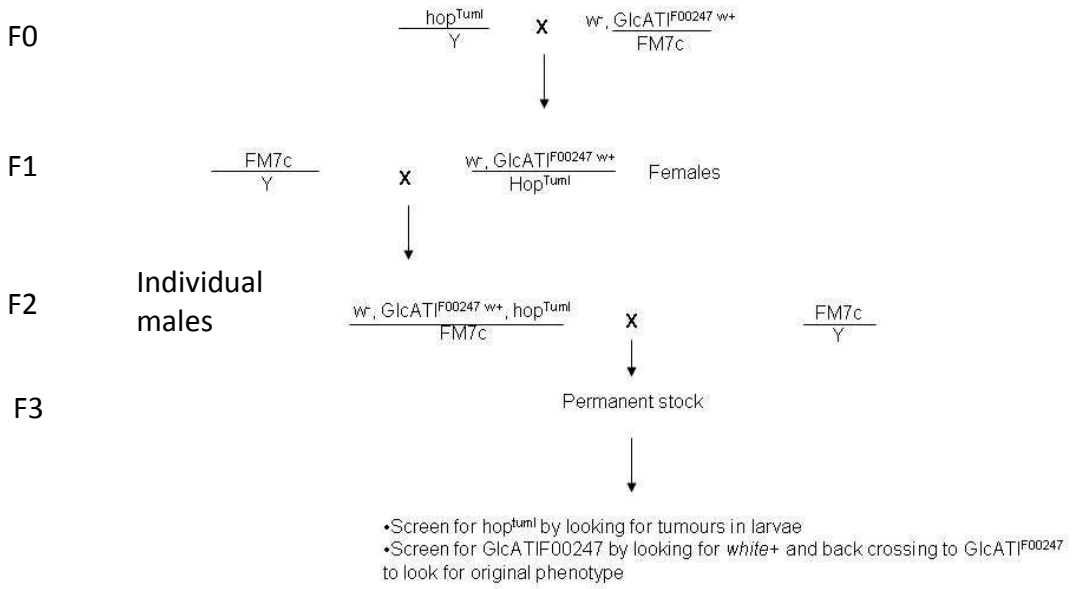


Loss of the *miniwhite* gene (w+) found in the PiggyBac element was used as an indication that the transposable element had been lost in the F3 cross. At this cross, individual [w-] females were back crossed to FM7c/Y to make a stock. In addition to the [w-] stocks, flies which had retained the insert ([w+2]) but had been through the same crosses were analysed as a control for the change in genetic background.

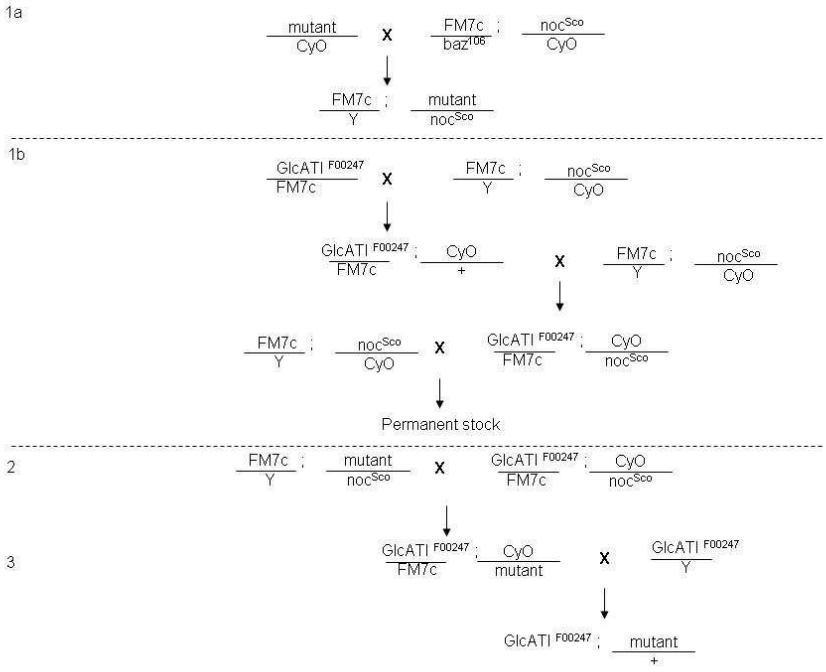
Generation of A2bp1^{KG06463}, Dad-LacZ/ TM6, Hu



