

Exploring how *Drosophila* midgut tumours respond to sex and reproduction

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Statement of Originality

I, Emily Ophelia Strachan, acknowledge that the work contained within this thesis is entirely my own. Other contributions are clearly indicated by acknowledgment or reference. I carried out this work jointly between the Metabolism and Cell Growth Group, led by Dr Susumu Hirabayashi, and the Gut Signalling and Metabolism Group, led by Prof Irene Miguel-Aliaga. Both groups are based at the Medical Research Council Laboratory of Medical Sciences (MRC LMS), and my work was funded by the MRC – UK Research and Innovation (UKRI). The main text has 1.5 line spacing and it does not exceed the 100,000-word limit.

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Abstract

Cancer risk, type and progression vary with sex, due to a range of genetic, hormonal and behavioural factors. Post pregnancy, this is modulated further due to changes in hormones, growth factors and the immune system. However, these factors are often overlooked in experimental disease models. Here I use the fruit fly *Drosophila melanogaster* as a model system to investigate how sex and reproduction affect tumour progression.

I compare several different genetic tumours generated in the progenitor cells of the adult fly midgut and show how sex and reproductive status affects tumour proliferation. I show tumours induced by simultaneous overexpression of oncogenic *Ras* (*Ras*^{G12V}) and downregulation of tumour suppressor *Apc* (*Apc*^{RNAi}) proliferate more in mated females. Unexpectedly, I found that the Hippo pathway midgut tumours, induced by overexpression of a constitutively active oncogene *yorkie* (*yki*^{3SA}) or downregulation of tumour suppressor *warts* (*wts*^{RNAi}), behave differently in response to sex and reproduction.

Unlike *Ras*^{G12V} *Apc*^{RNAi} tumours, Hippo pathway tumours exhibited unusually high proliferation in virgin females and lose many of their transcriptional post-mating differences. Furthermore, I observe that male-biased sugar metabolism is suppressed in highly proliferative *yki*^{3SA} male tumours, but not in non-proliferative *wts*^{RNAi} male tumours. I investigate the expression of several sexually dimorphic sugar enzymes and show their expression changes in both gut region and cell type in tumours. I also show that these male-biased sugar genes are downregulated in healthy female flies after mating.

Thus, I demonstrate that the tumour response to sex and reproductive status is dependent on genetic model, with different Hippo pathway tumours losing dependency on sex and reproductive cues. This reveals a new potential function for the Hippo pathway in the adult midgut in modulating post-mating remodelling. These findings highlight the importance of considering tumour genotype, sex and reproductive status when studying cancer.

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List of Abbreviations

20HE	20-Hydroxy-Ecdysone
Acon	Aconitase
AcsI	Acyl-CoA synthetase long-chain
Acsx	Acyl-CoA synthetase
Ald	Aldolase
AMOT	Angiomotin
Amy-d	Amylase-distal
Amy-p	Amylase-proximal
Amyrel	Amylase-related
Aos	Argos
Apc	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
App	Approximated
ATPCL	ATP citrate synthase
Babo	Baboon
BCAA	Branched chain amino acids
BDRC	Bloomington <i>Drosophila</i> Stock Centre
Bgm	Bubblegum
BMP	Bone morphogenetic protein
BP	Biological process
Br	Broad
Brat	Brain tumour
Burs	Bursicon
COSMIC	Catalogue Of Somatic Mutations In Cancer
Cp36	Chorion protein 36
Crb	Crumbs
CySC	Cyst stem cell
Dally	Division abnormally delayed
Daw	Dawdle
Dco	Discs overgrown
DE	Differentially expressed
Dgl	Discs large
DGRC	<i>Drosophila</i> Genomics Resource Centre
Diap1	Death-associated inhibitor of apoptosis 1
Dilps	<i>Drosophila</i> insulin-like peptides
DI	Delta
Dome	Domeless
Dpp	Decapentaplegic
Ds	Dachsous
DSS	Dextran sodium sulfate
Dsx	Doublesex
EB	Enteroblast
EC	Enterocyte
Ecc	<i>Erwinia carotovora carotovora</i>
Ecl	Ecdysone importer
ECM	Extracellular matrix
EcR	Ecdysone receptor

EE	Enteroendocrine
EGFR	Epidermal growth factor receptor
Eig	Ecdysone-induced gene
Eip	Ecdysone-induced protein
Eno	Enolase
Esg	Escargot
Esg^{SHOF}	Escargot Shutoff
Ex	Expanded
Fas3	Fasciclin 3
FC	Fold-change
Fj	Four-jointed
Fpb	Fructose biphosphatase
Fru	Fruitless
Fs(1)M3	Female sterile (1) M3
Ft	Fat
G6PD	Glucose-6-phosphate dehydrogenase
Gapdh	Glyceraldehyde 3 phosphate dehydrogenase
Gce	Germ cell-expressed bhlh-PAS
GFP	Green fluorescent protein
Glut1	Glucose transporter 1
GO	Gene ontology
GSC	Germline stem cell
Gst	Glutathione-S-Transferase
Hex-A	Hexokinase
Hop	Hopscotch
Hpo	Hippo
HRP	Horse radish peroxidase
Hth	Homothorax
Idgf1	Imaginal disc growth factor 1
IGF	Insulin growth factor
ImpL2	Ecdysone-inducible gene L2
Indy	I'm not dead yet
ISC	Intestinal stem cell
JAK-STAT	Janus kinase-signal transducer and activator of transcription
JH	Juvenile hormone
Jhamt	Juvenile hormone acid O-methyltransferase
Jub	Ajuba
Kbr	Kibra
KDM	Lysine demethylase
Kdn	Knockdown
Kr-h1	Kruppel homologue 1
L(3)mbt	Lethal(3)malignant brain tumour
LacZ	B-galactosidase
Lan	Laminin
Lats	Large tumour suppressor kinase
Ldh	Lactate dehydrogenase
Lft	Lowfat
Lgl	Lethal (2) giant larvae
Lola	Longitudinals lacking

Mad	Mothers against dpp
Mal	Maltase
MAP4K	Mitogen-activated protein kinase kinase kinase kinase 4
MAPK	Mitogen-activated protein kinase
MARCM	Mosaic analysis with a repressible cell marker
Mats	Mob as tumour suppressor
Mct1	Monocarboxylate transporter 1
Med	Medea
Mer	Merlin
Met	Methoprene-tolerant
MF	Molecular function
MMP	Matrix metalloproteinases
Mop	Myopic
Msl-2	Male specific lethal 2
Msn	Misshapen
Mst	Macrophage stimulating
NK	Natural killer
NPF	Neuropeptide F
Obst-A	Obstructor-A
Out	Outsiders
P.e.	<i>Pseudomonas entomophila</i>
Pap	Papilin
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCB	Pyruvate carboxylase
PCD	Programmed cell death
Pdp	Pyruvate dehydrogenase phosphatase
PDZ	Primary decidual zone
PG	Prothoracic gland
Pgi	Phosphoglucose isomerase
Pgk	Phosphoglycerate kinase
Pglym	Phosphoglyceromutase
pH3	Phospho-Histone H3
Ph⁵⁰⁵	Mutant allele for ph proximal and ph distal
Phf7	PHD finger protein 7
Pnt	Pointed
PPP	Pentose phosphate pathway
Pros	Prospero
PTTH	Prothoracicotropic hormone
Pvf1	PDGF- and VEGF-related factor 1
PyK	Pyruvate kinase
PCA	Pyruvate carboxylase
RASSF	Ras association family member
Rdo	Reduced ocelli
ReDDM	Repressible Dual Differential stability cell Marker
RFP	Red fluorescent protein
RNA-Seq	RNA-sequencing
RNAi	RNA interference
ROS	Reactive oxygen species

Sav	Salvador
Scrib	Scribble
Sd	Scalloped
SDS	Sodium dodecyl sulfate
Smad	Suppressor of Mothers against Dpp
Spn88Eb	Serpin 88eb
SRY	Sex-determining region Y gene
PP2A	Protein phosphatase 2A
STRIPAK	Striatin-interacting phosphatase and kinase
Su(H)	Suppressor of hairless
Sut	Sugar transporter 1
Sxl	Sex lethal
Tai	Taiman
Taz	Transcriptional coactivator with PDZ-binding motif
TCGA	The Cancer Genome Atlas
TEAD	Transcriptional enhanced associate domain
TFG-β	Transforming growth factor-β
Tgi	Tondu-domain-containing Growth Inhibitor
Tj	Traffic-jam
TLR7	Toll-like receptor 7
Tobi	Target of brain insulin
Tra	Transformer
Treh	Trehalase
Tret	Trehalose transporter
ts	Temperature sensitive
Tsh	Teashirt
Tub	Tubulin
UAS	Upstream activating sequence
Upd	Unpaired
usp	Ultraspiracle
VDRC	Vienna <i>Drosophila</i> Research Centre
VM	Visceral muscle
Vm26Aa	Vitelline membrane 26Aa
Vn	Vein
Wg	Wingless
Wts	Warts
Yap	Yes-associated protein 1
Yki	Yorkie
Yp	Yolk protein
Zpg	Zero population growth
Zyx	Zyxin

Chapter 1: Introduction

1.1 Cancer, sex and reproductive status

1.1.1 Cancer risk, type and progression varies with sex

Cancer risk, type and progression varies with biological sex. Males have both a higher incidence of cancer (Strand and Micchelli, 2011) and higher mortality (Bray *et al.*, 2018). This male bias is very consistent across different datasets regardless of geographical region or wealth disparity (Bray *et al.*, 2018; Ferlay *et al.*, 2019). Most cancer types have a higher relative risk in males, including lung, liver, colorectal, skin, and haematological malignancies. Only thyroid cancer is consistently more likely in females across all geographical regions and economic groups, although anus and gallbladder may be slightly more likely in females (Bray *et al.*, 2018; Ferlay *et al.*, 2019). Data from UK cancer incidence in 2016 – 2018 also confirms thyroid cancer is the only cancer with a clear female bias, with males having higher risk of lung, bowel, kidney, head and neck and leukaemia amongst others (**Figure 1.1**). By far the most common two cancers in the UK are both sex specific: prostate, exclusively found in males, and breast cancer, found almost exclusively in females, with only 0.68% of cases found in males (**Figure 1.1**; Cancer Research UK, 2020). Generally, advanced age increases further male incidence (Edgren *et al.*, 2012).

Many factors have been suggested to contribute towards sex differences in cancer incidence and mortality. Lifestyle conveys some risk, with males generally having higher smoking and alcohol consumption rates and increased risk of exposure to environmental carcinogens (Edgren *et al.*, 2012). However, differences at the genetic, cellular and organismal level play a major role.

Sex hormones have many different effects on the body throughout the lifetime of a person. These hormones include circulating oestrogen, progesterone and testosterone. Generally, androgens suppress the immune system, whereas oestrogens enhance it, and progesterone is anti-inflammatory (Beagley and Gockel, 2003; Taneja, 2018). Their effect varies by tumour origin: oestrogen suppresses liver and colon tumours, both male-biased cancers. However, oestrogen enhances thyroid cancer, notably a female-biased cancer. Breast cancer is highly responsive to both oestrogens and androgens, although in females some androgens are also converted to oestrogens by aromatase (Yager and Davidson, 2006). Obesity also increases

expression of aromatase, increasing levels of oestrogens (Endogenous Hormones Breast Cancer Collaborative Group, 2003). This further contributes to the higher oestrogen levels that are associated with risk of developing breast cancer, especially post menopause (Onland-Moret *et al.*, 2003).

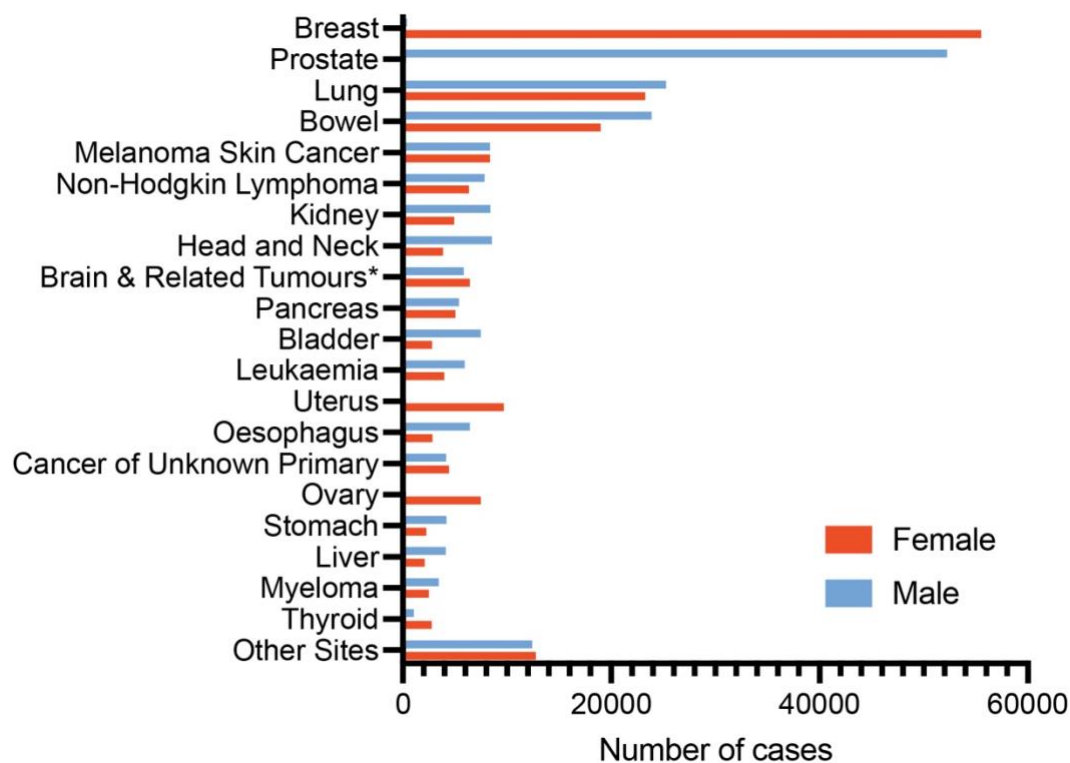


Figure 1.1. The 20 most common cancer in the UK shows males have higher incidence of most cancers.

Data from Cancer Research UK 2016 – 2018.

*Brain & Related Tumours includes brain, other CNS (central nervous system) and intracranial tumours. This category includes tumours that are malignant and non-malignant but only the malignant tumours are included in 'all cancers combined' total. Therefore the 'all cancers combined' total is not equal to the sum of all data in this chart.

Adapted from Cancer Research UK 'Cancer incidence for common cancers', published 2020.

Cancer cells undergo huge metabolic reprogramming, but their initial sex-biased metabolic differences are rarely taken into account. Even before gonad formation, the differential effects of sex chromosome dosage can be seen in embryos. Glucose and pyruvate uptake are increased in early male embryos (Ray *et al.*, 1995). Male bovine embryos have increased glucose consumption and females have increased expression of pentose phosphate pathway (PPP) genes. The key PPP gene glucose-6-phosphate dehydrogenase (G6PD) is on the X chromosome, and has been found to

have higher expression in both mouse and bovine female embryos (Williams, 1986; Kimura, Iwata and Thompson, 2008).

In adulthood, males have increased blood serum glycolysis, gluconeogenesis and pyruvate metabolism, as well as higher levels of many carbohydrate-related sub-pathways. Males also have increased serum amino acid levels, especially branched chain amino acids (BCAA; Krumsiek *et al.*, 2015). Female serum has increased branched and medium chain fatty acids, whereas males have increased serum sterols and steroids, degradation products from androgen hormones (Krumsiek *et al.*, 2015). These factors culminate in two different biases for energy post exercise and fasting: males favour carbohydrate utilisation whereas females favour fatty acids (Hedrington and Davis, 2015). However, each tissue has its own unique metabolic profile, which varies both with sex (Mauvais-Jarvis, 2015) and age (Palmer and Jensen, 2022).

The metabolism of reactive oxygen species (ROS) is also highly influenced by sex. ROS are generated in the mitochondria as a result of oxidative phosphorylation. In two murine models males produce more ROS than females in their brain mitochondria (Borrás *et al.*, 2003) despite females having higher mitochondrial respiratory activity, which may be due to oestrogen promoting antioxidant production (Borrás *et al.*, 2005; Khalifa *et al.*, 2017). Human males have higher plasma oxidative stress markers, and this increases with age (Ide *et al.*, 2002). In cancer, ROS can act as a signalling molecule to promote proliferation, angiogenesis and invasion. However, at higher levels ROS are very detrimental to the cell, resulting in senescence, apoptosis and necrosis (Reczek and Chandel, 2017).

The X chromosome plays a prominent role in tumour suppression. Several tumour suppressors including lysine demethylases *KDM6A* and *KDM5C* are encoded on the X chromosome, and frequently have loss of function mutations in male tumours. *KDM6A* and *KDM5C* both often escape X-chromosome inactivation, leading to increased expression in females (Dunford *et al.*, 2017). These genes contribute to epigenic methylation changes. Sex specific methylation patterns are found in mice and humans across many tissues, especially liver, muscle, brain and blood (Reizel *et al.*, 2015; McCormick *et al.*, 2017; Davegårdh *et al.*, 2019; Grimm *et al.*, 2019). Male cells also often lose their Y chromosome both with age and tumourigenesis, and in cancer

this is associated with poor prognosis (Huh, Moon and Chung, 2007; Abdel-Hafiz *et al.*, 2023). The differential impact of the sex chromosomes and the impact of their mutation or loss should be considered when looking at how cancer changes with sex.

As well as chromosomal intrinsic factors, systemic immunity contributes to cancer formation and outcome, and varies with sex. Females have higher levels of circulating B cells and CD4⁺ T cells, whereas males have higher levels of CD8⁺ T cells (Lee *et al.*, 1996; Lisse *et al.*, 1997; Uppal, Verma and Dhot, 2003). Males have more natural killer (NK) cells, whereas females have more macrophages and neutrophils (Lee *et al.*, 1996). Females have greater type I IFN responses to Toll-like receptor 7 (TLR7) ligands, pattern recognition receptors that boost their ability to detect tumours. Many of the genes needed in this response are X-chromosomal (Berghöfer *et al.*, 2006; Griesbeck *et al.*, 2015).

As males and females have different baseline metabolisms, metabolic reprogramming that occurs during cancer formation starts from a different baseline. This may involve different mutations and may leave the cancer vulnerable to different treatments. Brain cancer incidence and mortality is increased in males (Edgren *et al.*, 2012). In male low-grade gliomas, high expression of glycolytic genes is correlated with decreased survival (Ippolito *et al.*, 2017). In glioblastoma tumour samples male specimens have higher levels of amino acid metabolites. Males with glioblastoma additionally have increased uptake of glutamine, and a mouse model showed that male transformed astrocytes are more dependent on glutamine and glutathione (Sponagel *et al.*, 2022).

Colorectal cancer is more likely in males, and males also have higher mortality. This trend is clearest in older age groups, as there are fewer sex differences before the age of 50 (White *et al.*, 2018). Much of this sex difference is attributed to lifestyle differences, as males consume more red meat (Page *et al.*, 2016) and alcohol (Schütze *et al.*, 2011) and smoke more tobacco (Chang *et al.*, 2014), all risk factors for colon cancer. As females are more likely to be older when diagnosed, they also have an increased risk of co-morbidities (Chacko, Macaron and Burke, 2015).

Therefore, males and females have many sex differences including energy metabolism, ROS metabolism and immunity. Both sex hormone levels and X

chromosomal genes explain this, although there may also be some cell-intrinsic differences. Males have both greater incidence and mortality in almost all cancers. Differential metabolism favouring glutamine in male glioblastoma has been showed to result in poor male prognosis. However, the male-bias towards cancer still requires more study.

1.1.2 Reproductive status further modulates cancer risk, type and progression

Pregnancy involves many changes in metabolism, immunity and organ remodelling, many of which are maintained throughout breastfeeding and must then be reversed at the end of weaning. Parity refers to the number of times a person has carried a pregnancy to at least 24 weeks, with nulliparous referring to someone who has never been pregnant.

The endocrine system plays a major part, with hormonal changes necessary to drive many of the changes that occur during pregnancy. Hormone increases include the sex steroid hormones oestrogen and progesterone, but also adrenal cortisol, pituitary prolactin and its pregnancy specific homologue placental lactogen (Grattan and Ladyman, 2020). These hormone-induced changes mainly occur in the cardiovascular system and mammary gland, as well as the brain, adipose tissue and pancreas. Early in gestation there is an increase in cardiovascular and circulatory function to support the increased oxygen consumption of the maternal organs and foetus (Sanghavi and Rutherford, 2014).

Although it is known mammals increase in weight during pregnancy, the size changes of individual organs have not been fully profiled, although animal models suggest many organs are affected. Rodent models show the liver increases in size during pregnancy, then during weaning undergoes involution via cell death and stromal remodelling (Kennedy, Pearce and Parrott, 1958; Hollister *et al.*, 1987; Dai *et al.*, 2011; Goddard *et al.*, 2017; Hyatt *et al.*, 2019). The pancreas increases in size and decreases after weaning (Jolicoeur, Assenlin and Morisset, 1980). Finally, the small intestine and colon also increases in length in rodents (Fell, Smith and Campbell, 1963; Hammond, 1997; Speakman, 2007). These changes in organ size may impact

tumour risk; a bigger organ that proliferates to grow may also have an increased risk of uncontrolled growth.

Pregnancy modulates metabolism. Lipids like cholesterol, fatty acids and lipoproteins are increased (Waage *et al.*, 2022). Glucose metabolism must be modulated, because of the increased energy demands (Most *et al.*, 2019). Late in pregnancy there are high levels of glucose and insulin resistance, and the pancreas compensates by increasing B cell mass and insulin release (Kampmann *et al.*, 2019). Postpartum, energy demands are reduced, and metabolism changes must be reversed. Weight gain, if not lost postpartum, may be a risk factor for cancer (Kinnunen *et al.*, 2004).

Pregnancy modulates immunity but does not necessarily suppress it. The endometrium around the foetus accumulates high levels of NK cells, macrophages and regulatory T cells that are necessary for full term pregnancy (Mor and Cardenas, 2010). Upon pregnancy and lactation, the mammary gland must not only proliferate but also differentiate to form a functional secretory tissue. This tissue then faces massive remodelling after pregnancy, rodent models have shown that there is apoptosis of 80 - 90% of the new mammary epithelium post-lactation (Strange *et al.*, 1992; Lund *et al.*, 1996; Watson and Kreuzaler, 2011). Remodelling to return to a non-secretory state involves extracellular matrix (ECM) and collagen remodelling and macrophage recruitment (O'Brien *et al.*, 2010).

As a result of the wide-ranging changes induced by pregnancy and lactation, risk of cancer is modulated in an interesting way. Breast cancer involves many of the processes needed for mammary gland remodelling during pregnancy and lactation, including increase in cell proliferation, reduced apoptosis and ECM remodelling (Slepicka, Cyrill and dos Santos, 2019). However, parity is actually associated with an overall reduced risk of breast cancer, with highest breast cancer risk in nulliparous females across a range of cohorts (MacMahon *et al.*, 1970; Albrektsen *et al.*, 2005; Palmer *et al.*, 2014). This benefit is clearest in those who undergo their first pregnancy before the age of 25, and subsequent births may further reduce the risk. Interestingly, in any pregnancy after the age of 25 there is a transient increased risk in breast cancer (Albrektsen *et al.*, 2005). Pregnancy greatly increases sex hormone levels, which may contribute to tumour growth, but two studies have highlighted increased levels of

oestrogen receptor negative (Phillips *et al.*, 2004) and progesterone receptor negative breast cancers (Colditz *et al.*, 2004) after recent births, suggesting other placental secreted factors could play a role. These postpartum breast cancers also have poorer prognosis (Olson *et al.*, 1998; Phillips *et al.*, 2004). Postpartum there is also a three-fold increase in risk of breast cancer metastasis (Callihan *et al.*, 2013), specifically to the liver. This is because when the liver undergoes involution after weaning, it creates a pro-metastatic environment due to its stromal remodelling and immune cell infiltration (Goddard *et al.*, 2017; Bartlett *et al.*, 2021).

Parity also decreases risk of both ovarian (Tsilidis *et al.*, 2011; Pearce *et al.*, 2013; Wu *et al.*, 2017) and endometrial cancer (Kelsey *et al.*, 1982; Dossus *et al.*, 2010; Karageorgi *et al.*, 2010). The ‘incessant ovulation’ theory hypothesises that this reduces cancer risk as pregnancy temporarily prevents ovulation, which reduces epithelial trauma, reduces gonadotropin levels and increases progesterone levels (Fathalla, 1971; Cramer and Welch, 1983). Alternatively the ‘foetal immunisation’ theory states that exposure to foetal and placental cells primes the immune system for similar proteins found in ovarian and endometrial tumours, resulting in tumour suppression (Janerich, 1980, 1994; Shields *et al.*, 1997; Katsanis *et al.*, 1998; Main *et al.*, 2022).

Whilst there is some discussion of the impact of parity on the risk of lung, thyroid and colorectal cancer, there are no conclusive trends (Troisi *et al.*, 2018). One study did find a protective effect of parity against developing colorectal cancer (Wernli *et al.*, 2009), and hypothesised that oestrogen released during pregnancy may inhibit colon growth, either directly or via a reduction in insulin-like growth factors (Newcomb, Pocobelli and Chia, 2008).

Thus, pregnancy causes many changes, including in metabolism, the immune system and remodelling of organs including the mammary gland. This is mediated by hormonal signals, and many changes are reversed when lactation ceases. Generally, pregnancy reduces the risk of breast, ovarian and endometrial cancer, but the exact mechanism is poorly understood. There is a lack of knowledge on both how the small intestine and colon are remodelled in pregnancy, and if that changes the risk of tumourigenesis. However, it is known pregnancy causes a short-term increase in

breast cancer risk, and in liver metastasis. Hence, more investigation into how reproduction may modulate cancer risk is important.

1.2 Common cancer pathways

1.2.1 Common signalling pathways mutated in human cancer

Cancer is driven by uncontrolled cellular division and cellular survival caused by dysregulation of a range of signalling pathways. Such erroneous regulation can be caused by gene mutation, duplication or deletion as well as chromosome rearrangement (Salk, Fox and Loeb, 2010; Ostroverkhova, Przytycka and Panchenko, 2023). The result includes either activation of proto-oncogenes or inactivation of tumour suppressors. This modulates not only cell proliferation, but the microenvironment around the cancer cells, including immune suppression and angiogenesis (Hanahan and Weinberg, 2011; de Visser and Joyce, 2023). Generally, signalling pathways that cause human cancer are highly conserved in *Drosophila* (Sonoshita and Cagan, 2017).

The Ras/Raf/MAPK pathway is frequently perturbed in cancer. This pathway is needed for proliferation, differentiation and cytoskeleton organisation (Sun *et al.*, 2015). Ras is a small GTPase that acts as a molecular switch, activating a cascade of kinases that phosphorylate several transcription factors that activate pro-survival genes. When turned on it causes hyperproliferation, and mutation or amplification of one or more Ras or Raf proto-oncogenes are very common in human cancer (Prior, Lewis and Mattos, 2012).

The Wnt/ β -catenin pathway is important for cell pluripotency and differentiation, especially during development. It is commonly perturbed in cancer, especially Adenomatous Polyposis Coli (APC). APC is needed to prevent β -catenin from entering the nucleus and activating a pro-survival and proliferation program (MacDonald, Tamai and He, 2009). Hence, APC is an important tumour suppressor. Mutations in APC are especially common in colon cancer (Zhao *et al.*, 2022).

The Notch pathway regulates cell-cell contacts between cells. Notch is a transmembrane receptor that can be cleaved, allowing its intracellular fragment to regulate the expression of genes related to proliferation, apoptosis or differentiation,

depending on cell type (Bray, 2006). Notch is often mutated in lymphoblastic leukaemia, but is also mutated in squamous cell and urothelial carcinomas (Aster, Pear and Blacklow, 2017).

1.2.2 The Hippo pathway in humans and *Drosophila*

The Hippo pathway is a highly conserved regulator of organ size and is of particular relevance to this thesis (**Figure 1.2**). When the Hippo pathway is active, Ste20 family kinase Hippo (Hpo, human MST1/2) activates downstream kinase Warts (Wts, human LATS1/2) aided by the scaffold proteins Salvador (Sav, human SAV1) and Mob-as-tumour-suppressor (Mats; human MOB1). Wts can then phosphorylate the transcriptional co-activator Yki (human YAP/TAZ), preventing it from entering the nucleus. Yki/YAP/TAZ are either degraded or retained in the cytoplasm (reviewed Pan, 2010; Zhao, Tumaneng and Guan, 2011; Valero, Pawlik and Anders, 2015; Xiao and Dong, 2021)

When the Hippo pathway is inactivated in *Drosophila*, Yki enters the nucleus together with transcriptional co-factor Scalloped (Sd) and promotes the expression of pro-growth genes (Goulev *et al.*, 2008; Wu *et al.*, 2008). This involves genes key to cell proliferation and growth (*CyclinE*, *bantam*) and inhibition of apoptosis (*diap1*) (Harvey, Pflieger and Hariharan, 2003; Udan *et al.*, 2003; Wu *et al.*, 2003; Nolo *et al.*, 2006; Thompson and Cohen, 2006). In humans, when the Hippo pathway is inactivated, YAP/TAZ works with the transcriptional co-factor TEAD to promote cell survival and growth. Less is known about the YAP/TAZ targets, but they include the genes Integrin $\beta 2$ (ITGB2) and Fibroblast Growth Factor 1 (FGF1; Hao *et al.*, 2008). Thus, an active Hippo pathway prevents Yki/YAP/TAZ from entering the nucleus and protects cells from unnecessary proliferation and cell death.

Three main pathways act upstream of Hippo in *Drosophila*: the atypical cadherin Fat-Dachs pathway (Lawrence and Casal, 2013; Strutt and Strutt, 2021), the upstream apical Kibra-Expanded-Merlin complex found in cell junctions (Grusche, Richardson and Harvey, 2010; Su *et al.*, 2017) and the apicobasal Scribble-Dlg polarity pathway at septate junctions (Enomoto and Igaki, 2011). These varied inputs allow the Hippo pathway to respond to cell growth and cell-cell contact.

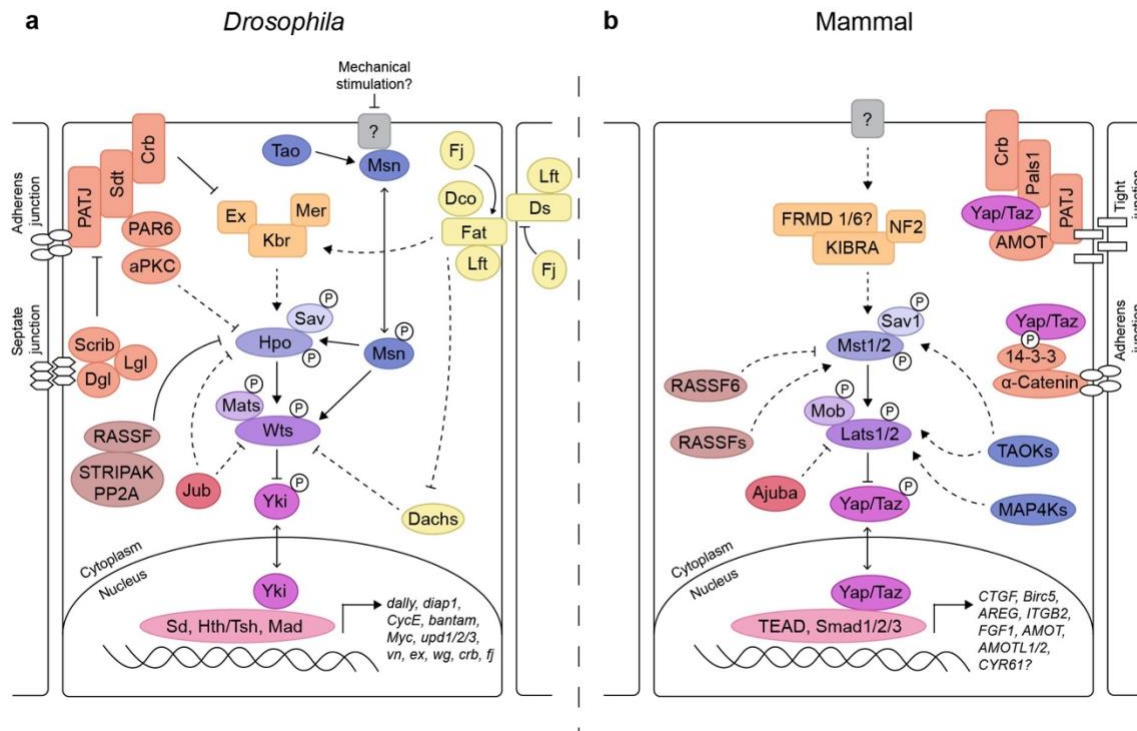


Figure 1.2. The *Hippo* pathway in *Drosophila* and mammals.

a. The *Drosophila* Hippo pathway. **b.** The mammalian Hippo pathway. Corresponding proteins in *Drosophila* a. and mammals b. are indicated by matching colours. Arrowed or blunted ends indicate activation or inhibition, respectively. Dashed lines indicate unknown or indirect mechanisms.

Adapted from Zhao, Tumaneng and Guan, 2011 Valero, Pawlik and Anders, 2015 and Xiao and Dong, 2021.

In mammals, the KIBRA-FRMD-NF2 complex acts upstream of the Hippo pathway (Zhang *et al.*, 2010), as does α-catenin at adherens junctions (Schlegelmilch *et al.*, 2011; Zhao, Tumaneng and Guan, 2011). However, less is known about the other inputs. The Hippo pathway in mammals can respond to cell-cell contact and the actin cytoskeleton, and mechanical and hormonal signals via G-protein-coupled receptors (Meng, Moroishi and Guan, 2016).

The Hippo pathway was first discovered in *Drosophila* but it has now been shown to be important in human cancer (Steinhardt *et al.*, 2008; Cho *et al.*, 2018; Kim and Myung, 2018). YAP is often amplified in human cancer, and has been linked to tumour metastasis (Zhao *et al.*, 2021).

1.3 *Drosophila* as a model organism to study cancer

Drosophila melanogaster has been used as a model organism for over a century. It can be used in its embryonic, larval and adult stages, to study topics ranging from chromatin remodelling, cell competition, inter-organ communication to organismal behaviour. The *Drosophila* genome shares a large number of homologs with the human genome, with conservation of around 75% of disease associated genes, and especially high conservation of genes related to cancer, nonmyelin-associated neurological disorders and other developmental defects (Reiter *et al.*, 2001).

As a model to study cancer, the fly has been a successful model for characterising tumour behaviour and identifying many important genes mutated in cancer. The earliest work involved the characterisation of dark spots on the bodies of mutant larvae, and identification of their similarity to vertebrate tumours (Stark, 1918, 1919). Even at this early stage of research, careful observations confirmed these tumours were hereditary, identified their likely tissue origins, described their metastasis and how larval density modulated tumour intensity (Wilson, 1924). Further characterisation of another tumorous mutant larvae lead to the identification of *lethal(2)giant larvae (lgl)*, the first tumour suppressor gene discovered in *Drosophila* (Gateff and Schneiderman, 1967, 1969, 1974). Development of genetic tools including germline genetic transformation via P-element insertion (Rubin and Spradling, 1982; Spradling and Rubin, 1982), targeted gene expression with UAS/Gal4 (Brand and Perrimon, 1993) and clone generation with FLP-FRT (Golic and Lindquist, 1989; Xu and Rubin, 1993) allowed researchers to generate tumour clones and understand the signalling pathways that led to oncogenic growth. In particular, this led to the discovery of the Hippo pathway (Justice *et al.*, 1995; Xu *et al.*, 1995), which was later shown to be frequently deregulated in human cancer (Harvey and Tapon, 2007). Other pathways important in human cancer first discovered in flies include Notch (Mohr, 1919) and Wingless (Wg; Sharma and Chopra, 1976).

Fly tumour models have investigated topics including cell signalling (Read, Bach and Cagan, 2004), neural stem cells (Caussinus and Gonzalez, 2005), epigenetic silencing (Ferres-Marco *et al.*, 2006), mitochondrial dysfunction (Ohsawa *et al.*, 2012), cachexia (Figuerola-Clarevega and Bilder, 2015; Kwon *et al.*, 2015) and the microbiome (Zhou

and Boutros, 2020). With the use of RNAi libraries and overexpression constructs, many different human cancer mutations can be modelled, creating customised fly ‘avatars’ that mimic patient mutations, in combination with large scale drug screening (Kasai and Cagan, 2010; Gladstone and Su, 2011; Gonzalez, 2013; Sonoshita and Cagan, 2017).

1.4 Sex determination in humans and *Drosophila*

1.4.1 Human sex determination

Human sex determination is based on the presence of a Y chromosome. In humans, rodents and most mammals, the Y chromosome encodes the transcription factor *Sex-determining region Y (SRY)* gene (Gubbay *et al.*, 1990; Koopman *et al.*, 1990; Sinclair *et al.*, 1990; Vining *et al.*, 2021). Sex determination can be split into two phases: the genetic chromosome to gonad formation, then the hormonal gonad to phenotype. In female gonadal cells with two X chromosomes, in the absence of *SRY* expression, the transcriptional programme of ovary formation is initiated. It was recently shown that other factors are needed to push the gonads towards the female state, including the Wilms tumour suppressor (WT1) splice isoform -KTS (Gregoire *et al.*, 2023). In male gonadal cells with one X and one Y chromosome, sufficient *SRY* within a certain developmental time window expression induces the transcriptional programme that results in testis formation.

The immediate targets of *SRY* are not known, and whilst it is associated with gonadal formation, some postulate it may have other roles in cell intrinsic (non-hormonal) sex determination in cell growth and organism behaviour (Gutiérrez-Adán *et al.*, 1997; Schmahl *et al.*, 2000; Dewing *et al.*, 2006). After gonad formation, it is generally regarded that steroid hormones released from the gonads mediate sex differences, rather than chromosomal sex. Thus oestrogen and testosterone act on cells via steroid receptors and activate female or male transcriptional programmes respectively (Williams and Carroll, 2009). Even pre-puberty, at 12 months of age boys have a lower percentage fat mass, and higher lean mass, reflecting trends in adulthood (Davis *et al.*, 2019). This is thought to be caused by the ‘mini-puberty’ period at 1-6 months of age that results in a period of high testosterone in boys (Renault *et al.*, 2020).

Adolescent puberty then causes a large hormonal shift that results in the development of secondary sex characteristics.

As females have two X chromosomes, and males only one, without intervention, females would have twice the X-linked gene expression of males. To bring gene expression to the same levels, females randomly silence an X chromosome, resulting in a single active X chromosome. Then, both males and females have a two-fold upregulation of the active X chromosome (Panning, 2008). However, as previously mentioned, around 15% of genes will escape X-inactivation, resulting in higher expression in females (Carrel and Willard, 2005).

Active maintenance of sex determining factors through adult life can also be seen in vertebrate gonads in mouse and fish models. Deletion of transcription factors like *Doublesex- and mab-3-related transcription factor 1 (Dmrt1)*, in the adult testis can cause these somatic cells to be feminised (Matson *et al.*, 2011; Masuyama *et al.*, 2012). Deletion of forkhead transcriptional regulator *Foxl2* in female ovarian follicles leads to masculinisation of the gene expression profile, including upregulation of SRY target *Sox9* (Uhlenhaut *et al.*, 2009).

1.4.2 *Drosophila* sex determination

Drosophila somatic sex determination is based on the ratio of X chromosomes, rather than the presence of the Y chromosome (reviewed in Cline, 1993; Salz and Erickson, 2010). During early development, in female cells with two X chromosomes to two autosomes (2X:2A), the *Sex lethal (Sxl)* gene is transcribed (Bell *et al.*, 1988; Cline, 1988; Nagoshi *et al.*, 1988; Belote *et al.*, 1989). This splicing factor will then generate the female-specific splicing product of transcription factor *transformer (tra)* (**Figure 1.3a**). Only females have a functional *tra* protein: Tra^F (Boggs *et al.*, 1987; McKeown, Belote and Baker, 1987). *tra* and *transformer-2 (tra-2)* mediate female-specific splicing of transcription factor *doublesex (dsx)* (Ryner *et al.*, 1996; Heinrichs, Ryner and Baker, 1998; Goodwin *et al.*, 2000; Lee *et al.*, 2000). These coordinate expression of genes needed for sexually dimorphic anatomy like genitals and body size, as well as behaviour (Taylor, 1992; Demir and Dickson, 2005; Chatterjee *et al.*, 2011; Rideout, Narsaiya and Grewal, 2016).

The ratio of X chromosomes can only be detected early in development, prior to the onset of dosage compensation, where 1X gene expression is increased to match 2X expression levels. Expression of *Sxl* is maintained in females throughout their lifetimes as it can initiate autoregulatory splicing to continue to generate new *Sxl* (Cline, 1988; Duffy and Gergen, 1991; Sánchez, Granadino and Torres, 1994; Jinks *et al.*, 2000; Sefton *et al.*, 2000). *Sxl* also represses the dosage compensation factor *male-specific lethal-2* (*msl-2*). *Drosophila* dosage compensation involves the two-fold upregulation of the X chromosome in males. Thus, females require repression of *msl-2* to prevent any additional upregulation of X-linked genes, as unlike in mammals there is no X chromosome silencing (Bashaw and Baker, 1995; Lucchesi and Kuroda, 2015).

In male cells with one X chromosome to two autosomes (1X:2A), the failure to express *Sxl* results in an absence of functional Tra^F protein (**Figure 1.3b**). This means the male isoforms of *dsx* and *fruitless* (*fru*; Nagoshi *et al.*, 1988; Burtis and Baker, 1989; Ryner *et al.*, 1996; Heinrichs, Ryner and Baker, 1998; Goodwin *et al.*, 2000; Lee *et al.*, 2000; Demir and Dickson, 2005) are produced, which have specific roles in generating sexual dimorphic features including neurophysiology and behaviour (Zhang *et al.*, 2020; Han *et al.*, 2022).