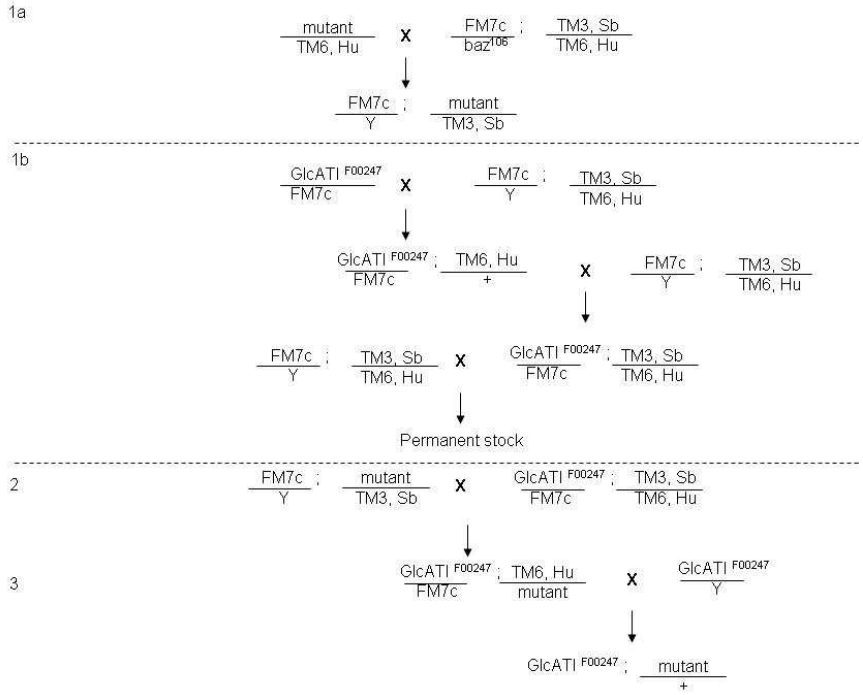
Generation of hop^{tuml}, GlcATI^{F00247}/FM7c for GlcATI^{F00247} genetic interactions.



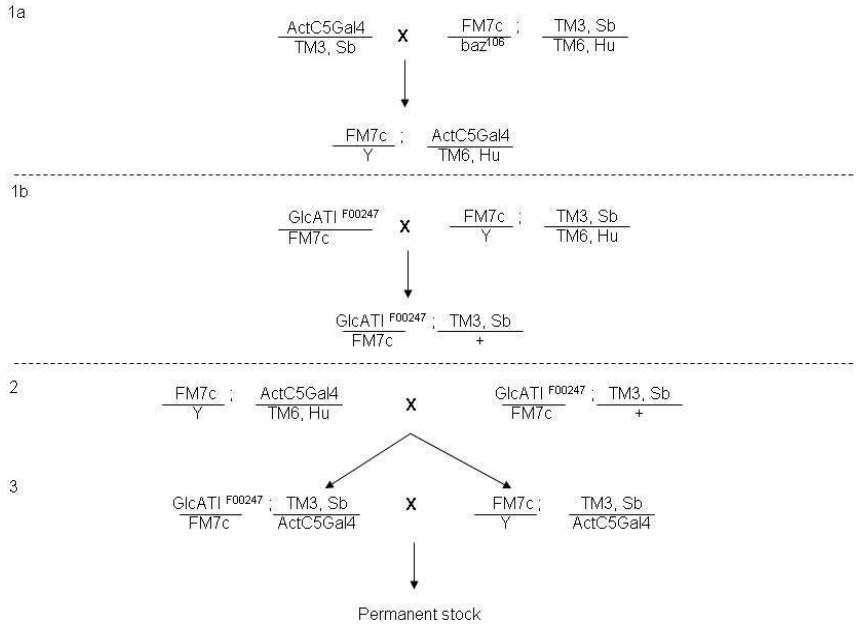
Crosses used in GlcATI^{F00247} genetic experiments The following crossing scheme was used to generate flies which were homozygous for *GlcATI^{F00247}* and heterozygous for second chromosome mutants (these were *tkv$^{Sz\text{-}1}$* and *Egfr^{t1}*). Step 1b was used to generate permanent stock of GlcATI^{F00247}flies *which were balanced* on the 1st and 2nd chromosome.



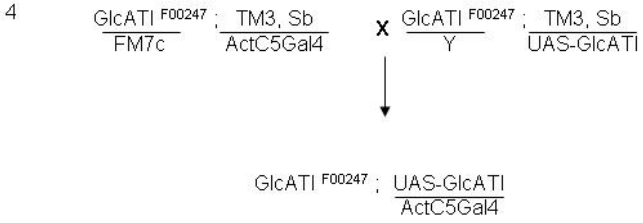
The following crossing scheme was used to generate flies which were homozygous for *GlcATI*^{F00247} and heterozygous for third chromosome mutants (these were *hh*^{AC},*hh*^{MRT} and *Stat92e*^{F}). Step 1b was used to generate permanent stock of *GlcATI*^{F00247}*flies* which were balanced on the 1st and 3rd chromosome.



Crossing scheme for the GlcATI^{F00247} rescue experiment. The following crossing scheme was carried out for both ActC5Gal4 and UAS-GlcATI construct which are both found on the 3rd chromosome;



Flies from the two permanent stocks generated in the above crossing scheme were used in the following cross;



Additionally, flies from the same permanent stocks were crossed to OregonR to generate the following controls; *GlcATI^{F00247}; UAS-GlcATI/+* and *GlcATI^{F00247}; ActinC5Gal4/+*. These were used as a control to rule out that the observed phenotypes were not caused by the genetic background (i.e. that out crossing *GlcATI^{F00247}* does not rescue the *GlcATI^{F00247}* phenotype).

Antibodies; dilutions, concentrations, antibody type. The following table summarises the antibodies used and their corresponding secondary antibody.

Antibodies	Type	Concentration	Source	Secondary
Fasciclin III	Monoclonal, supernatant	1/20	DSHB	Donkey anti-mouse IgG Cy3
αSpectrin	Monoclonal, supernatant	1/20	DSHB	Donkey anti-mouse IgG Alexa488
Coracle	Polyclonal, antisera	1/10 000	R. Fehon	Donkey anti-guinea pig IgG RRX
Bag of marbles	Polyclonal, antisera	1/500	D. McKearin	Donkey anti-rat IgG Cy5
βgal	Monoclonal, supernatant	1/1000	Promega	Donkey anti-mouse IgG Cy3
118-Sex lethal	Monoclonal, supernatant	1/50	DSHB	Donkey anti-mouse IgG Cy3

The following immunofluorescence protocol was carried out for Bag of Marbles. Ovarioles were dissected as described in main text and fixed in a solution consisting of 300 µl 4% Formaldehye and 900 µl Heptane for 30 minutes at room temperature. Samples were subsequently rinsed with phosphate buffered saline (PBS) and incubated for 1 hour at room temperature with 5% normal donkey serum diluted in PBS. Rat anti-bag of marbles was added at 1/500 and incubated overnight at room temperature. This was then washed three times with 0.1% PBS Tween 20 and the donkey anti-rat RRX secondary was added for two hours at room temperature. Samples were then incubated with DAPI containing mountant overnight and then were subsequently mounted and analysed as described in main text.

First egg chamber staging. The first egg chamber adjacent to the germarium was staged using criteria found in published literature (King, 1957). These included;

Stage	Features
2	Oocyte indistinguishable from nurse cells. Adjacent stalks are a cluster of cells
3	Oocyte appears smaller than nurse cells. Posterior stalk is straight, anterior stalk is still clustered
4	Nurse cells take on characteristic "blob" like appearance as they become polyploid. Oocyte is easily identifiable, due to it being much smaller than nurse cells.
5	Nurse cells become large and "speckled." Egg chamber becomes oval shaped.
6	Nurse cells are all same size, but anterior end becomes narrower.