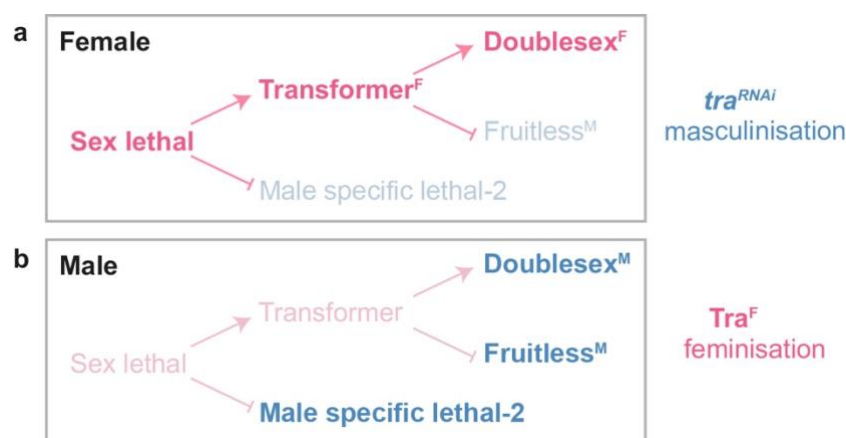


Figure 1.3. Sex determination in *Drosophila*.

a. In females, chromosomal sex initiates expression of Sex lethal, which leads to female splicing isoforms of Transformer (Tra) and Doublesex. This can be 'masculinised' by downregulation of *tra*. **b.** In males, chromosomal sex does not cause Sex lethal expression, resulting in the male splicing isoforms of Doublesex and Fruitless. This can be 'feminised' by expression of the female isoform of Transformer (Tra^F).

1.5 Sexual dimorphism and reproductive remodelling of the fly midgut

1.5.1 Function of the midgut

The *Drosophila* digestive tract is formed of the foregut, midgut and hindgut (**Figure 1.4a**). The foregut includes the oesophagus, crop and proventriculus. After entry through the oesophagus, food enters the crop, a Diptera structure important for early digestion, antimicrobial activity and regulation of food intake (Stoffolano and Haselton, 2013; Hadjieconomou *et al.*, 2020).

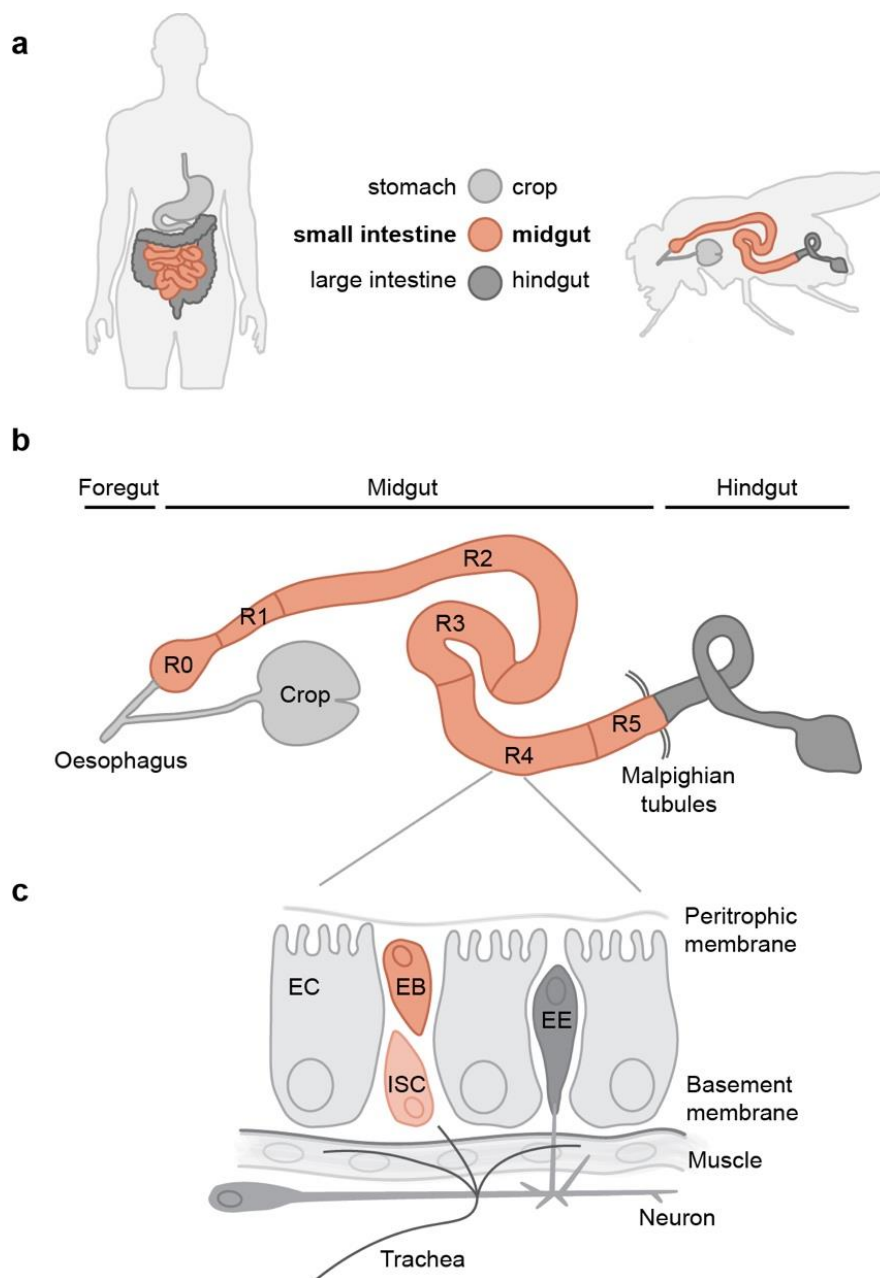


Figure 1.4 Structure of the *Drosophila* midgut.

a. The human small intestine is equivalent to the *Drosophila* midgut. **b.** Regionality of the *Drosophila* gut showing R0 – R5 of the midgut. **c.** Structure of the *Drosophila* midgut epithelium, supported by the muscle, neuronal innervation and tracheal network. ISC – intestinal stem cell, EB – enteroblast, EE cell – enteroendocrine cell, EC – enterocyte.

Adapted from Miguel-Aliaga, Jasper and Lemaitre, 2018.

It then enters the proventriculus, a bulb-like structure that regulates the passage of food (King, 1988), and a source of antimicrobial peptides from the peritrophic matrix (Tzou *et al.*, 2000). Next food enters the midgut. This is the site of nutrient digestion and absorption and the longest portion of the gut. Between the midgut and hindgut are the Malpighian tubules, an excretory organ that regulates ion balance and fluid secretion via the hindgut. Finally, the hindgut is formed of the valve-like pylorus, ileum and rectal ampulla. Here the water and ion balance is regulated prior to excretion (**Figure 1.4b**; Cohen *et al.*, 2020 and Dow, Simons and Romero, 2022).

The midgut is comprised of a single-layered epithelium, made mainly of enterocytes (EC), large endoreplicating cells specialised in nutrient absorption (**Figure 1.4c**). Between these cells are the smaller enteroendocrine (EE) cells, capable of releasing peptide hormones to modulate nutrient sensing, digestion and energy storage (Chopra, Kaushik and Kain, 2022). These cells are replenished by the progenitor cells: intestinal stem cells (ISC) and enteroblasts (EBs) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). ISC are small multipotent progenitors, which can divide to generate EBs, which begin to endoreplicate and form ECs (Xiang *et al.*, 2017; Rojas Villa, Meng and Biteau, 2019). ISC can also divide into pre-EE cells, a specialised progenitor which can then develop into an EE cell (Biteau and Jasper, 2014; Zeng and Hou, 2015; Chen *et al.*, 2018; He *et al.*, 2018). A subpopulation of EE cells can also dedifferentiate into ISC in response to diet (Nagai *et al.*, 2023). The ISC niche is supported by numerous signalling pathways including Notch (Ohlstein and Spradling, 2007), JNK (Biteau, Hochmuth and Jasper, 2008), Tor (Amcheslavsky *et al.*, 2011; Kapuria *et al.*, 2012; Quan *et al.*, 2013) and Hippo (Karpowicz, Perez and Perrimon, 2010; Ren *et al.*, 2010; Shaw *et al.*, 2010; Staley and Irvine, 2010).

The midgut epithelium is supported by several other tissues (**Figure 1.4c**). Wrapped around the midgut is a sheath of visceral muscle important for peristalsis as well as supporting the ISC niche through Wg, EGFR, Insulin and JAK-STAT signalling (Lin, Xu and Xi, 2008; Jiang and Edgar, 2009; Biteau and Jasper, 2011; O'Brien *et al.*, 2011; Xu *et al.*, 2011). Wg has also been reported to be produced by the EBs, with a much stronger effect on ISC proliferation (Cordero *et al.*, 2012). The midgut is surrounded by the tracheal system, a network of oxygen transporting tubes similar to the mammalian respiratory system (Hayashi and Kondo, 2018). However they also

provide important signals like *Drosophila* BMP ligand Decapentaplegic (Dpp) to surrounding cells to maintain homeostasis (Li *et al.*, 2013). Trachea expand in response to oxidative stress, infection and tumourigenesis to support ISC proliferation (Perochon *et al.*, 2021; Tamamouna *et al.*, 2021). Gut-associated haemocytes, a macrophage-like cell, can induce ISC proliferation through Dpp (Ayyaz, Li and Jasper, 2015) and JAK-STAT signalling (Chakrabarti *et al.*, 2016). There are enteric neurons near the proventriculus and crop, and at the pylorus and ampulla (Miguel-Aliaga, Jasper and Lemaitre, 2018). Cholinergic neurons have been shown to facilitate return to homeostasis after midgut injury via Ca^{2+} currents through the ECs (Petsakou *et al.*, 2023).

The midgut can be divided into many different regions depending on morphology, gene and protein expression (Murakami *et al.*, 1994; Buchon *et al.*, 2013; Marianes and Spradling, 2013), but throughout my thesis I have referred to regions R0-R5 (**Figure 1.4b**) (Buchon *et al.*, 2013). R0 represents the proventriculus. The R1-R2 regions of the anterior midgut are especially high in digestive enzymes for more complex macromolecules, and antimicrobial peptides. The R3 middle region has a highly acidic pH and contains specialised iron, copper and large flat cells (Strand and Micchelli, 2011). The R4-R5 regions of the posterior midgut contain some digestive enzymes for simple molecules, but are generally more specialised for absorption and in mated females have higher levels of lipid storage (Buchon *et al.*, 2013; Marianes and Spradling, 2013). As well as digestion being highly regionalised, the ISC also differ between regions. The posterior ISC in R4 are most likely to divide, followed by R2 then R5 (Marianes and Spradling, 2013). These ISC have the highest levels of EGFR and JAK-STAT signalling, which may be responsible for their higher rates of proliferation (Dutta *et al.*, 2015). However, others have suggested the high proliferation of R4 ISC is due to their location in the absorptive region of the gut with the most highly available nutrients (Marianes and Spradling, 2013). The midgut is generally a flat single layer, but has some folded regions in R4 that resemble the villi of a mammalian gut (Buchon *et al.*, 2013).

1.5.2 The midgut as a model for colorectal cancer

Cancer of the colon and rectum are common in humans, whereas cancer of the small intestine is incredibly rare (Bray *et al.*, 2018; Ferlay *et al.*, 2019). Several factors have been proposed to explain this, including faster transit through the small intestine, resulting in shorter exposure of luminal epithelium to carcinogens, and rapid epithelial turnover outpacing tumour development. Additionally, the bacterial load is lower than in the large intestine and varies dramatically by strain, potentially reducing bacterial carcinogenic compounds (Jensen *et al.*, 2023). The small intestine also has an extensive lymphoid tissue network that may suppress tumourigenesis (DiSario *et al.*, 1994; Ciresi and Scholten, 1995; Hatzaras *et al.*, 2007). Therefore, it may be questioned why a tumour model of the *Drosophila* small intestine is being used.

Although functionally, the *Drosophila* midgut is closest to the human small intestine (nutrient digestion and absorption), and the hindgut and ampulla to the colon and rectum (water balance and faeces storage), the midgut resembles the colon in many ways. The midgut is of endodermal developmental origin, whereas the hindgut is of ectodermal origin. However, the human small intestine, colon and rectum are all of endodermal origin (Miguel-Aliaga, Jasper and Lemaitre, 2018). Thus, the midgut better reflects the developmental origin of the human colon. Additionally, although the hindgut has a band of stem cells at the midgut-hindgut boundary, it does not proliferate in response to damage or tumourigenesis (Losick, Fox and Spradling, 2013; Sawyer, Cohen and Fox, 2017). The midgut however has stem cells that rapidly turnover the epithelium (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). The stem cells in the mammalian colon do also proliferate to facilitate epithelial turnover, although not as fast as small intestinal stem cells (Barker *et al.*, 2007). Therefore, the midgut makes a good model for the human colon, where tumours are common.

There are five pathways most often mutated in human colorectal cancer: Ras/MAPK, PI3K/Akt, Wnt, p53 and TGF- β . Although the genetic combination to modulate these pathways using *Ras*^{G12V}, *pten*^{RNAi}, *Apc1*^{RNAi}, *p53*^{RNAi} and *Med*^{RNAi} respectively was initially tested in the hindgut (Bangi *et al.*, 2016), modulation of these pathways in the midgut ISC/EB leads to increased midgut proliferation and tumourigenesis (Wang *et al.*, 2013; Martorell *et al.*, 2014; Ayyaz, Li and Jasper, 2015; Strilbytska *et al.*, 2020;

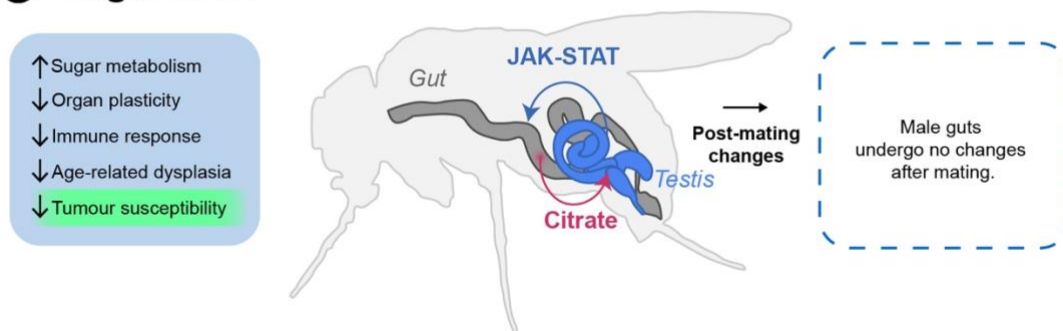
Zipper *et al.*, 2022). The midgut also offers opportunities to generate tumours in multiple ways, including in the ISC and EB progenitor cells with the *escargot* (*esg*) Gal4 driver (**Section 3.1.1**). This also includes lineage tracing with *esg*^{ReDDM} (Antonello *et al.*, 2015) or *esg*^{flipout} (Jiang *et al.*, 2009). Alternatively, *esg*-driven MARCM clones can be used (Lee and Luo, 1999; Yu *et al.*, 2009; Martorell *et al.*, 2014), which cannot be generated in the hindgut as there are no mitotic cells.

1.5.3 The gut and sex

The midgut is highly responsive and adaptable to change, as it directly interacts with the environment, via the passage of food and pathogens. It responds robustly to nutritional fluctuations (Choi, Lucchetta and Ohlstein, 2011; O'Brien *et al.*, 2011; Bonfini *et al.*, 2021), infection (Buchon *et al.*, 2009; Onuma *et al.*, 2023) and tissue damage (Amcheslavsky, Jiang and Ip, 2009) and its ability to do so is modulated by aging (Guo *et al.*, 2014; Wu *et al.*, 2021). Internal and external factors modulate this ability. One key internal factor is sex. Male and female flies have striking differences

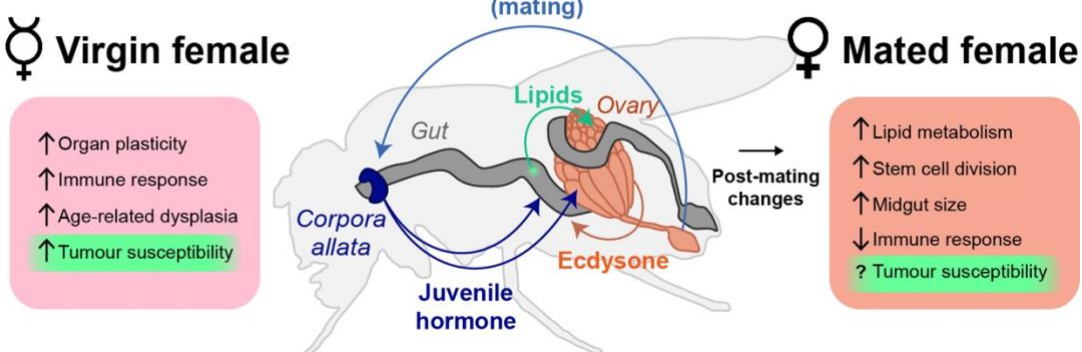
a

♂ Virgin male



b

♀ Virgin female



♀ Mated female

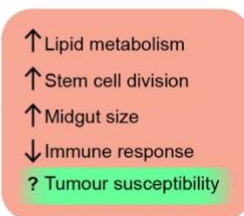


Figure 1.5. Summary of sex and reproductive changes in the *Drosophila* midgut (previous page).

a. Male midguts have reduced organ plasticity, leading to reduced dysplasia and tumour susceptibility. JAK-STAT signalling from the testis promotes carbohydrate metabolism in the posterior midgut. This results in citrate being transported from the midgut to the testes to promote sperm production. **b.** Females have increased organ plasticity, leading to increased tumour susceptibility compared to males. The mating signal sex peptide acts on the *corpora allata* (an endocrine gland similar in function to the thyroid). This triggers an increase in juvenile hormone which travels to the gut and ovaries. Simultaneously mating triggers hormonal signal ecdysone (20HE) from the ovaries and other sources to the gut. This triggers post-mating changes such as lipid trafficking from the gut to the ovaries.

Schematic created using findings from Reiff et al., 2015; Hudry, Khadayate and Miguel-Aliaga, 2016; Regan et al., 2016; Ameku et al., 2018; Hudry et al., 2019; Ahmed et al., 2020; White et al., 2021; Gordon, Wolfner and Lazzaro, 2022; Zipper et al., 2022.

in body size and reproductive organs (reviewed in Shingleton and Veal, 2023), but these differences are present in organs and tissues not traditionally thought of as sex organs.

Male midguts have much higher levels of sugar metabolism gene expression than females, and also have a different pattern of sugar metabolism protein expression. Whilst males and females have expression of the various amylases, maltases and glycolysis genes in the R1 and R2 regions of the gut, expression in the R4 region is much higher in males. This is due to communication between the midgut R4 region and the hub of the testis. *Drosophila* JAK-STAT ligand Unpaired (Upd) released from the testis hub increases JAK-STAT signalling in the R4 region of the midgut, which increases sugar metabolism in order to promote citrate production (**Figure 1.5a**). Citrate is important for sperm production, and also promotes feeding (Hudry *et al.*, 2019).

Female midguts are longer and their ISC proliferate more when challenged by damage, infection and tumourigenesis (Hudry, Khadayate and Miguel-Aliaga, 2016). This makes female flies less susceptible to infection, but as a trade-off causes a large increase in age-related dysplasia (**Figure 1.5b**; Regan *et al.*, 2016). These age-related pathologies can be reduced with dietary restriction, causing a female-specific lifespan increase (in comparison to mated females, Regan *et al.*, 2016).

Some stem cells have been shown to use a non-canonical sexual differentiation pathway, different from the pathway defined in **Section 1.4.2**. In the gonads, both the germline cells and the surrounding somatic tissue must have their sexual identity specified, and a mismatch can lead to improper gametogenesis and infertility. In the male somatic CySC, loss of the transcriptional repressor *chinmo* causes feminisation, creating ovary-like female somatic stem cells within the testes (Ma, Wawersik and Matunis, 2014). This is because loss of *chinmo* causes upregulation of *tra*, resulting in increased production of Tra^F and downstream Dsx^F. This is Sxl-independent but does require the *tra*-splicing factors Virilizer (Vir) and Female lethal (2)d (Fl(2)d) (Grmai *et al.*, 2018).

In the midgut, female ISC use a non-canonical sexual differentiation pathway involving *tra*, but not the *tra* binding partner *tra2* or the downstream *tra* targets *dsx* and *fru*. Instead, female transcriptional activity is mediated at the expression level by a range of *tra* targets including positive regulators *Imaginal disc growth factor 1* (*Idgf1*) and *Serpin 88Eb* (*Spn88Eb*) and negative regulator *reduced ocelli* (*rdo*; Hudry, Khadayate and Miguel-Aliaga, 2016).

When ISC and EB are masculinised by *tra* or *Sxl* downregulation, female midguts have reduced proliferation in response to dextran sodium sulfate (DSS)-induced damage and tumourigenesis caused by *Notch* downregulation (Hudry, Khadayate and Miguel-Aliaga, 2016). Modulation of *tra* also affects midgut length, with 20 days (d) of *tra* downregulation able to reduce the longer virgin female midguts to a male-like length. Virgin females have longer R3 regions, and this can also be reduced to shorter male-like length with *tra* downregulation (Hudry, Khadayate and Miguel-Aliaga, 2016). Ahmed and colleagues corroborated this by looking at the *tra* dependence of proliferation triggered by several different sources. They also showed in virgin females that downregulation of *tra* in ISC and EC prevented proliferation induced by sodium dodecyl sulfate (SDS) damage and *Pseudomonas entomophila* (*P.e.*) bacterial infection, but interestingly not proliferation induced by 20-Hydroxy-Ecdysone (20HE) supplementation (Ahmed *et al.*, 2020). Additionally, it is known males proliferate less than mated females in response to both *Erwinia carotovora* (*Ecc*; Regan *et al.*, 2016) and *P.e.* bacterial infection (Ahmed *et al.*, 2020).

Some work has been done on sex differences on *esg^{ts}*-driven *Notch^{RNAi}* tumours, showing males are very resistant to tumour formation (Hudry, Khadayate and Miguel-Aliaga, 2016), and that this can be overcome by supplementation with 20HE (Ahmed *et al.*, 2020). Female tumourigenesis caused by *Notch^{RNAi}* can be reversed by masculinisation with downregulation of *tra* or *Sxl* (Virgin females; Hudry, Khadayate and Miguel-Aliaga, 2016) or blocking ecdysone signalling with dominant negative EcR (Mated females; Ahmed *et al.*, 2020). In midgut *Apc-Ras* MARCM clones (null mutant *Apc* and constitutively active *Ras*), virgin females have more proliferation than males, which can be reduced with *Sxl^{RNAi}* (Hudry, Khadayate and Miguel-Aliaga, 2016).

Therefore, the sex of the *Drosophila* midgut is determined by a non-canonical sex determination pathway, which affects the midgut length, ISC proliferative potential and cellular metabolism. This results in a propensity for females to both develop age-related dysplasia and more proliferative genetic tumours.

1.5.4 The gut post-mating

Females require more responsive ISC as their midguts must be remodelled after mating to prepare for energy intensive egg laying. These post reproductive changes are mediated by two hormones: the sesquiterpenoid juvenile hormone (JH) and steroid hormone 20-Hydroxy-Ecdysone (20HE). After mating, sex peptide from the male triggers a large increase in circulating JH released from the endocrine *corpus allatum*. Two proteins form the receptor that binds JH, *methoprene-tolerant (met)* and *germ cell-expressed bHLH-PAS (gce)* (Jindra, Palli and Riddiford, 2013; Jindra *et al.*, 2015), and both are required for JH-induced effects in the midgut (Reiff *et al.*, 2015). These effects include an increase in midgut *kruppel homologue 1 (kr-h1)* which mediates an increase in ISC proliferation and an increase in midgut lipid metabolism (Reiff *et al.*, 2015).

Mating additionally triggers an increase in 20HE in the ovaries and haemolymph (Ameku *et al.*, 2018; Zipper *et al.*, 2020). 20HE binds to the heterodimer of *Ecdysone receptor (EcR)* and *ultraspiracle (usp)* (Ahmed *et al.*, 2020). One of the key genes upregulated in the midgut post-mating is nuclear receptor *Ecdysone-induced protein 75B (Eip75B)*. The *Eip75B-A* and *Eip75B-C* isoforms specifically promote

differentiation into EC, allowing the midgut to greatly increase in size post-mating. When these factors are downregulated, the gut still proliferates post-mating but the total number of EC is reduced, resulting in a shorter gut (Zipper *et al.*, 2020).

When virgin flies are supplemented with 20HE, it can also induce midgut proliferation not just in females but also in males. This is independent of *tra*. 20HE also induces the expression of *Eip75B*, which targets effectors including by repressing nuclear receptor *Hormone receptor 3 (Hr3)*. *broad (br)* is another *EcR* target important for ISC proliferation (Ahmed *et al.*, 2020). Downregulation of ecdysone synthesis enzymes *disembodied (dib)* and *spook (spo)* in the ovary is sufficient to reduce midgut ISC proliferation post-mating (Ahmed *et al.*, 2020). Post-mating midgut EE cells release *neuropeptide F (NPF)*, which stimulates ovary GSC proliferation, both increasing egg production and likely 20HE levels (Ameku *et al.*, 2018). Thus, the source of 20HE is likely the ovary. However, previous experiments in the Miguel-Aliaga lab in which we downregulated 20HE production genes in the ovary did not prevent the effects of 20HE in the gut, hinting there may be another source. It has been proposed this may be the gut, but 20HE production genes are lowly expressed in the midgut. Interestingly, the pupal skeletal muscle has recently been shown to be an alternative source of 20HE that drives ISC division in the pupae (Zhang *et al.*, 2023).

Ahmed and colleagues found JH receptors *gce* and *met* are required for 20HE induced proliferation and for maintenance of ISC in homeostasis. Thus the effects of these two hormonal signals are tightly intertwined, with both being key for post-mating midgut remodelling (Ahmed *et al.*, 2020). These two hormones work together in other contexts, for example in moulting and metamorphosis across many different insect species (Truman and Riddiford, 2019).

Females have reduced survival after infection with several different bacterial species including *P. rettgeri* and *P. aeruginosa* 2 - 3 h after mating (Fedorka *et al.*, 2007; Short and Lazzaro, 2010). This is mediated by sex peptide and accessory gland peptides (Short, Wolfner and Lazzaro, 2012), which act via JH (Schwenke and Lazzaro, 2017). Females must be producing eggs to show a reduction in immune defence post-mating, hinting energy intensive egg laying may be prioritised over immune function. This immune suppression lasts at least 10 d (Gordon, Wolfner and Lazzaro, 2022). It has

been suggested that immune suppression may be a result of the fat body being incapable of simultaneously producing proteins for both reproductive output and infection (Gupta *et al.*, 2022). I have not found any studies in which midgut proliferation from bacterial infection has been directly compared between virgin females and mated females. However, midgut proliferation in mated females after *P.e.* infection is dependent on both EcR and its downstream target Eip75B (Ahmed *et al.*, 2020).

The midgut increases in size for the first six days post-mating, then plateaus until ten days post-mating, before decreasing. However, it still maintains a longer gut than an age matched virgin female. The majority of the growth increase is in the posterior midgut (White *et al.*, 2021). As well as the increase in ISC proliferation and EC differentiation, Sex peptide from the male triggers large transcriptional changes in the mated female gut. As mentioned previously an increase in lipid metabolism is key to increased egg production. There are also increases in amino acid transport, chitin metabolism and serine-type endopeptidases. There is a decrease in sugar metabolism, including galactose metabolism, glucose transport and maltase expression, and also Glutathione-S-Transferase (Gst; White *et al.*, 2021). Notably, the sugar metabolism significantly downregulated post-mating involves the same genes that are key to male-biased citrate metabolism (Hudry *et al.*, 2019).

Very little work has been done to assess the impact of these reproductive changes on midgut tumourigenesis. Most work on midgut tumours has either used mated females, or has used females with an undeclared mating status. Some of this work involves the use of *esg^{ts}* to express transgenes in the midgut ISC and EB in an adult-specific manner (**Section 1.7**). It has been shown virgin females are very resistant to *esg^{ts}* induced *Notch^{RNAi}* tumours compared to mated females (Ahmed *et al.*, 2020). In midgut *Apc-Ras* MARCM clones, downregulation of *juvenile hormone acid o-methyltransferase (jhamt)*, one of the enzymes necessary for JH biosynthesis, greatly reduces tumour clone size in females (mating status undisclosed). As does downregulation of JH receptor *gce* and JH induced transcription factor *taiman (tai)*, but not JH receptor *met*. This hints that the autocrine signal of JH is necessary to sustain tumour growth in *Apc-Ras* midgut clones (Rahman *et al.*, 2017). It has also been shown that *esg^{ts}*-driven *Notch^{RNAi}* tumours in mated females are sensitive to 20HE signalling, resulting in reduced tumour growth when *EcR* or *Eip75B-A/C* are

downregulated. Increased 20HE signalling caused by overexpression of *Ecdysone Importer (Ecl)* causes massive increase in *Notch^{RNAi}* tumour growth in mated females (Zipper *et al.*, 2020).

Therefore, mating in female flies results in many changes to the midgut, including an increase in gut size, increased ISC proliferation and increased lipid metabolism. These changes, coupled with the pro-mitotic effects of 20HE and JH hints that mating status should affect tumour formation in mated versus virgin females. The *Drosophila* midgut is an ideal system to understand tumour formation and behaviour. Its sex and mating-induced changes also make it a fascinating model to probe the impact of sex and reproduction in cancer.

1.6 Effect of sex and reproductive status on non-midgut *Drosophila* tumours

Although my project focuses on the midgut, other non-gonadal organs have been found to have sex differences in flies. Even at the larval stage, before sexual maturity, key differences are found. Male larvae have higher levels of fat (Diaz *et al.*, 2023), a feature which is lost by adult eclosion (Wat *et al.*, 2020), and then reversed by adult maturity, when females have higher fat levels (Sieber and Spradling, 2015; Rideout, Narsaiya and Grewal, 2016). These whole body effects may also impact the midgut, as communication between the fat body and midgut has been shown to be important in maintaining homeostasis (Scopelliti *et al.*, 2019; Yang *et al.*, 2019; Zhao and Karpac, 2020).

Drosophila male and female brains show dramatic differences, in the number and function of neurons (Lee *et al.*, 2000; Lee, Hall and Park, 2002; Stockinger *et al.*, 2005; Rideout *et al.*, 2010; Pavlou *et al.*, 2016), as well as gross brain anatomy (Cachero *et al.*, 2010), in both larval and adult stages. Interestingly, a larval brain tumour model using *lethal(3)malignant brain tumour (l(3)mbt)* mutants showed males developed malignant neoplasms more often than females. This was not found in alternative larval brain tumour model *brain tumour (brat)*. Sex differences in *l(3)mbt* tumours were caused by male-biased expression of chromatin reader *PHD finger protein 7 (Phf7)*, a

gene usually responsible for activating the male-transcriptional programme in the male germline (Molnar *et al.*, 2019).

Regarding the impact of reproduction on tumour formation, many tumours have been found to be influenced by 20HE or JH, two hormones high in mated females (Rahman *et al.*, 2017; Zipper *et al.*, 2020). 20HE has a key role in controlling development in larvae, when several pulses control moulting and then finally metamorphosis (Kozlova and Thummel, 2000). In a prothoracic gland (PG) tumour model, tumour growth induced by the constitutive activation of *yki* (*yki*^{3SA}) was suppressed by decreased 20HE levels caused by simultaneous downregulation of *EcR*. However, proliferation in the wing disc was not affected by downregulation of *EcR* levels, showing the tissue origin affects responsiveness to 20HE (Mansilla *et al.*, 2016).

Alternatively, 20HE has been shown to mediate the differentiation and elimination of one type of tumour. The mutant *ph*⁵⁰⁵ (a mutant allele for *ph proximal* and *ph distal*) causes large eye disc tumours when MARCM clones are generated. However, when larvae harbouring these eye disc tumours pupate and eclose into adults, the *ph*⁵⁰⁵ cells are eliminated within several days of adulthood. This was shown to be because the 20HE pulse at metamorphosis suppressed the tumourigenic nature of the *ph*⁵⁰⁵ cells. Co-expression of *ph*⁵⁰⁵ with *EcR*^{DN} resulted in survival of the tumour cells and expansion into aggressive adult tumours. 20HE mediates this reprogramming via miRNA *let-7* and its target gene *chinmo*, as together these cause the differentiation of the *ph*⁵⁰⁵ cells and loss of neoplastic state. Thus in this context, 20HE exposure causes tumour suppression (Jiang *et al.*, 2018).

1.7 The Gal4/UAS and TARGET systems in *Drosophila*

This project utilises the Gal4/UAS and TARGET systems (Brand and Perrimon, 1993). Gal4 is a yeast transcription factor, either inserted into an endogenous enhancer region (enhancer trap) or fused to enhancer fragments of a chosen gene (transgene). Thus, Gal4 expression is limited to that of its enhancer region or fusion sequence, usually resulting in expression in a particular cell type at a particular developmental period. Gal4 induces the expression of genes with an Upstream Activating Sequence (UAS). UAS sequences are used to drive overexpression of a gene of interest,

downregulation with an RNAi sequence or expression of a marker such as GFP or LacZ.

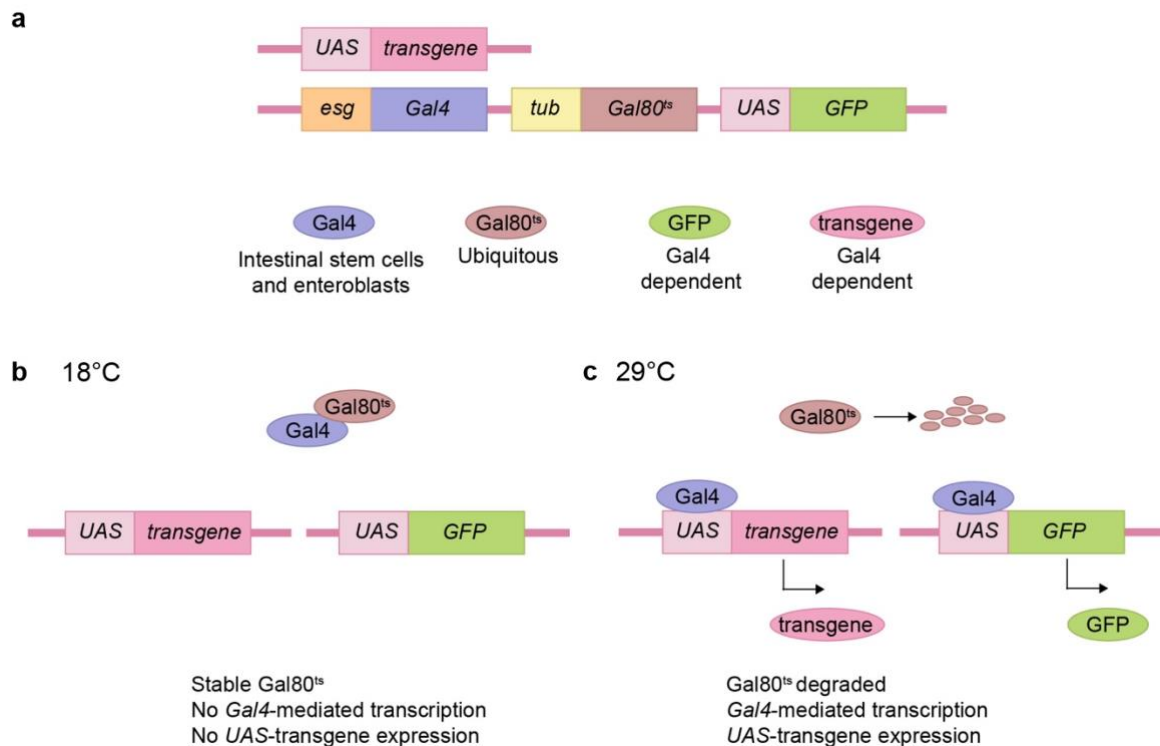


Figure 1.6. The Gal4/UAS/Gal80^{ts} expression system.

a. The yeast transcription factor Gal4 is under the control of *escargot* (*esg*), resulting in Gal4 expression in intestinal stem cells (ISC) and enteroblasts (EB). A temperature sensitive form of the repressor Gal80 (Gal80^{ts}) is expressed ubiquitously by the tubulin promoter. GFP and other transgenes are fused to an Upstream Activating Sequence (UAS), meaning they are only expressed in the presence of Gal4. **b.** At 18°C, Gal4 is expressed specifically in ISC and EB and Gal80^{ts} is expressed ubiquitously. As Gal4 is repressed by Gal80^{ts} the transgenes under control of the UAS promoter will not be expressed. **c.** After temperature increase to 29°C, Gal80^{ts} is unstable and repression is relieved, resulting in Gal4-mediated transgene expression in ISC and EB.

The Gal4/UAS system can be made inducible by the ubiquitously expressed temperature sensitive (*ts*) *tubulin-Gal80^{ts}*. At 18°C Gal80^{ts} prevents expression of Gal4, ensuring transgene expression is prevented through the embryonic and larval stages. When flies are switched to 29°C, Gal80^{ts} is degraded, and adult specific induction of Gal4 allows temporal control of gene expression. This system is referred to as the TARGET system (McGuire, Mao and Davis, 2004).

1.8 Thesis aims

It is known in humans that cancer risk, type and progression is influenced by both sex and reproductive status. However, sex and reproductive status is often not taken into account in experimental models to study cancer.

Here, I aim to profile how different genetic tumour models in the *Drosophila* midgut change with sex and reproductive status, and why they may respond differently. This will consider the ways sex and reproduction affect the midgut, to see if the tumours respond to these factors, or overcome them.

To address this question, I induced several different genetic tumours in the *Drosophila* midgut in virgin males, virgin females and mated females, and quantified the tumour proliferation (**Chapter 3: Results I**). I then focused on one of the genetic tumours, *wt^s^{RNAi}*, and characterised how this tumour responded to genetic masculinisation and feminisation, and its dependency on its downstream Hippo pathway component *yki* (**Chapter 4: Results II**). After this I returned to three of the genetic midgut tumours, and profiled how they change in virgin males, virgin females and mated females using RNA-sequencing (**Chapter 5: Results III**). Finally, I used my RNA-sequencing data to explore some of the sex and reproductive differences affected by tumour generation, including Hippo pathway activity and carbohydrate metabolism (**Chapter 6: Results IV**).

Chapter 2: Material & Methods

2.1 Fly husbandry and genetics

2.1.1 Fly maintenance

Flies were raised on a standard cornmeal/agar diet: 6.65% organic corn maize (Dove's Farm), 7.1% dextrose (Tate and Lyle), 5% autolysed yeast powder (Kerry), 0.66% technical agar no.3 (Oxoid, LP0013) supplemented with 2.2% nipagin (Sigma, H3647) and 3.4% propionic acid (Sigma, P1386). All experimental flies were kept in incubators or temperature-controlled rooms at 65% humidity on a 12 hr light/dark cycle. For temperature sensitive experiments with the TARGET system (*esg^{ts}*) flies were raised at 18°C. Density controlled crosses were set up with 8 virgin females and 5 males. After eclosion, virgin flies were aged for 6 - 7 d, then mated for 24 hr (for the mated female condition), with a ratio of at least 2:1 males to females. Flies were then switched to 29°C incubators for transgene induction for the number of days indicated in the figure legends. Flies were transferred to new food every 2 - 3 d and fly density was maintained at 20 flies per vial.

2.1.2 Fly stocks

Drivers: *esg⁵¹³⁰-Gal4* (DGRC 104863; Hayashi *et al.*, 2002), *esg⁷³⁹⁷-Gal4* (DGRC 114268; Hayashi *et al.*, 2002), *tubulin-Gal80^{ts}* (BDRC: 7017).

UAS transgenes: *UAS-Dcr2* (24646; Dietzl *et al.*, 2007), *UAS-CD8::RFP* (II - 27398; III - 27399; Ye *et al.*, 2007), *UAS-CD8::GFP* (32186; Lee and Luo, 1999), *Su(H)GBE-LacZ* (Furriols and Bray, 2001), *UAS-mCherry^{RNAi}.Valium10* (TRiP control, BDSC: 35787; Neumüller *et al.*, 2012), *UAS-attP-VIE-260B* (KK control, VDRC: 60100; Dietzl *et al.*, 2007), *UAS-Ras^{G12V}* (Karim and Rubin, 1998), *UAS-yki^{3SA}* (gifted by Dr. Nic Tapon, Oh and Irvine, 2009), *UAS-Apc^{RNAi}* (VDRC GD 7748), *UAS-wts^{RNAi}* (VDRC KK106174), *UAS-Notch^{RNAi}* (VDRC GD27229), *UAS-mats^{RNAi}* (VDRC GD17716), *UAS-10XStat92E::GFP* (BDSC: 26198 (Bach *et al.*, 2007), *UAS-Mal-A1::GFP* (VDRC 318296), *UAS-Amy-p::GFP* (Hudry *et al.*, 2019), *UAS-bantam-sensor::GFP* (Brennecke *et al.*, 2003), *UAS-yki^{RNAi}* (VDRC GD50253, gifted by Dr. Nic Tapon), *UAS-tra^F* (Ferveur *et al.*, 1995), *UAS-tra^{RNAi}* (BDSC: 28512, TRiP.JF03132).

Controls: *w¹¹¹⁸* (GD control, VDRC: 60000; Kurkulos *et al.*, 1991), Oregon-R (Lindsley and Grell, 1967).

2.2 Immunostaining and imaging

2.2.1 Immunostaining

Midguts or male reproductive tracts were dissected in PBS and mounted on poly-D-lysine coated slides (Sigma, P1524). They were then fixed for 30 min in 4% formaldehyde in PBS, incubated for 15 min in PBT (0.2% Triton X-100 in PBS) to permeabilise the tissue, then blocked for 90 min with PBTN (2% horse serum in PBT), and stained with the primary antibody overnight at 4°C in PBTN. After washing with PBT, slides were stained with secondary antibody in PBTN for 2 hr at room temperature, then washed and mounted in Vectashield with DAPI (Vector Labs).