7	Egg chamber elongates and anterior nurse cell nuclei are noticeably smaller than posterior nuclei.
8	Yolk becomes visible at posterior end. Follicle cell layer still covers whole egg chamber
9	Follicle cells begin to migrate from the anterior end over the oocyte. The yolk fills 1/3 of the egg chamber.
10a	Yolk fills 1/2 of the egg chamber. Follicle cell layer over oocyte becomes columnar epithelial like. Follicle cell migration between oocyte and nurse cells is not cisible.
10b	As for 10a but now follicle cells begin to migrate between nurse cell and oocyte.
11	Oocyte is not 3/4 of the egg chamber. Follicle cell layer thins over oocyte.
12	Oocyte fills entire egg chamber. Nurse cells are shrunken at the pointed anterior tip.
13-14	Chorion and dorsal appendages form. Follicle cells and nurse cells undergo apoptosis.

Phenol:Chloroform Genomic DNA extraction. After incubation with RNaseA, samples were spun down and the supernatant was transferred to a fresh eppendorf. A 24:25:1 mix of Phenol; Chloroform; Isoamylalcohol (Sigma-Aldritch) was added to the supernatant. This was mixed well and spun down. This process was repeated and then the supernatant was transferred to a new eppendorf where it was subsequently mixed with chloroform alone. The supernatant was again transferred to a new eppendorf which was then treated with 2 supernatant volumes of 100% ethanol and 1/10th supernatant volume of sodium acetate. This was finally washed with ethanol and allowed to air dry at room temperature.

RNA extraction. The QIagen RNA extraction kit (QIAgen, West Sussex, UK) was used to extract RNA. Tissue (ovaries, heads or whole flies, as appropriate) was mashed in an eppendorf containing buffer RLT with 6% β-mercaptoethanol using a sterile, RNase free pestle. The mixture was then transferred to a sterile QIagen Shredder column and spun down. 70% ethanol diluted in diethylpyrocarbonate (DEPC) water was applied to the column and this was spun down again. Buffer RW1 was added to the flow-through which was then transferred to a RNeasy column which was spun down again. 10 µl of RNase free DNase (QIagen) was added to the column and incubated for 15 minutes at

room temperature. This was further washed with buffer RW1 and spun again. Buffer RPE was applied to the column and this was spun twice, with the flow through being discarded after each spin. The collection tube was replaced with a new eppendorf and then distilled, sterile water was added to the column. After spinning, the eppendorf was labelled appropriately and stored at -20°C. RNA quantities were estimated using agarose gel electrophoresis.

PCR and RT PCR programs and reaction mix. The following table summarises the *Tm* values of all the primers used and the techniques they were used for.

Name	**Sequence**	**Technique**	***Tm***
15F1	ACA ACT TGG CGC TCT TCT GT	PCR	51
15R1	CGA ATT CAA CAG GCC AAT CT	PCR	51
GlcATIF	GAC AGC TCG CCG ATT TGT TTG	PCR	56
GlcATIRC	GCC TGC GGA TTC CTG ATG AAG	PCR	56
PBac3F	GAA AAG GTC CAA AGT CGC AA	PCR	56
5r2	TCC AAG CGG CGA CTG AGA TG	PCR	56
38F1	CAG CAC GCG AAG ATG AAT AA	PCR	51
38R1	TTT TGG CCC ACT GTT CTA GG	PCR	51
RP49F	AGA TGA CCA TCC GCC CAG CAT	PCR/RTPCR	55
RP49RC	CGA CCG TTG GGG TTG GTG AG	PCR/RTPCR	55
F2	GTT ATT TAG GCA CAC AGC TCG C	RTPCR	55
R2	GCC TGC GGA TTC CTG ATG AAG	RTPCR	55
LP20	CGC CGT TGG GGT TGG TGA GA	RTPCR	55
LP21	GCG GCT CCG GTG AGT TTT GTA	RTPCR	55

Standard reaction mix for PCR; 1 Unit Taq, 2.5 µl each primers (25pmol), 1 µl dNTP mix (10mM of each NTP), 5 µl of 10x reaction buffer, 0.5 µg of genomic DNA. Made up to 50 µl with distilled water.

Standard PCR program; 95°C for 2 minutes, 95 °C for 1 minutes, *Tm* value for 30 seconds, 72 °C for 1 minute per Kb of sequence. Repeat from second step 34 times. Final extension at 72 °C for 5 minutes.

Standard RT PCR mix; 1 µl of RT platinum Taq mix (Invitrogen, Life Technologies, Manchester, UK), 25 µl of 2x reaction mix, 1 µg of RNA, 1 µl of each primer (10 uM). Made up to 50 µl with distilled water.

Standard RT PCR program; 50°C for 30 minutes, 94 °C for 2 minutes, 94 °C for 15 seconds, *Tm* value for 30 seconds, 72 °C for 1 minute per Kb of sequence. Repeat from second step 16 or 40 times. Final extension at 72 °C for 7 minutes. 17 cycles was used for the amplification of $GlcATI^{F00247}$ mRNA; this was to ensure that the PCR was not saturated so any differences in RNA levels could be observed. 40 cycles was used for the $GlcATI^{F00247}$ RT PCR demonstrating that the insert was still present in the mRNA message. This was to saturate the PCR so smaller quantities of the final sample could be run in the gel and thus, better resolution could be obtained.

pUASp-GH05057 GlcATI rescue construct generation.

Electrophoresis; PCR amplified GH05057 GlcATI fragment was run in a 1% agarose gel in 1x Tris acetate EDTA buffer (40 mM Tris acetate and 1 mM EDTA). Bands were illuminated for extraction using a transilluminator (UVItec, 365nm) and the desired band was extracted using a scalpel. DNA was then purified using the QIagen quick gel extraction kit (QIagen). Three gel volumes of buffer QG was added to the gel which was then mixed. Following this, one gel volume of isopropanol was added. The sample was then spun down and in a QIaquick spin column which was placed in a 1.5 ml eppendorf. The flow through was discarded and the column washed with buffer QG. This was centrifuged again and buffer PE was added. This was centrifuged again and the collection tube replaced. This was spun and again, the collection tube was replaced. DNA was eluted from the column using distilled water.

Digestions; Restriction digests of both the pUASP vector and GH05057 GlcATI PCR product were carried out at 37°C for 2 hours. Each reaction consisted of the following; 1

µg of DNA, 10 units of appropriate enzyme, 2 µl of appropriate restriction buffer, 0.5 µl of bovine serum albumin as required, and 20 µl of distilled water. After digestion of vectors, 1 unit of calf intestinal phosphatase (New England Biolabs) was added to the vector to prevent re-ligation. These were gel purified as described above to remove overhang fragments. Uncut vector treated in the same manner (i.e. reaction mixture with no restriction enzyme) was used as a control for the digestion reactions.

***Ligations*;** Gel purified GlcATI DNA fragments and vector were ligated using the Rapid Ligation Kit (Roche Diagnostics). The reaction mix consisted of the following; 5 µl of 2x reaction buffer and 1µl of T4 DNA ligase (Rapid Ligation Kit, Roche Diagnostics). This was performed at room temperature for 10 minutes. Vectors and fragments were also treated separately as a control. The ligation was confirmed using a restriction digest enzyme which would cut in both the insert and the vector, thus giving a unique set of digestion bands during electrophoresis.