Primary Antibodies

Phospho-Histone H3 (Ser10), New England Biolabs, 1:1500 (rabbit)

Anti-GFP antibody (ab6673), Abcam, 1:1000 (goat)

Anti-RFP antibody (ab62341) Abcam 1:1000 (rabbit)

Anti-Fas3 antibody 7G10 serum (DSHB) 1:50 (mouse)

Anti-Prospero MR1A concentrate (DSHB) 1:1000 (mouse)

Secondary Antibodies

Anti-Rabbit Cy3, Jackson ImmunoResearch, 1:300

Anti-Rabbit Cy5, Jackson ImmunoResearch, 1:300

Anti-Mouse Cy3, Jackson ImmunoResearch, 1:300

Anti-Mouse Cy5, Jackson ImmunoResearch, 1:300

Anti-Goat FITC, Jackson ImmunoResearch, 1:300

Anti-Horseradish Peroxidase (HRP) Rhodamine (TRITC)-conjugated 1:300

2.2.2 Microscopy

Confocal microscopy

Images of the midgut (gut region indicated in figure legend) or male reproductive tract (region indicated in figure legend) were taken with the Leica SP8 DLS Confocal at 20X magnification with 1024 x 1024 pixel resolution. Z-stacks were taken with a z-step size of 4 µm. Images were analysed with FIJI ImageJ (v2.1.0/1.53d; Schindelin *et al.*, 2012), Adobe Photoshop (24.4.1) and Adobe Illustrator (27.5).

Microscopy

Midgut lengths were manually calculated in FIJI (v2.1.0/1.53d; Schindelin *et al.*, 2012) using images taken on a Nikon Eclipse 50i. Length was defined as being from the proventriculus to the midgut-hindgut border, as defined by the region of increased DAPI intensity.

pH3⁺ cell counts were quantified using a Nikon Eclipse 50i and Leica DM4B II. pH3⁺ cells were counted across the entire midgut, and pH3⁺ cells in the Malpighian tubules were excluded.

Measurement of intensity across midgut length

The intensity of GFP was measured along gut length in FIJI from maximum projections of z-stacks. Measurements were taken along a 30 pixel-wide line drawn manually using the freehand line tool through the centre of the midgut tube along the gut length. Using a landmark placed manually at the R3 region, gut length was adjusted relatively to give a percentage position along gut length with R3 aligned between sexes at 50% gut length. In R, intensity values for multiple flies were binned into 40 categories along gut length and the mean and standard deviation found for each bin. Please note this experiment, imaging and quantification was performed by Pedro Gaspar (**Figure 6.11d**).

2.3 Molecular biology

2.3.1 Determination of *esg-Gal4* insertion site

DNA extraction

This protocol was adapted by Pedro Gaspar.

Genomic DNA was extracted from one male fly per sample. Each fly was squished in 200 μ L of homogenisation buffer (160mM Sucrose, 80mM EDTA (pH 8.0), 100 mM Tris (pH 8.0), 0.5% SDS, 0.10 mg/ml Proteinase K) and incubated at 58°C overnight. A standard salt extraction protocol was then followed (Miller, Dykes and Polesky, 1988).

PCR reaction

A PCR reaction was set up using the Q5® High-Fidelity DNA Polymerase following the manufacturer's instructions (New England Biolabs, M0491). PCR products were run on a 0.8% agarose gel, and the strongest band cut and purified using QIAquick PCR Purification Kit following the manufacturer's instructions (QIAGEN, 28104).

<i>esgNP5130 gen rev</i>	GCTCATTACACGAAGCTGCCAAAAG
<i>esgNP5130 gen fwd</i>	TCGACGCATATACCTGAATGAACATGC
<i>esgNP7397 gen rev</i>	GGCGTTCCGCCGAGAGTATG
<i>esgNP7397 gen fwd</i>	GGCGAAACCTGTTTAGCCGAGTG
<i>pGawB gen rev</i>	CAGATTGTTTAGCTTGTTTCAGCTGCG
<i>pGawB gen fwd</i>	CCACTAGTGGATCTGGATCCTC

Sequencing

Sanger sequencing was carried out by GeneWiz using the *pGawB gen fwd* primer.

2.4 RNA sequencing data

This protocol was adapted from Jake Jacobson and Bruno Hudry by Pedro Gaspar.

For Oregon-R dataset: Dissection and extraction was performed by Tomotsune Ameku, sequencing was performed by Laurence Game and Ivan Andrew (MRC-LMS Genomics Facility) and data analysis was performed by Yi-Fang Wang (MRC-LMS Bioinformatics Facility) and Tomotsune Ameku.

For tumour dataset: Dissection and extraction was performed by me, sequencing was performed by Laurence Game and Ivan Andrew (MRC-LMS Genomics Facility) and data analysis was performed by Yi-Fang Wang and Jesús Urtasun Elizari (MRC-LMS Bioinformatics Facility).

2.4.1 Fly maintenance for RNAseq

For the Oregon-R dataset: 5 d old virgin male, virgin female and mated female flies (3 d post-mating) were raised at 25°C. Flies were dissected from fresh.

For the tumour dataset: flies were raised as described in **Section 2.1.1**, then flash frozen with liquid nitrogen and stored at -80°C until dissection date. Flies were dissected from frozen.

2.4.2 Dissection and tissue lysis

Dissection area and tools were prepared using RNaseZap (Invitrogen, AM9780) and DEPC-treated water. Samples were randomly selected on each dissection day to avoid batch effects and there were four replicates of each condition. 30 guts were pooled and combined with 180 µL Trizol and a steel ball. Tissue was lysed in the TissueLyser (2 min at 25 Hz) and stored at -80°C before RNA extraction.

2.4.3 RNA extraction

Dissection area and tools were prepared using RNaseZap (Invitrogen, AM9780) and DEPC-treated water. RNA extraction was performed over three days, with samples randomly assigned to each day to avoid batch effect. 200 µL chloroform was added, samples were inverted and mixed for 15 sec, incubated for 3 min and centrifuged at 12,000 G for 15 min at 4 °C. The clear aqueous phase was removed and added to an equal volume µL ice-cold isopropanol and RNA extraction was performed with the QIAGEN RNeasy Mini Kit (Qiagen, 74104).

2.4.4 RNA sequencing

Sequencing was performed by Laurence Game and Ivan Andrew at the MRC-LMS Genomics Facility. RNA quality was assessed using the Agilent 2100 RNA 6000 Nano assay and libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit with NEBNext Poly(A) mRNA Magnetic Isolation Module following the manufacturer's instructions.

Library quality was evaluated using the Agilent 2100 High-Sensitivity DNA assay, and their concentrations measured using the Qubit™ dsDNA HS Assay Kit. Libraries were pooled in equimolar quantities and sequenced on a Nextseq P3-100 to generate a minimum of 1.2 billion paired end 60 bp reads per sample.

2.4.5 Alignment and DESeq2

FasQC (v0.11.9) was used to generate FastQC results. Reads were aligned to *Drosophila* (dm6) using STAR (Dobin et al., 2013). Using MultiQC (v1.9) a summary report was generated for the FastQC and STAR alignment results. The count matrix was prepared by Principal Component Analysis (PCA) using R (v3.6.0, R Core Team, 2021). Normalisation and differential expression analysis was performed with the DESeq2 R Bioconductor package (Love, Huber and Anders, 2014). Differentially expressed genes were defined with an adjusted p-value (p-adj) ≤ 0.05 .

2.4.6 Gene Ontology Analysis

Gene Ontology Analysis was performed with goseq (Young *et al.*, 2010), using the DE gene lists with a threshold of 1.5 FC and p-adj ≤ 0.05 .

2.4.7 Data Plotting

PCA plot was produced with R (v4.1.2, R Core Team, 2021). Bubble plots and heat maps were plotted with by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang *et al.*, 2023). Bar plots were plotted with GraphPad Prism (v9.5.1).

2.5 Statistical analysis

Parametric tests (midgut length)

For pairwise comparisons the t-test was performed and for multiple comparisons an ordinary one-way ANOVA followed by Dunn's multiple comparisons test was performed.

Non-parametric tests (pH3 counts)

For pairwise comparisons the Mann-Whitney test was performed and for multiple comparisons the Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed.

All data if not stated otherwise was presented using GraphPad Prism v9.5.1 for Mac OS, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com (GraphPad Prism, 2023). For each test, significance is as follows:

ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Chapter 3: Results I

3.1 Introduction

Cancer susceptibility and progression changes with both sex and reproductive status across a range of different tissues. How this changes with tumours with different genetic origins has not been studied in depth. This chapter explores the relationship between different genetic tumours and sex and reproductive status, using the *Drosophila* midgut as a model. I describe an investigation into the expression of different *esg-Gal4* drivers, used to generate tumours by stimulating the proliferation of the intestinal stem cell (ISC) and enteroblast (EB) populations. I use quantification of the number of phospho-Histone H3-positive (pH3⁺) cells as a marker of proliferating cells to compare the different tumours. I compare tumours in virgin males versus virgin females, to study sex, and in virgin females versus mated females to study reproduction. I show that tumours with different genetic origins have different levels of proliferation depending on their sex and reproductive status. I compare this across different lengths of time of tumour induction and show how different genetic tumour models behave over time.

3.1.1 *esg-Gal4^{ts}* as a tool to express genes in the *Drosophila* midgut stem cells

To generate tumours in the adult midgut I used *esg-Gal4^{ts}*. *esg* is a member of the highly conserved Slug/Snail family of transcription factors (Whiteley *et al.*, 1992; Nieto, 2002). *esg-Gal4* induces Gal4 expression in the midgut intestinal stem cells (ISC), and transient progenitor enteroblasts (EB) (Micchelli and Perrimon, 2006; Jiang and Edgar, 2009). In these cells the function of *esg* is to maintain stemness in ISC and EB by suppressing pro-differentiation genes like the enterocyte (EC) marker *Pdm1* (Korzeliuss *et al.*, 2014; Loza-Coll *et al.*, 2014). However, *esg* is important in stem cells of other tissues, including the somatic hub cells, somatic cyst stem cells (CySC) and germline stem cells (GSC) of the testes (Kiger, White-Cooper and Fuller, 2000; Voog *et al.*, 2014). Both CySC and hub cells are maintained by *esg*-mediated suppression of the insulin signalling pathway (Sênos Demarco *et al.*, 2022). *esg* has been shown to be very male-biased in early gonad development and is only expressed in the developing testes, with no *esg* transcript found in developing ovaries (Streit *et al.*, 2002; Le Bras and Van Doren, 2006). However, some *esg-Gal4* expression has been found in some of the escort cells of the adult ovary germarium (Reiff *et al.*, 2015; Ahmed *et al.*, 2020).

There is also a small amount of *esg-Gal4* expression in the adult brain within a few cells of the suboesophageal ganglion (Nagai *et al.*, 2023).

3.1.2 Quantifying tumour proliferation

A common way of quantifying tumour proliferation is by counting the number of pH3⁺ cells. This histone mark has been shown to closely correlate with the condensation of mitotic and meiotic chromosomes that occurs during cell division across many species (Nagai *et al.*, 2023). Antibodies against specific phosphorylated residues, like Ser-10 (Paulson and Taylor, 1982; Hendzel *et al.*, 1997) and Ser-28 (Goto *et al.*, 1999) allow mitotic and meiotic cells to be identified (Hendzel *et al.*, 1997; Wei *et al.*, 1999). This stain is used to mark proliferating cells in *Drosophila* (Su *et al.*, 1998) and its use as a marker of proliferation is conserved in mammalian tissue and tumour samples (Brenner *et al.*, 2003; Ribalta *et al.*, 2004).

In the *Drosophila* midgut the number of mitotic cells, as marked by the pH3 staining, has been shown to be linked to circadian rhythms (Karpowicz *et al.*, 2013). As such, when the gut is responding to damage the peak of pH3⁺ cells is at dawn. Although this has not been shown to occur in a tumour context, I endeavoured to always perform my pH3 dissections at the same time of day in the early afternoon.

3.1.3 Generating genetic tumours in the *Drosophila* midgut

Tumours can be generated in the midgut using *esg-Gal4^{ts}*, by either downregulating tumour suppressors or overexpressing oncogenes. This uses expression of *esg-Gal4* in combination with *tub-Gal80^{ts}*, a temperature sensitive Gal4 repressor that is degraded at 29°C, allowing adult specific transgene expression (**Section 1.7**). This will be referred to as tumour induction throughout my thesis.

Previous studies using *esg-Gal4^{ts}* have induced tumours using *Notch^{RNAi}* (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Patel, Dutta and Edgar, 2015), constitutively active *Ras^{G12V}* (Doggett *et al.*, 2015; Lee *et al.*, 2020) often in combination with null alleles or knockdown of *Apc* (Martorell *et al.*, 2014) and modulations of the Hippo pathway such as constitutively active *yorkie* (*yki^{3SA}*; Song *et al.*, 2019; Bajpai *et al.*, 2020; Lee *et al.*, 2021). Such genetic modulations generally

result in hyperproliferation across the midgut, as well as some multi-layered tissue, and depending on the model, systemic effects like organ wasting (Kwon *et al.*, 2015). Throughout my thesis, I will be referring to this hyperproliferation as a tumour.

I initially chose to look at *Ras*^{G12V} *Apc*^{RNAi} tumours because of the relevance of these mutations to human colon cancer (**Section 1.2.1**). I also chose to look at several Hippo pathway tumours, a pathway often deregulated in human cancer (**Section 1.2.2**).

3.1.4 Notch tumours in the *Drosophila* midgut

Notch is a ligand key to fate determination in the midgut epithelium. After ISC divide, if their progeny has low Notch signalling it differentiates into an EE cell (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Sustained downregulation with *Notch*^{RNAi} or *Notch*^{DN} (dominant negative) with *esg*^{ts} results in massive overgrowth of EE-precursor and EE cells, and prevention of differentiation into the EC lineage (Patel, Dutta and Edgar, 2015). These tumours are sometimes referred to as endocrine or neuroendocrine tumours, and require cell autonomous Spitz/EGFR signalling to maintain their high proliferation. They have been noted as being quite stochastic, as not all experimental animals will develop them despite all having the same block to differentiation. Efficiency of tumour induction can be increased if the gut has an additional stress, for example infection with *Pseudomonas aeruginosa* (Apidianakis *et al.*, 2009) increasing JAK-STAT signalling with *Upd1* overexpression or the pro-mitotic transgene combination of *CyclinE* and *string* overexpression (Patel, Dutta and Edgar, 2015).

3.1.5 *Ras* and *Apc* tumours in the *Drosophila* midgut

Ras85D (henceforth *Ras*) is a small GTPase and proto-oncogene that controls a range of signalling networks as a molecular switch in the Ras/Raf/MAPK pathway. It has a highly conserved function in mediating cell growth, proliferation, differentiation and migration (Molina and Adjei, 2006). *Ras*^{G12V} (also called *Ras*^{ACT}) is a oncogenic form of *Ras*, with a single mutation that causes constitutive activation (Bishop and Corces, 1988; Karim and Rubin, 1998). It protects cells from apoptosis and promotes neoplastic growth (Brumby and Richardson, 2003; Uhlirova, Jasper and Bohmann, 2005). In midgut MARCM clones, *Ras*^{G12V} produces small clones that disappear over

time independently of apoptosis (Martorell *et al.*, 2014). These disappearing cells were investigated further by another group that expressed *Ras*^{G12V} in the midgut with *esg*^{ts}. They showed although the ISC and EBs initially proliferate, they will then produce actin and cortactin rich protrusions allowing them to invade through the basement membrane and visceral muscle, leading to the disappearance of *Ras*^{G12V} cells across the midgut. These cells can be found in the haemolymph but do not metastasise (Lee *et al.*, 2020). Expressing *Ras*^{G12V} alone with *esg*^{ts} can have a somewhat mild effect on proliferation, but this is greatly enhanced with *Pseudomonas aeruginosa* infection or a mild increase in JNK pathway activity in a *puckered* (*puc*) mutant background (Apidianakis *et al.*, 2009).

Apc is a large multidomain protein and tumour suppressor that binds to many protein partners to mediate the Wnt signalling pathway and cellular adhesion (Fearnhead, Britton and Bodmer, 2001). In FLIP/FRT clones in the midgut *Apc1* and *Apc2* mutants increase ISC proliferation by activating Wingless (Wg, the *Drosophila* Wnt) signalling (Lee *et al.*, 2009). In midgut MARCM clones, *Apc* mutant alleles produce small clones that increase in size slightly over time (Martorell *et al.*, 2014). Such clones have also been shown to non-autonomously activate JNK signalling to induce chronic EGF signalling (Ngo *et al.*, 2020).

In combination, *Ras*^{G12V} overexpression and *Apc* mutation or downregulation in midgut MARCM clones causes much larger overgrowths than each gene in isolation (Martorell *et al.*, 2014). *Ras*^{G12V} further promotes Wg signalling induced by *Apc* loss (Wang *et al.*, 2013). The clones lose cell polarity, become multi-layered and begin to extrude from the visceral muscle (VM). Most are negative for ISC marker Delta (DI), EC marker Pdm1 and EE marker Prospero (Pros), indicating a build-up of partially differentiated EB-like cells (Martorell *et al.*, 2014).

3.1.6 *yki* and *wt*s tumours in the *Drosophila* midgut

yki is a transcription factor regulated by the highly conserved Hippo pathway. When the Hippo pathway is downregulated, *yki* can induce transcription of genes important for cell proliferation, apoptosis and organ size (Liu, Liu and Song, 2021). The triple mutant *yki*^{3SA} (*yki*^{S111A-S168A-S250}, also called *yki*^{ACT}) is a constitutively active oncogene

that causes massive tissue overgrowth (Oh and Irvine, 2009). In the midgut, when expressed with *esg^{ts}* it causes large amounts of ISC proliferation. However, unlike *Ras^{G12V}*, the majority of cells maintain their adherens junctions and do not delaminate and leave the midgut. The tumours are heterogenous, with some cells with larger nucleus size becoming positive for the EC marker Pdm1 and other maintaining an EB-like undifferentiated state. However, a subset of cells with high expression of matrix metalloproteinases (MMP) can basally delaminate, cross the VM and extracellular matrix (ECM) and disseminate (Pranoto, Lee and Kwon, 2023).

esg^{ts}-driven *yki^{3SA}* midgut tumours additionally secrete cachectic ligands including Pvf1, Upd3 and ImpL2. This causes systemic organ wasting, including ovary atrophy, and fat body and muscle degeneration, as well as metabolic defects including hyperglycaemia (Kwon *et al.*, 2015; Song *et al.*, 2019; Ding *et al.*, 2021). *yki^{3SA}* tumours produce Vein (Vn) ligand to trigger cell autonomous EGFR/MAPK induced proliferation and production of the Pvf1 ligand, which contributes to wasting (Song *et al.*, 2019). ImpL2 mediates wasting by reducing insulin/IGF signalling. In the midgut tumour itself, the deleterious effects of high ImpL2 are prevented by Wg upregulation (Lee *et al.*, 2021).

wts is a Hippo pathway kinase that alongside *mats* is responsible for phosphorylating and inactivating *yki*, thus preventing increases in proliferation and tissue size. Downregulation with *esg^{ts}* using *wts^{RNAi}* or in MARCM clones with the *wts^{X1}* loss of function allele causes a large increase in ISC proliferation, and acts via *yki* (Karpowicz, Perez and Perrimon, 2010; Ren *et al.*, 2010; Shaw *et al.*, 2010; Staley and Irvine, 2010). Thus, this genetic tumour model behaves similarly to a *yki* overexpression tumour. No-one has studied if *wts* downregulation has the same metabolic effects as *yki* overexpression. *wts* may have some functions independent of *yki*, but this remains to be further investigated (Hao *et al.*, 2020).

3.1.7 Previous work on the effect of sex and reproduction on midgut tumourigenesis

As described in **Section 1.5.2**, females are generally more prone to midgut tumours than males. This has been shown for *esg^{ts}*-driven *Notch^{RNAi}* (Hudry, Khadayate and

Miguel-Aliaga, 2016; Ahmed *et al.*, 2020) and *Apc-Ras* MARCM clones (Hudry, Khadayate and Miguel-Aliaga, 2016). Other conditions that trigger high proliferation also agree with this. Females have a greater proliferative response than males in response to damage inducing agents DSS (using virgin females; Hudry, Khadayate and Miguel-Aliaga, 2016) and SDS (using mated females; Ahmed *et al.*, 2020). Mated female midguts also proliferate more than males in response to *Ecc* (Regan *et al.*, 2016) and *P.e* infection (Ahmed *et al.*, 2020).

Mating induces many changes in the female midgut, as described in **Section 1.5.3**. However, only one study has compared virgin and mated females midgut tumourigenesis: *esg^{ts}*-driven *Notch^{RNAi}* tumours, where mated females midguts proliferate more than virgin females (Ahmed *et al.*, 2020). There is evidence that these *Notch^{RNAi}* tumours in mated females are very sensitive to 20HE (Ahmed *et al.*, 2020; Zipper *et al.*, 2020). Midgut *Apc-Ras* clones are also sensitive to JH signalling in females (mating status undisclosed; Rahman *et al.*, 2017). As these studies show increased 20HE or JH promotes tumourigenesis, and mating induces the titres of both these hormones, it is expected mated females will proliferate more than virgin females.

This work seeks to investigate how both sex and reproductive status affects tumour formation, firstly by quantifying tumour proliferation across several genetic tumour models.

3.2 Characterisation of *esg* drivers

3.2.1 Characterisation of *Gal4* insertion of *esg* drivers

As I began accumulating fly stocks, I set out to identify which *esg-Gal4* enhancer trap my lab was using. The NP consortium generated different insertions into the *esg* gene locus, and several are commonly used within the fly community (Hayashi *et al.*, 2002). However, it is often not specifically labelled which construct is being used. The two most commonly used are the *esg^{NP7397}* insertion (Jiang *et al.*, 2009; Perochon *et al.*, 2021) and the *esg^{NP5130}* insertion (Tamamouna *et al.*, 2021).

Together with our senior research scientist Pedro Gaspar, we designed two primers sets for the two likely insertions, *esg^{NP7397}* and *esg^{NP5130}*, used alongside the pGawB

plasmid primers, from the plasmid used originally to generate the stock. Using two positive controls of *esg*^{NP7397} and *esg*^{NP5130} from the DGRC-Kyoto Stock Centre, we analysed five lines: *esg-flipout* (*esg*^{F/O}; Jiang *et al.*, 2009), two fly lines from our lab stocks (*esg*-IMA 1 and 2), the lineage tracing *esg*^{ReDDM} (Antonello *et al.*, 2015) and the *esg-Gal4* line kindly gifted to us by the Cordero lab (*esg*-JC; Perochon *et al.*, 2021).

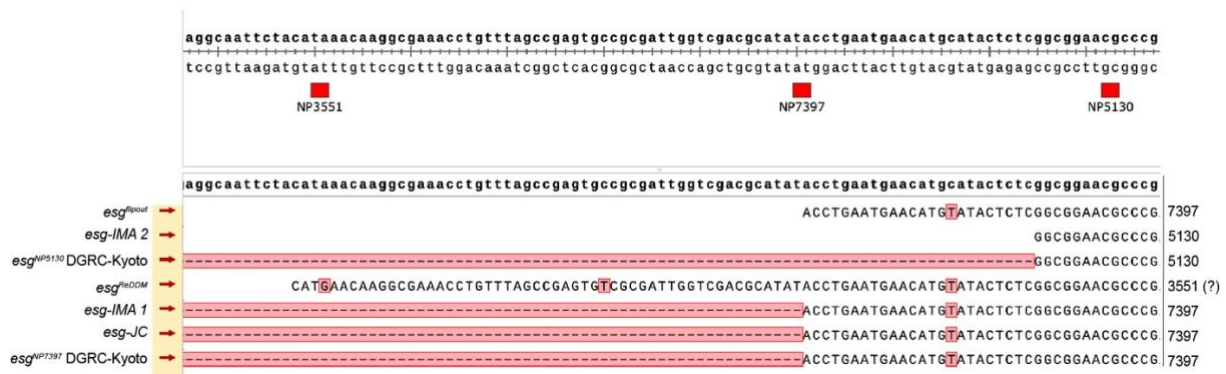


Figure 3.1. Identification of insertion site of *esg*-Gal4.

Blast alignment of sequencing of *esg* locus area for several fly stocks with unknown *esg* insertion. Insertion sites of *Gal4* have been identified as three different insertions: *esg*^{NP7397}-*Gal4*, *esg*^{NP5130}-*Gal4* and *esg*^{NP3551}-*Gal4*. Sequencing performed in collaboration with Pedro Gaspar.

We sent these for Sanger sequencing, and identified that we were using the *esg*^{NP7397}-*Gal4* in our main lab stock IMA 1, which is also used in *esg*^{F/O} and in the stock used in the Cordero lab (**Figure 3.1**). We found our other lab stock IMA 2 matches the sequence for the *esg*^{NP5130} control, but that this control insertion is 8bp from the Flybase annotation (**Figure 3.1**). We also found the *esg*^{ReDDM} stock uses another different insertion, likely to be *esg*^{NP3551} but 2bp away from the Flybase annotation (**Figure 3.1**).

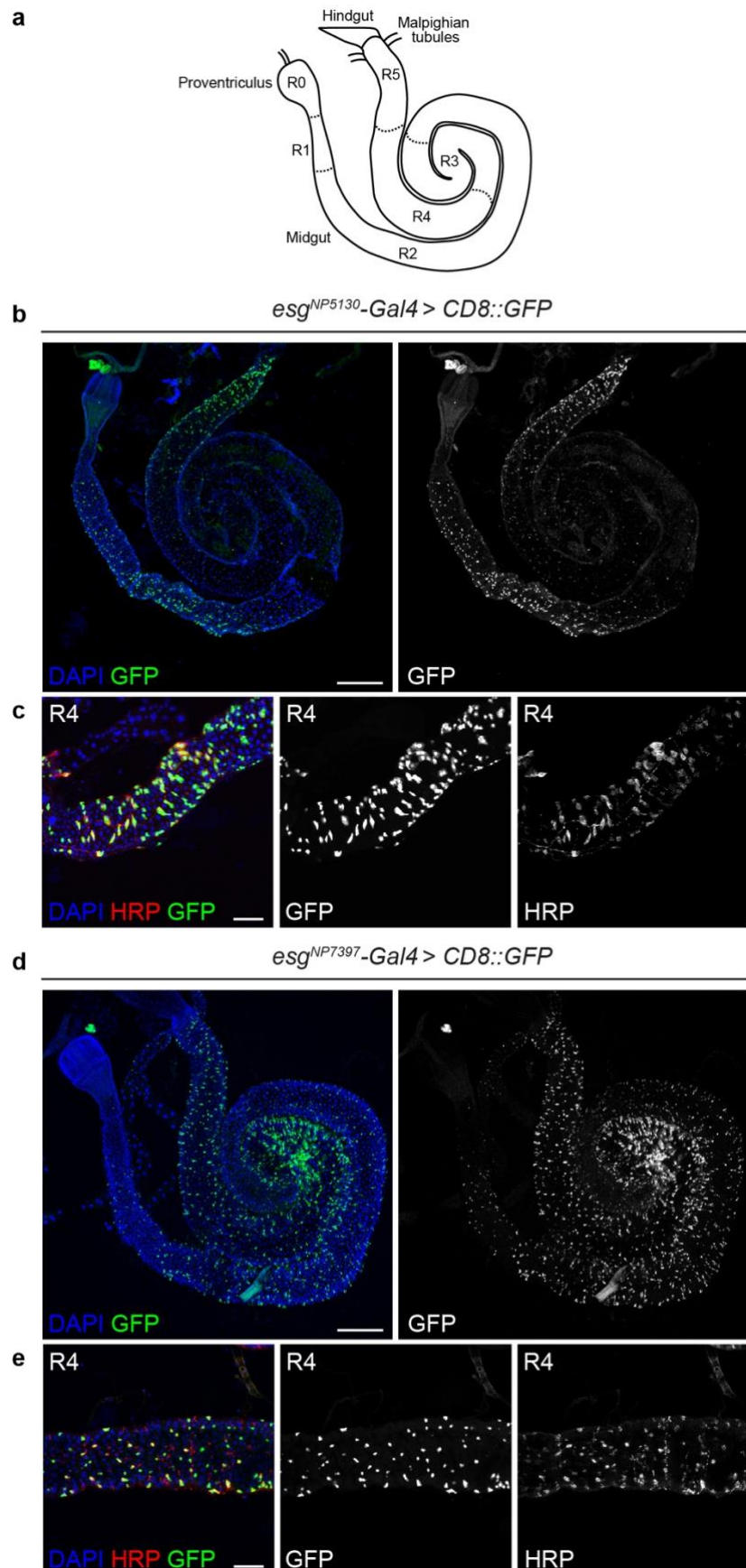
3.2.2 Characterisation of *esg*-*Gal4* expression pattern

As the exact location of the *Gal4* insertion determines the expression pattern, we also set out to verify any differences in the expression pattern of *esg*^{NP7397}-*Gal4* and *esg*^{NP5130}-*Gal4*. Pedro Gaspar performed the dissection and imaging of midguts for this section (**Section 3.2.2**). Horseradish peroxidase (HRP) stain was used to label all ISC and EB in the midgut, and the *esg*-*Gal4* flies were crossed to *CD8::GFP* to identify which cells are labelled. This interestingly showed that in both drivers, not all ISC and

Figure 3.2. Midgut expression pattern of *esg^{NP5130}-Gal4* and *esg^{NP7397}-Gal4*.

a. Schematic of the midgut regions when the midgut is mounted in a spiral shape. The gut begins at the proventriculus (foregut). The midgut regions R0-R5 are labelled with dotted lines, followed by the Malpighian tubules and the hindgut. **b.** Immunostaining showing expression of *esg^{NP5130}-Gal4 > CD8::GFP* across the whole midgut. **c.** Immunostaining showing expression of *esg^{NP5130}-Gal4 > CD8::GFP* in the R4 of the midgut. **d.** Immunostaining showing expression of *esg^{NP7397}-Gal4 > CD8::GFP* across the whole midgut. **e.** Immunostaining showing expression of *esg^{NP7397}-Gal4 > CD8::GFP* in the R4 of the midgut. HRP (horseradish peroxidase) staining marks ISC and EB. Scale bar = 200 μ m in whole midgut images (b, d), and 50 μ m in R4 images (c, e).

Imaging performed by Pedro Gaspar.



EB are *esg*/GFP⁺ (**Figure 3.2**). This is especially apparent in *esg*^{NP7397}-*Gal4*, where there are many HRP⁺ GFP⁻ cells in R4 (**Figure 3.2e**). There was also a difference in the distribution of *esg*⁺ cells across the midgut. *esg*^{NP7397}-*Gal4* labelled more uniformly, with higher levels of *esg* expression in R3 (**Figure 3.2d**). However, *esg*^{NP5130}-*Gal4* has almost no expression in the R2/R3 and R3 regions, but very strong expression in R1/R2 and R5 (**Figure 3.2b**). These images also reveal strong *esg* expression in several large nuclei cells by the proventriculus of the gut (**Figures 3.2b, d**), which may be the garland nephrocytes, although I have not verified this.

Ultimately, I decided to continue using *esg*^{NP7397}, due to its more uniform expression and its continuity with the work the Miguel-Aliaga lab has previously published (Hudry, Khadayate and Miguel-Aliaga, 2016). From here onwards, all use of *esg*-*Gal4* unless otherwise stated will be *esg*^{NP7397}-*Gal4*.

3.3 Characterisation of tumour response to sex and reproductive status

3.3.1 Initial characterisation of tumour response to sex and reproductive status

After crossing *esg*-*Gal4*, *tubulin*-*Gal80*^{ts} (hereafter, *esg*^{ts}) and *UAS* flies, virgin male and female flies were collected and aged at 18 °C for at least 7 d to ensure full gut development. After 1 d mating if appropriate, tumour transgenes were induced for 4 - 12 d by switching to 29 °C (**Figure 3.3a**). Fly midguts were dissected, fixed and stained with pH3 to identify cells undergoing mitosis. The total number of pH3⁺ cells across the midgut was counted, and used as a measurement of tumour proliferation. Virgin females were compared separately to virgin males and to mated females, to allow separate analysis of the effects of sex and mating respectively.

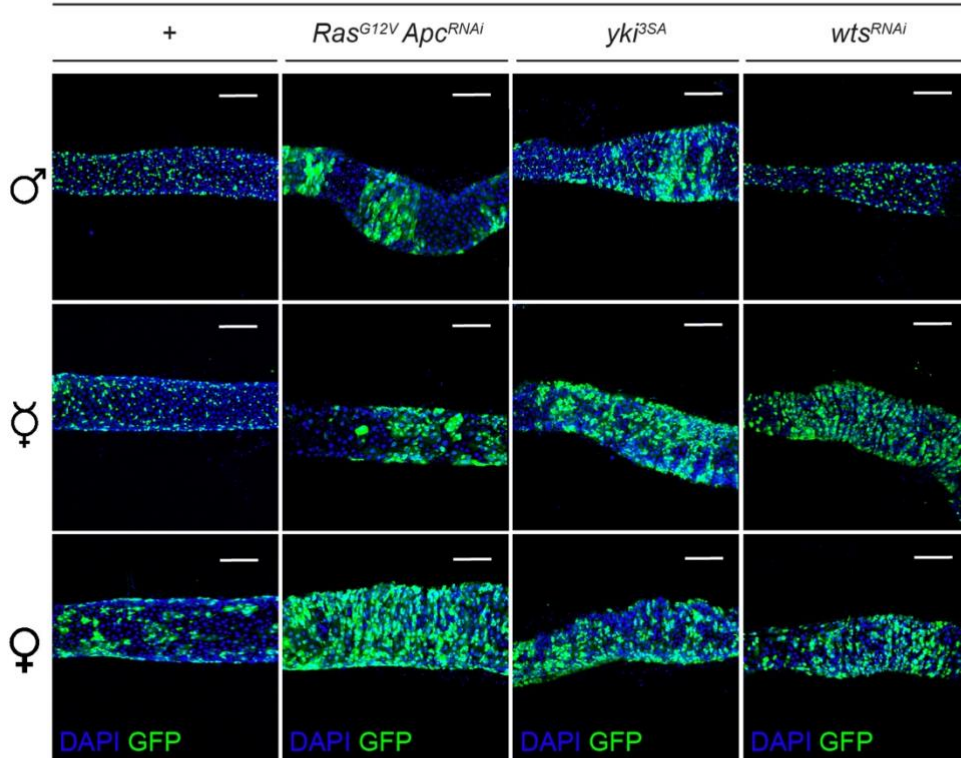
In a control gut, expressing only GFP in their ISC and EBs for 6 d, there is very low proliferation in both virgin males and females. In mated females there is a small increase in proliferation and an increase in GFP⁺ ISC and EC (**Figures 3.3b, c**), necessary for the expansion of the gut as shown in previous reports (Reiff *et al.*, 2015).

Protocol for tumour induction

Experimental timeline diagram showing the progression from cross set up to dissection at 18 °C and 29 °C. The timeline includes stages for cross set up, eclosion, maturation, and tumour induction. A specific 1-day mating period is highlighted in red.

18 °C		29 °C	
cross set up	eclosion	maturation	tumour induction
21 d	8 d	4 - 10 d	dissection
21 d	8 d	4 - 10 d	
21 d	7 d	1 d (mating) 4 - 10 d	
larvae		adult	

Tumour growth in R4 of midgut

$$esg^{ts} >$$


Midgut mitoses

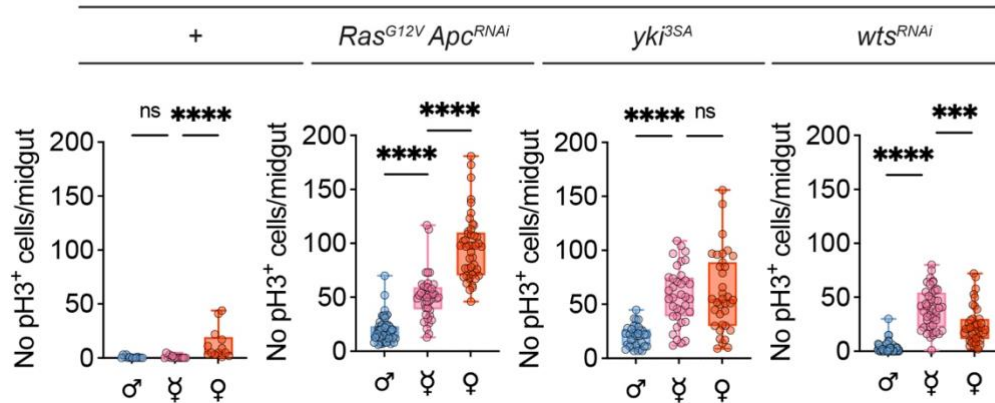
$$esg^{ts} >$$


Figure 3.3. Different genetic tumours respond differently to sex and reproduction (previous page).

a. Protocol for inducing midgut tumours in virgin male, virgin female and mated female adult flies. **b.** Immunostaining of midgut tumours driven by *esg^{ts}* crossed to the GFP control (+), *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* or *wts^{RNAi}* induced for 6 d. **c.** Quantification of mitosis for the above conditions measured by the number of pH3⁺ cells per midgut. ♂ - virgin males, ♀ - virgin females, ♀ - mated females. The Mann-Whitney statistical test is used to separately compare sex and reproduction. ns $p > 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale bar = 100 μ M.

At 6 d of tumour induction, the combined model of *Ras^{G12V}* and *Apc^{RNAi}* shows an increase in proliferation in all conditions, with the lowest proliferation in males and highest in mated females (**Figures 3.3b, c**). All conditions show expanded ISC and EB populations in the R4 region, especially the mated females.

For the constitutively active *yki^{3SA}* at the 6 d timepoint, males and females both had increased proliferation, but the difference in proliferation was lost between virgin and mated females (**Figures 3.3b, c**). Again, all conditions show expanded R4 ISC and EB populations.

After this unexpected loss of response to reproductive status in *yki^{3SA}* mated female proliferation, I tested another Hippo pathway member, downregulation of the *wts* kinase with *wts^{RNAi}*. Here, unexpectedly, there was almost no response for the virgin males, who had only slightly more proliferation than control males. Their ISC and EB populations appear evenly distributed like the control male. Additionally, virgin females had more proliferation than mated females, reversing the trend seen in the control and *Ras^{G12V} Apc^{RNAi}* model (**Figures 3.3b, c**).

These results indicate that the effect of sex and reproductive status on midgut tumour proliferation is dependent on the genetic tumour model.

3.3.2 Characterisation of tumour response to sex and reproductive status across time

As each genetic tumour model varies in its magnitude of proliferation, I further investigated how sex and reproductive status changes proliferation over several timepoints after tumour induction.

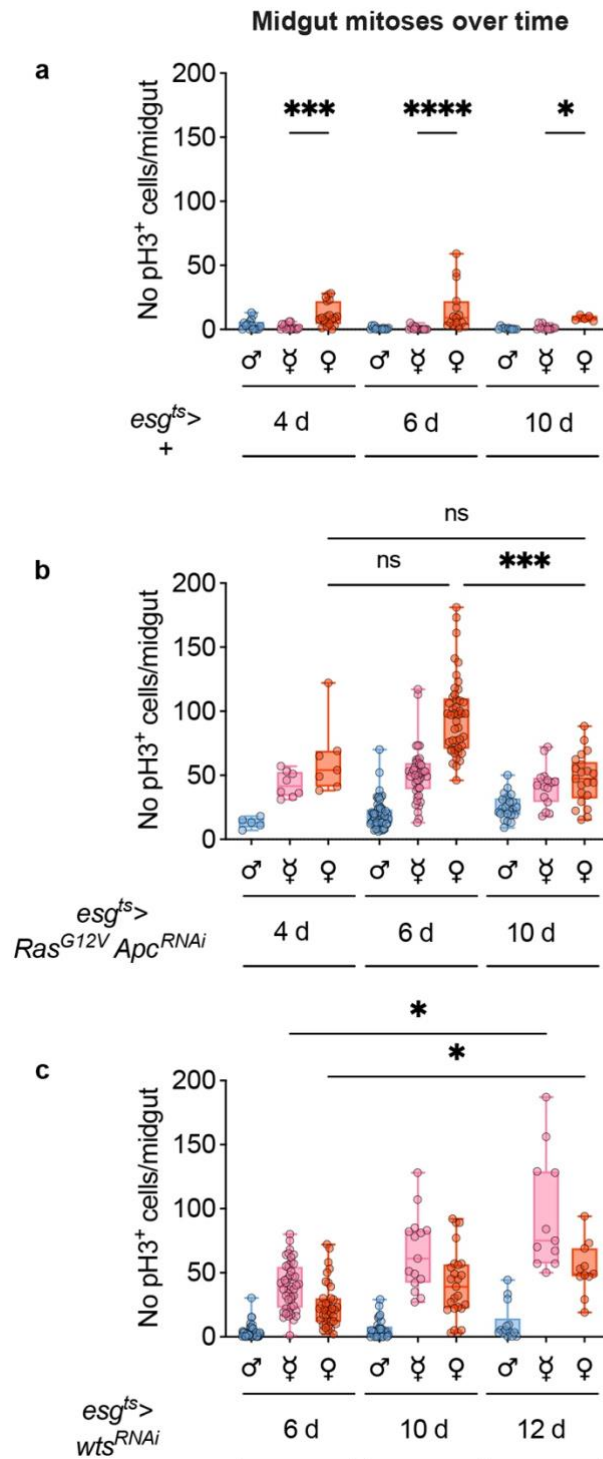


Figure 3.4. Genetic tumours respond to sex and reproduction differently over time.

Quantification of tumours driven by *esg^{ts}* with mitosis measured by number of pH3⁺ cells per midgut in virgin males, virgin females and mated females. **a.** The GFP control (+) induced for 4 d, 6 d and 10 d shows no significant changes within each condition over time. **b.** The *Ras^{G12V} Apc^{RNAi}* condition induced for 4 d, 6 d and 10 d shows the mated female peak of proliferation at 6 d is transient. **c.** The *wts^{RNAi}* condition induced for 6 d, 10 d and 12 d shows mated and virgin females continue to proliferate over time. Data for 6 d timepoint is the same data as in

Figure 3.3. ♂ - virgin males, ♀ - virgin females, ♀ - mated females. The Kruskal-Wallis statistical test is used to compare across reproduction and time, ns $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$.

I also assessed *Ras*^{G12V} *Apc*^{RNAi} at 4 d and 10 d timepoints, and *wt*^{sRNAi} for 10 d and 12 d timepoints. The control condition shows no significant changes across time, and at 10 d there is still more proliferation in the mated female than the virgin female, although less than at the 6 d timepoint (**Figure 3.4a**). The 12 d timepoint is missing for the control condition.

The *Ras*^{G12V} *Apc*^{RNAi} tumours only have a difference between virgin and mated female proliferation at the 6 d timepoint. At 4 d, there is not yet a difference, and at 10 d, the proliferation in the mated female is greatly reduced. Males have a slight increase in proliferation from 4 d to 10 d (**Figure 3.4b**).