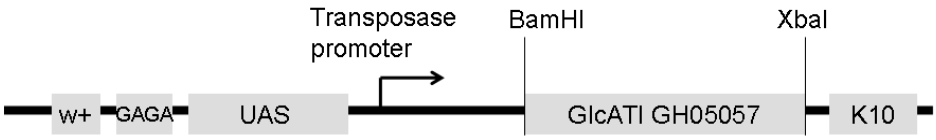
***E. coli transformation*;** *Eschericha coli* XL10 gold ultracompetent cells (Stratagene) were used for amplification of the ligated vector-fragment (pUASP-GH05057). Cells were allowed to thaw on ice. 100µl of cells were incubated with 0.5 µl of β-mercaptoethanol for 10 minutes on ice. The cells were then inoculated with 2 µl of ligation mixture and left to incubate on ice for 30 minutes. A 30 second heat shock was carried out at 42°C. The cells were then allowed to settle for 2 minutes on ice before 1 ml of Luria Bertani (LB) broth was added. This was then agitated for 1 hour at 37°C before being spread onto 50 µg/ml ampicillin agar plates which were then incubated at 37°C overnight.

Plasmid purification*;* Plasmids were isolated from agar plate colonies and added to 5 ml of LB broth containing ampicillin at 50 µg/ml. Samples were transferred to a 15 ml Falcon tube and centrifuged at 300rpm for 10 minutes. Qiagen miniprep plasmid kit was used to extract plasmid DNA as follows. The supernatant was discarded and the pellet

resuspended in 250 µl of resuspension buffer was added to the cells which was transferred to a 1.5 ml eppendorf. 250 µl of lysis buffer was then added to the samples. This was then centrifuged for 10 minutes at 13000 rpm. 300 µl of buffer N3 was added to precipitate proteins. The sample was then spun for 10 minutes at 13000 rpm (Eppendorf 5415D) and the supernatant was transferred to a column which was then treated with 0.5 ml of buffer PE. This was centrifuged again and the supernatant discarded. A new collection tube was added and the DNA was eluted using distilled water. Midi preps (PureLink HiPure Plasmid DNA purification kit, Invitrogen) were carried out in a similar manner except volumes were larger and, rather than centrifugation, samples were drained through a column on the bench.

Sequencing; The vector was sequenced using the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosciences) to ensure there were no mutations which had occurred during PCR or subsequent plasmid amplification in E. coli. The reaction mix contained the following; 2 µl of ready Reaction mix, 1x Big Dye Terminator v3.1 buffer, 1µl of primer (3.2 pmol), 300 ng of DNA template, made up to a volume of 20 µl with water. The PCR cycle use was; 96°C for 2 minutes, 96°C for 30 seconds, 50°C for 15 seconds and 60°C extension for 4 minutes. The last three steps were repeated 34 times. Resulting DNA was purified using 100% ethanol and was air dried at room temperature. The samples were sequenced at the University of Manchester Sequencing Facility and SeqEdit was used to analyse sequence data. The sample sequence was aligned with the GH05057 vector DNA to check for mutations using ClustalW.

Map of pUASP GH05057. GlcATI GH05057 was inserted downstream of the Transposase promoter which is found in pUASP. W+ was used as the marker for injection into *Drosophila embryos.*



In situ probe generation. *E. coli* JM109 cells were used for the amplification of pOT2 GH05057 using the protocol outlined above, but substituting the ampicillin plates for 34 µg/ml chloramphenicol plates. Plasmid DNA was extracted using QIagen midiprep plasmid kit. 3 µg of plasmid was digested with either XhoI (for T7) or EcoRI (for SP6).

***T7/SP6 reaction mixture*;** pOT2 contains both a T7 and SP6 promoter. Labelling and transcription of GlcATI GH05057 probe was carried out simultaneously as follows. For T7; 2 µl of Dig labelling mix (Roche), 2 µl of transcription buffer, 1.5 µl of T7 polymerase, 1 µl of RNase, 1 µg of DNA, made up to 20 µl with distilled water. For SP6; 2 µl transcription buffer, 2 µl Dig labelled mix (Roche), 1.5 µl of SP6 (Roche), 1 µg of DNA, made up to 20 µl with distilled water. These were incubated at 37°C for 2 hours. 2 µl of the reaction mix was removed after this time for electrophoresis. Probes were precipitated as described in main text.

pOT2 GH05057 vector. Probe was transcribed with either EcoRI digested, Sp6 polymerase or XhoI digested, T7 polymerase to produce GlcATI GH05057 in situ probe.