However, *wt*^{sRNAi} tumours have a different response. By the 10 d and 12 d timepoints, the majority of the male midguts still have no proliferation, although there are a few that have high proliferation. Both virgin and mated females continue to proliferate with time, with especially high virgin female proliferation (**Figure 3.4c**).

Thus, despite the *Ras*^{G12V} *Apc*^{RNAi} tumours inducing more proliferation than the *wt*^{sRNAi} tumours, the tumour behaviour over time is markedly different. *Ras*^{G12V} *Apc*^{RNAi} maintain their high mated female proliferation only at 6 d of induction, whereas *wt*^{sRNAi} continue to proliferate over time, especially in the virgin condition. The failure of *Ras*^{G12V} *Apc*^{RNAi} tumours to continue to proliferate over time may be linked to the observation that *Ras*^{G12V} cells can disseminate from the midgut (Lee *et al.*, 2020), which may be highest in the condition with the most proliferation. It may also imply that female *Ras*^{G12V} *Apc*^{RNAi} tumours are influenced by the transient hormonal changes induced by mating and thus decrease in proliferation over time (White *et al.*, 2021). However, *wt*^{sRNAi} continues to increase with time, potentially implying independence of the hormonal input of mating.

3.3.3 Characterisation of tumour response to sex and reproductive status across additional models

I looked at three further genetic models to see if they shared any similarity with my results. *Ras*^{G12V} showed proliferation in all conditions, with a significant increase in

proliferation in virgin females compared to virgin males, and the highest proliferation in mated females (**Figure 3.5a**).

Notch^{RNAi} tumours are the predominant *Drosophila* midgut model. At 6 d of tumour induction, I found them stochastic, with most guts having no proliferation, and some beginning to have high numbers of pH3⁺ cells. The mated females had the most guts with higher proliferation, but not enough to be significant (**Figure 3.5b**).

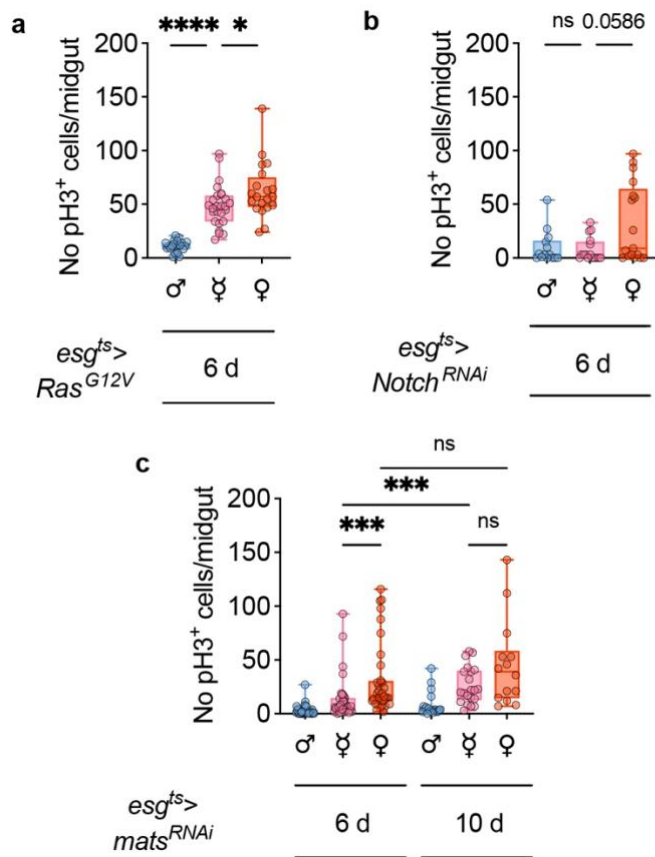


Figure 3.5. Other genetic tumours and their responses to sex and reproduction.

Quantification of tumours driven by *esg*^{ts} with mitosis measured by the number of pH3⁺ cells per midgut in virgin males, virgin females and mated females. **a.** The *Ras*^{G12V} condition has low male proliferation, higher virgin female proliferation and the highest proliferation in mated females after tumour induction for 6 d **b.** The *Notch*^{RNAi} condition is highly stochastic and shows low virgin male and virgin female proliferation, but higher mated female proliferation. **c.** The *mats*^{RNAi} condition shows lower proliferation than other conditions, with a reproductive difference at 6 d but not 10 d of tumour induction. ♂ - virgin males, ♀ - virgin females, ♀ - mated females. The Mann-Whitney statistical test is used to separately compare sex and reproduction for **a** and **b**, and the Kruskal-Wallis statistical test is used to compare across reproduction and time for **c**. ns *p* > 0.05, * *p* ≤ 0.05, *** *p* ≤ 0.001, **** *p* ≤ 0.0001.

Regulatory protein *mats* is the coactivator of *wts*. Downregulation of *mats* at 6 d led to limited proliferation of virgin males and females, and some proliferation of mated females. As this proliferation was less robust than *wts*^{RNAi}, I also quantified the 10 d timepoint. At 10 d, the males were quite resistant to tumourigenesis, with the majority

only having a few mitotic cells. The virgin and mated females had some proliferation, with higher mated female proliferation at 6 d but not 10 d. Between the 6 d and 10 d timepoint, only the virgin female had a significant increase in proliferation (**Figure 3.5c**).

Hence, analysis of these additional models supports the view that most genetically induced tumours have low male proliferation and high mated female proliferation, and that Hippo pathway tumours escape this pattern.

3.3.4 Characterisation of tumour growth across the midgut and midgut length

After investigating these further tumour models, I chose to focus on how sex and reproductive status modulates *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} and *wt*^{sRNAi} tumours. Although my pH3⁺ cell counts are from the entire midgut, my imaging previously focussed on the midgut R4 (**Section 3.3.1**). As my *esg*^{ts} driver is expressed in ISC and EB across the entire midgut (**Section 3.2.2**), I wanted to see how the different regions of the gut proliferate (**Figure 1.4b**).

Across the midgut of the *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} and *wt*^{sRNAi} tumour models, I see ISC and EB expansion across R2, R4 and R5, with less growth in R1 and R3 (**Figure 3.6a**). Each of my genetic tumour models follows this pattern, with the most ISC and EB expansion in R4, then R5 and R2. There is consistently less growth in R1 and R3 (**Figure 3.6a**), although ISC and EB in R3 do begin to expand with the models with higher proliferation like *Ras*^{G12V} *Apc*^{RNAi} tumours (**Figure 3.6a**). The *wt*^{sRNAi} males that show almost no proliferation have guts very similar to the control males although slightly wider (**Figure 3.6a**). They have evenly distributed ISC and EB, with mainly single progenitors and no patches of GFP indicating clusters of proliferating cells. *yki*^{3SA} tumours in females have more severe overgrowth than *wt*^{sRNAi} tumours in females, reflecting their higher pH3 counts. These observations would be complemented by quantifications of GFP across R2, R3 and R4.

As it is known that gut length is sexually dimorphic (Hudry, Khadayate and Miguel-Aliaga, 2016) and changes with reproduction (Reiff *et al.*, 2015), and I have shown that two Hippo pathway tumours do not respond to reproduction, I measured midgut

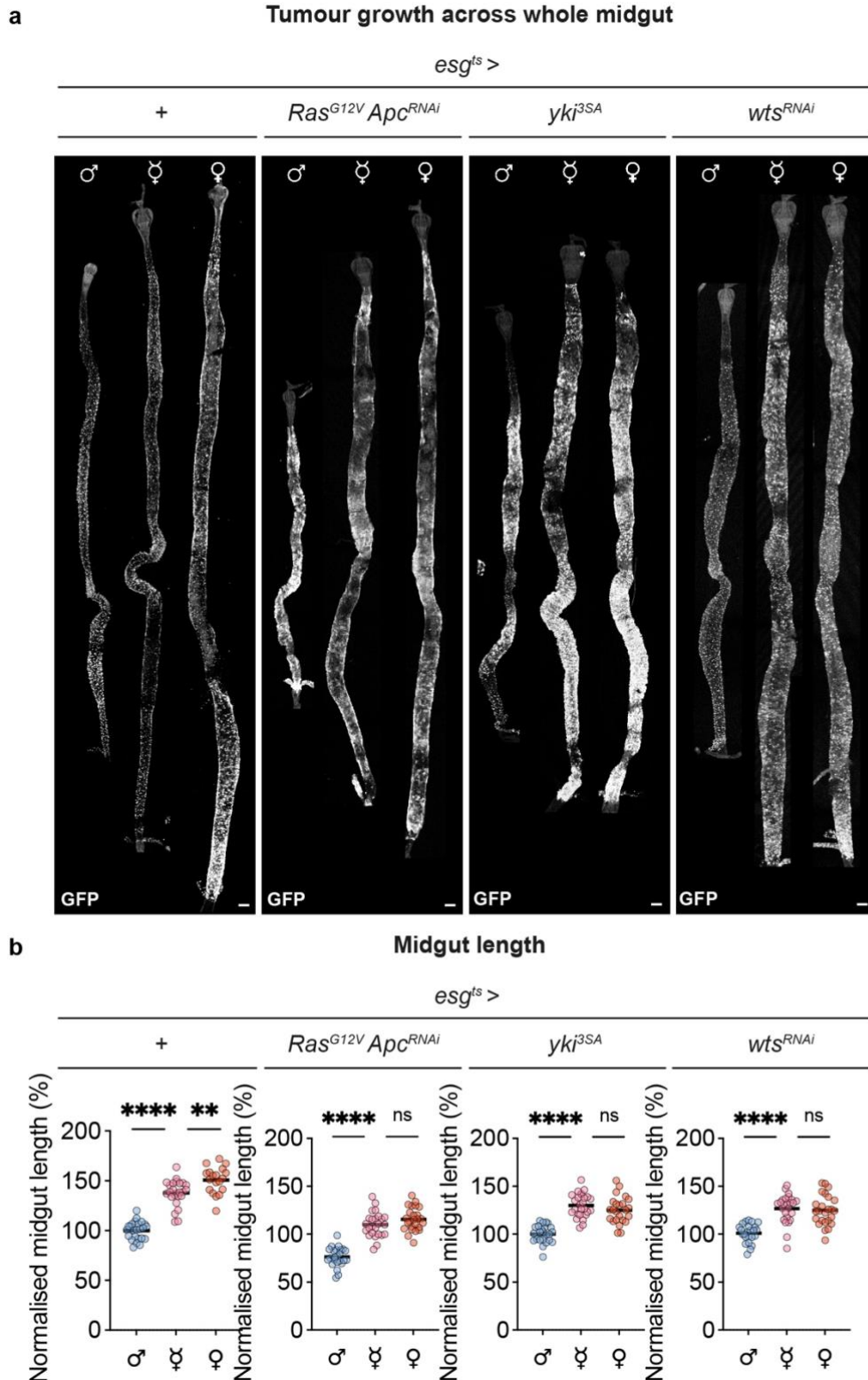


Figure 3.6. Tumour growth across the midgut and total midgut length changes for different genetic tumours depending on sex and reproductive status.

a. Images of whole midgut with tumours driven by *esg^{ts}* crossed to the GFP control (+), *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* or *wtS^{RNAi}*, in virgin males, virgin females and mated females. **b.** Quantification of the whole midgut length of the above conditions, normalised as a percentage of GFP control (+) virgin male. ♂ - virgin males, ♀ - virgin females, ♀ - mated females. The unpaired t-test was used to separately compare sex and reproduction. Scale bar = 100 μ M. ns $p > 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

length after 6 d of tumour induction, and normalised them to the length of the control male midgut. This showed several interesting observations. The *Ras*^{G12V} *Apc*^{RNAi} males have much shorter guts of just 76% of the control male guts, whereas the *yki*^{3SA} and *wts*^{RNAi} males had lengths of 100% and 101% respectively (**Figure 3.6b**). This is despite both *Ras*^{G12V} *Apc*^{RNAi} and *yki*^{3SA} males having high proliferation, and *wts*^{RNAi} males having almost no proliferation, hinting there may be some uncoupling of proliferation and midgut length.

At my timepoint of 6 d of tumour induction, which is 7 d post-mating, the virgin female control gut is significantly shorter than the mated female midgut at 138% and 151% of the virgin male control respectively (**Figure 3.6b**).

The *Ras*^{G12V} *Apc*^{RNAi} virgin female midgut is slightly shorter than the mated female but it does not reach significance. Like the *Ras*^{G12V} *Apc*^{RNAi} virgin males, the *Ras*^{G12V} *Apc*^{RNAi} virgin female (109%) and mated female (116%) are shorter than the virgin female (138%) and mated female (151%) controls (**Figure 3.6b**). Thus, this tumour models results in a shorter midgut despite having high levels of proliferation.

The *yki*^{3SA} and the *wts*^{RNAi} virgin female midgut length trends higher than the mated but is not significant, with 130% and 125% for *yki*^{3SA} virgin and mated female respectively, and 127% and 125% for *wts*^{RNAi} virgin and mated female respectively (**Figure 3.6b**). Once again, the Hippo pathway tumours have lost their reproductive differences, with slightly but not significantly longer guts in the virgin females.

Thus, quantifying gut length has revealed that depending on genetic tumour model, gut length and proliferation are uncoupled. This could be better described with length quantification of different gut regions, as R3 seems to be reduced in *Ras*^{G12V} *Apc*^{RNAi} tumours. Finally, although none of the tumour conditions have significantly different lengths between virgin and mated females, Hippo pathway tumours have longer midguts in virgin females, similar to their equal or higher proliferation in virgin females.

3.4 Discussion

In this chapter I have begun to investigate how several different genetic tumour models in the adult *Drosophila* midgut, induced in ISC and EB with the *esg^{ts}* system, change with sex and reproductive status.

3.4.1 Effect of the *esg-Gal4* driver on tumourigenesis

Several different *esg-Gal4* constructs are commonly used, each inserted into a different region of the locus upstream of the *esg* gene. I started by characterising several different *esg-Gal4* stocks we had in the lab, with known or unknown insertion site. This was in collaboration with Pedro Gaspar. We identified several stocks, and importantly identified our commonly used lab stock as *esg^{NP7397}* (**Section 3.2.1**). We then verified the expression pattern of *esg^{NP7397}-Gal4* and *esg^{NP5130}-Gal4*, and showed that although *esg^{NP7397}-Gal4* was expressed in more ISC and EB overall, *esg^{NP7397}-Gal4* had more uniform expression across all gut regions (**Section 3.2.2**). As I henceforth only used *esg^{NP7397}-Gal4*, I do not know if different *esg* insertions would modulate the tumour phenotype. I think it is likely it would modulate number of pH3⁺ cells, as the number of ISC and EB expressing the transgenes that induce proliferation appear different in *esg^{NP7397}-Gal4* and *esg^{NP5130}-Gal4*, although I did not quantify this.

3.4.2 Male tumourigenesis depends on the genetic tumour model

To study the effect of sex, I collected virgin male and female flies, and compared them directly. There is no evidence of changes in midgut proliferation or length in males after mating (Miguel-Aliaga lab, unpublished). As summarised in **Section 1.5.2**, virgin male midguts do not proliferate as much as virgin females in response to damage from DSS, tumourigenesis induced by *Notch^{RNAi}* (Hudry, Khadayate and Miguel-Aliaga, 2016) or infection with *P.e.* (Ahmed *et al.*, 2020) or *Ecc* (compared to mated females; Regan *et al.*, 2016). Thus, it was expected that in genetic tumour induction, virgin males would always proliferate less than both virgin and mated females.

In control conditions, virgin males and virgin females have almost no midgut proliferation, whereas mated females have a small increase in proliferation that fuels the expansion of the midgut size post-mating (**Section 3.3.1**; Reiff *et al.*, 2015; Hudry, Khadayate and Miguel-Aliaga, 2016).

It was expected that for tumourigenesis, males would have the lowest proliferation, followed by virgin females, then mated females with the highest. *Ras^{G12V} Apc^{RNAi}* tumours exemplified this pattern. All *yki^{3SA}* conditions responded robustly to tumourigenesis, including the virgin males. However, for *wt^{sRNAi}* tumours, the males did not respond to tumourigenesis, with guts that looked remarkably like the controls (**Section 3.3.1**). Both these Hippo pathway tumours did not behave as expected with regard to reproductive status (**Section 3.4.3**).

The proliferation seen in *Ras^{G12V} Apc^{RNAi}* and *yki^{3SA}* males, although lower than the virgin females, is in contrast to previous work on *Notch^{RNAi}* tumours, where the males showed almost no proliferation (Hudry, Khadayate and Miguel-Aliaga, 2016). It agrees with previous work on *Apc-Ras* MARCM clones that do show proliferation in males, with smaller clone size than in virgin females (Hudry, Khadayate and Miguel-Aliaga, 2016).

The *Ras^{G12V} Apc^{RNAi}* condition had an interesting phenotype with regard to midgut length (**Section 3.3.4**). In *Ras^{G12V} Apc^{RNAi}* males especially, midgut length was much shorter than the controls. However, *yki^{3SA}* males that also have some midgut proliferation, do not have a reduced midgut length, nor do *wt^{sRNAi}* males that have no proliferation. Thus, in a tumour situation, length and proliferation are no longer coupled, and this is dependent on the genetic tumour model. This would also be better explored with a measurement of total gut area, as tumourigenesis causes multilayering that may explain why excess proliferation does not cause a length increase. I did not measure changes in cell death, nor cell dissemination. ISC and EB expressing *Ras^{G12V}* are known to disseminate from gut (Lee *et al.*, 2020), which may explain a reduction in gut length, but this does not explain why it is so pronounced in the virgin male condition.

Cell dissemination has been measured in two different *esg^{ts}* driven tumours. In *Ras^{G12V}* tumours, where GFP⁺ cells have been seen to disappear with time, these cells have been shown to basally disseminate from the midgut into both the body cavity of the fly and the lumen of the midgut. This can be quantified by counting the number of GFP⁺ cells detected on the surface of the midgut overlapping the VM (in a given region,

for example the posterior midgut). It can also be quantified by examining the integrity of the intestinal muscles and counting the number of disrupted muscles. This must be done by extensive confocal imaging of many posterior midguts for each condition and is very time consuming. For a mated female after 2 d of tumour induction, approximately 2 – 8 disseminated cells can be counted on the surface of the posterior midgut. Additionally, GFP⁺ cells can be seen in the haemolymph of flies bearing *esg^{ts}* driven *Ras^{V12G}* tumours but not in control flies (Lee *et al.*, 2020). In *esg^{ts}* driven *yki^{3SA}* tumours, which persist over time, a small subset of GFP⁺ cells can be detected outside of the intestinal VM. These can also be manually counted after confocal imaging of the entire posterior midgut, looking for cells that are outside the VM. For a mated female after 6 d of tumour induction, approximately 5 – 6 disseminated cells can be counted (Pranoto, Lee and Kwon, 2023). Due to its time-consuming nature, I chose not to pursue dissemination as a readout.

Ras^{G12V} Apc^{RNAi} males and females have expanded ISC and EB in their R3 region, an area normally very resistant to tumourigenesis (**Section 3.3.4**). It could be that this region is truncated or missing, and should be checked with an R3 specific stain like a Cut or Labial antibody (Buchon *et al.*, 2013; Marianes and Spradling, 2013). This would also allow the separate quantification of changes in anterior (R1-R2) and posterior (R4-R5) gut length across all conditions.

The second interesting finding is that *wts^{RNAi}* males have almost no proliferation even after 12 d of tumour induction (**Section 3.3.2**). This suggests that the Hippo pathway may be more active in male ISC, resulting in low Yki activity and a reduction in its pro-mitotic and anti-apoptotic target genes. Males are known for having low midgut proliferation in response to damage and infection (Hudry, Khadayate and Miguel-Aliaga, 2016; Regan *et al.*, 2016; Ahmed *et al.*, 2020). High basal levels of the Hippo pathway may explain this. Thus, disruption of the Hippo pathway has a weaker response. However, *yki^{3SA}* males do have a robust proliferative response. This may be because the overexpression of this construct overrides any sex differences in basal levels of the Hippo pathway. Most *wts^{RNAi}* males have very little proliferation at the later timepoints of 10 d and 12 d. Some begin to have larger levels of proliferation, showing that *wts* downregulation is capable of having a phenotype in males, but that they remain very resistant to tumour formation.

As *mats* is a core component of the Hippo pathway, it was expected that *mats*^{RNAi} and *mts*^{RNAi} would have similar phenotypes. For the virgin males, they both had low proliferation even at the later 10 d timepoint (**Section 3.3.3**). As both will downregulate the basal levels of Hippo pathway activity, this fits the model that downregulation reflects how this pathway functions physiologically, and that males may have higher basal Hippo pathway activity.

Males are unlikely to ever proliferate as much as virgin or mated females, because they have much shorter guts, and thus fewer ISC (Hudry, Khadayate and Miguel-Aliaga, 2016). Thus, another way to compare tumour severity would be to normalise their average pH3⁺ cell counts by midgut length, to adjust for this difference.

3.4.3 Reproductive status does not affect Hippo pathway tumour proliferation

To study the effect of reproduction I mated half the females for 1 d before tumour induction. This ensures the gut is already underway in the mating induced changes, such as increased ISC proliferation, expansion of the posterior gut region and increases in EC lipid metabolism (Reiff *et al.*, 2015). There were alternate approaches I could have taken. For example, I could have mated the flies 7 d before tumour induction, to ensure all post-mating changes in the gut had taken place. This would have tested more if a gut that had already expanded its ISC and EB populations and increased in size would respond the same to tumourigenesis. Post-mating changes such as the increase in gut length persist for at least 14 d (White *et al.*, 2021), so the virgin and mated females would be likely to respond differently, but potentially in different ways to near-concurrent mating and tumour induction.

As we know midgut tumours proliferate in response to JH and 20HE, and that these hormones are increased after mating, it was expected mated females would always proliferate more than virgin females. This is seen in *Ras*^{G12V} *Apc*^{RNAi} tumours (**Section 3.3.1**), and also *Ras*^{G12V} alone (**Section 3.3.3**). However, the higher *Ras*^{G12V} *Apc*^{RNAi} mated female proliferation was only found at the 6 d timepoint and is no longer significantly different from virgin females at 10 d (**Section 3.3.2**). This may imply the mated proliferation increase is additive and dependent on the mating triggered

increase in JH and 20HE that promote proliferation, which may begin to subside by 10 d. However, the length of time that the mating-induced proliferation persists for is not fully characterised. It is known the gut continues to increase in length until approximately 10 d post-mating (White *et al.*, 2021).

Alternatively, work by the Kwon lab shows that *esg^{ts}*-driven *Ras^{G12V}* tumours begin to disseminate and leave the midgut, resulting in a large reduction in GFP⁺ ISC and EB cells and proliferation (Lee *et al.*, 2020). It may be that as the most proliferative condition, the *Ras^{G12V} Apc^{RNAi}* mated females are affected by this most, and thus see a large reduction in proliferation with time as the cells leave the midgut. I have not characterised any differences in dissemination with sex and reproduction in any of my genetic tumour models.

Although I initially investigated *Notch^{RNAi}* tumours, I found them stochastic, with some flies having normal guts with no proliferation, and some having upwards of 50 pH3⁺ cells (**Section 3.3.3**). This may have been resolved by using a longer tumour generation time of 14 – 20 d (Hudry, Khadayate and Miguel-Aliaga, 2016; Ahmed *et al.*, 2020). Many papers instead assess tumour frequency, with phenotypes sorted by their severity, to account for the bimodal distribution of pH3⁺ cell counts (Patel, Dutta and Edgar, 2015; Ahmed *et al.*, 2020). It has been shown however that virgin females are very resistant to *Notch^{RNAi}* tumours, and at 14 d of tumour induction most virgin females have no proliferation. Although my data did not reach significance at the 6 d time point, I did see a trend for mated female proliferation, with the virgin female and males having similar lower levels. This is markedly different from the two Hippo pathway tumours, where virgin females have a robust response and high proliferation.

I looked at two Hippo pathway tumours, downregulation of *wts*, and overexpression of *yki^{3SA}*. All *yki^{3SA}* conditions responded robustly to tumourigenesis, with similar proliferation in virgin and mated females, and lower male proliferation. For *wts^{RNAi}* tumours, the males did not respond to tumourigenesis, and the virgin females unexpectedly proliferated more than the mated females (**Section 3.3.1**).

Both *yki^{3SA}* and *wts^{RNAi}* did not have the expected increase in proliferation in the female response to mating. These results suggests that the Hippo pathway may be important

for reproductive remodelling in the midgut, as its disruption results in the same amount of proliferation in *yki*^{3SA} and a reversed response in *wts*^{RNAi} virgin and mated females. Thus, it could be that either disruption of the Hippo pathway prevents reproductive remodelling, or that it mimics it, causing more proliferation in the *wts*^{RNAi} virgin females that have not yet been mated. This may be because the *wts*^{RNAi} mated females already have a downregulated Hippo pathway, thus further downregulation has less effect. When *wts*^{RNAi} tumours are induced for 10 d and 12 d, both female conditions continue to proliferate (**Section 3.3.2**). This may imply the growth caused by downregulation of *wts* is not dependent on the hormonal changes brought on by mating, as it continues to increase past the peak of the reproductive changes (White *et al.*, 2021).

For females, *mats*^{RNAi} shows increased proliferation in both virgin and mated flies, but does not show the same large virgin female increase that *wts*^{RNAi} shows (**Section 3.3.3**). However, the proliferation induced by *mats* downregulation is much lower than *wts*, suggesting that the mating-induced increase in proliferation is not surpassed. Additionally, virgin female proliferation continues to increase at the 10 d timepoint in both conditions, which could mean this proliferation is independent of the mating-induced proliferation (**Section 3.3.3**). Alternatively, the role of *wts* in the post-mating response could be independent of *mats*.

After mating, the gut increases in length in females (Reiff *et al.*, 2015). At the 6 d timepoint, the *Ras*^{G12V} *Apc*^{RNAi} mated female trends longer than the virgin female, and the *yki*^{3SA} and *wts*^{RNAi} virgin females trend longer than the mated females (**Section 3.3.4**). However, none of these comparisons are significant. All of the tumour conditions have shorter midguts than the control, showing excess proliferation is uncoupled from midgut length. A better measure would be total midgut area, as this would take into account the increased width seen across many of the conditions. This difference is also more profound between the control virgin and mated females. The pattern of midguts with Hippo pathway tumours trending longer in virgin females than mated females again suggests that the Hippo pathway may be important for post-mating remodelling and is disrupted.

Midgut *yki*^{3SA} tumours are known for the wasting effects they have on other organs (Kwon *et al.*, 2015; Song *et al.*, 2019). I did see some bloating and in virgin males,

virgin females and mated females, and ovary wasting in females. I did not quantify if this changes between conditions. I did also observe some bloating in *wt^s^{RNAi}* females, indicating as expected that this tumour has similar effect on physiology as *yki* overexpression.

Thus, in this chapter I demonstrated that (a) males are only resistant to some genetic tumours and (b) reproductive status does not affect the proliferation of females with Hippo pathway tumours. After investigating a range of different genetic tumours, I chose to focus mainly on the *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* and *wt^s^{RNAi}* tumours, at the 6 d timepoint, to try and characterise further why they respond differently to sex and reproduction.

Chapter 4: Results II

4.1 Introduction

I have shown proliferation of genetic midgut tumours is dependent on sex and reproductive status. In this chapter I will focus on an interesting sex difference that I found: whilst *esg^{ts}*-driven *yki^{3SA}* and *wts^{RNAi}* induce proliferation in both virgin and mated females, only *yki^{3SA}* induces proliferation in males (**Section 3.3.1**). Males with *wts^{RNAi}* appear almost wild-type, with some proliferation at later timepoints like 12 d of tumour induction (**Section 3.3.2**).

Previous work in *esg^{ts}*-driven tumours caused by *Notch* downregulation shows males are almost entirely resistant to tumourigeneses, whereas mated females respond robustly. This can be overcome in males with overexpression of a pro-proliferative ligand such as Spitz (Hudry, Khadayate and Miguel-Aliaga, 2016). Additionally, masculinisation of the ISC and EB with the downregulation of female splicing factors *tra* or *Sxl* prevents proliferation of virgin female *Notch^{RNAi}* tumours (Hudry, Khadayate and Miguel-Aliaga, 2016). We do not know if Hippo pathway tumours respond to masculinisation or feminisation. Most literature agrees that in the midgut Wts acts as a kinase to inhibit Yki activity, but new research suggests Wts may act via the transcription factor Lola in a Yki-independent manner (Hao *et al.*, 2020).

Thus, several questions remain outstanding regarding why males get *yki^{3SA}* tumours, but are very resistant to *wts^{RNAi}* tumours. For example: (a) does *wts* function independently of *yki* in the midgut, and does this change with sex, and (b) do Hippo pathway tumours respond to feminisation and masculinisation like *Notch^{RNAi}* tumours?

4.1.1 The Hippo pathway in *Drosophila*

The Hippo pathway, as summarised in **Section 1.2.2** is a highly conserved pathway, important in regulating cell proliferation, apoptosis and organ growth generally (reviewed in Pan, 2010). With an active Hippo pathway, upstream kinase Hpo activates kinase Wts, which then phosphorylates Yki, preventing it from entering the nucleus. With an inactive Hippo pathway, in the absence of functional Hpo and Wts, Yki can enter the nucleus and transcribe genes important for cell proliferation and growth (*CyclinE*, *bantam*, *Myc*, *Upd1/2/3*, *Vein*) and apoptosis (*diap1*) (Harvey, Pfleger

and Hariharan, 2003; Udan *et al.*, 2003; Wu *et al.*, 2003; Nolo *et al.*, 2006; Thompson and Cohen, 2006).

The Hippo pathway has been explored in many tissues in *Drosophila*, such as in the development and differentiation of neuroblasts in the optic lobe of the larval brain via Notch crosstalk (Reddy, Rauskolb and Irvine, 2010) or in the mechanosensing follicular epithelial cells that mediate cellular morphogenesis of the adult ovary (Fletcher *et al.*, 2018). In each tissue, different Hippo pathway components are needed, with differing severity of phenotypes. For example, in imaginal disc, loss of *hpo* has a much stronger phenotype than loss of upstream components *mer* and *kibra* (Hamaratoglu *et al.*, 2006; Baumgartner *et al.*, 2010), but in the ovarian follicle cells all three have similarly severe phenotypes when lost (Dong *et al.*, 2015; MacDougall *et al.*, 2001; Polesello and Tapon, 2007; Yu *et al.*, 2008).

4.1.2 The Hippo pathway in *Drosophila* midgut ISC/EB

In the midgut, the Hippo pathway is important in both ISC and EB, and in EC. Antibody staining show Yki is present at higher levels in the ISC and EB of the midgut compared to EC and EE, although this has only been shown in females (mating status not disclosed; Karpowicz, Perez and Perrimon, 2010). Downregulation of *yki* in the ISC and EB with *esg^{ts}* does not result in a smaller midgut, unlike downregulation in the wing disc which results in severely undersized wings (Karpowicz, Perez and Perrimon, 2010). Analysis of *yki* mutant clones in the midgut shows these cells can divide and reach a comparable size to control clones, thus *yki* is not necessary for proliferation during normal midgut homeostasis (Karpowicz, Perez and Perrimon, 2010; Ren *et al.*, 2010). Additionally, because of the cytoplasmic localisation of Yki in midgut ISC and EB, it is assumed that Yki is inactive during normal midgut homeostasis. When the gut is damaged, cell contact cues disrupt the Hippo pathway components, derepressing Yki and causing increasing proliferation (Karpowicz, Perez and Perrimon, 2010).

Depletion of JAK-STAT or EGFR signalling in ISC greatly reduces proliferation caused by *hpo* downregulation (Karpowicz, Perez and Perrimon, 2010; Ren *et al.*, 2010). Yki can only induce proliferation in ISC when the JAK-STAT pathway is active. Although

Yki induces Upd production, the JAK-STAT pathway can induce proliferation completely independently of Yki (Karpowicz, Perez and Perrimon, 2010).

The microRNA *bantam* is a key gene transcriptionally regulated by Yki that mediates cell growth. It is highly expressed in ISCs, EBs and EEs. Overexpression of Yki cannot cause midgut proliferation if *bantam* is downregulated (Huang *et al.*, 2014). Expanded (Ex) is an apical cell-cell junction protein that inhibits Yki activity via Hpo and Wts, and is mainly expressed in EC, not progenitors (Huang *et al.*, 2014). As it is one of the Yki transcriptional targets, it allows the Hippo pathway a mechanism of negatively regulating itself (Hamaratoglu *et al.*, 2006). Diap1 mediates cell apoptosis, and is expressed in both progenitors and ECs (Goyal *et al.*, 2000; Harvey, Pfleger and Hariharan, 2003).

It is unclear if Yki is necessary in ISC to drive proliferation in response to all stimuli. Certainly downregulation of the Hippo pathway (*hpo^{RNAi}* or *wts^{RNAi}*) in ISC and EB requires *yki* in order to induce proliferation (Karpowicz, Perez and Perrimon, 2010; Ren *et al.*, 2010). Karpowicz and colleagues showed downregulation of *yki* in ISC and EB greatly reduces proliferation in response to damage with DSS, and partially reduced proliferation caused by bacterial infection with *Pseudomonas* (Karpowicz, Perez and Perrimon, 2010). Ren and colleagues agreed that ISC and EB *yki* downregulation prevents DSS induced damage, but found it does not prevent proliferation caused by bleomycin or infection with *Pseudomonas*. When Hippo pathway downregulation with *wts^{RNAi}* and DSS treatment are combined, there is no significant increase in proliferation. Thus response to DSS requires cell autonomous Yki in ISC and EB (Ren *et al.*, 2010). However, Shaw and colleagues completely suppressed *Pseudomonas* induced proliferation with ISC and EB downregulation of *yki* (Shaw *et al.*, 2010). Thus, it cannot be concluded that damage including bleomycin and *Pseudomonas* infection stimulate ISC entirely through a Yki-independent mechanism.

4.1.3 The Hippo pathway in *Drosophila* midgut EC

Hippo signalling also plays a role in the midgut ECs. Loss of Hippo signalling in ECs causes a large ISC/EB specific increase in expression of several pro-mitotic ligands

including the JAK-STAT ligands Upd1-3 and the EGFR ligands Vn, Spi and Keren (Krn). This dramatically increases ISC proliferation in a cell non-autonomous manner (Ren *et al.*, 2010; Shaw *et al.*, 2010; Staley and Irvine, 2010). Downregulation of *yki* in EC mildly reduces midgut proliferation caused by DSS, bleomycin or *P.e* infection, indicating the non-cell-autonomous role of the Hippo pathway in EC is not essential but does contribute to ISC proliferation (Shaw *et al.*, 2010; Staley and Irvine, 2010; Huang *et al.*, 2014). When *bantam* is downregulated in EC, the ISC/EB can still proliferate, meaning it is not needed for the extrinsic EC-mediated effect on progenitor proliferation (Huang *et al.*, 2014).

4.1.4 The function of the Hippo pathway the *Drosophila* midgut

The Hippo pathway can affect progenitor proliferation from both the EC, where its repression induces production of pro-mitotic ligands to stimulate ISC proliferation, and in ISC and EB themselves, where it induces expression of Hippo pathway targets that induce cell proliferation like *bantam*. However, the exact function of the Hippo pathway in the midgut is not fully understood. For example, downregulation of *yki* either from progenitors or in mutant clones does not lead to any significant tissue size defects (Karpowicz, Perez and Perrimon, 2010).

One explanation of the function of the Hippo pathway in the midgut involves the Ste20 family kinase Misshapen (Msn) in sensing food intake and organ resizing. The Hippo pathway kinase Tao-1 activates Msn, which in turn phosphorylates Wts, allowing it to inactivate Yki. This is independent of the Hpo kinase, and is important in preventing ISC proliferation (Li *et al.*, 2014). Interestingly, Msn membrane localisation and phosphorylation is greatly decreased when flies are fed yeast particles, which stretches their midguts. This in turn decreases Wts activity, allowing Yki to induce proliferation and production of ligands such as Upd3. Thus food intake-induced epithelial stretching reduces Msn membrane localisation, leading to an increase in Yki activity and ISC proliferation (Li *et al.*, 2018). This is interesting, as after mating food intake dramatically increases (Carvalho *et al.*, 2006; Hadjieconomou *et al.*, 2020), which could contribute to inhibition of the Hippo pathway after mating in order to increase Yki-mediated progenitor proliferation.

Recently, a paper was published that claims to have found *wts* downregulation mediated ISC proliferation is independent of *yki*, using female flies with an undisclosed mating status. They instead show *wts* function is dependent on *lola*, a transcription factor known for its role in axon guidance and germline and neuronal stem cell identity (Hao *et al.*, 2020). *Wts* and *Lola* interact independently of phosphorylation, and *lola* overexpression prevents *wts* downregulation induced proliferation in both *esg^{ts}* and MARCM clones. Downregulation of *lola* increases midgut proliferation, and activates JAK-STAT, EGFR and *bantam* signalling in a similar way to Hippo pathway inactivation (Hao *et al.*, 2020). However, this directly contradicts the work of Ren and colleagues, who show expression of *wts^{RNAi}* and *yki^{RNAi}* in ISC and EB completely prevents midgut proliferation (female flies, undisclosed mating status, Ren *et al.*, 2010).

4.1.5 Sex determination in the *Drosophila* midgut

As introduced in **Section 1.4.2**, *Drosophila* sex determination involves female-specific transcription of the splicing factor *Sxl*, which generates the female isoform of *tra* which with *tra2* generates the female specific isoform of *dsx*. In males, in the absence of *Sxl*, male isoforms of *dsx* and *fru* are transcribed (Boggs *et al.*, 1987; Bell *et al.*, 1988; Cline, 1988; McKeown, Belote and Boggs, 1988; Nagoshi *et al.*, 1988; Belote *et al.*, 1989). Dosage compensation only occurs in males, where *msl-2* causes a two-fold upregulation of X-chromosomal genes (Bashaw and Baker, 1995; Lucchesi and Kuroda, 2015).

4.1.6 The role of sex-determining factors in midgut EC

In the adult midgut, the *tra* gene is ubiquitously expressed in all epithelial cells. The *Sxl* and *Msl-2* proteins are expressed in all female or male cells respectively. The *Tra* protein expressed in males however is not functional. Downstream of *tra*, *Fru^M* is absent in the midgut, and *Dsx^{M/F}* is only expressed in EC, and not in ISC, EB or EE (Hudry, Khadayate and Miguel-Aliaga, 2016).

Some work has been done into the function of *Tra^F* in the EC. These cells have large nuclei caused by several rounds of endoreplication. Females have larger EC than males, and this can be reduced in females by EC-specific (*mex1*) *tra* downregulation, but not increased in males with *tra^F* overexpression (Regan *et al.*, 2022). Thus *tra* is

necessary but not sufficient for larger female EC size. Female flies have increased intestinal dysplasia with age, as do male flies with EC-specific (*Myo1A*) *tra*^F overexpression (Regan *et al.*, 2016). This dysplasia is caused by females having more responsive ISC, and is thus a trade-off with better ability to regenerate the gut.

Males are more vulnerable to infection, potentially as their ISC proliferate less in response to damage and infection. However, EC-feminised males are still vulnerable to infection like control males, thus the sex of ISC, and other tissues more broadly like the fat body or haemocytes, also play a key role in sex differences in immunity (Regan *et al.*, 2016).

4.1.7 The role of sex-determining factors in midgut ISC

Both *tra* and *Sxl* are actively maintained and needed in female ISC to give them their female identity, in particular their enhanced homeostatic and regenerative proliferation compared to males. Downregulation of these factors with *esg*^{ts} in females will masculinise the ISC and EB, causing them to proliferate less in response to DSS damage, and 20 d of downregulation is sufficient to reduce the length of the female gut. Overexpression of these factors in males will feminise the ISC and EB, increasing regenerative proliferation. However, the sex of the ISC is independent of canonical Tra binding partner Tra2. Three regulators of sex differences in ISC proliferation have been identified: positive regulators *Imaginal disc growth factor 1* (*Idgf1*) or *Serpin 88Eb* (*Spn88Eb*) and negative regulator *reduced ocelli* (*rdo*; Hudry, Khadayate and Miguel-Aliaga, 2016). Downregulation of *tra* in ISC and EB is sufficient to decrease *Pseudomonas* infection induced proliferation in virgin females, but not sufficient to decrease proliferation induced by 20HE feeding. Males fed 20HE will also have a large increase in midgut mitosis (Ahmed *et al.*, 2020).

Some work has been done on the role of ISC intrinsic sex and midgut tumourigenesis. For *esg*^{ts}-induced tumours, masculinisation of ISC and EB with downregulation of *tra* or *Sxl* completely ablates female proliferation with *Notch*^{RNAi} tumours. Females with *Apc-Ras* MARCM clones have higher midgut proliferation than males, and this can be reduced with *Sxl* downregulation. Although it is known *tra*^F overexpression sensitises

males to proliferate in response to DSS damage, it has not been published if it can increase tumourigenesis (Hudry, Khadayate and Miguel-Aliaga, 2016).

4.2 Effects of sex and reproduction on the Hippo pathway

4.2.1 The Hippo pathway is slightly downregulated post-mating in females

The Miguel-Aliaga lab has an RNAseq dataset of Oregon-R virgin males, virgin females and mated females (3 d post-mating), carried out by Tomotsune Ameku. This dataset reveals that after mating in females the Hippo pathway is transcriptionally downregulated. This includes several inputs into the Hippo pathway, including the Fat-Dachs, Scribble-Dlg and Kibra-Merlin-Expanded pathways. Generally, virgin males have highest levels of these pathways, followed by virgin females, then mated females. Yki does appear to have the highest expression levels in virgin males, but as Yki is regulated at a protein level by phosphorylation and subcellular localisation, its transcript expression level may not be meaningful.

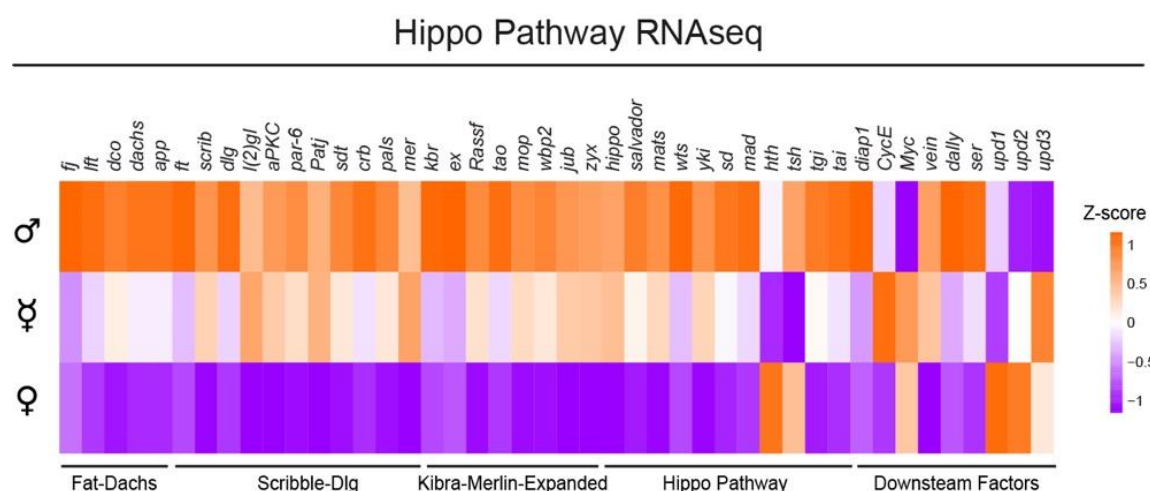


Figure 4.1. The Hippo pathway is transcriptionally downregulated in females post-mating.

Heatmap showing expression of Hippo pathway genes including upstream and downstream pathways from Oregon-R RNAseq dataset of virgin males, virgin females and mated females (3 d post-mating).

♂ - virgin males, ♀ - virgin females, ♀ - mated females.

N.B. Oregon-R dataset generated by Tomotsune Ameku with bioinformatics analysis performed by Tomotsune Ameku and Yi-Fang Wang at the LMS Bioinformatics Core Facility.

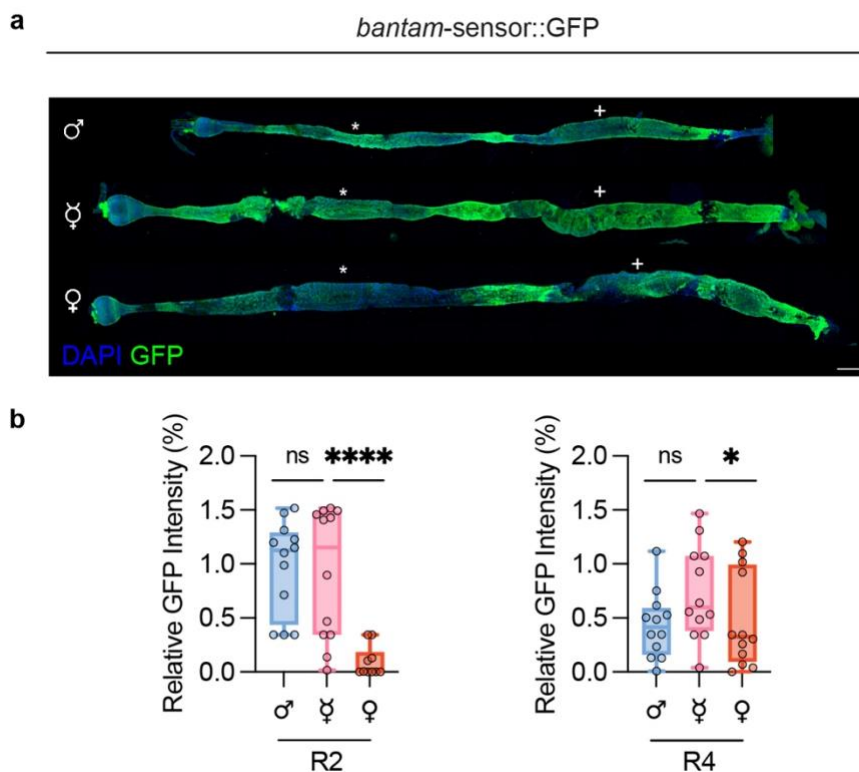
Heatmap was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

The genes downstream of the Hippo pathway should generally be high when Hippo is downregulated. For example, the *upd1* and *upd2* ligands are highest in mated females (**Figure 4.1**). However, the *upd* genes, which in a bulk RNAseq dataset will be mainly

from the EC, have other non-Hippo inputs and are generally higher in proliferative situations, such as the proliferation induced by mating. Some exceptions are Yki-target genes *diap1*, *CyclinE*, *vn* and *dally*, which may be expected to be higher in mated females and lower in virgin males, but instead show the opposite trend.

However, although this data shows a trend in a suppression of the Hippo pathway after mating, none of these individual genes are significantly different. Additionally, much of the Hippo pathway regulation is at a protein level via phosphorylation. Hence, although this data suggests there may be differences in the Hippo pathway post-mating, this must be further investigated with for example, protein trap readouts or antibody staining.

Figure 4.2. microRNA *bantam* is increased in females after mating.



a. Immunostaining of midguts showing expression of microRNA *bantam* in virgin males, virgin females and mated females (3 d post-mating). This shows *bantam* is increased after mating, especially in the R2 region of the gut. The *bantam-sensor::GFP* inversely correlates with *bantam* expression.

b. Quantification of GFP intensity of *bantam-sensor::GFP* in the R2 and R4 of the midgut. ♂ - virgin males, ♀ - virgin females, ♀ - mated females. R2 is indicated by *, R4 is indicated by +. The Mann-Whitney statistical test is used to separately compare sex and reproduction. ns $p > 0.05$, * $p \leq 0.05$, **** $p \leq 0.0001$. Scale bar = 200 μ M.

4.2.2 microRNA *bantam* is increased post-mating in females

The expression levels of the microRNA *bantam* can be monitored with the *bantam-sensor::GFP* (Brennecke *et al.*, 2003; Thompson and Cohen, 2006). The expression of GFP from this construct is inversely correlated to *bantam* expression, so high GFP

Tumour growth in R2 and R4 of midgut

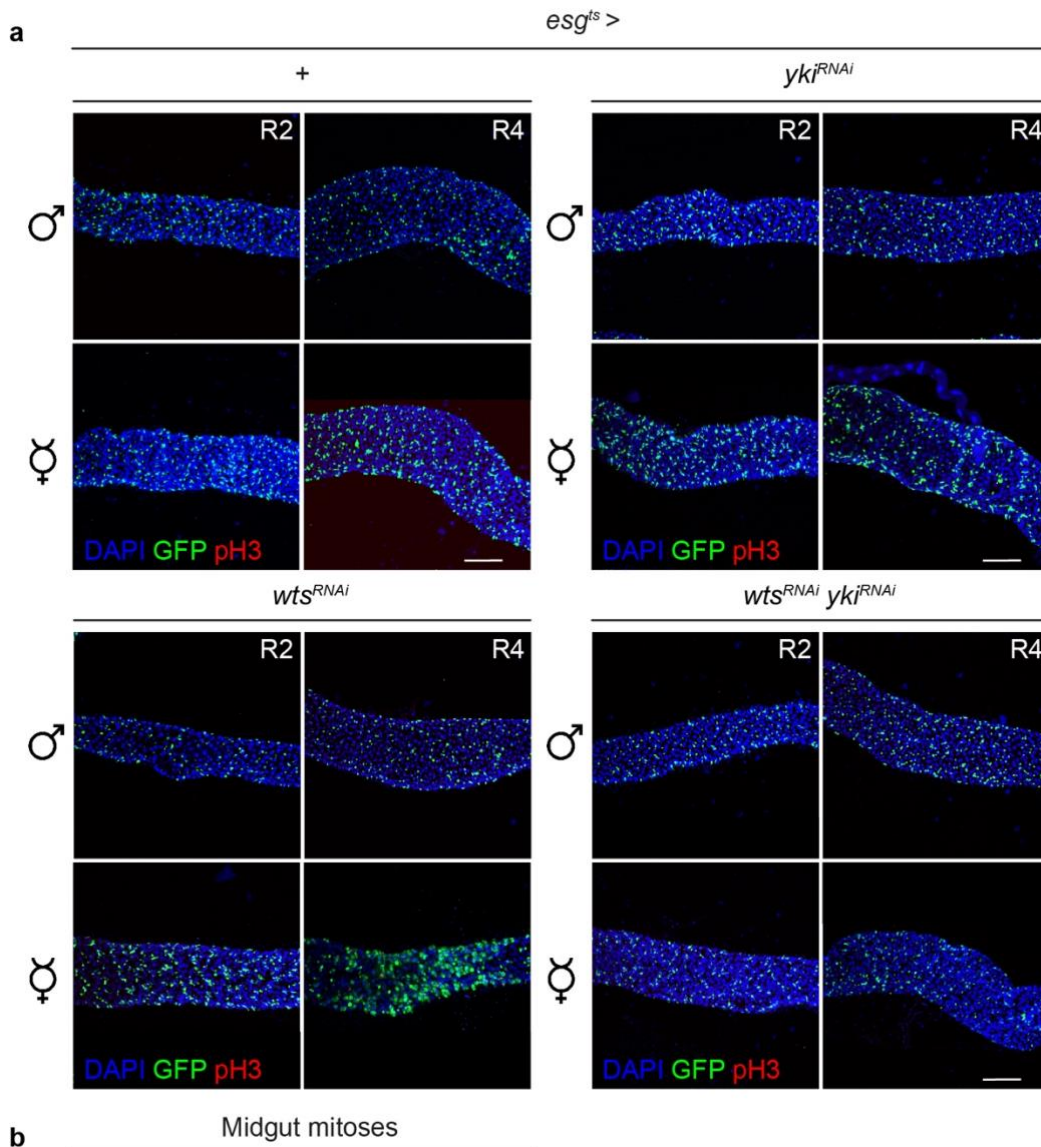


Figure 4.3. Virgin male and virgin female *wt^{sRNAi}* tumours cannot proliferate with simultaneous downregulation of *yki*.

a. Immunostaining of the R2 and R4 region of midgut tumours in virgin males and virgin females, showing *esg^{ts}* driving the GFP control (+), *yki^{RNAi}*, *wt^{sRNAi}* or *yki^{RNAi} wt^{sRNAi}* for 10 d.

b. Quantification of mitosis for the above conditions measured by the number of pH3⁺ cells per midgut. ♂ - virgin males, ♀ - virgin females. The Kruskal-Wallis statistical test is used to compare sex and experimental condition. ns $p > 0.05$, **** $p \leq 0.0001$. Scale bar = 100 μ M.

relates to low *bantam* expression (**Figure 4.2a**). It has been shown that midgut EC have low *bantam* (high GFP), but ISC, EB and EE have high *bantam* (low GFP; Huang *et al.*, 2014). Quantification of GFP intensity shows *bantam* is higher in mated females, especially in the R2 region, but also in R4 (**Figures 4.2b**).

Overall GFP intensity mainly represents the contribution of *bantam* from the large EC. Thus, it will also be important to check if there are any differences in ISC/EB or EE *bantam* levels by co-expressing or staining appropriate markers, and quantifying intensity per cell. Therefore, this RNAseq transcriptional and *bantam*-sensor analysis points to a modulation of the Hippo pathway with mating in females, but more Hippo pathway readouts and more detailed analysis is needed.

4.3 *wts* does not act independently of *yki* in midgut tumours

As *yki*^{3SA} and *wts*^{RNAi} tumours both behave differently to each other with respect to sex, I wanted to check if downregulation of *wts* can induce proliferation without *yki*. Indeed, I found almost no proliferation in virgin male and female midguts with co-expression of *wts*^{RNAi} and *yki*^{RNAi} for 10 d (**Figure 4.3**). This difference is particularly profound in virgin females. Virgin males are highly resistant to proliferation but will usually have a few animals with high levels of midgut pH3⁺ cells. In the *wts*^{RNAi} *yki*^{RNAi} males, all males have virtually no proliferation. Thus, *wts* downregulation-induced proliferation in the midgut acts via *yki* in both virgin males and females.

4.4 Effect of ISC/EB sex in *wts* downregulated tumours

4.4.1 *wts*^{RNAi} male tumours partially respond to feminisation with overexpression of *tra*^F

Overexpression of *tra*^F with *esg*^{ts} will feminise the ISC and EB and their response to pro-mitotic stimuli. Alone, expression of *tra*^F does not increase midgut proliferation. In combination with *wts*^{RNAi} male guts have increased proliferation compared to control guts after 10 d, although this proliferation is still much lower than the female proliferation, and some males still have no increase in mitosis. Females with *wts*^{RNAi} and *tra*^F also have slightly increased proliferation, but this is not significant (**Figures 4.3a, b**).

Tumour growth in R2 and R4 of midgut

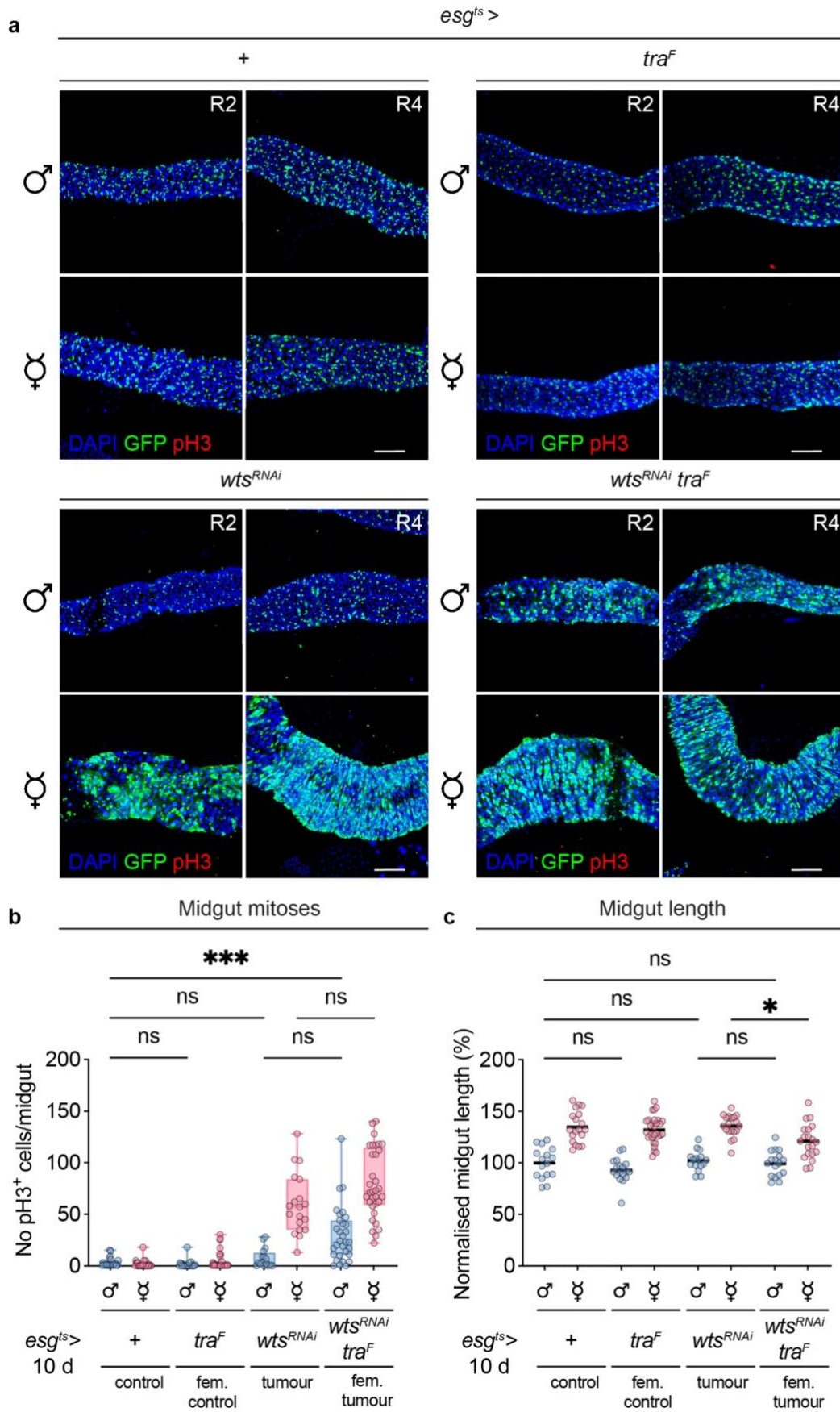


Figure 4.4. *wts^{RNAi}* male tumours partially respond to feminisation with overexpression of *tra^F* (previous page).

a. Immunostaining of the R2 and R4 region of midgut tumours in virgin males and virgin females, showing *esg^{ts}* driving the GFP control (+), *tra^F*, *wts^{RNAi}* or *wts^{RNAi} tra^F* for 10 d **b.** Quantification of mitosis for the above conditions measured by the number of pH3⁺ cells per midgut. **c.** Quantification of length for the above conditions normalised to the GFP control (+) virgin male. Fem – feminisation, ♂ - virgin males, ♀ - virgin females. The Kruskal-Wallis statistical test is used to compare sex and experimental condition. ns $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$. Scale bar = 100 μ M.

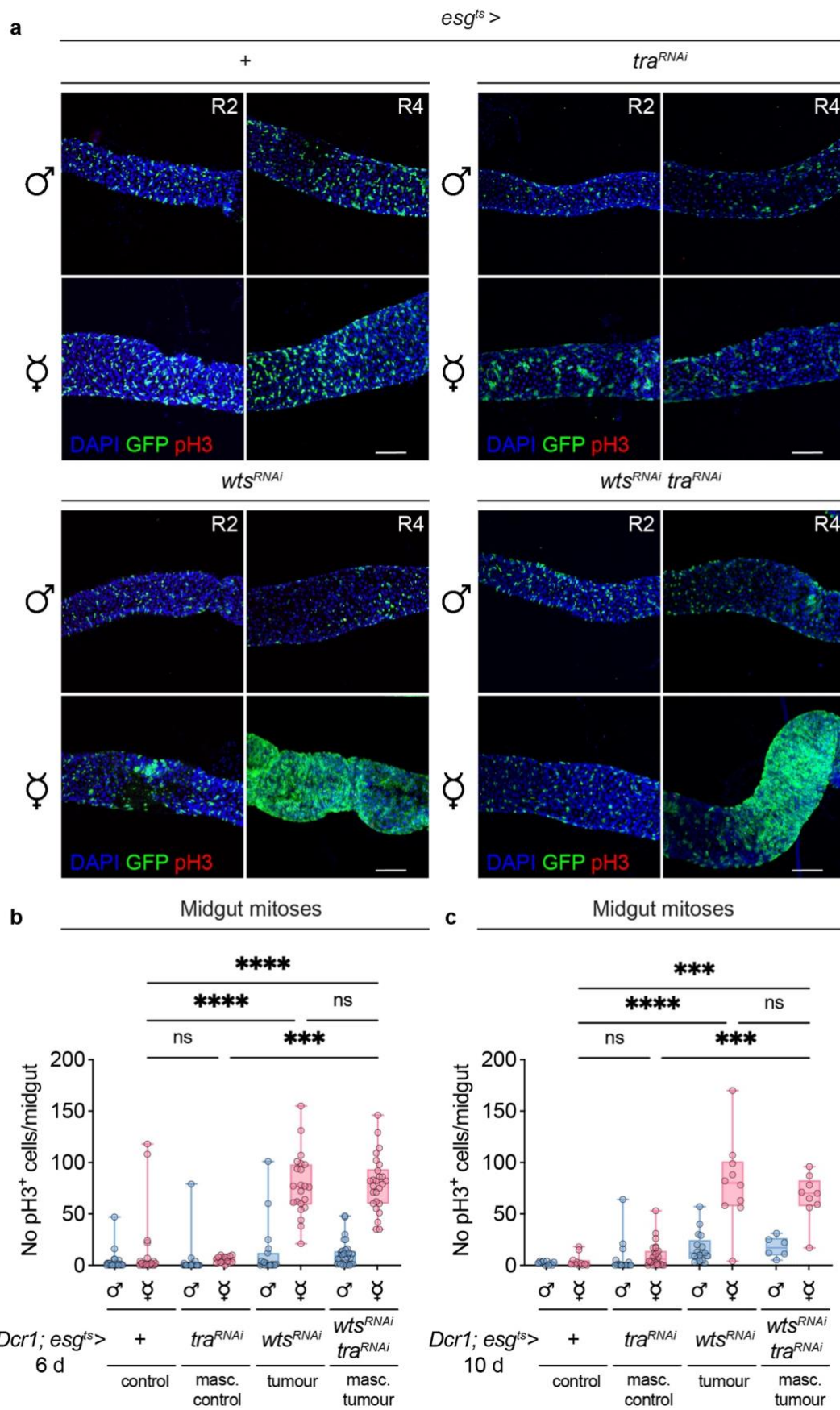
Females maintain significantly longer guts than males in all conditions. Male gut lengths do not increase with feminised ISC and EB. Females with *wts^{RNAi}* and *tra^F* have slightly shorter guts, potentially as they have increased gut hyperproliferation, which I previously showed may reduce gut length (**Section 3.3.4**).

4.4.2 *wts^{RNAi}* females do not respond to masculinisation with *tra^{RNAi}*

Downregulation of *tra* with *esg^{ts}* will masculinise the ISCs and EBs and their response to pro-mitotic stimuli. The *tra^{RNAi}* construct I am using has been shown to require *Dcr1* (Hudry, Khadayate and Miguel-Aliaga, 2016). Alone, *tra^{RNAi}* induced for 6 d does not decrease proliferation as baseline proliferation is almost zero (**Figure 4.5a**), but does decrease female midgut length (**Figure 4.5d**). However, I do see a slight proliferation increase in this condition, although it is not significant, and the flies appeared unhealthy, with some bloating and increased time spent stationary on the food. This *tra^{RNAi}* induced proliferation is more obvious but still not significant in the 10 d condition (**Figure 4.5c**). As I have used *Dcr1*, the *wts^{RNAi}* tumours have a stronger phenotype than previous experiments in both males and females (**Figures 4.5a, b, c**). Surprisingly, for both 6 d and 10 d of tumour induction, I do not see a decrease in proliferation in female tumours expressing both *wts^{RNAi}* and *tra^{RNAi}* (**Figures 4.5b, c**).

Gut length is decreased in females in *tra^{RNAi}* and *wts^{RNAi}*, and in the combined condition *tra^{RNAi} wts^{RNAi}*. The reduction in midgut length in *tra^{RNAi}* females implies the construct is working and the ISC are masculinised, but as I showed previously (**Section 3.3.4**) in hyperproliferative situations the midgut length often decreases. Thus, this reduction in midgut length may be because the *tra^{RNAi}* construct has made the flies stressed and has increased midgut proliferation. A slight increase in proliferation can be seen in the condition with *tra^{RNAi}* alone. Thus, without further testing of alternative *tra^{RNAi}* constructs or *Sxl^{RNAi}* I cannot say if the *wts^{RNAi}* tumours have been successfully masculinised.

Tumour growth in R2 and R4 of midgut



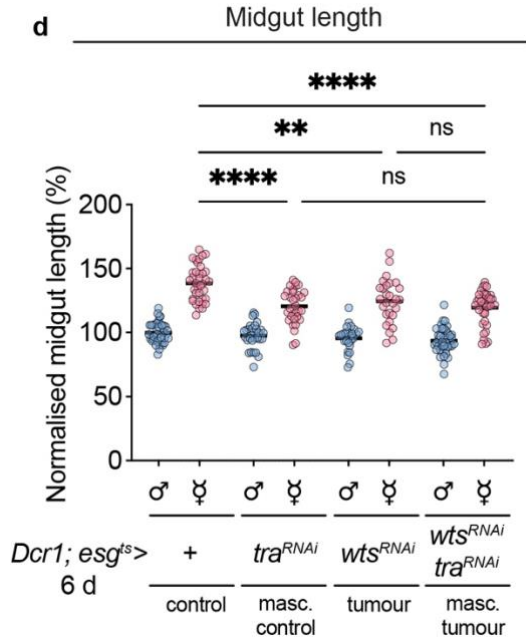


Figure 4.5. *wts*^{RNAi} female tumours do not respond to masculinisation with downregulation of *tra*.

a. Immunostaining of the R2 and R4 region of midgut tumours in virgin males and virgin females, showing *Dcr1; esg^{ts}* driving the GFP control (+), *tra*^{RNAi}, *wts*^{RNAi} or *wts*^{RNAi} *tra*^{RNAi} for 10 d. **b.** Quantification of mitosis for the above conditions induced for 6 d measured by the number of pH3⁺ cells per midgut. **c.** Quantification of mitosis for the above conditions induced for 10 d measured by the number of pH3⁺ cells per midgut. **d.** Quantification of length for the above conditions induced for 6 d normalised to the GFP control (+) virgin male. Masc – masculinisation, ♂ - virgin males, ♀ - virgin females. The Kruskal-Wallis statistical test is used to compare sex and experimental condition. ns p > 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001. Scale bar = 100 μM.

Thus, I have shown males that are usually resistant to *wts*^{RNAi} induced midgut proliferation, will have an increase in proliferation if they have feminised ISC and EB via *tra*^F overexpression. However, virgin females will proliferate with *wts*^{RNAi} tumours regardless of having masculinised ISC and EB with *tra*^{RNAi}. This may suggest that *wts*^{RNAi}-induced proliferation is insensitive to the sex of the ISC/EB, but other masculinisation conditions such as *Sxl*^{RNAi} need to be tested.

4.5 Discussion

4.5.1 *wts* is dependent on *yki* in midgut tumours in both males and females

In the canonical Hippo pathway, the *wts* kinase is responsible for preventing *yki* from entering the nucleus and activating pro-proliferative and anti-apoptosis genes. Previously, *wts* downregulation has been assumed to always induce proliferation via *yki* activation. However, Hao and colleagues recently showed *wts*^{RNAi} still induces proliferation with *yki*^{RNAi}, directly contradicting the work of Ren and colleagues, who showed this same experiment resulted in no proliferation. Hao and colleagues also showed there is an increase in proliferation with *wts*^{X1} *yki*^{RNAi} and *wts*^{RNAi} *yki*^{B5} clones, compared to control clones. Because I was also looking at *wts*^{RNAi} tumours, and had shown they have interesting sex differences, I decided to also perform this experiment, in virgin males and virgin females. My results agreed with the earlier work of Ren and

colleagues, as I showed *wt*s downregulation cannot cause proliferation in the absence of *yki* (Ren *et al.*, 2010). The best way to show this would be to repeat a clonal experiment like *wt*s^{RNAi} *yki*^{B5}. Combining two RNAi constructs can be inconclusive, as if either gene is not fully downregulated, it could still have an effect. Hao and colleagues also showed *wt*s interacts with the transcription factor *lola*, and that *lola* overexpression rescues *wt*s^{RNAi} induced proliferation. I cannot rule out if *lola* does have some interesting effect in the midgut related to sex or reproduction, and indeed it has been suggested it may be an important transcription factor in post-mating midgut remodelling (**Section 5.5.3**).

Simultaneous downregulation of *wt*s and *yki* may be worth repeating in mated females. Neither of the aforementioned papers disclose the mating status of their females. It is potentially possible that *wt*s can act independently of *yki* only in mated female guts, where *wt*s may be responsible for repressing some other transcription factor.

Damage-induced proliferation caused by bacterial infection and DSS has been described as *yki* dependent but this varies by lab. For example, Shaw and colleagues claim to completely prevent *Pseudomonas* infection induced proliferation with *yki* downregulation, but Ren and colleagues claim the midguts can still proliferate (Ren *et al.*, 2010; Shaw *et al.*, 2010). A potential issue with this series of experiments is they all rely on *yki*^{RNAi} constructs, which may not fully deplete *yki* in ISC and EB. Thus, even low levels of *yki* remaining could induce cell proliferation. This highlights how such experiments require the use of a loss of function mutant like *yki*^{B5} in a clonal experiment to verify their findings. However, such experiment would not rule out non-cell autonomous effects of the surrounding Yki intact tissue on proliferation. Another issue is the variability of proliferation assays across different labs, which tend to vary based on fly diet, food freshness, fly density and microbiota.

4.5.2 Role of sex determination in Hippo pathway tumours

Midgut ISC sex determines its ability to proliferate in response to stimuli including DSS (Hudry, Khadayate and Miguel-Aliaga, 2016), bacterial infection (Ahmed *et al.*, 2020) and *Notch*^{RNAi} tumours (Hudry, Khadayate and Miguel-Aliaga, 2016), but not 20HE

feeding (Ahmed *et al.*, 2020). Additionally, MARCM clones of *Ras*^{G12V} *Apc*^{2N175K} *Apc*^{Q8} have reduced proliferation in females when masculinised with *Sxl* downregulation.

Because only a few genetic tumours have been tested for their dependence on ISC sex, I decided to try sex manipulations on a Hippo pathway tumour: *wtS* downregulation. This unusual tumour has higher proliferation in virgin females (**Section 3.3**), unlike *Notch*^{RNAi} tumours where mated females have higher proliferation (Ahmed *et al.*, 2020). However, both share the characteristic of having virtually no effect on male midgut proliferation, unlike *Ras*^{G12V} *Apc*^{RNAi} and *yki*^{3SA} tumours, where males get smaller tumours, but are not resistant to proliferation.

It is known that *tra*^F overexpression in males with *esg*^{ts} feminises the ISC and EB, allowing them to proliferate robustly in response to damage with DSS. It has not been previously tested if this also makes males more susceptible to tumourigenesis, although feminisation of EC makes males more susceptible to age-related intestinal dysplasia (Regan *et al.*, 2016). I have shown that co-expression of *wtS*^{RNAi} and *tra*^F in ISC/EB causes an increase in midgut proliferation in males. The majority of midguts now have pH3⁺ cells, unlike *wtS*^{RNAi} where males are resistant to proliferation. There does appear to be a small but non-significant increase in virgin female proliferation, which may be because there are some titration effects of increasing Tra above physiological levels. These *wtS*^{RNAi} *tra*^F virgin female guts are also slightly shorter than control guts, unlike *wtS*^{RNAi} virgin females.

It is not known exactly how feminisation of ISC would make males more susceptible to *wtS*^{RNAi} tumours. For example, do female ISC have different Hippo pathway activity, thus feminised ISC are more sensitive to *wtS* downregulation?

This question is difficult to answer given my masculinisation results, where downregulation of *tra* and *wtS* for 6 d or 10 d did not change virgin female tumour proliferation. This is in contrast to previous work showing *Notch*^{RNAi} tumours can be prevented in females with 15 d of simultaneous *tra* or *Sxl* downregulation (Hudry, Khadayate and Miguel-Aliaga, 2016). I did not perform the *tra* downregulation in *Notch* tumours myself, and so I cannot directly compare these results. Both tumours have different mechanisms of causing proliferation. Tumours induced by *Notch*

downregulation are composed of pre-EE and EE cells. Tumour induced by *wt*s downregulation have not been fully characterised, but it is suggested they do not have differentiation defects (Shaw *et al.*, 2010).

As males are usually resistant to *wt*s^{RNAi} tumours, why is this not the case for virgin females with masculinised ISC and EB? This result cannot be fully considered until this experiment is repeated with an alternative *tra*^{RNAi} line, and with *Sxl*^{RNAi}. Additionally, epistasis limits the conclusions that can be drawn from experiments with two RNAi constructs. For example, if *wt*s is downregulated at a faster rate than *tra*, the ISC may have already begun to rapidly proliferate before the ISC can begin to express the male transcriptional programme. Alternatively, even a small amount of Tra^F remaining may continue to activate the pro-proliferative female programme in the extreme stress caused by Hippo pathway downregulation. Thus, this question cannot be concluded without a clonal experiment, for example a MARCM clone *tra*¹ null allele with *wt*s downregulation. This would ensure the complete absence of the female transcriptional program within the clone and could show in that situation if *wt*s downregulation results in any proliferation. It is unlikely that *wt*s is upstream of *tra*, as *tra* is one of the earliest factors in sex determination. However, it is possible that other cell types or tissues are important in Hippo pathway-mediated proliferation.

The exact mechanism by which *tra* increases female ISC proliferation is not fully understood. We know three mediators of it: *ldgf1*, *Spn88Eb* and *rdo*. Further work into these factors and other potential mediators of sex differences in the midgut is needed. Chitinase-like protein *ldgf1* and endopeptidase inhibitor *Spn88Eb* are both higher in adult female trachea compared to larvae of mixed sex, raising the possibility they could play a role in modulating proliferation from another tissue (Bossen *et al.*, 2023). It could be important to profile the response of other tissues to *wt*s downregulation in ISC and EB, like the muscles and trachea, and see if this varies with sex and reproduction.

4.5.3 A potential new function for the Hippo pathway in the midgut

Although modulations of the Hippo pathway in the midgut have phenotypes (modulation of proliferation), the function of the Hippo pathway in the midgut is not fully understood. Yki does seem to need to be present in most proliferative situations, as

its downregulation at least partially reducing proliferation in response to bacterial infection, bleomycin damage and DSS damage, although this varied by the lab carrying out the experiment (Karpowicz, Perez and Perrimon, 2010; Ren *et al.*, 2010; Shaw *et al.*, 2010).

Wts and Yki have been shown to be involved in a mechanosensing pathway in the gut that involves the Hippo pathway kinase Tao-1 and Msn, which like Hpo is a Ste20-like kinase. After feeding of yeast particles, which causes midgut stretching, Msn subcellular localisation in ISC and EB changes from the membrane to the cytoplasm, preventing it from inhibiting Yki via Wts. Thus Yki can induce ISC proliferation (Li *et al.*, 2018). After mating, food intake increases dramatically (Carvalho *et al.*, 2006; Hadjieconomou *et al.*, 2020), which may stimulate a similar midgut stretching.

I showed that transcriptionally there is a slight downregulation of the Hippo pathway in mated females, but it is not significant (**Section 4.2.1**). I did show however that there is a significant increase in *bantam-sensor::GFP* signal in mated females, especially in the R2 region of the gut (**Section 4.2.2**). This hints that the Hippo pathway may be suppressed after mating in females. It would be interesting to investigate if this suppression is caused by increased food intake changing Msn localisation and thus increasing Yki activity.

Several papers have linked the Hippo pathway with hormonal signalling in other tissues. During the programmed cell death (PCD) of larval metamorphosis, 20HE has been shown to actually increase *hpo* levels, resulting in reduced Yki activity and a reduction in inhibitor of apoptosis (*IAP*) expression. This results in an increase in caspase activity and transition into PCD (Dong *et al.*, 2015).

In the larval wing disc, Taiman (Tai), an ecdysone transcriptional co-activator binds to Yki to induce the expression of an alternate set of Yki-responsive genes. Rather than the canonical Yki targets like *ex*, *CyclinE* and *bantam* this includes the germline stem cell factors *nanos* and *piwi*, and secreted insulin-like peptide *dilp-8* and 20HE target gene *Eig71Ee*. In *yki^{S168A}* (constitutively active single mutant) expressed in wing discs Tai is important in determining the transcriptional output, hinting that in other tumour models Tai may influence the impact of *yki* overexpression (Zhang *et al.*, 2015). Maybe

in the gut, after mating the increased 20HE levels induce more Tai-mediated Yki target gene expression.

Finally, in the prothoracic gland, several factors feed into control of 20HE production needed to coordinate growth, including *Drosophila* insulin-like peptides (Dilps) and the brain neuropeptide prothoracicotropic hormone (PTTH). High Dilps and PTTH factors act via Wts, which then inhibits Yki and its target *bantam*, which prevents 20HE production. 20HE is needed to increase disc size without increasing overall larval size, thus coordinating their growth (Moeller *et al.*, 2017). Thus, Wts increases 20HE production in this context. If this is the case in the midgut, then maybe *wts* downregulation leads to a prevention in 20HE production post-mating, thus preventing the post-mating remodelling. Although the connection between the Hippo pathway and 20HE is likely to be dependent on the tissue, it is another interesting avenue to pursue, for example by quantifying 20HE titres after midgut ISC/EB *wts* downregulation.

Chapter 5: Results III

5.1 Introduction

After profiling the proliferation of control guts, *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} and *wtS*^{RNAi} tumours guts in virgin males, virgin females and mated females, I found that (a). the difference in midgut stem cell proliferation normally apparent between virgin and mated females was lost in Hippo pathway tumours *yki*^{3SA} and *wtS*^{RNAi} (**Section 3.3.1**), and (b). the midguts of *wtS*^{RNAi} males did not proliferate, whereas those of *Ras*^{V12} *Apc*^{RNAi} and *yki*^{3SA} males did (**Section 3.3.1**). I additionally found differences in the length of the midgut after tumour induction, and the regionality of tumour formation (**Section 3.3.4**). I then found that tumours caused by *wtS*^{RNAi} in virgin males and females require *yki* to proliferate (**Section 4.3**). However, I found that whilst males with feminised ISC/EB would proliferate with *wtS*^{RNAi}, (**Section 4.4.1**) females with masculinised ISC/EB proliferate regardless (**Section 4.4.2**). Finally, I found that in Oregon-R control midguts, there is some evidence that Hippo pathway activity is reduced after mating, but this needs further investigation (**Section 4.2**).

In this Chapter, in order to understand more about why different genetic tumours behave differently, I carry out an RNA-sequencing (RNA-seq) experiment to investigate the transcriptional changes. I then examine which sex and reproductive transcriptional changes have been lost in the tumours.

5.1.1 Sex differences in the midgut

Male and female midguts have many sex differences. The female gut is longer, and has ISC that proliferate more both during homeostasis and in response to damage, including DSS, bacterial infection and *Notch*^{RNAi} tumour induction (Hudry, Khadayate and Miguel-Aliaga, 2016). Many of these sex differences are mediated by the intrinsic sex of the ISC and its non-canonical Tra splicing pathway (**Section 4.1.7**). The male midgut has a strong enrichment in EC sugar metabolism mediated by the communication between the midgut and the testis (Hudry, Khadayate and Miguel-Aliaga, 2016).

5.1.2 Post-mating changes in the female midgut

Mating induces many changes in the female midgut via male-derived Sex peptide. This acts on the *corpora allata* to release juvenile hormone (JH; Moshitzky *et al.*, 1996)

that then acts on the midgut (Reiff *et al.*, 2015). Levels of 20-Hydroxy-Ecdysone (20HE; the active form of ecdysone) also increase in the ovaries and haemolymph after mating and act in the midgut to drive proliferation and differentiation (Zipper *et al.*, 2020).

There are many transcriptional changes induced by Sex peptide, including an increase in serine-type endopeptidases and a decrease in the carbohydrate metabolic genes and Glutathione-S-transferase (Gst) genes associated with detoxification (White *et al.*, 2021). Previous work by the Dominguez and Miguel-Aliaga labs showed that after mating JH causes an increase in ISC proliferation resulting in an increased midgut length and width, especially in the R4 region (Reiff *et al.*, 2015). Lipid metabolism is also altered, including upregulation of expression of genes related to fatty acid synthesis and activation, like long chain fatty acid CoA ligases *bubblegum* (*bgm*) and *Acyl-CoA synthetase long-chain* (*Acs1*). Mated females have increased lipid accumulation in the posterior gut. These changes in lipid metabolism are important for egg production. Mating also increases the number of midgut EE cells, especially the subset of EE cells that express the hormone Bursicon α (Burs) (Hadjieconomou *et al.*, 2020). Finally mating also mediates communication from the midgut to the ovary. Sex peptide triggers EE cells to produce peptide hormone neuropeptide F (NPF) that travels to the ovary to drive germline stem cell (GSC) proliferation (Ameku *et al.*, 2018).

5.2 RNA sequencing and Gene Ontology Analysis

5.2.1 Sample preparation and RNA-sequencing

Different genetic tumours behave differently depending on their sex and reproductive status (**Section 3.3**). To probe this further, I performed a bulk RNA-seq experiment on whole midguts to investigate these differences on a transcriptional level.

As I was interested in studying three different genetic tumours, in virgin males, virgin females and mated females, I already had nine conditions. With 30 flies per condition, performed in quadruplicate, this already involved the dissection of over 1000 flies. To also have a control would have been 12 conditions, and I wanted to ensure the quality of my dissections and RNA extractions. Thus, I opted not to add the control condition, and to instead use several bulk RNAseq control experiments the lab had previously done. For clarity, I will only refer to one of them: Tomotsune Ameku's profiling of

Oregon-R virgin male, virgin female and mated female fly midguts. However, when I discuss a transcriptional feature associated with sex or mating it is conserved within all the other control datasets. These include the virgin male versus virgin female dataset created by Bruno Hudry (*w*, *Su(H)GBE-LacZ/w*; *esg^{NP7397}-Gal4*, *UAS-CD8::GFP*, *tub-Gal80^{ts/+};+/-*; Hudry, Khadayate and Miguel-Aliaga, 2016), as well as an unpublished virgin female versus mated female RNAseq dataset (*w¹¹¹⁸*) prepared by Jake Jacobson (unpublished). These combined datasets allow us to focus on sex and reproductive differences common between them.

However, they do not take into account several different variables. For example, the Hudry dataset is of the correct genetic background (*Su(H)GBE-LacZ*; *esg^{ts} > GFP*), but the Jacobson dataset is *w¹¹¹⁸* and the Ameku dataset is Oregon-R. The Jacobson and Ameku datasets were performed at 25°C, and the Hudry dataset, like my own was performed at 29°C. Most importantly, for the mated female conditions, there is a discrepancy in the number of days post-mating. Both mating datasets (Jacobson and Ameku) are 3 d post-mating, whereas my flies are 7 d post-mating. I froze my flies after tumour induction, to ensure I could dissect them without rushing and damaging the tissue or contaminating the RNA. This will potentially reduce the amount of RNA recovered, resulting in a lower coverage of reads. As the control datasets were dissected from fresh flies, this is another factor that is different between them. Other factors to consider are the different yeast batches for the food, differences in the person dissecting and the amount the gut is damaged in the process, and differences in who extracted the RNA and its resulting integrity. Thus, none of these datasets can be directly compared.

Regardless, taken together with our existing knowledge they can be used to broadly characterise the expected differences in gene expression in response to sex and reproduction. For example, we can consider the well characterised increase in sugar gene metabolism in males compared with virgin females (Hudry et al., 2019), and the increase in lipid metabolism in mated females compared with virgin females (Reiff et al., 2015). Additionally, any interesting finding from this comparison has or will be verified experimentally using the correct control animals, as well as qPCR.

Thus, the samples sequenced in my RNA-seq experiment were: *Ras*^{V12}*Apc*^{RNAi}, *yki*^{3SA} and *wt*^{RNAi} in virgin males, virgin females and mated females, a total of 9 conditions. The tumours were induced for 6 d, as this marked the highest point of difference in proliferation for the *Ras*^{V12}*Apc*^{RNAi} virgin and mated females, whilst still having a significant response in the other tumour conditions. Flies were flash frozen after 6 d of tumour induction, then dissected with each different condition randomly assigned to a dissection day. The hindgut and crop were removed, as *esg* is not expressed there, thus they do not directly contribute to the tumour.

Midgut dissections, RNA extraction and processing was performed by me. Library preparation and HiSeq sequencing was performed by Laurence Game and Ivan Andrew at the LMS Genomics Core Facility. Bioinformatics analysis was performed by Yi-Fang Wang and Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility.

For the Oregon-R control dataset of virgin male, virgin female and mated female fly midguts, Tomotsune Ameku performed the RNA extraction and processing, HiSeq sequencing was performed by Laurence Game and Ivan Andrew at the LMS Genomics Core Facility and bioinformatics analysis was performed by Yi-Fang Wang and Tomotsune Ameku at the LMS Bioinformatics Core Facility.

5.2.2 Data processing and principal component analysis

FasQC (v0.11.9) was used to generate FastQC results. Reads were aligned to *Drosophila* (dm6) using STAR (Dobin et al., 2013). MultiQC was used to generate a summary report for the FastQC and STAR alignment results. The count matrix was prepared by Principal Component Analysis (PCA), a method of visualising correlation between samples.

The PCA plot revealed good clustering for each group (**Figure 5.1a**). The most distant cluster was the *wt*^{RNAi} virgin males, the condition with the least proliferation. The *Ras*^{G12V} *Apc*^{RNAi} and *yki*^{3SA} males cluster closer to the other conditions, but still distinctly. The *Ras*^{G12V} *Apc*^{RNAi} virgin and mated females cluster nearby in two separate groups. For *yki*^{3SA} virgin and mated females however, the groups have some overlap. Finally, for the *wt*^{RNAi} virgin and mated females, the groups completely overlap. This

reflects the proliferation data, where *Ras*^{G12V} *Apc*^{RNAi} females retain a reproductive difference, but Hippo pathway tumours lose it. The PCA plot shows the Hippo pathway tumours are transcriptionally similar between virgin female and mated females. This could mean that disturbing the Hippo pathway either prevents normal post-mating changes in the mated female, or induces them in the virgin female.

5.2.3 Differential expression analysis with DESeq2

For the comparison of the number of differentially expressed (DE) genes I used a p-adjusted (p-adj) value threshold of less than 0.05, and a fold-change (FC) value of threshold of more than 1.5 (**Figure 5.1b**). The comparison with the most DE genes was between *wt*^{RNAi} virgin males and *wt*^{RNAi} virgin females (upDE: 2224, downDE: 2623). This is followed by *Ras*^{G12V} *Apc*^{RNAi} virgin males versus *Ras*^{G12V} *Apc*^{RNAi} virgin females (upDE: 1357, downDE: 1612), and then the *yki*^{3SA} virgin males and *yki*^{3SA} virgin females are the most similar of the sex comparisons (upDE: 914, downDE: 928).

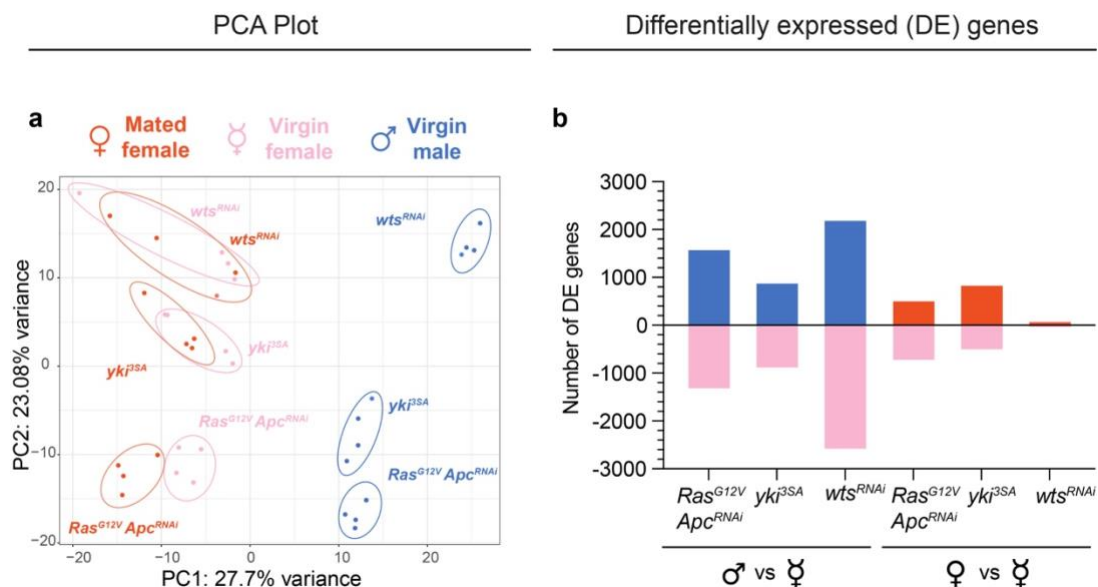


Figure 5.1 Principal component analysis (PCA) and number of differentially expressed (DE) genes in RNA-seq.

a. PCA plot of RNA-sequencing data of whole midguts with *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} and *wt*^{RNAi} tumours induced for 6 d, in virgin males, virgin females and mated females. **b.** Number of differentially expressed genes with respect to sex (virgin male minus virgin female) and reproduction (mated female minus virgin female) for the conditions described in a. ♂ - virgin males, ♀ - virgin females, ♀ - mated females.

Midgut dissections, RNA extraction and processing was performed by me. Library preparation and HiSeq sequencing was performed by Laurence Game and Ivan Andrew at the LMS Genomics Core Facility. Bioinformatics analysis was performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility.

The comparison with the least DE genes is *wt^{sRNAi}* virgin females versus *wt^{sRNAi}* mated females (upDE: 106, down: 33). There are more reproductive differences between *yki^{3SA}* virgin females versus *yki^{3SA}* mated females (upDE: 866, downDE: 545) and *Ras^{G12V} Apc^{RNAi}* virgin females versus *Ras^{G12V} Apc^{RNAi}* mated females (upDE: 541, downDE: 766). It is expected there will be generally fewer DE genes for the reproductive differences than the sex differences, and it is interesting how transcriptionally similar the *wt^{sRNAi}* virgin and mated females are.

5.2.4 Gene Ontology (GO) analysis of RNA-seq

In order to further probe the many DE genes in my comparisons, we used Gene Ontology (GO) analysis to identify different pathway enrichments. GO analysis uses pre-defined gene sets that have a common biological function and calculates their enrichment or depletion within a comparison of two conditions. We used the DE gene list with the same threshold as the DEseq2 analysis ($p\text{-adj} \leq 0.05$, $FC \geq 1.5$), and performed the analysis with the R Bioconductor package goseq (Young *et al.*, 2010).

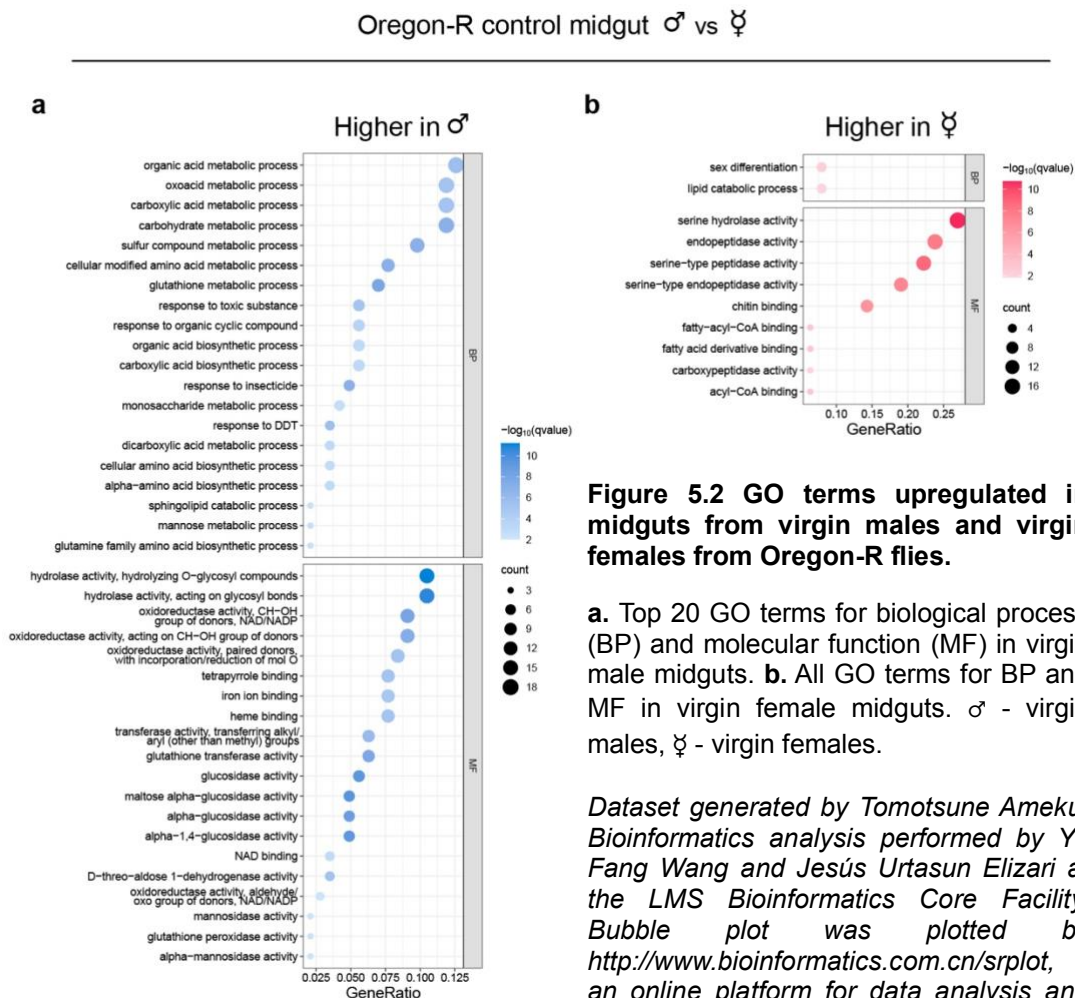


Figure 5.2 GO terms upregulated in midguts from virgin males and virgin females from Oregon-R flies.

a. Top 20 GO terms for biological process (BP) and molecular function (MF) in virgin male midguts. **b.** All GO terms for BP and MF in virgin female midguts. ♂ - virgin males, ♀ - virgin females.

Dataset generated by Tomotsune Ameku. Bioinformatics analysis performed by Yi-Fang Wang and Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang *et al.*, 2023).

5.3 Transcriptional sex differences in tumourigenesis

5.3.1 Sex differences in control midguts

As I did not prepare a control in my RNAseq data, I initially looked at the GO analysis of an RNAseq dataset of whole Oregon-R midguts performed in the Miguel-Aliaga lab by Tomotsune Ameku. This were reanalysed with the same pipeline as my RNA-seq, and the analysis of the top GO terms is my own. I also referenced several other control datasets the lab has used in the past, and the features I refer to that change with sex and reproduction are generally conserved between these different datasets. For clarity, I have displayed only the Oregon-R control, and looked at the top 20 biological process (BP) and top 20 molecular function (MF) terms. The GO terms were sorted into up (higher in virgin males) and down (lower in virgin males), and throughout I have referred to up as ‘virgin male-enriched’ and down as ‘virgin female-enriched’.

In this Oregon-R control dataset, comparing the Oregon-R virgin males versus the Oregon-R virgin females, two of the defining BP of the male transcriptome are sugar gene metabolism (*carbohydrate metabolic process*) and glutathione metabolism (*glutathione metabolic process*; **Figure 5.2a**). Carbohydrate metabolism involves the upregulation of sugar digestion, glycolysis, and some TCA cycle genes to produce citrate, important for spermatogenesis (Hudry *et al.*, 2019). These genes can also be seen in the MF terms involving *hydrolase activity* and several *glucosidase* categories. The importance of glutathione metabolism in males is not fully understood, but similar DE genes are also found in the male-enriched *cellular modified amino acid metabolic process* and *sulfur compound metabolic process*. This includes many genes from the Glutathione-S-transferase (Gst) families (*GstE*, *GstD*, *GstD*). The other highly enriched categories of *organic acid*, *oxoacid* and *carboxylic acid metabolic process* include many DE genes involved in NAD/NADP reduction (*Idh*, *Men*, *CG8665*). These processes can also be seen in several MF involving *oxidoreductase activity* and *NAD/NADH* (**Figure 5.2a**).

In the Oregon-R control condition, virgin females have much higher expression of serine peptidases like *highroad*, and the serine protease (*sp*) and *Jonah* families (*serine hydrolase activity*; **Figure 5.2b**). The exact function of these serine peptidases is poorly defined and some hypothesise a role in digestion and immunity (Ross *et al.*,

2003; Kumar *et al.*, 2021). Virgin females also have higher expression of lipid metabolism genes (*lipid catabolic process*) and of female specific proteins like the yolk proteins (*yp*), *tra* and *Sxl* (*sex differentiation*). There is also an increase in *acyl-CoA binding* because of several *acyl-Coenzyme A oxidase (Acbp)* genes. Peritrophic membrane components like *Peritrophin* family genes are also upregulated in the *chitin binding* category (**Figure 5.2b**).

Thus, there are several GO terms enriched in virgin males and virgin females from the Oregon-R control dataset that are conserved in other Miguel-Aliaga control datasets and can be used to compare to my tumour dataset to see if sex differences are maintained.

5.3.2 Sex differences in *Ras*^{G12V} *Apc*^{RNAi} males and females

Males expressing *Ras*^{G12V} *Apc*^{RNAi} in their midgut ISC and EB have tumours, but they proliferate less than virgin or mated female tumours. I took the GO terms that I described from the Oregon-R virgin male versus virgin female dataset, and compared them to the *Ras*^{G12V} *Apc*^{RNAi} virgin male versus *Ras*^{G12V} *Apc*^{RNAi} virgin female comparison. Transcriptionally, these males maintain many of the sex differences as the Oregon-R males, including *carbohydrate metabolic process* and *glutathione metabolic process* and other related terms involving the *Gst* genes (**Figure 5.3a**). They also maintain the NAD/NADH-related terms *organic acid*, *oxoacid* and *carboxylic acid metabolic process*. These males now have enrichment of *acyl-CoA-metabolic processes*, a term enriched in the Oregon-R virgin females. However, the genes enriched in this term are different, and many involve *Acyl-CoA synthetase (Acsx)* family genes. *Ras*^{G12V} *Apc*^{RNAi} males also have enriched *metal ion transmembrane activity* mainly composed of enriched calcium (Ca) transporters.

Ras^{G12V} *Apc*^{RNAi} virgin females maintain enriched *lipid catabolic process*, although they have more lipid related terms than the Oregon-R control (**Figure 5.3b**). They also maintain *serine hydrolase activity* and *chitin binding*. Although they have lost the term *sex differentiation*, they have higher levels of *yp1-3*, *tra* and *Sxl*.

Thus, *Ras*^{G12V} *Apc*^{RNAi} males maintain most sex differences, but have an enrichment of the female-biased term *acyl-CoA-metabolic processes*, and *Ras*^{G12V} *Apc*^{RNAi} virgin females maintain all expected female-biased terms.

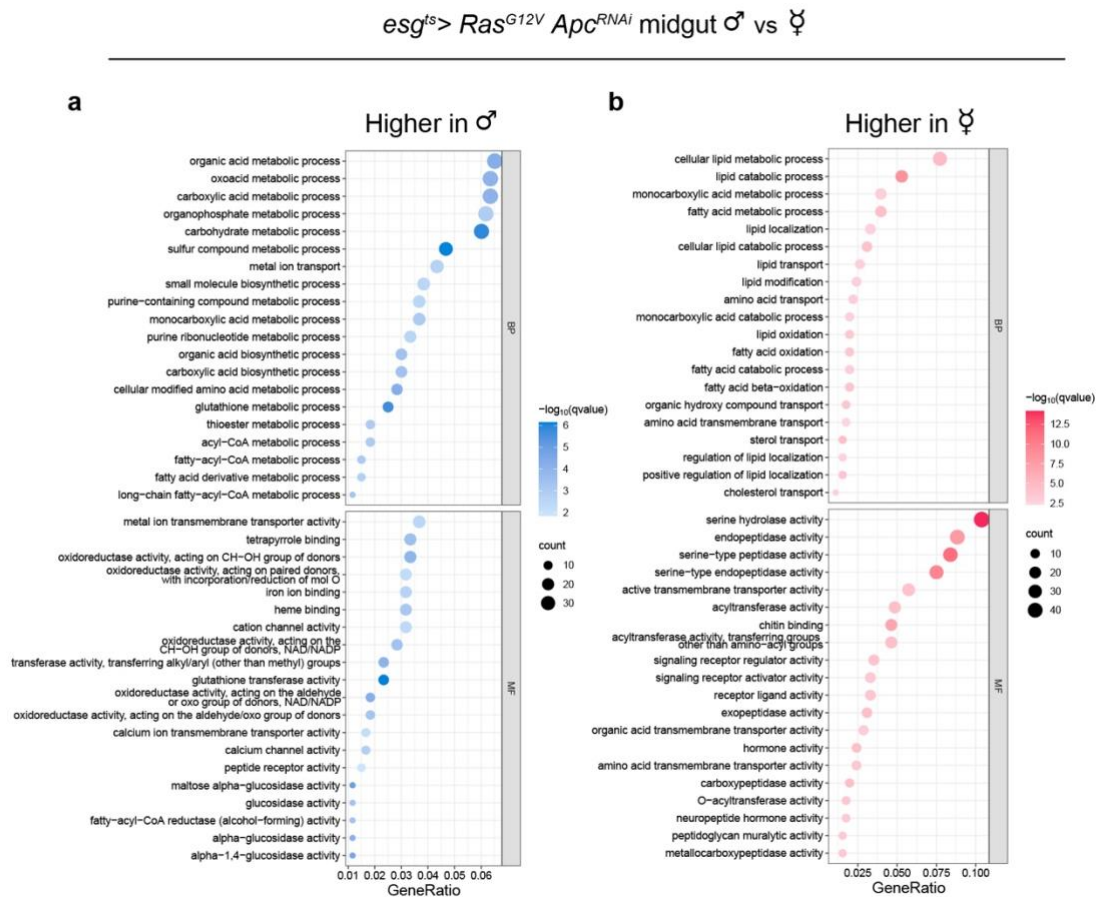


Figure 5.3. GO terms upregulated in midguts from virgin males and virgin females expressing *esg^{ts} > Ras^{G12V} Apc^{RNAi}* for 6 d.

a. Top 20 GO terms for biological process (BP) and molecular function (MF) in virgin male midguts. **b.** Top 20 GO terms for BP and MF in virgin female midguts. ♂ - virgin males, ♀ - virgin females.

Dataset generated by me. Bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

5.3.3 Sex differences in *yki*^{3SA} males and females

Males with *yki*^{3SA} midgut tumours proliferate less than the highly proliferative *yki*^{3SA} virgin or mated females. Here however, comparing *yki*^{3SA} virgin males versus *yki*^{3SA} virgin females, males have lost many of their sexually dimorphic features, and have fewer GO categories (**Figure 5.4a**). They no longer have enhanced *organic acid*, *oxoacid* and *carboxylic acid metabolic process*, although they have two

oxidoreductase activity and *NAD/NADH related MF term*. They have also lost *glutathione metabolic process* and related terms *cellular modified amino acid metabolic process* and *sulfur compound metabolic process*. However, they do maintain *carbohydrate metabolic process*, although all of the *hydrolase activity* and several *glucosidase* categories are no longer enriched. Unlike the Oregon-R control, they have increased *cell adhesion* genes including extracellular matrix (ECM) adhesion mechano-effector *Vinculin* and ECM component *Laminin* (*LanB1/2*). They also have *embryonic organ development*, a term that includes receptor tyrosine kinase ligands *Pvf1/2*, morphogenic ligand *robo1/2* and ECM proteinase *MMP1*.

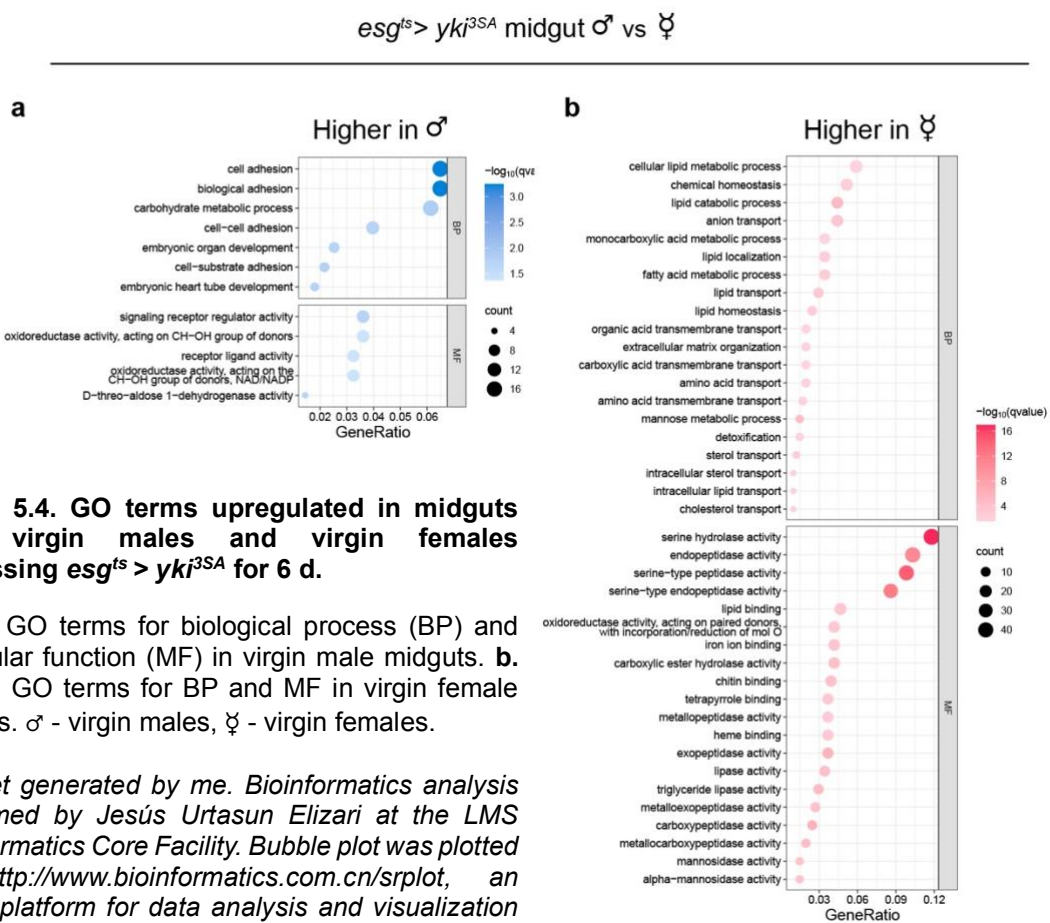


Figure 5.4. GO terms upregulated in midguts from virgin males and virgin females expressing *esg^{ts} > yki^{3SA}* for 6 d.

a. Top GO terms for biological process (BP) and molecular function (MF) in virgin male midguts. **b.** Top 20 GO terms for BP and MF in virgin female midguts. ♂ - virgin males, ♀ - virgin females.

Dataset generated by me. Bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

yki^{3SA} virgin females maintain sex differences including *lipid catabolic process*, and also have more lipid related terms than the Oregon-R (**Figure 5.4b**). They maintain higher *serine hydrolase activity* and *chitin binding*. Unlike the control, they also have the term *extracellular matrix organization* which includes *papilin* (*pap*), an ECM protein

that modulates cell rearrangements and chitin modifying protein *obstructor-A (obst-A)*, as well as several eggshell vitelline membrane proteins *female sterile (1) M3 (fs(1)M3)*, *Vitelline membrane 26Aa (Vm26Aa)* and *Chorion protein 36 (Cp36)*.

Thus *yki*^{3SA} virgin males maintain some male-biased features, but lose their *acid metabolic process* terms and some of their sugar metabolism MF terms, and have additional *cell adhesion* terms. *yki*^{3SA} virgin females maintain their sex differences, and have additional enrichment in *extracellular matrix organization*.

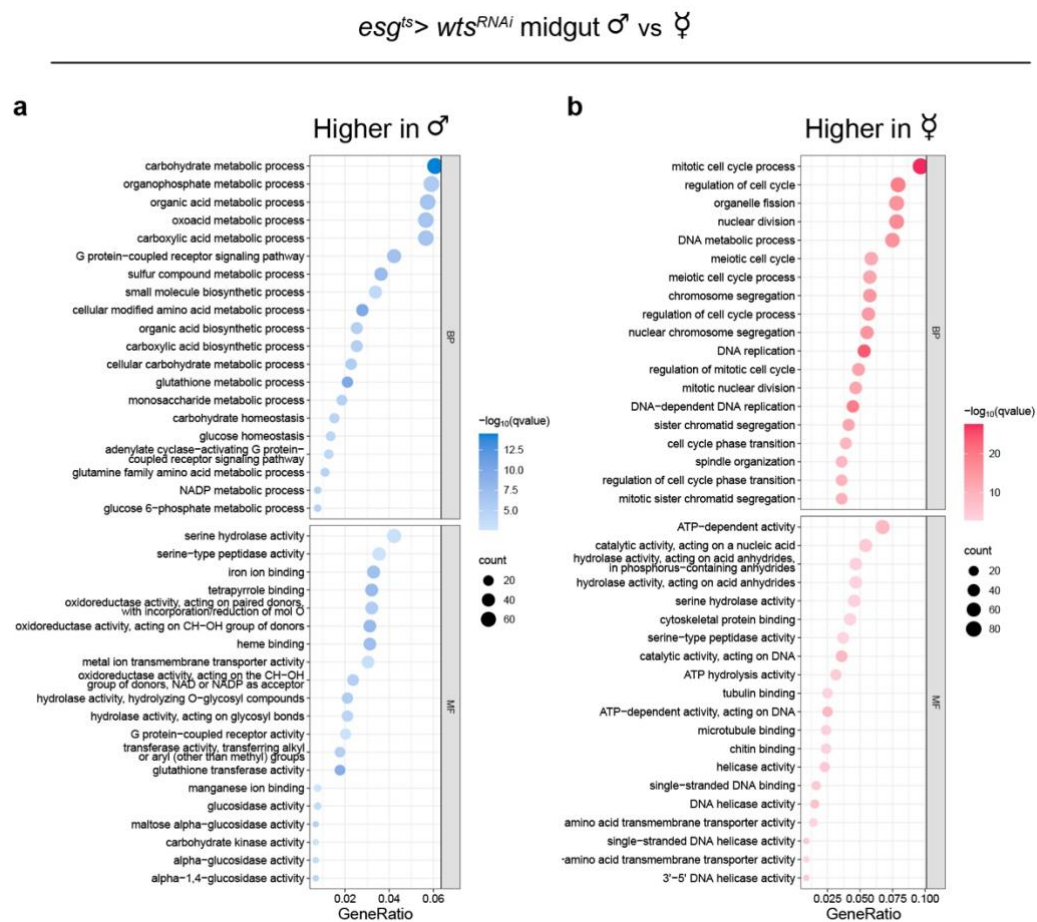


Figure 5.5. GO terms upregulated in midguts from virgin males and virgin females for expressing *esg*^{ts} > *wts*^{RNAi} for 6 d.

a. Top 20 GO terms for biological process (BP) and molecular function (MF) in virgin male midguts. **b.** Top 20 GO terms for BP and MF in virgin female midguts. ♂ - virgin males, ♀ - virgin females.

Dataset generated by me. Bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

5.3.4 Sex differences in *wt^{sRNAi}* males and females

Unlike females with *wt^{sRNAi}* midgut tumours, which proliferate robustly, males expressing *wt^{sRNAi}* in their midgut ISC and EB are resistant to tumour formation, and most will have no proliferation. Comparing *wt^{sRNAi}* virgin males versus *wt^{sRNAi}* virgin females, the males maintain higher sugar gene metabolism (*carbohydrate metabolic process*), as well as related terms including *glucose homeostasis* and *organophosphate metabolic process* (**Figure 5.5a**). Like Oregon-R control males, they have enriched *glutathione metabolic process* and related *cellular modified amino acid metabolic process* and *sulfur compound metabolic process*. They also maintain enriched *organic acid, oxoacid* and *carboxylic acid metabolic process*. Unlike the Oregon-R control, they have an enriched *G protein-coupled receptor signaling pathway*, especially including genes involved in cAMP metabolism.

The main dominating feature enriched in *wt^{sRNAi}* virgin females is mitosis and DNA replication (*mitotic cell cycle, regulation of cell cycle, DNA replication*) which is expected as the *wt^{sRNAi}* virgin females proliferate much more than the virgin males (**Figure 5.5b**). Lipid metabolism is enriched in *wt^{sRNAi}* virgin females, but is not in the top 20 enriched MF or BP terms. *Serine hydrolase activity* remains enriched, as does *chitin binding*.

Thus *wt^{sRNAi}* males maintain sex differences, especially sugar metabolism, with some additional cAMP metabolism. *wt^{sRNAi}* virgin females also maintain their sex differences, although some are masked by incredibly high levels of mitosis and DNA replication-related terms.

5.4 Reproductive differences in tumourigenesis

5.4.1 Reproductive differences in control midguts

To study reproduction, I sorted the top 20 MF and BP GO terms into up (higher in mated females) and down (lower in mated females), and throughout I have referred to up as ‘mated female-enriched’ and down as ‘virgin female-enriched’. In the Oregon-R control condition comparing virgin females and mated females, the main feature of the virgin female transcriptome is sugar metabolism (*carbohydrate metabolic process*), which can also be seen in the many *glucosidase* MF terms (**Figure 5.6a**). They also

feature several MF terms referring to steroid and estradiol dehydrogenase activity (*steroid dehydrogenase activity*, *estradiol 17-beta-dehydrogenase activity*).

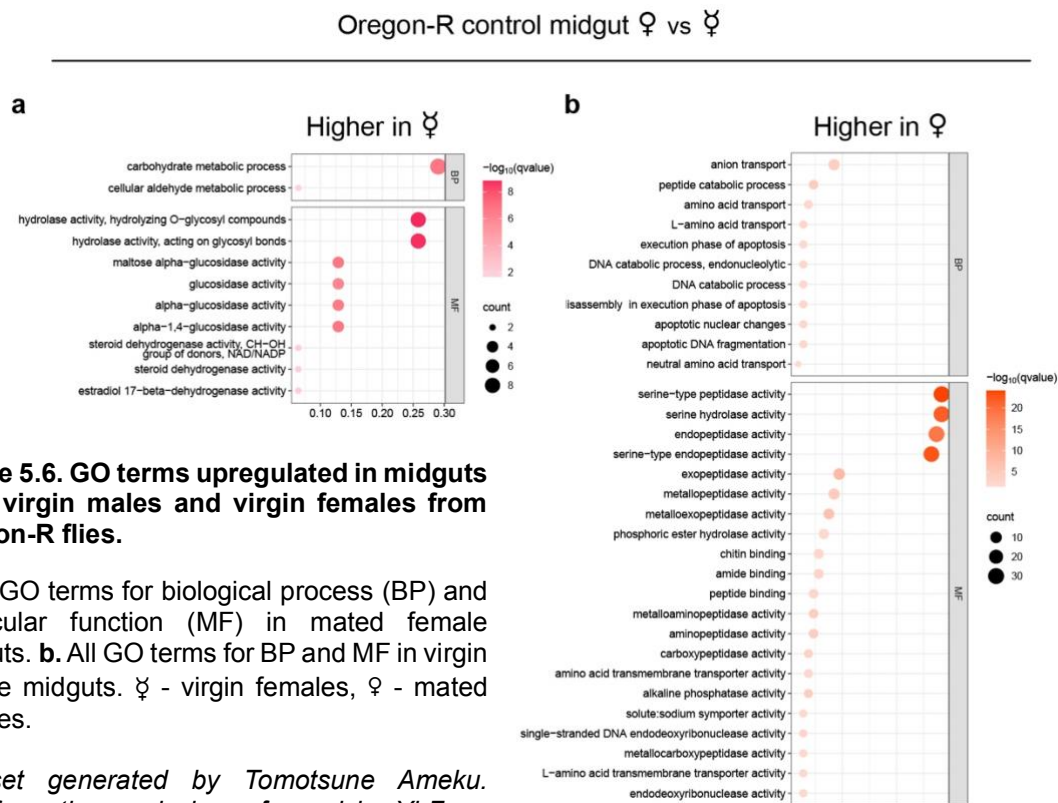


Figure 5.6. GO terms upregulated in midguts from virgin males and virgin females from Oregon-R flies.

a. All GO terms for biological process (BP) and molecular function (MF) in mated female midguts. **b.** All GO terms for BP and MF in virgin female midguts. ♂ - virgin females, ♀ - mated females.

Dataset generated by Tomotsune Ameku. Bioinformatics analysis performed by Yi-Fang Wang and Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

The Oregon-R mated control condition shows enrichment of many MF peptidase related terms (*serine-type peptidase activity*, *metallopeptidase activity*, *peptide catabolic process*), *chitin binding* well as some DNA and apoptosis related terms (*DNA catabolic process*, *execution phase of apoptosis*; **Figure 5.6b**). Many *amino acid transport* related terms are enriched, including *L-amino acid* and *neutral amino acid transport*, and also more broadly *anion transport*.

5.4.2 Reproductive differences in *Ras*^{G12V} *Apc*^{RNAi} virgin and mated females

Ras^{G12V} *Apc*^{RNAi} virgin females with midgut tumours proliferate less than *Ras*^{G12V} *Apc*^{RNAi} mated females. Comparing *Ras*^{G12V} *Apc*^{RNAi} virgin females versus *Ras*^{G12V}

Apc^{RNAi} mated females, I show that for virgin females carbohydrate metabolic process remains the most enriched term, alongside *glucose metabolism*, and many MF terms related to *glucosidase activity* (**Figure 5.7a**). Additionally, several *organic acid* terms are enriched, with many *NAD/NADH* MF terms, as well as *lipid homeostasis*.

Ras^{G12V} Apc^{RNAi} mated females do not have enrichment of peptidases, DNA or apoptosis related terms like the Oregon-R control (**Figure 5.7b**). Instead, they have a strong *fatty acid metabolic process* signature, and the terms *neurotransmitter transport* and *signal release from synapse*. They do have enriched *chitin binding* like the control.

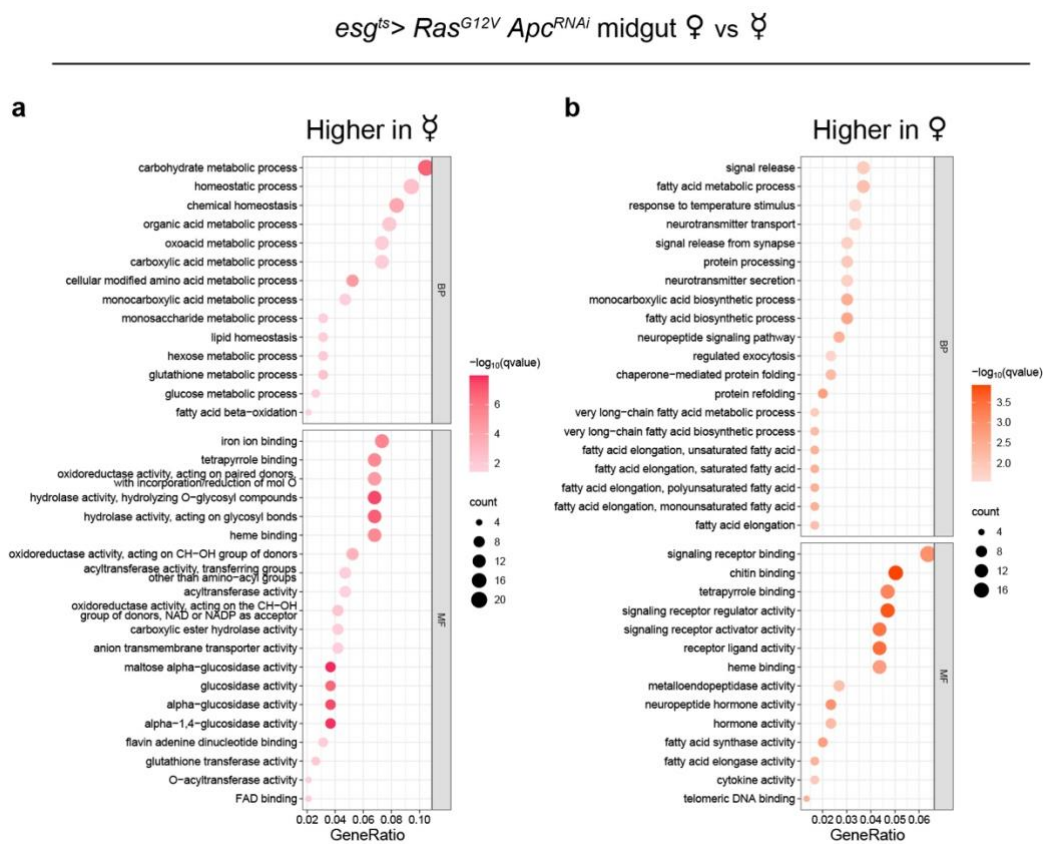


Figure 5.7. GO terms upregulated in midguts from mated females and virgin females expressing *esg^{ts} > Ras^{G12V} Apc^{RNAi}* for 6 d.

a. All GO terms for biological process (BP) and molecular function (MF) in mated female midguts. **b.** All GO terms for BP and MF in virgin female midguts. ♂ - virgin females, ♀ - mated females.

Dataset generated by me. Bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

Thus, the *Ras*^{G12V} *Apc*^{RNAi} virgin females maintain many virgin transcriptional signatures like *carbohydrate metabolism*, but the *Ras*^{G12V} *Apc*^{RNAi} mated females only have a few mated-like enrichment signatures like *chitin binding*.

5.4.3 Reproductive differences in *yki*^{3SA} virgin and mated females

yki^{3SA} virgin females have the same amount of proliferation as mated females, implying they may have lost some reproductive differences. Comparing *yki*^{3SA} virgin females versus *yki*^{3SA} mated females, virgin females do not have enrichment of any of the terms found in Oregon-R virgin females (**Figure 5.8a**). Instead, they have enrichment in a range of terms related to *regulation of cell differentiation*, *cell population proliferation* and *cell fate commitment*. Additionally, several *negative regulation of developmental process* related terms are enriched. This includes upregulation of genes such as *pointed (pnt)*, which can trigger cellular senescence upon *Ras* activation and *argos (aos)*, a negative regulator of EGFR signalling. Many of these terms more specifically regulate *neurogenesis* and *dorsal/ventral pattern formation*.

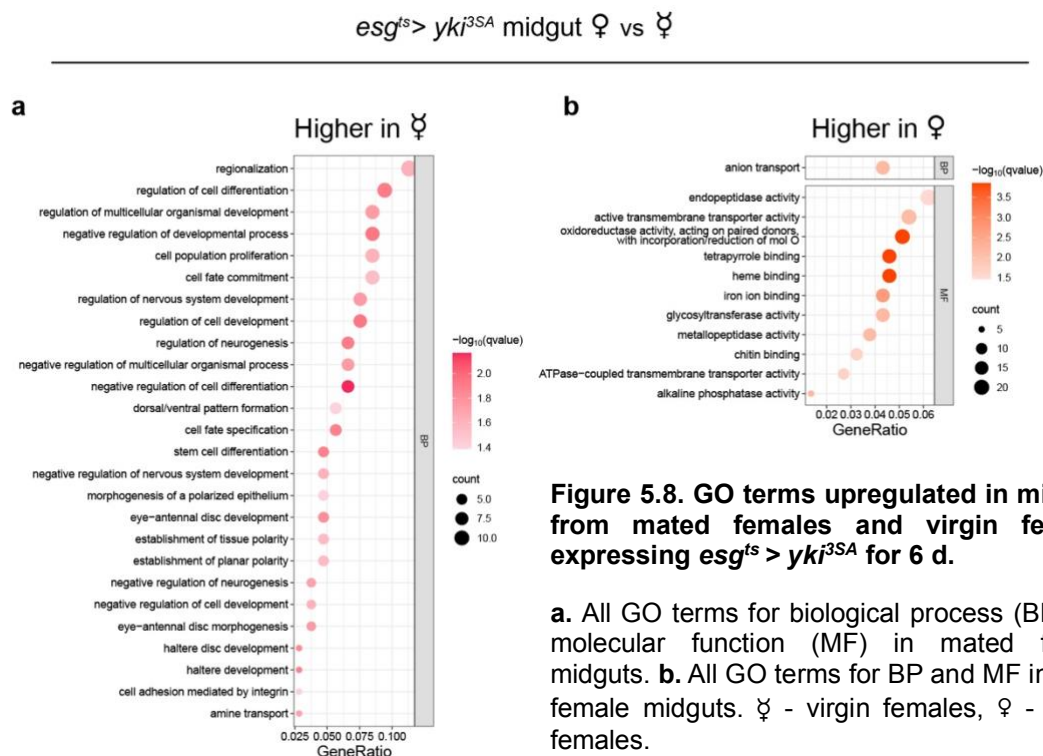


Figure 5.8. GO terms upregulated in midguts from mated females and virgin females expressing *esg*^{ts} > *yki*^{3SA} for 6 d.

a. All GO terms for biological process (BP) and molecular function (MF) in mated female midguts. **b.** All GO terms for BP and MF in virgin female midguts. ♂ - virgin females, ♀ - mated females.

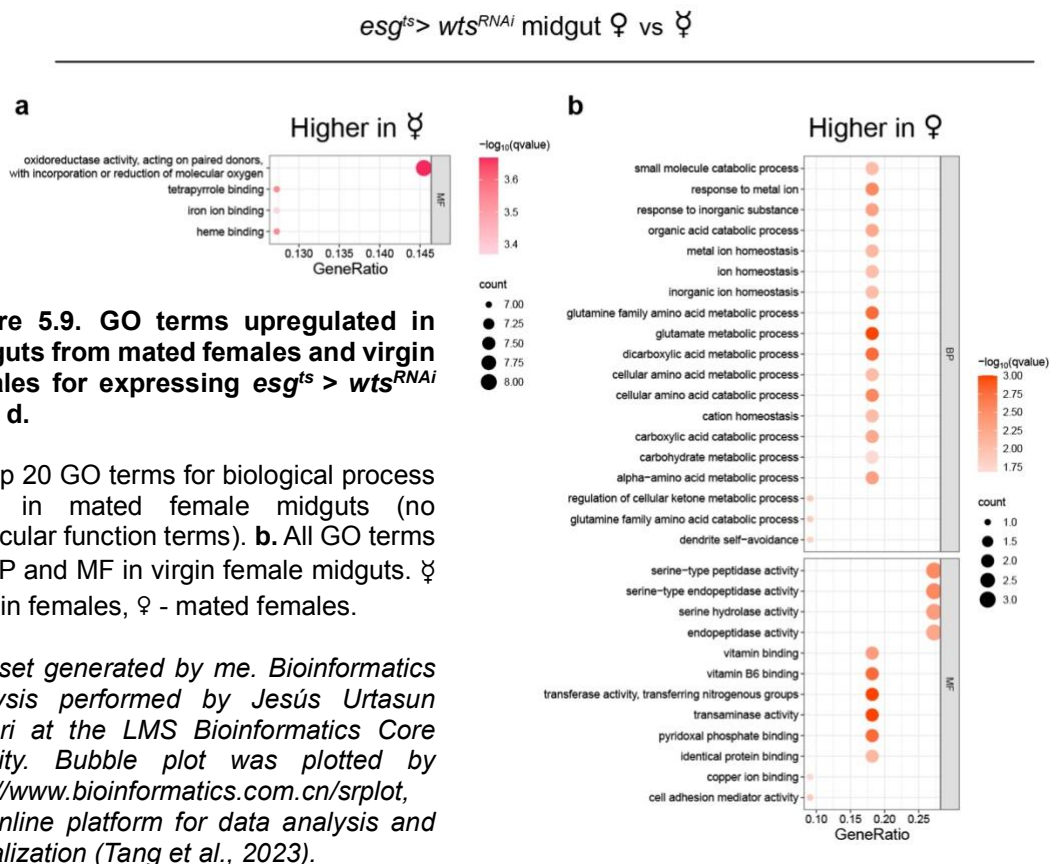
Dataset generated by me. Bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Bubble plot was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

Mated females expressing *yki*^{3SA} have fewer enriched GO terms (**Figure 5.8b**). They have some mating associated terms, including *anion transport*, *chitin binding* and some peptidase related terms like *endopeptidase activity*.

Thus, *yki*^{3SA} virgin females do not maintain any virgin-enriched features, but mated females have mating-associated terms including *chitin binding* and *endopeptidase activity*.

5.4.4 Reproductive differences in *wtS*^{RNAi} virgin and mated females

Unusually, virgin female *wtS*^{RNAi} midgut tumours proliferate more than mated female *wtS*^{RNAi} midgut tumours. Comparing *wtS*^{RNAi} virgin females versus *wtS*^{RNAi} mated females shows there are very few enriched terms in *wtS*^{RNAi} virgin females (**Figure 5.9a**). The main term is related to *oxidoreductase activity*, with some *iron ion binding* and similar MF terms. The only genes in their categories however are seven *Cytochrome P450 (Cyp)* genes, whose function is broadly uncharacterised, and may be important in insecticide tolerance and stress response.



There are more GO categories enriched in *wts^{RNAi}* mated females, but none of them have more than three genes per group (**Figure 5.9b**). They involve mainly *glutamine*, *glutamate* and *amino acid* metabolic process. The two genes that fuel these terms are *Gabat*, an enzyme that breaks down the neurotransmitter GABA, and *Oat*, an enzyme needed for arginine catabolism. There are three *serine-type peptidase activity* enzymes, but these are not amongst those typically upregulated after mating.

Therefore, at a transcriptional level, *wts^{RNAi}* virgin and mated females are almost identical, with very few GO categories enriched in either condition.

5.4.5 Hippo pathway tumours lose reproductive differences in females

Many transcriptional changes in the midgut post-mating have been profiled. In addition to an increase in lipid metabolism (Reiff *et al.*, 2015), there is an increase in amino acid transporters, chitin metabolism and serine-type endopeptidases (White *et al.*, 2021). There is also a decrease in sugar metabolism, particularly glucose metabolism and transport, galactose metabolism and maltase expression, as well as a decrease in *Gst* expression (White *et al.*, 2021). White and colleagues compiled a subset of genes they found to change with mating, which I have used to assess how mated the guts are.

Using this subset of genes, I compared how their expression changed in the Oregon-R control dataset (**Figure 5.10a**), and my tumour dataset (**Figure 5.10b**). For my dataset, I mated the females for 1 d before tumour induction for 6 d. This is a later timepoint post-mating than the Oregon-R control dataset (3 d post-mating) and the reference gene list from White *et al* (2 d post-mating; White *et al.*, 2021), thus not all of the post-mating changes in the midgut will be found at this timepoint. Interestingly, in the Oregon-R control dataset, factors that increase after mating (amino acid transport, chitin, peptidases and lipid metabolism) are expressed at similar levels in virgin females and males, and then increase dramatically only in mated females. However, factors that decrease after mating (sugar metabolism and glutathione-S-transferases) are markedly higher in males than virgin females, then decrease further in mated females.

For the tumour dataset, *Ras*^{G12V} *Apc*^{RNAi} females do have an increase in chitin metabolism after mating, but not the other expected increases in gene expression. They do however maintain a reduction in carbohydrate metabolism and Glutathione-S-transferase expression after mating. This is similar to what I found in the GO analysis (Section 5.4.2). For both *yki*^{3SA} and *wtS*^{RNAi} there is no difference in carbohydrate metabolism

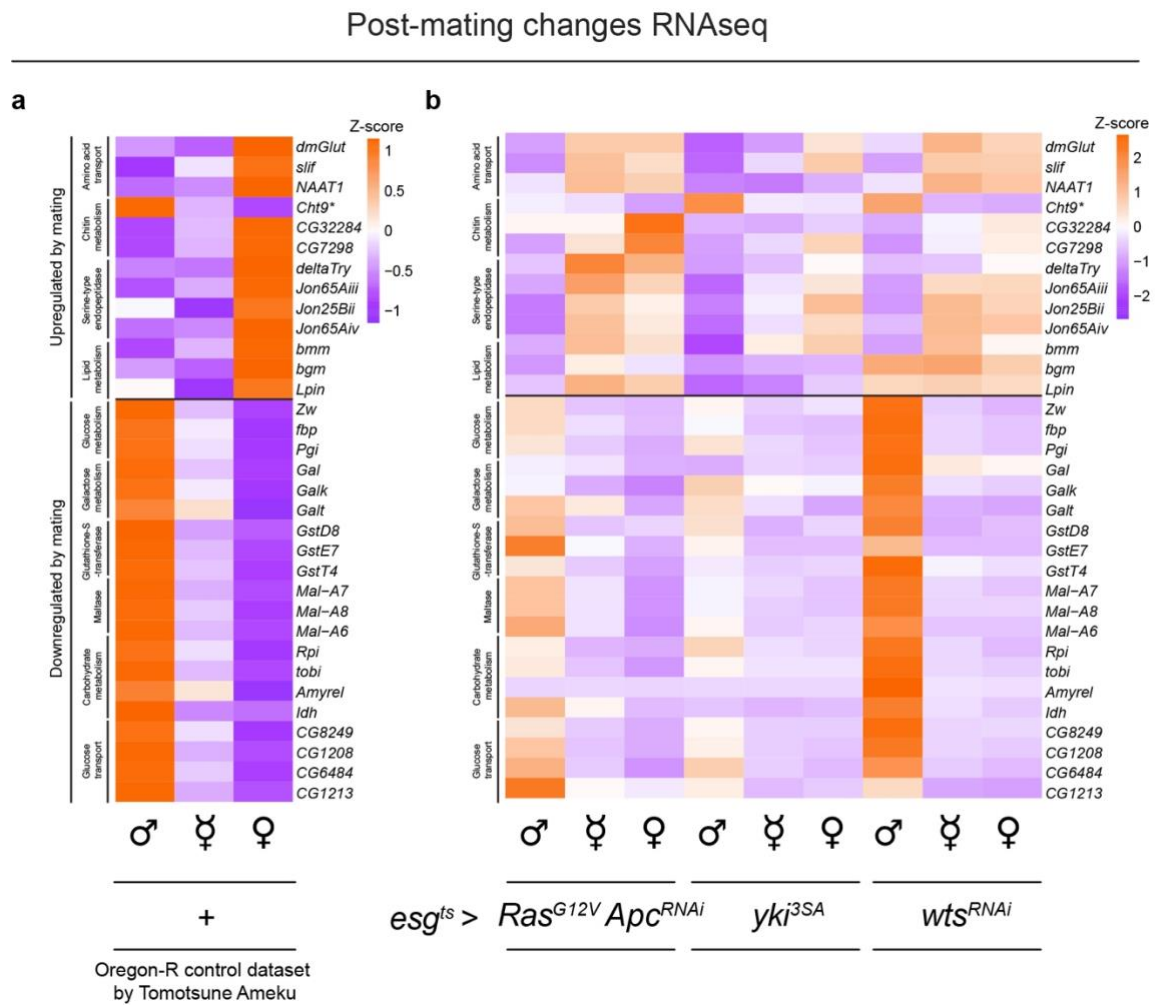


Figure 5.10. Many mating induced transcriptional changes are lost after midgut tumour generation with *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} or *wtS*^{RNAi}.

a. Mating-induced changes in gene expression in Oregon-R virgin males, virgin females and mated females 3 d post-mating. **b.** Mating-induced changes in gene expression in midguts expressing *esg*^{ts} driving *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} or *wtS*^{RNAi} in virgin males, virgin females and mated females. An asterisk indicates a gene that negatively regulates a process, and hence is expected to change expression in the opposite direction.

Oregon-R dataset generated by Tomotsune Ameku with bioinformatics analysis performed by Yi-Fang Wang at the LMS Bioinformatics Core Facility. Tumour dataset generated by me, with bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility.

Heatmap was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

and glutathione-S-transferases after mating, again echoing my GO analysis results (**Section 5.4.2 - 3**). The *yki*^{3SA} female midguts have increased endopeptidase expression after mating, but *wts*^{RNAi} females are remarkably similar in their expression of all genes expected to change with mating. Thus, although none of the tumour models have all the expected transcriptional changes associated with mating, *Ras*^{G12V} *Apc*^{RNAi} preserves the most differences, whereas the midguts of *yki*^{3SA} and *wts*^{RNAi} tumours are transcriptionally very similar between virgin and mated females.

5.5 Discussion

In this Chapter I performed RNA-seq analysis on whole midguts with *esg*-driven *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} and *wts*^{RNAi} tumours, in virgin males, virgin females and mated females, to determine potential mechanisms for how sex and reproductive status affects midgut tumourigenesis. I identified male-biased sugar metabolism as being lost in male tumours that proliferate (*Ras*^{V12} *Apc*^{RNAi} and *yki*^{3SA}) and maintained in the non-proliferative tumour (*wts*^{RNAi}). I finally looked further into how reproductive status affects female tumour proliferation by comparing their transcriptomes to identify which GO terms associated with reproductive are lost or maintained.

5.5.1 Many sex differences are lost after tumour formation

There are many interesting sex differences between virgin male and female midguts that have never been fully explored. Control male midguts have a strong enrichment in *Gst* genes and *glutathione metabolic process*, as well as the terms related terms *cellular modified amino acid metabolic process* and *sulfur compound metabolic process* (**Section 5.3.1**). These genes are also further downregulated in females after mating. Males with *yki*^{3SA} midgut tumours no longer have enrichment of this term compared to virgin females of the same genotype (**Section 5.3.3**), but males with *Ras*^{G12V} *Apc*^{RNAi} maintain this term (**Section 5.3.2**). The function of these genes is not fully understood. Indeed, the more proliferative gut of a mated females would surely have greater need of the ability to process toxic dietary compounds and oxidative stress (Tu and Akgül, 2005). Instead, mated females have increased risk of gut dysplasia resulting in reduced lifespan, potentially as a consequence of a poorer ability to deal with such stress (Chapman *et al.*, 1995; Wang, Salmon and Harshman, 2001;

Ahmed *et al.*, 2020). The role of oxidative stress in midgut tumours would be an interesting avenue to follow.

Virgin males with *Ras*^{G12V} *Apc*^{RNAi} tumours have some lipid metabolism genes enriched compared to virgin females of the same genotype (**Section 5.3.2**). These *Acsx* family genes are predicted to have a role in fatty-acyl-coA biosynthesis. Other work in the Miguel-Aliaga lab has shown the importance of the tumour suppressor *Myc* in midgut lipid homeostasis (Amourda *et al.*, in preparation). Some of these lipid genes may play a role in providing energy to the proliferative tumour cells. As lipid metabolism and storage is usually female biased, especially after mating, this may also be a factor that enables female midgut tumours to proliferate more.

5.5.2 Role of mating-induced changes in tumourigenesis

Virgin and mated female midguts have many transcriptional differences (**Section 5.4.1**). Previously profiled is the increase in lipid metabolism, and previously noted but not investigated is an increase in chitin metabolism, amino acid transporters and serine-type endopeptidases. There is also a decrease in sugar metabolism and glutathione metabolism (White *et al.*, 2021).

The function of several of these factors in the mated gut is not fully understood. Chitin binding includes genes in the *Peritrophic* family, essential for formation of the peritrophic membrane. This barrier is composed of chitin and glycoproteins, and forms a physical barrier to protect the midgut epithelium. It also has an important role in antimicrobial immunity (Kuraishi *et al.*, 2011). Several of these chitin binding genes upregulated in Oregon-R mated females (compared to virgin females) have been shown to be involved in the immune response (*CG7298*, *CG6933*, *CG32302*, *CG10725*; Vodovar *et al.*, 2005; Rynes *et al.*, 2012; Broderick, Buchon and Lemaitre, 2014). However, mating actually suppresses female immunity, and increases risk of bacterial infection (Gordon, Wolfner and Lazzaro, 2022). Thus, these chitin binding genes may have roles in modulating the peritrophic matrix to increase nutrient absorption, maybe to the detriment of immune defence. Creating a leaky gut may also promote tumour formation, leading to bacterial dysbiosis and increased gut proliferation.

The serine-type peptidases are a diverse range of genes thought to have both roles in digestion and immunity. In the skeletal muscle several have been found to be important in preventing protein aggregation, and decrease dramatically with aging, resulting in pathology (Jiao et al., 2023).

One observation I have made is that factors that increase in the midgut after mating are initially expressed at similar levels in virgin males and virgin females, then increase after mating in females (**Section 5.4.5**). These may reflect functions that are important for oogenesis needs, and thus virgin females would benefit from keeping these processes at low levels until they are ready to commit to energy intensive egg laying. These increased features include lipid metabolism, key for egg laying (Reiff *et al.*, 2015), but also amino acid transporters and endopeptidases, which may be important for the protein rich diet females favour after mating (Ribeiro and Dickson, 2010; Vargas *et al.*, 2010). Chitin metabolism and endopeptidases are important in immunity, which also decreases markedly after mating (Gordon, Wolfner and Lazzaro, 2022).

However, factors that decrease after mating are highest in males, lower in virgin females and lower still in mated females (**Section 5.4.5**). This may reflect processes that are important for female sexual identity, as regardless of mating they must be lower in virgin females than virgin males. This includes carbohydrate metabolism and glutathione metabolism. In the next Chapter I explore the role of carbohydrate metabolism in proliferation and tumourigenesis further.

5.5.3 Hippo pathway tumours lose most reproductive differences

One of the big questions of this chapter was why Hippo pathway tumours lose their reproductive differences in proliferation. For example, is it because downregulation of the Hippo pathway prevents the gut remodelling from taking place, keeping the mated females in a virgin-like state? Alternatively, is this downregulation obligatory for reproductive remodelling and has thus already occurred in mated females, meaning further downregulation of the Hippo pathway has less of an effect on proliferation?

Because mated females respond to *Ras*^{G12V} *Apc*^{RNAi} tumours as expected, with more proliferation than in virgin females and males, we assumed they would also have many

of the expected reproductive transcriptional differences. *Ras*^{G12V} *Apc*^{RNAi} mated females maintain a suppressed sugar metabolism and glutathione metabolism compared to virgin females. They also have the expected increase in chitin binding, and some evidence of higher lipid metabolism: the GO term *higher fatty acid metabolic process* despite not having an increase in the other mated-biased genes *bmm*, *bgm* and *Lpin* (**Section 5.4.2**). However, they do not increase their amino acid metabolism or serine-type endopeptidase expression. Thus, to a certain extent, generation of *Ras*^{G12V} *Apc*^{RNAi} tumours also either disrupts reproductive remodelling, or mimics it. However, this tumour still maintains more DE genes than *yki*^{3SA} and *wts*^{RNAi} tumours (**Section 5.2.3**).

The *yki*^{3SA} midgut tumours do have some transcriptional differences between virgin and mated females, despite having no difference in proliferation (**Section 5.4.3**). For example, the mated females have enriched *chitin binding* and *endopeptidase* GO terms, terms associated with the mated Oregon-R control. However, the majority of terms enriched in virgin females are related to *regulation of cell differentiation*, *cell population proliferation* and *cell fate commitment*. I do not know why the virgin *yki*^{3SA} female would have enrichment in these terms compared to the mated *yki*^{3SA} female, but it is known that after mating cell differentiation into EC is encouraged by JH and 20HE exposure (Zipper *et al.*, 2020). There is some evidence that the Hippo pathway interacts with 20HE (Dong *et al.*, 2015; Zhang *et al.*, 2015; Moeller *et al.*, 2017) (**Section 4.5.3**), and to find a direct link would also provide a clear function for the Hippo pathway in the midgut.

The *wts*^{RNAi} midgut tumours are transcriptionally almost identical between virgin and mated females, with only 106 DE genes compared to 541 for *Ras*^{G12V} *Apc*^{RNAi} and 866 for *yki*^{3SA} virgin and mated females (**Section 5.2.3**). Of these 106 DE genes most are broadly uncharacterised, and there are very few GO categories enriched (**Section 5.4.4**). This is despite *wts*^{RNAi} virgin females actually having more proliferation than mated females.

As I did not use a control for this experiment, and the *Ras*^{G12V} *Apc*^{RNAi} tumours do not appear to have all the expected reproductive transcriptional changes, it has been difficult to determine if the female Hippo pathway tumours are more virgin-like or more

mated-like. For example, if lipid metabolism is high in both virgin and mated females, it is not possible for me to tell from the RNAseq data if the virgin lipid metabolism is higher than expected, or if the mated lipid metabolism is lower than expected. Thus, in order to understand how the Hippo pathway is connected to reproductive remodelling in the midgut, further functional experiments are needed.

Several interesting avenues could be pursued. White and colleagues showed that as well as the binding motif for *GATAe* (**Section 6.3.3**) being enriched in DE genes in virgin and mated female midguts, so is *lola* (White *et al.*, 2021). As previously described, *lola* is a transcription factor mainly characterised for its role in neuronal fate. Downregulation of *lola* in the midgut ISC/EB induces proliferation and has a similar effect to Hippo pathway downregulation. Lola has been shown to interact directly with Wts to prevent midgut proliferation in a Yki-independent manner (Hao *et al.*, 2020). Although I showed that in my experimental conditions midgut *wts* downregulation induced proliferation is dependent on *yki* (**Section 4.3**), it does not rule out an interesting role for *lola* in reproductive remodelling of the gut. If Wts and Lola do interact, then maybe downregulation of *wts* prevents Lola-mediated post reproductive remodelling.

Chapter 6: Results IV

6.1 Introduction

In the previous Chapter, I used RNAseq to profile the midguts of flies with *Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA} and *wt*^{RNAi} tumours in virgin males, virgin females and mated females (**Section 3.3**). One of the interesting sex differences I found was that the gene ontology (GO) terms for carbohydrate metabolism, which is normally higher in males than females, is less sexually dimorphic in midguts with tumours that proliferate (*Ras*^{G12V} *Apc*^{RNAi}, *yki*^{3SA}) compared to tumours that do not (*wt*^{RNAi}; **Section 5.3**). Additionally, I saw that similar carbohydrate metabolism terms are reduced further in control virgin females after mating (**Section 5.4.1**).

In this Chapter I firstly characterise that this loss in sugar gene expression is not caused by testes dysregulation, as the connection between the midgut and testes is important for male-biased sugar gene expression. I also confirm that in control conditions, these sugar genes and their reporters are downregulated after mating in females. Finally, I characterise some of the changes in sugar gene expression in midguts with tumours, including the amylase Amy-p and the maltase Mal-A1, and suggest potential experiments to test if they contribute to proliferation.

6.1.1 Sugar metabolism in the male midgut

In a previous study published by the Miguel-Aliaga lab, it was shown that in order to generate citrate for sperm production male flies have increased sugar metabolism in the midgut (Hudry *et al.*, 2019). This includes increased digestive enzymes like the amylases and maltases, glucose transporters, glycolysis enzymes, TCA cycle enzymes required to generate citrate and citrate transporters. The Amylase family consists of three members, Amylase-proximal (Amy-p), Amylase-distal (Amy-d) and Amylase-related (Amyrel): α -amylases that hydrolyse α -1-4-glycosidic bonds in starch, generating maltose. Maltose is broken down into glucose by the Maltase family, Mal-A1-A8, all of which are highly expressed in male midguts, and Mal-B1-B2 which have no sexual dimorphism in midgut expression. Other glucosidases like *target of brain insulin (tobi)* are highly expressed in males, as are enzymes that utilise pyruvate to generate citrate, like Pyruvate carboxylase (PCA) and the citrate transporter I'm not dead yet (Indy). Many of these proteins have been verified to be expressed sex-specifically in the posterior R4 portion of the male gut, including Mal-A1, Mal-A3, Mal-

A7, Amy-p, Trehalase (Treh), Hexokinase (Hex-A), Phosphoglucose isomerase (Pgi) and Ldh (Lactate dehydrogenase) (Hudry *et al.*, 2019). This is at least partly mediated by the *Drosophila* JAK-STAT ligand Unpaired (Upd) from the apical tip of the testis, which activates R4 midgut EC JAK-STAT signalling, increasing sugar gene expression. These R4 EC have increased levels of cellular citrate, which is important for sperm production and to promote feeding in males (Hudry *et al.*, 2019).

6.1.2 The male reproductive tract

The male reproductive tract is comprised of several organs that contribute to sperm production and reproduction. The testis is where sperm is produced, and has at its apical tip the hub region (Hardy *et al.*, 1979). The accessory glands produce many seminal proteins needed for fertility (Gillott, 1996). The seminal vesicles are where the accessory gland proteins and the sperm are stored, before passage through the ejaculatory duct during reproduction (McCullough *et al.*, 2022).

The apical tip of the testis contains the hub cells and the stem cell niche of the somatic cyst stem cells (CySCs) and germline stem cells (GSCs). The hub cells are terminally differentiated somatic cells, responsible for maintaining the two stem cell populations (Spradling *et al.*, 2008; Greenspan, De Cuevas and Matunis, 2015). There are 10-15 stromal cells within the hub region, with one hub per testis tip (Hardy *et al.*, 1979). They can be identified by Fas3 staining. Each GSC is enclosed by two CySC, which ensure correct self-renewal and differentiation (Le Bras and Van Doren, 2006). Both divide asymmetrically, with one remaining in contact with the hub, and the other beginning to differentiate (Kitadate and Kobayashi, 2010; DiNardo *et al.*, 2011). The GSC becomes a gonialblast, and the CySC becomes a somatic cyst cell. After four rounds of mitosis, the cells undergo meiosis and eventually terminal differentiation, forming mature spermatids (White-Cooper, 2010). The hub of the testis must be present in order to mediate an increase in midgut sugar metabolism via JAK-STAT signalling (Hudry *et al.*, 2019).

Sugar Metabolism RNAseq

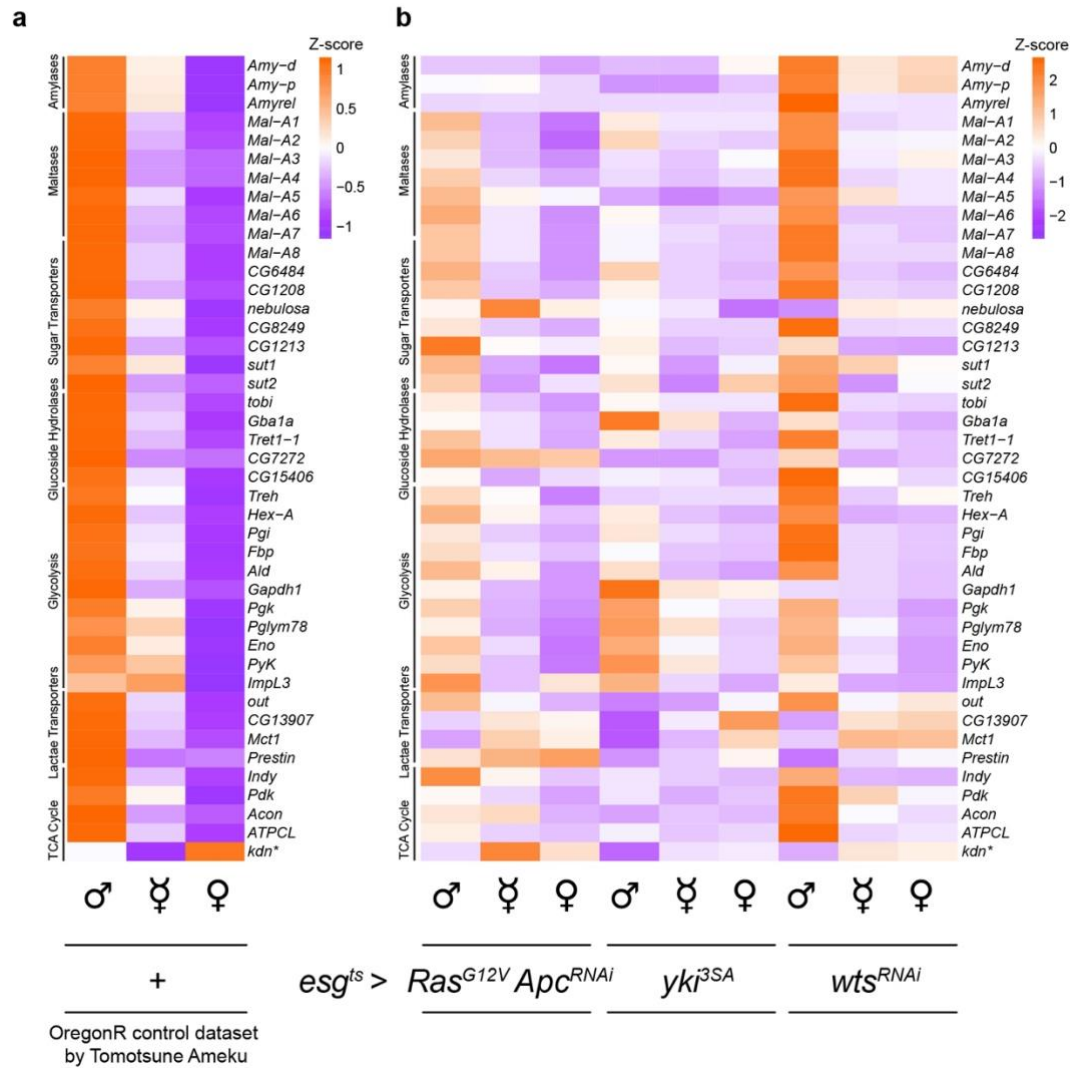


Figure 6.1. Male-biased sugar metabolism is lost in flies with $Ras^{G12V} Apc^{RNAi}$ and yki^{3SA} midgut tumour, but maintained in non-proliferative wts^{RNAi} midguts

a. Sugar gene expression in midguts from Oregon-R virgin males, virgin females and mated females 3 d post-mating. **b.** Sugar gene expression in midguts expressing esg^{ts} driving $Ras^{G12V} Apc^{RNAi}$, yki^{3SA} or wts^{RNAi} in virgin males, virgin females and mated females. σ - virgin males, ♀ - virgin females, ♀ - mated females. An asterisk indicates a gene that negatively regulates a process, and hence is expected to change expression in the opposite direction.

Oregon-R dataset generated by Tomotsune Ameku with bioinformatics analysis performed by Yi-Fang Wang at the LMS Bioinformatics Core Facility. Tumour dataset generated by me, with bioinformatics analysis performed by Jesús Urtasun Elizari at the LMS Bioinformatics Core Facility. Heatmap was plotted by <http://www.bioinformatics.com.cn/srplot>, an online platform for data analysis and visualization (Tang et al., 2023).

6.2 Sugar metabolism in tumourigenesis

6.2.1 Expression of male-biased sugar metabolism genes inversely correlates with proliferation

High sugar metabolism is a key feature of the male midgut, and the GO term analysis shows that although it is maintained in all male tumours compared to the virgin female of the same genotype, it is suppressed slightly in *yki^{3SA}* males (**Section 5.3.3**). Using the shortlist of sugar genes known to be enriched in the male midgut from Hudry et al (2019), I compared the gene expression across the Oregon-R control dataset and my tumour dataset.

These male-biased genes include the digestive enzyme families the amylases and maltases, several sugar transporters, most glycolysis enzymes and some TCA cycle enzymes involved in generating citrate (Hudry *et al.*, 2019). Although it was previously shown that these genes are higher in males than virgin females, I show that these genes are further downregulated in females after mating (**Figure 6.1a**). Previously White and colleagues had shown that some carbohydrate metabolism related terms including glucose metabolism, galactose metabolism and the maltase family are reduced after mating (White *et al.*, 2021).

Across the tumour models, *wts^{RNAi}* males maintain high sugar gene expression, but both *Ras^{G12V} Apc^{RNAi}* and especially *yki^{3SA}* males have lower expression, though not as low as virgin females (**Figure 6.1b**). Thus, in males with tumours (*Ras^{G12V} Apc^{RNAi}* and *yki^{3SA}*) sugar gene expression is suppressed, but in males without tumours (*wts^{RNAi}*) sugar gene expression is maintained.

6.2.2 Assessment of testes hub and proliferation

Because the testis hub must be present in males to induce high midgut sugar metabolism (Hudry *et al.*, 2019), I set out to ensure the testis is intact in each tumour condition. As introduced in **Section 3.1.1**, *esg* is known to be important in the maintenance of the CySC and GSC in the testis (Kiger, White-Cooper and Fuller, 2000; Voog *et al.*, 2014).

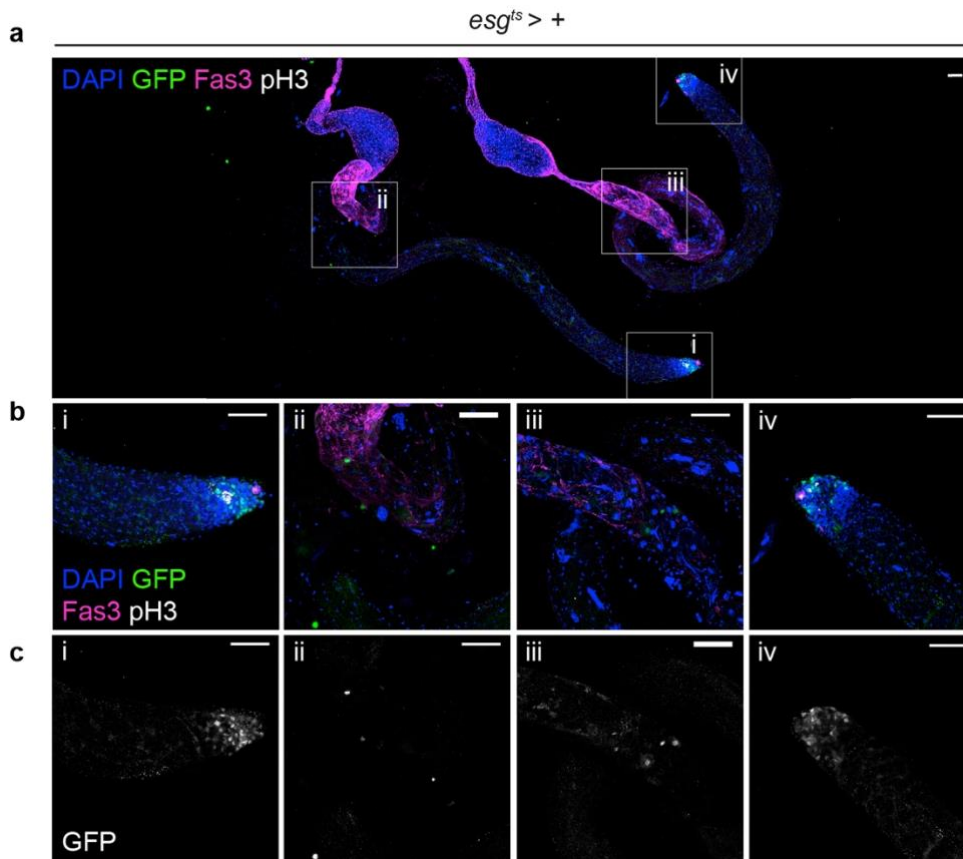


Figure 6.3. esg^{ts} is expressed in the testes tip and seminal vesicles of flies expressing the GFP control (+) for 6 d.

a. Immunostaining of the male reproductive tract for flies expressing $esg^{ts} > CD8::GFP$ for 6 d shows esg expression in the apical tip of the testis and the seminal vesicles. **b.** Magnification of sections i-iv showing from left to right: apical tip of testis, seminal vesicles, seminal vesicles, apical tip of testis. **c.** Panels from **b** with the GFP channel only. Fas3 marks the hub cells in the apical tip of the testis and the septate junctions of the seminal vesicles. pH3 marks cells undergoing mitosis. Scale bar = 50 μ M.

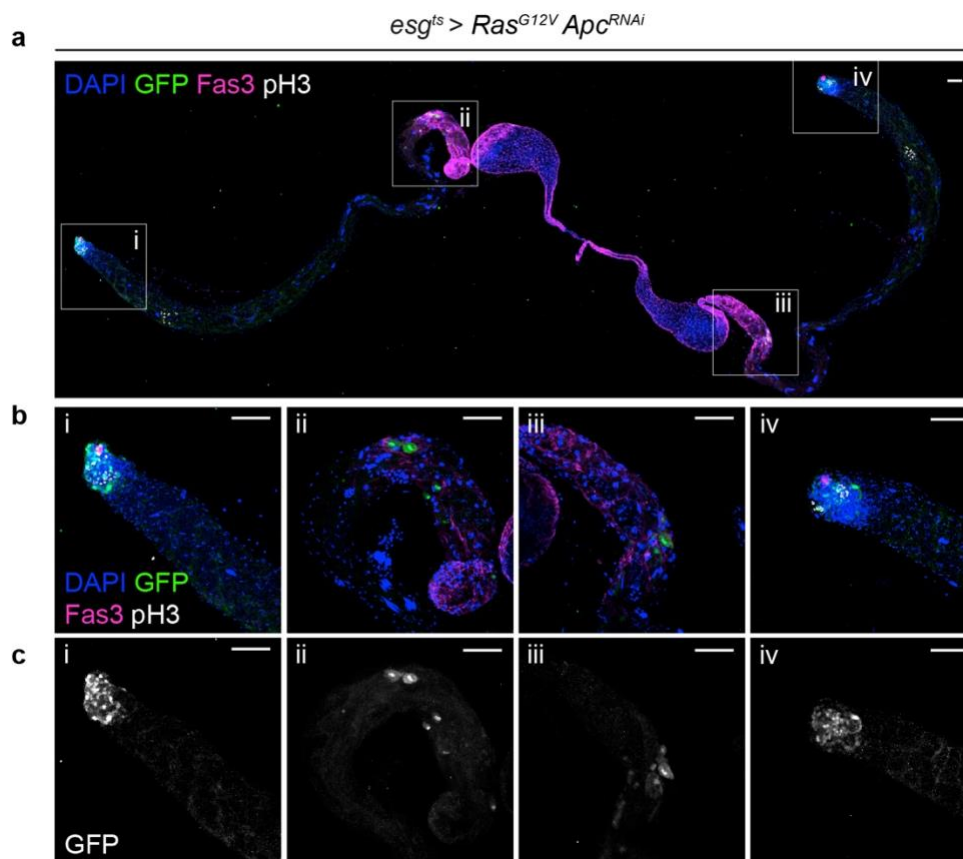


Figure 6.2. esg^{ts} is expressed in the testes tip and seminal vesicles of flies expressing $Ras^{G12V} Apc^{RNAi}$ for 6 d.

a. Immunostaining of the male reproductive tract for flies expressing $esg^{ts} > Ras^{G12V} Apc^{RNAi}$ for 6 d shows esg expression in the apical tip of the testis and the seminal vesicles. **b.** Magnification of sections i-iv showing from left to right: apical tip of testis, seminal vesicles, seminal vesicles, apical tip of testis. **c.** Panels from **b** with the GFP channel only. Fas3 marks the hub cells in the apical tip of the testis and the septate junctions of the seminal vesicles. pH3 marks cells undergoing mitosis. Scale bar = 50 μ M.

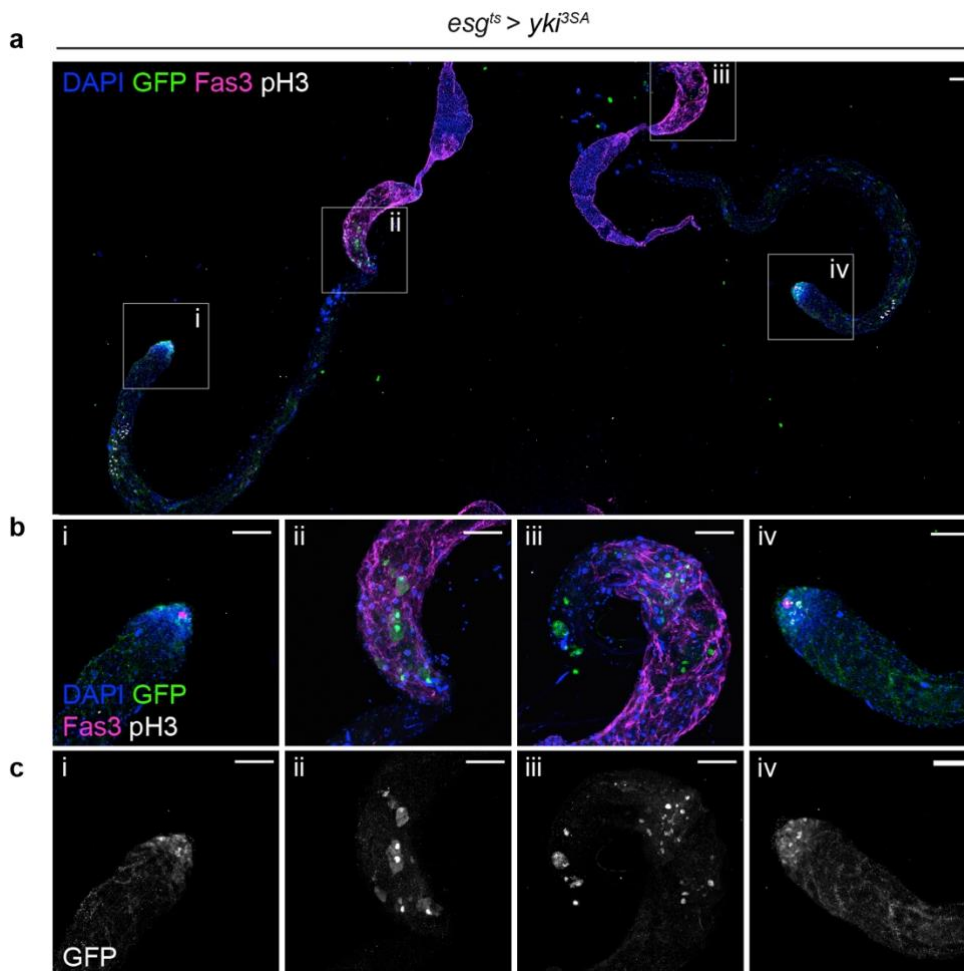


Figure 6.4. *esg^{ts}* is expressed in the testes tip and seminal vesicles of flies expressing *yki^{3SA}* for 6 d.

a. Immunostaining of the male reproductive tract for flies expressing *esg^{ts} > yki^{3SA}* for 6 d shows *esg* expression in the apical tip of the testis and the seminal vesicles. **b.** Magnification of sections i-iv showing from left to right: apical tip of testis, seminal vesicles, seminal vesicles, apical tip of testis. **c.** Panels from b with the GFP channel only. Fas3 marks the hub cells in the apical tip of the testis and the septate junctions of the seminal vesicles. pH3 marks cells undergoing mitosis. Scale bar = 50 μ M.

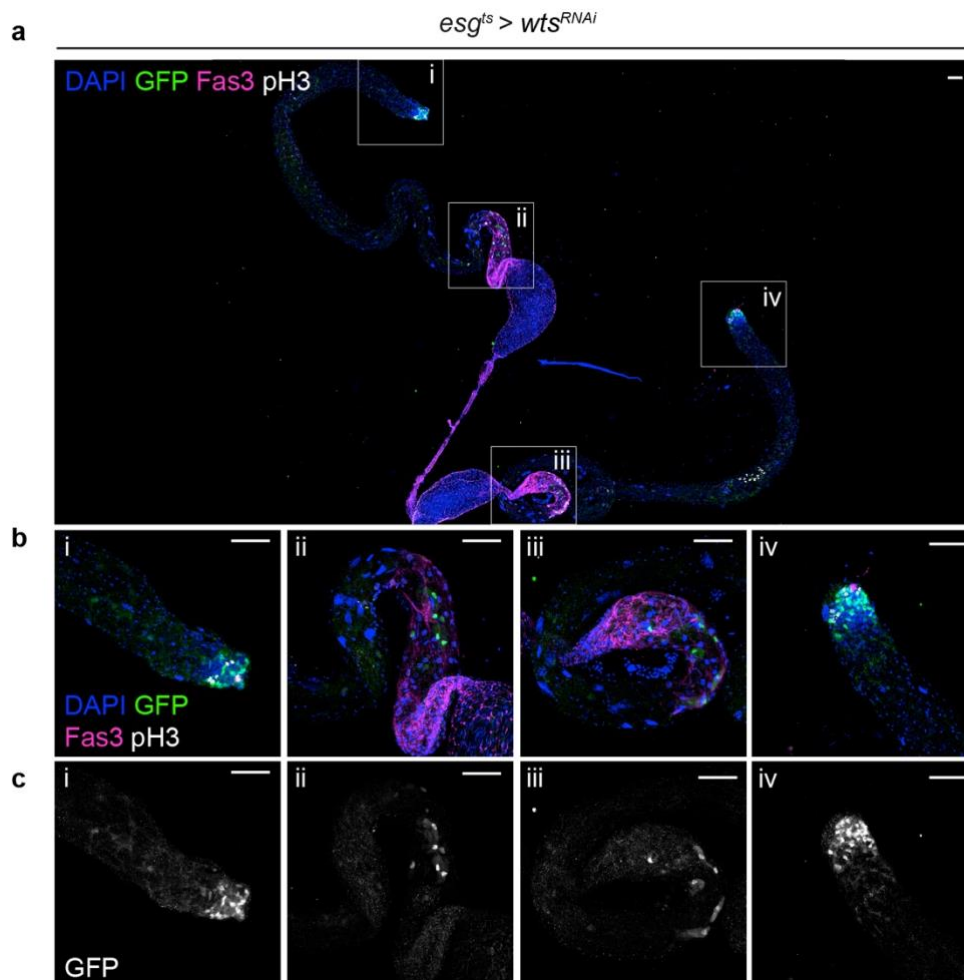


Figure 6.5. *esg^{ts}* is expressed in the testes tip and seminal vesicles of flies expressing *wts^{RNAi}* for 6 d.

a. Immunostaining of the male reproductive tract for flies expressing *esg^{ts} > wts^{RNAi}* for 6 d shows *esg* expression in the apical tip of the testis and the seminal vesicles. **b.** Magnification of sections i-iv showing from left to right: apical tip of testis, seminal vesicles, seminal vesicles, apical tip of testis. **c.** Panels from b with the GFP channel only. Fas3 marks the hub cells in the apical tip of the testis and the septate junctions of the seminal vesicles. pH3 marks cells undergoing mitosis. Scale bar = 50 μ M.

After checking flies expressed *esg^{ts} > CD8::GFP*, I found that there was a small amount of GFP expression in the hub of the testis, and some small bright cells in the seminal vesicles (**Figure 6.2**). As there is some *esg* expression in the male reproductive tract, it is possible that the tumour transgenes may be expressed highly enough to impact the function of the testes.

This is important as JAK-STAT ligands from the testis hub induce the increased sugar metabolism in the posterior midgut to produce citrate for sperm production. I additionally dissected the reproductive tract from males with tumours induced for 6 d for *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* and *wt^{sRNAi}*.

I found expression in the tip of the testis and some bright GFP⁺ cells in the seminal vesicles. This was present at similar levels in *Ras^{G12V} Apc^{RNAi}* (**Figure 6.3**), *yki^{3SA}* (**Figure 6.4**) and *wt^{sRNAi}* (**Figure 6.5**).

I stained for Fascillin3 (Fas3), to check the hub cells at the apical tip of the testis were intact, and found the control (**Figure 6.2**), *Ras^{G12V} Apc^{RNAi}* (**Figure 6.3**), *yki^{3SA}* (**Figure 6.4**) and *wt^{sRNAi}* (**Figure 6.5**) all had intact hubs.

Strong DAPI should only be present at the apical tip of the testis, where the densely packed stem cell niche and hub is. Increased DAPI throughout the length of the testes is a sign of cell overgrowth. The control (**Figure 6.2**) has high DAPI signal only in the apical tip, as does *Ras^{G12V} Apc^{RNAi}* (**Figure 6.3**), *yki^{3SA}* (**Figure 6.4**) and *wt^{sRNAi}* (**Figure 6.5**).

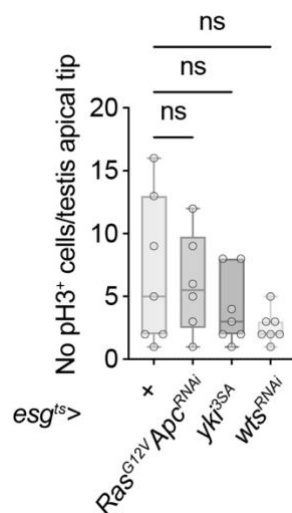


Figure 6.6. There is no change in mitosis in the testes of male flies with *esg^{ts}* midgut tumours.

Quantification of testes proliferation by the number of pH3⁺ cells in the apical tip of the testis, in flies expressing *esg^{ts}* with the GFP control (+), *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* or *wt^{sRNAi}* induced for 6 d. The Mann-Whitney statistical test is used. ns p > 0.05.

I also stained with pH3 to mark proliferating cells. Usually in the testes, there is mitosis in the apical tip, where spermatogonia are generated from division of the CySC and GSC. In the tip of the testes, I found no significant difference in the number of pH3⁺ cells (**Figure 6.6**), although *wt^s^{RNAi}* trended downwards.

6.2.3 Assessment of testes hub JAK-STAT activity

In order to induce male-biased sugar metabolism in the gut, the testes hub must be intact, and JAK-STAT signalling must be present. Using the 10XStat92E::GFP reporter I checked JAK-STAT activity in the control and in two tumour models: *yki^{3SA}* and *wt^s^{RNAi}*. Both the control, *yki^{3SA}* and *wt^s^{RNAi}* have 10XStat92E::GFP in the hub cells of the testes (**Figure 6.7**). I did not check the *Ras^{G12V} Apc^{RNAi}* tumour model as it was genetically difficult.

Therefore, despite small amounts of *esg* expression in the apical tip of the testis, the hub cells are intact, there is no excessive proliferation, and JAK-STAT signalling is intact. Thus, I am reassured that the loss of sugar metabolism in males with tumours is not due to testis dysfunction.

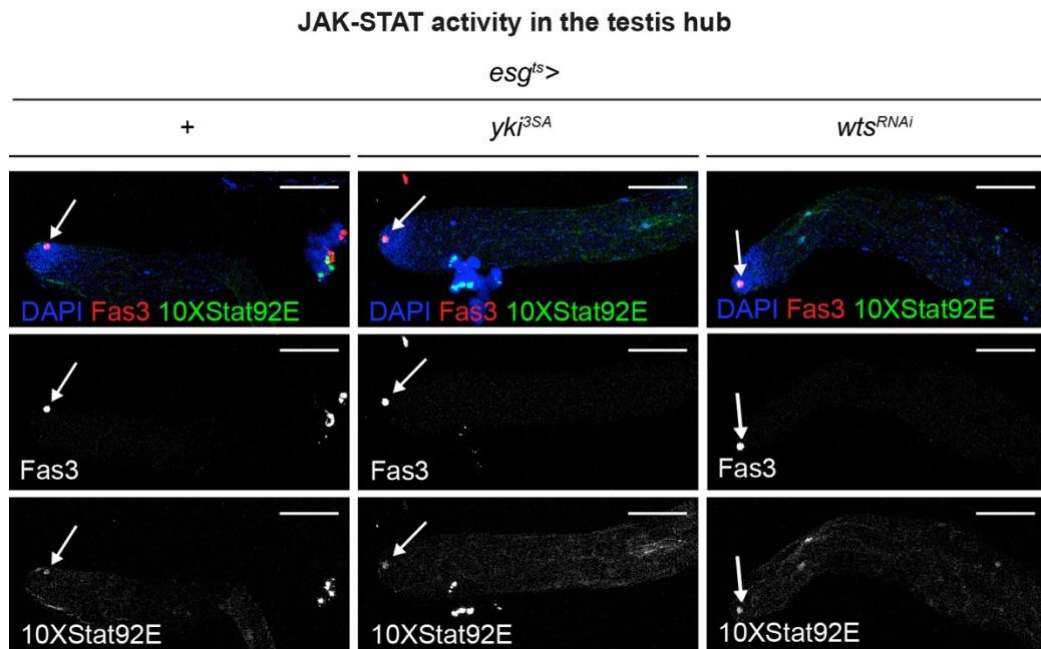


Figure 6.7. JAK-STAT signalling is intact in the testes hub for flies expressing *esg^{ts}* > *yki^{3SA}* and *wt^s^{RNAi}*.

Immunostaining of the apical testis tip for flies expressing *esg^{ts}* with the GFP control (+), *yki^{3SA}* or *wt^s^{RNAi}* shows JAK-STAT activity is present in the testis hub cells in all conditions. Fas3 marks testis tip hub (indicated by arrow), 10XStat92E::GFP reporter marks JAK-STAT activity. Scale bar = 100 μM.

Sugar enzyme expression in the midgut

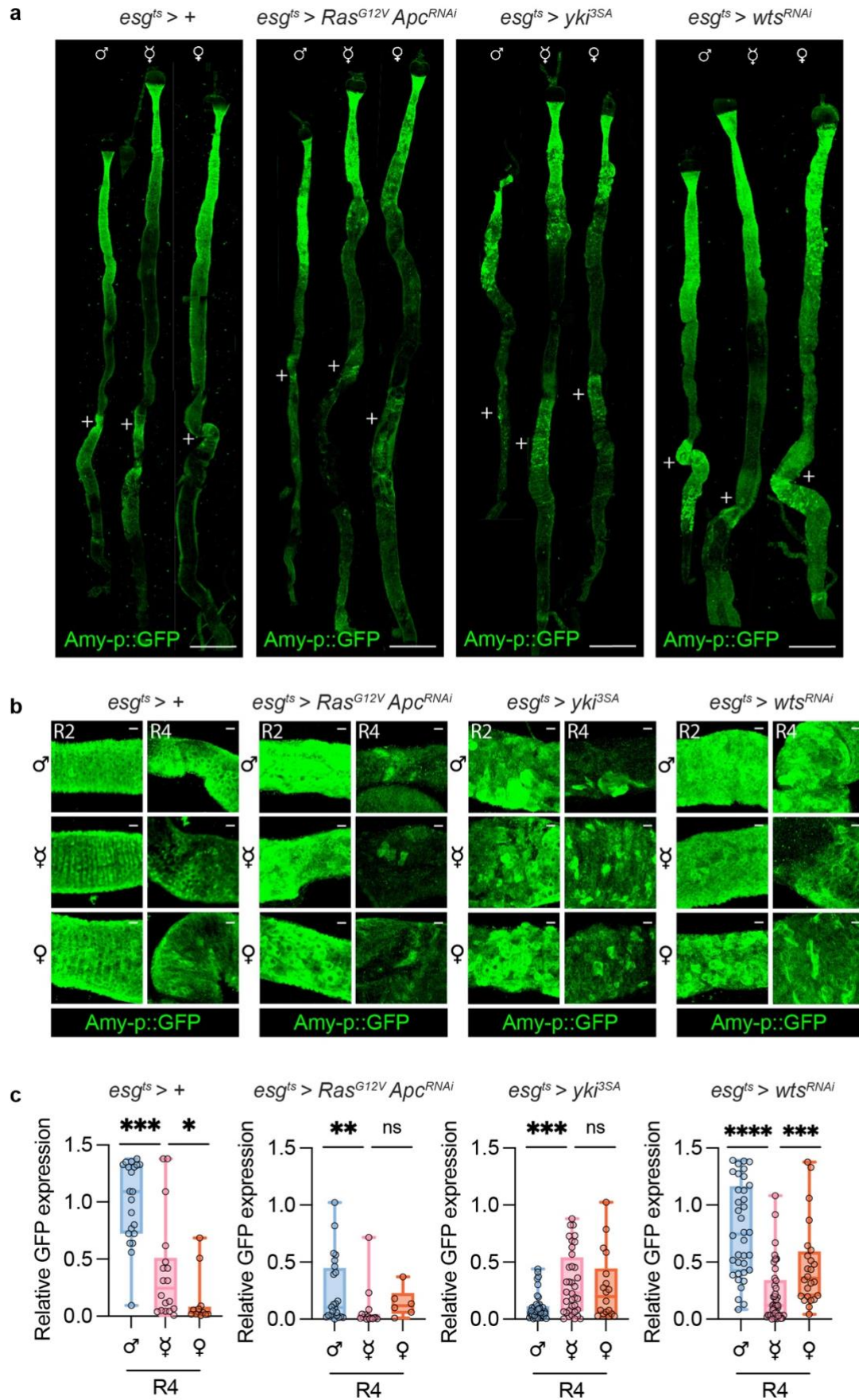


Figure 6.8. The male-biased Amy-p is lost in the R4 region of males with tumours (previous page).

a. Representative midgut expression of Amy-p::GFP reporter in virgin male, virgin female and mated female for *esg^{ts}* > control (+), *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* and *wts^{RNAi}* for 6 d of tumour induction **b.** Magnified insert of Amy-p::GFP reporter in the R2 and R4 region of the midgut for the conditions described in a. **c.** Quantification of the relative GFP expression in the R4 region of the midgut for the conditions described in a. R4 region is marked by +. Scale bar in a. is 500 μ m, scale bar in b. is 100 μ m. The Mann-Whitney statistical test is used to separately compare sex and reproduction. σ - virgin males, ϕ - virgin females, ϕ - mated females. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

6.2.4 Male-biased sugar gene Amy-p is lost in *yki^{3SA}* and *Ras^{G12V} Apc^{RNAi}* males

Several protein reporters exist for the male-biased sugar genes. Here, I checked the midgut expression of Mal-A1::GFP and Amy-p::GFP. I chose these reporters as they also have a very sexually dimorphic expression pattern: they are expressed in males and females in R1-R2, but only in males in R4 (Hudry *et al.*, 2019). Thus, I sought to check if tumourigenesis changes this sex difference in expression levels and localisation.

Amy-p is an amylase expressed highly in male midguts. I found Amy-p::GFP in the R1-R2 region of virgin males, virgin females, and additionally mated females. I found it is expressed in a narrow band in R4 in males, and at lower levels in virgin females, with even lower expression in mated females (**Figure 6.8**). It is strongly and uniformly expressed in all cells of these specific region (**Figure 6.8b**). In *Ras^{G12V} Apc^{RNAi}* midgut tumours, it remains present in R1- R2, and is lost in R4 in virgin males, virgin females and mated females (**Figure 6.8**). In *yki^{3SA}* midgut tumours it is also maintained in R1- R2, and is almost lost in virgin males in R4, where it is expressed strongly in some cells (**Figure 6.8**). In *yki^{3SA}* virgin females it is expressed at similar level to the control, but in mated females its expression is closer to virgin female levels. In males expressing *wts^{RNAi}* in their midgut, a condition without increased proliferation, Amy-p::GFP levels remain high like the control males in both R1/R2 and R4 (**Figure 6.8**). Virgin females with *wts^{RNAi}* tumours have levels similar to the control across the gut, but mated females have more expression than virgin females (**Figure 6.8**). The tumour conditions show variation in the levels of Amy-p::GFP, with a salt-and-pepper expression pattern, with some cells with very high expression next to cells with very weak expression (**Figure 6.8b**). Because it was genetically difficult, I did not have the tumour labelled in these conditions, so I could not directly check if any ISC and EB expressed Amy-p::GFP, or just the surrounding EC.

Therefore, Amy-p is present in the R2 region of males, virgin females and mated females regardless of the presence of a tumour. Amy-p is highest in the R4 of males, and lowest in the R4 of mated female, revealing a reproductive input into its expression. It is lost in the R4 of males with tumours, and its reproductive differences in R4 expression are lost after tumour generation in females.

6.2.5 Male-biased sugar gene Mal-A1 localises to EC in area of high proliferation

Mal-A1 is a maltase also known to be extremely male biased, with a sexually dimorphic expression pattern (Hudry *et al.*, 2019). In the control males, Mal-A1::GFP is expressed in a broad band from R1-R2, and another broad band across all of R4. For this reporter, I focused only on sex differences, and only looked at virgin females. Control virgin females also express a broad weakly expressed band of Mal-A1::GFP from R1-R2 (**Figure 6.9a**) In R4 there are occasionally a few large cells with high Mal-A1::GFP expression. The salt-and-pepper expression correlates with areas of high ISC/EB expansion, which can be found normally in the R4/R5 of control virgin females (**Figure 6.9b**).

For *yki^{3SA}* midgut tumours, where many transcriptional sex differences are lost, virgin males and females look similar. There is broad weak Mal-A1::GFP expression across R1-R2, but salt-and-pepper strong expression in R4 and sometimes in R2, in regions where the ISC/EB proliferate most (**Figure 6.9a**).

In the control males and females, although there is Mal-A1::GFP expression across the midgut epithelium, there are also some smaller cells with much higher levels of Mal-A1::GFP in R1/R2. These cells are not *esg/RFP⁺*, so I stained with Prospero and confirmed they are EE (**Figure 6.10a**). It is possible that these high levels of Mal-A1 are not because these cells are metabolising lots of sugar, but instead they are sequestering Mal-A1 away to prevent sugar digestion.

Sugar enzyme expression in the midgut

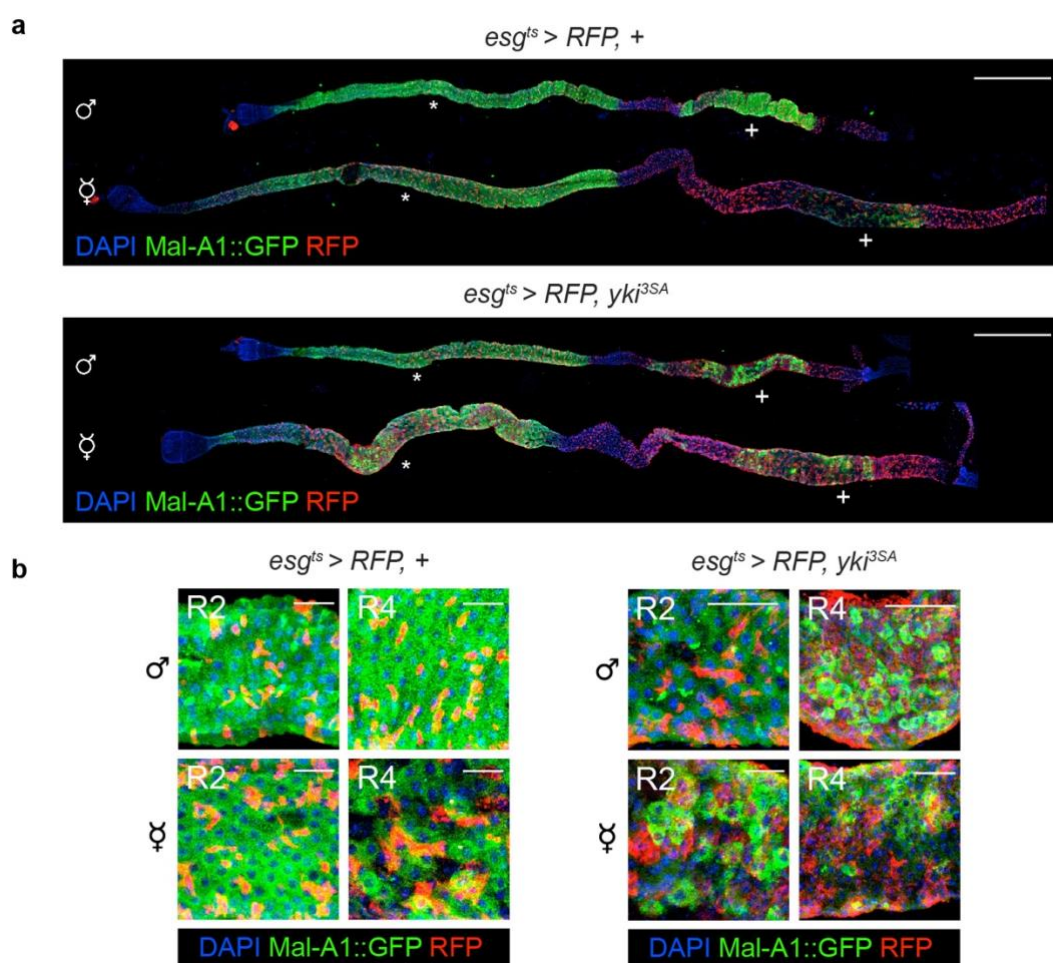


Figure 6.9. The male-biased Mal-A1 changes cellular localisation in guts with tumours.

a. Representative midgut expression of Mal-A1::GFP reporter in virgin male, virgin female and mated female for *esg^{ts} > RFP* control (+) and *yki^{3SA}* for 6 d of tumour induction. **b.** Magnified insert of Mal-A1::GFP reporter in the R2 and R4 region of the midgut for the conditions described in a. R2 is marked by * and R4 is marked by +. Scale bar in a. is 500 μ m, scale bar in b. is 50 μ m. σ - virgin males, ϕ - virgin females.

In the *yki^{3SA}* tumour condition, there are also cells with higher levels of Mal-A1::GFP, but these cells are larger and there are more of them (**Figure 6.10b**). To determine the identity of these cells I stained with Prospero. For this *yki^{3SA}* midgut there is a large area of high proliferation and ISC/EB overgrowth in R2, as well as a smaller one in R4. Although there are Prospero⁺ cells in the R1 and R2/R3 portion of midgut before the main tumour mass, this area of ISC/EB growth has almost no EE cells. Instead, these large cells with high expression of Mal-A1::GFP⁺ are likely to be EC due to their large nuclear size. These resemble the EC expressing Mal-A1::GFP at very high levels in a salt-and-pepper pattern that are seen in the virgin female control guts. Once again,

these cells could be sequestering Mal-A1 as the tissue is stressed due to tumour formation, preventing sugar digestion.

Sugar enzyme expression in the midgut

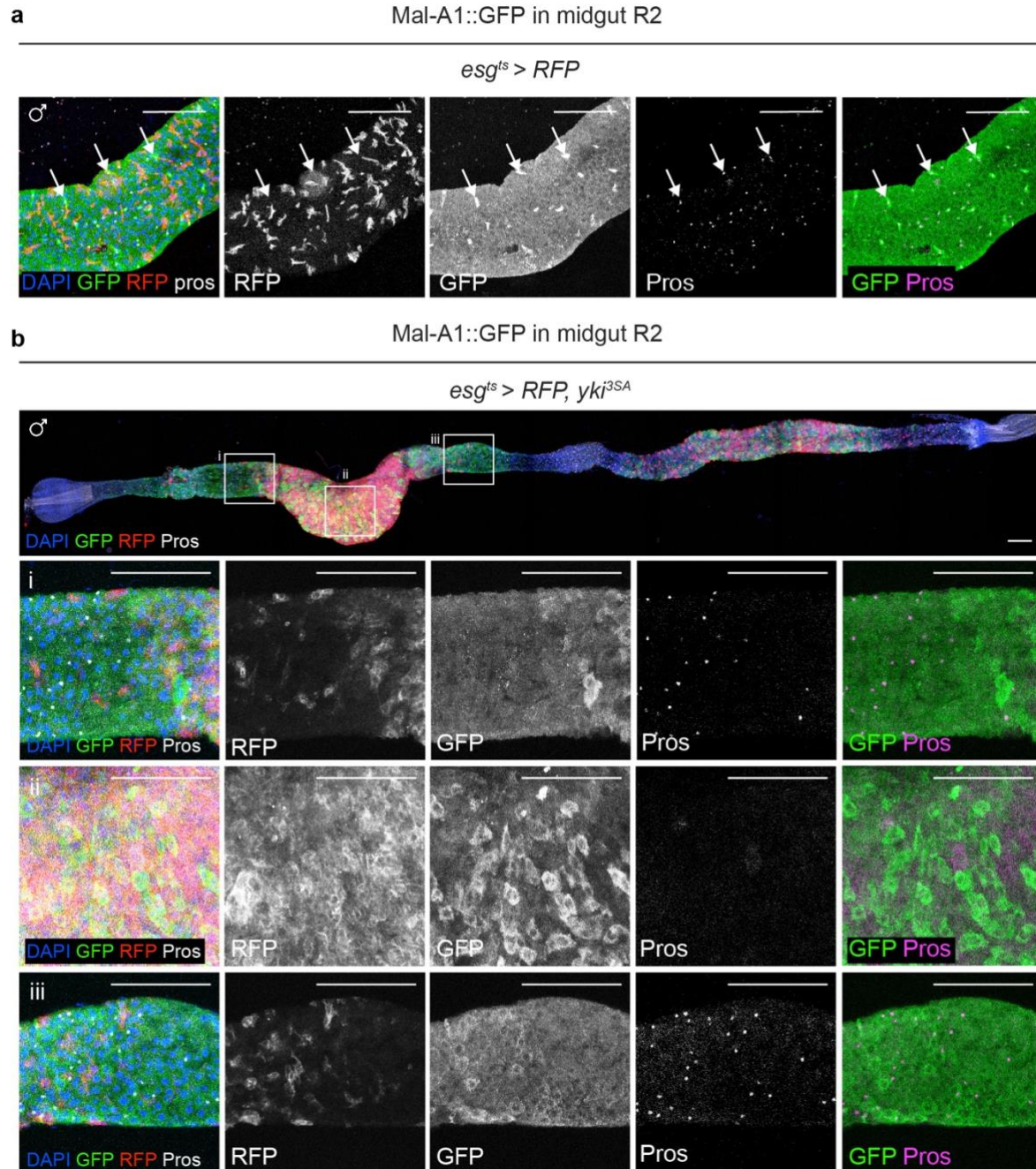


Figure 6.10. Mal-A1 is expressed strongly in some EE in control conditions, but in EC in tumour induction.

a. The R2 of a midgut expressing *esg^{ts} > RFP* stained with EE marker Prospero (Pros) to reveal some EE cells have high Mal-A1::GFP expression. **b.** Whole midgut expressing *esg^{ts} > RFP, yki^{3SA}* stained with EE marker Pros. Three magnified sections show **i.** R2 region before the main area of tumour proliferation, **ii.** the main tumour mass in R2 and **iii.** the R2/R3 region immediately after. The scale bar is 100 μ m. ♂ - virgin males.

Some of these *yki*^{3SA} cells may be *esg* > *RFP*⁺ and Mal-A1::GFP⁺ but this needs to be further investigated (**Figure 6.10b**). If they are, this implies they are tumour-derived ISC/EB that have potentially partially differentiated or begun to endoreplicate as indicated by their large size. However, the majority of cells with high levels of Mal-A1::GFP appear between the tumour cells, implying there may be a tumour non-cell autonomous effect on the surrounding tissue to cause the sequestering of Mal-A1.

Thus, the digestive enzyme Mal-A1 is usually expressed in R1/R2 in males and females, and R4 only in males, and can be found at high levels in EE in the R2 of control guts. In a *yki*^{3SA} tumour context, Mal-A1 has now been shown to be expressed in high levels in EC in a salt-and-pepper pattern in areas of high proliferation across the gut.

6.2.6 JAK-STAT signalling reporter does not show sexual dimorphism

It is known in males that Upd from the testis activates JAK-STAT signalling in the EC of R4, increasing expression of many sugar metabolism genes. It was previously shown that the 10XStat92E::GFP reporter is expressed in a narrow band in the R4 of the male midgut, as well as being expressed in ISC and EB across the guts of both males and females (Hudry *et al.*, 2019). Hence, I decided to check if this band of JAK-STAT signalling is lost in males with tumours. However, I did not see this band of expression in the control males or females, and instead only saw expression in the ISC and EB across the gut (**Figure 6.11**). There is some slightly stronger expression in the R3 region, but this is similar in males and virgin females. This was quantified by my colleague Pedro Gaspar as a control for a different experiment, confirming that under our conditions there is no sexual dimorphism in this reporter (**Figure 6.11d**).

In *yki*^{3SA} midgut tumours, there is increased JAK-STAT activity across all midgut regions that have more ISC and EB proliferation (**Figure 6.12**), but this remains absent from areas without proliferation like R3. This occurs in both virgin males and females. Some of the JAK-STAT signal comes from striated patches parallel across the midgut, which may indicate muscle signal (**Figures 6.12c, d**). Although there is high JAK-STAT signalling, this resolution is not sufficient to determine the EC contribution. This

requires further work to investigate if the EC JAK-STAT signal contributes to sugar gene expression in a tumour context.

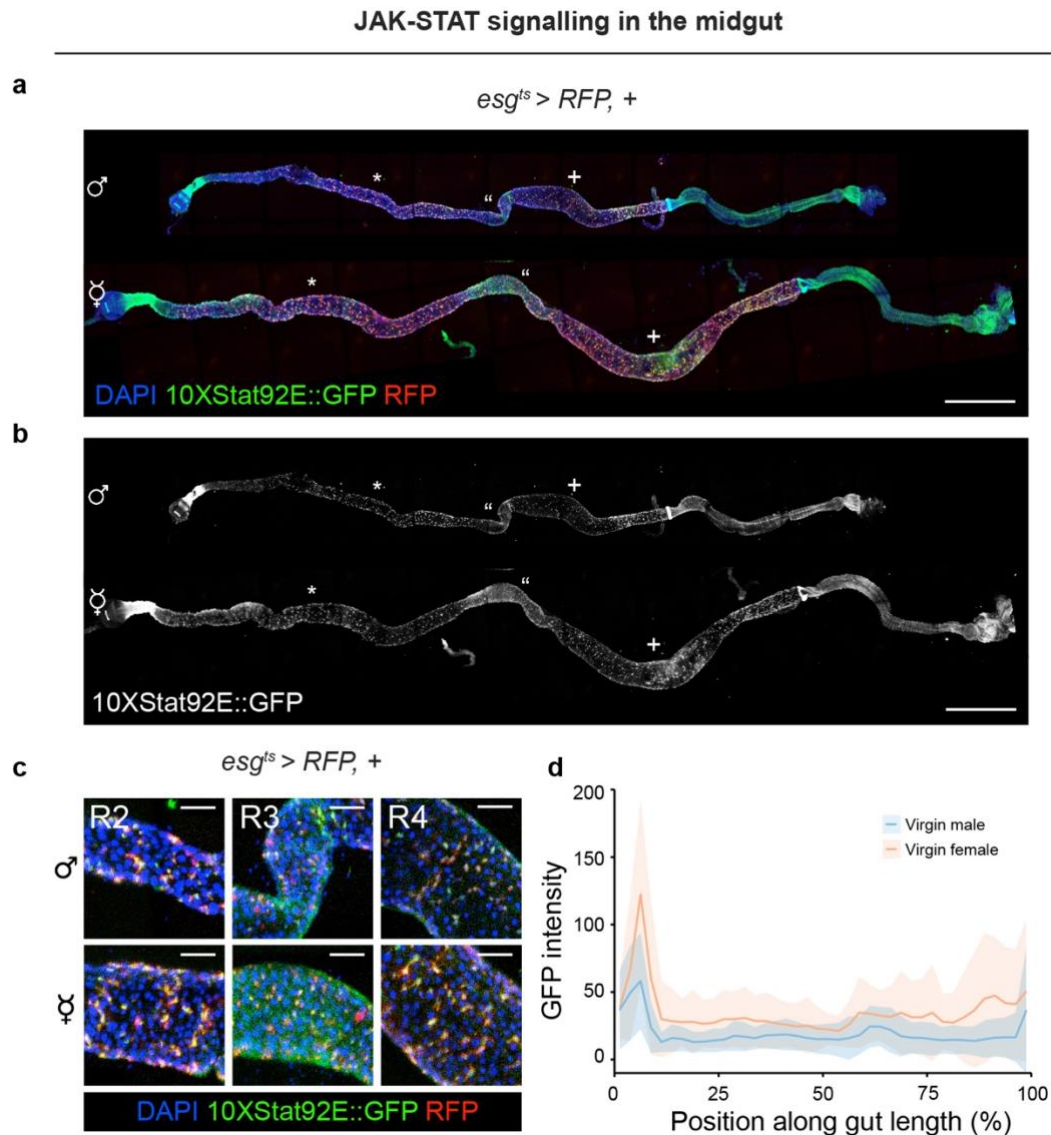


Figure 6.11. The JAK-STAT reporter 10XStat92E::GFP is not sexually dimorphic in controls and does not have a male-specific band in R4.

a. 10XStat92E::GFP reporter in virgin male and virgin female midguts expressing $esg^{ts} > RFP$. **b.** The GFP channel from **a.** showing 10XStat92E::GFP. **c.** Images from R2, R3 and R4 from **a.** **d.** Quantification of 10XStat92E::GFP in virgin males and virgin females expressing $hand-Gal4 > +$. Scale bar in **a.** and **b.** is 500 μ m, scale bar in **b.** is 100 μ m. R2 indicated by *, R3 by " and R4 by +.

The quantification of 10XStat92E::GFP was performed by Pedro Gaspar as a control for a different experiment.

JAK-STAT signalling in the midgut

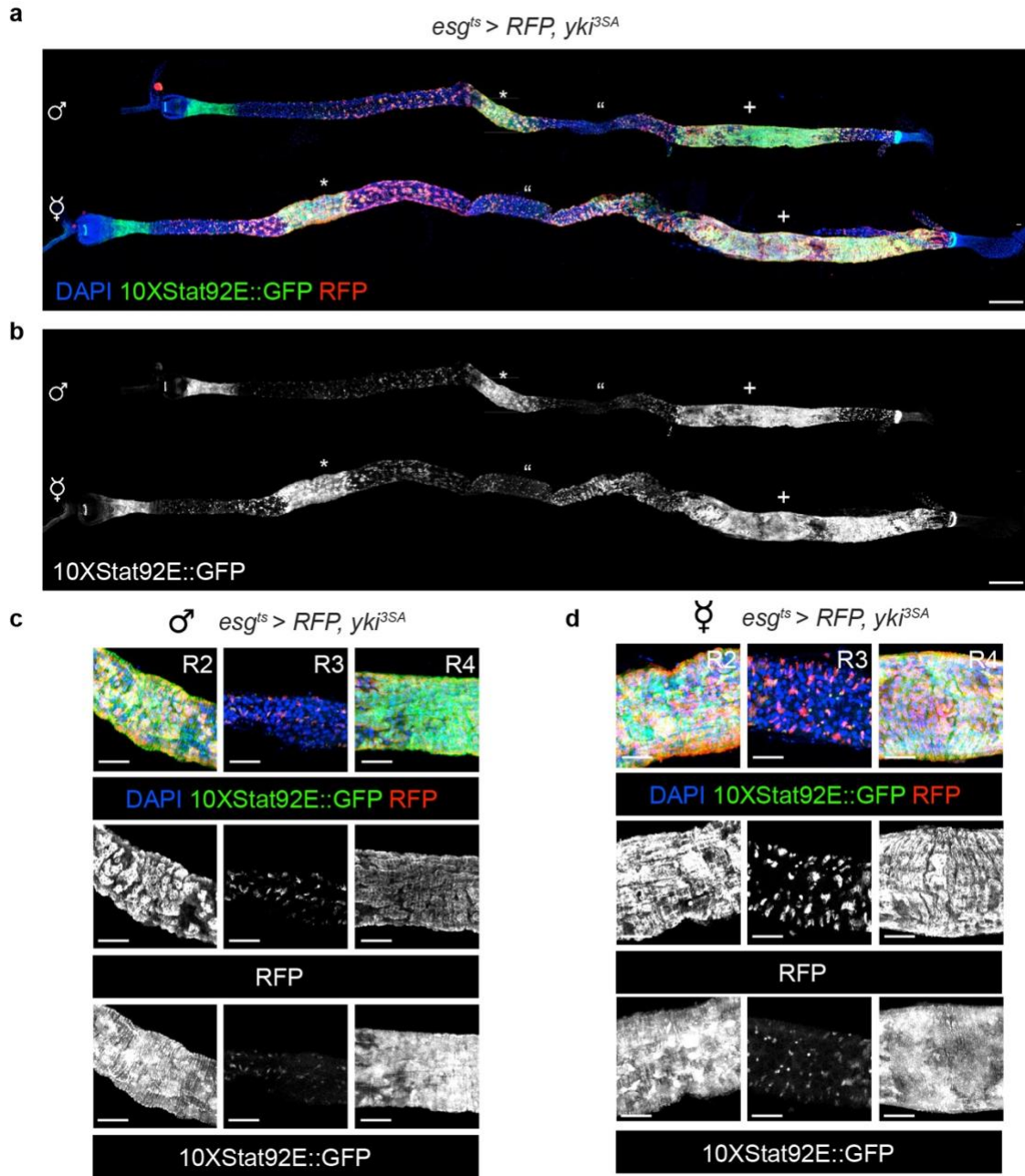


Figure 6.12. The JAK-STAT reporter 10XStat92E::GFP is expressed in areas of high proliferation in both virgin males and virgin females with midgut *yki^{3SA}* tumours.

a. 10XStat92E::GFP reporter in virgin male and virgin female midguts expressing *esg^{ts} > yki^{3SA}*. **b.** The GFP channel from a. showing 10XStat92E::GFP. **c.** Images from R2, R3 and R4 from a. showing the *esg^{ts} > yki^{3SA}* virgin male condition with separate GFP and RFP channels. **d.** Images from R2, R3 and R4 from a. showing the *esg^{ts} > yki^{3SA}* virgin female condition with separate GFP and RFP channels. Scale bar in a. and b. is 200 μm, scale bar in c. and d. is 50 μm. R2 indicated by *, R3 by " and R4 by +.

6.3 Discussion

In this chapter I explored further the expression of male-biased sugar genes, investigated if the testis of tumour bearing males were disrupted, and considered the role this metabolism may have in tumourigenesis.

6.3.1 Midgut tumours do not impact the male testes

It has now been shown that *esg-Gal4* is expressed in several tissues other than the midgut ISC and EB. I characterised both expression in the tip of the testes and some small bright cells in the seminal vesicles (**Section 6.2.2**). Without further staining, I cannot conclude if the *esg* expression is in the CySC or GSC, as both are in the same region of the testes tip. However, with Fas3 staining I was able to show that some of the *esg-Gal4* staining is in the quiescent circle of hub cells. These hub cells must be intact for JAK-STAT signalling from the testes to mediate male-biased sugar metabolism (Hudry *et al.*, 2019).

As I am investigating the effect of sex on tumourigenesis, and male-biased gut metabolism is dependent on its relationship with the testes, the presence of *esg* expression in the testes required further investigation. It could be possible that tumour transgenes are expressed in high enough levels in the testes to prevent the masculinisation of the sugar-biased metabolism of the male gut. For example, if JAK-STAT signalling was interrupted (Hudry *et al.*, 2019).

The niche around the hub in the apical tip of the testis contains two stem cell populations that can divide: CySC and GSC. I counted mitotic cells in the apical tip, and found no significant difference across the control and the three tumour models, although *wtS^{RNAi}* may trend downwards (**Section 6.2.3**). The concern was that the off-target expression of tumourigenic transgenes in the testes tip would induce excessive proliferation but this is not the case.

It is known that the hub of the testes must be intact in order to induce the JAK-STAT signalling that increases sugar gene metabolism in the males (Hudry *et al.*, 2019). For example, in *zero population growth (zpg)* males, which lack the GSC but do have an intact hub (Tazuke *et al.*, 2002; Gilboa *et al.*, 2003; Smendziuk *et al.*, 2015) male

midgut sugar metabolism is high. However, *shutoff* (*esg^{SHOF}*) males without the hub lack the male-biased high sugar metabolism in the midgut (Hudry *et al.*, 2019). Hence, I checked with the Fas3 stain to ensure that the hub in the apical tip was intact, with a circular shape. I also looked at the DAPI stain to ensure the brighter cycling stem cells were only in the apical tip, as testes with excessive proliferation from the stem cells have expansion of high DAPI signal throughout the testes (Loza-Coll *et al.*, 2019). I found that at 6 d of tumour induction, control, *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* and *wt^{sRNAi}* testes all had intact hubs, and high DNA density only in the hub region. Hence, according to Hudry *et al.*, 2019, the hub should be able to masculinise the midgut in all the tumour models.

I additionally checked the testis hub for JAK-STAT activity with the 10XStat92E::GFP reporter (**Section 6.2.3**). Both the control, *yki^{3SA}* and *wt^{sRNAi}* have JAK-STAT activity in the hub cells, all at similarly low levels. I did not check *Ras^{G12V} Apc^{RNAi}* as it was difficult genetically. However, I have shown no differences in JAK-STAT signalling in a condition where the midgut does proliferate (*yki^{3SA}*) and two conditions that do not proliferate (control and *wt^{sRNAi}*).

Although I did not check *esg* expression in the ovary, it has been shown by other authors that *esg-Gal4* expressed in a subset of escort cells in the germaria of the ovaries (Reiff *et al.*, 2015; Ahmed *et al.*, 2020). They suggested for some of their experiments with EcR this may have impacted fecundity. I did not use fecundity as a readout, but the post gut remodelling is dependent on 20HE most likely released from the ovary. Therefore, impact of ovary transgene expression in the loss of reproductive differences in Hippo pathway tumours cannot be ruled out. However, *Ras^{G12V} Apc^{RNAi}* tumours with the same *esg* driver maintain many of their reproductive differences, despite also potentially having ovary transgene expression.

6.3.2 Sugar metabolism in EE cells and after mating

I show one of the key transcriptional features lost from *yki^{3SA}* and *Ras^{V12} Apc^{RNAi}* male tumours but maintained in non-proliferative *wt^{sRNAi}* midguts is the male-biased sugar metabolism (**Section 6.2.1**). One of the key sex differences of the male gut is

enhanced sugar metabolism, including increased digestive enzymes, glycolysis and some TCA cycle enzymes in order to generate citrate (Hudry *et al.*, 2019).

With protein-trap reporters that are usually only expressed in the male, and not the female R4, I show the amylase Amy-p is lost in males with tumours (**Section 6.2.5**), and the maltase Mal-A1 changes its expression levels in both females and upon tumour induction (**Section 6.2.6**).

As these are both mainly expressed in EC and not the tumour itself, it means that it is possible they may be lost as a side effect of *esg*-driven tumour formation, where there is a reduced proportion of EC as the ISC and EB-derived cells take over the midgut. However, it does certainly look like Mal-A1 is not lost from the gut, but instead is expressed very strongly in some EC surrounding the tumour in a salt-and-pepper pattern (**Section 6.2.6**). This may be because Mal-A1 is sequestered into these cells, preventing its secretion and ability to digest carbohydrates. Sequestering this enzyme would provide a faster way of regulating digestion and energy metabolism than degrading the protein, and may also be a reversible process. This could potentially be the midgut response to stress, in order to prioritise energy intensive processes like ISC renewal rather than digestion.

Mal-A1 is also expressed in a subset of control virgin male and female gut R1/R2 EE, as shown by its colocalization with Pros. EE cells sense many environmental stimuli and secrete peptide hormones to mediate midgut contractions (LaJeunesse *et al.*, 2010; Vanderveken and O'Donnell, 2014), lipid homeostasis (Song, Veenstra and Perrimon, 2014) and ISC proliferation (Amcheslavsky *et al.*, 2014).

However, it is unclear why a digestive enzyme would be found in such high levels in EE cells under physiological circumstances. Although I observed there were more EE with high Mal-A1 expression in the R2 of the midgut, I did not sufficiently test if there are sex differences, or changes after mating in females. Whilst the EE cells are thought to control expression of many proteases, the production of lipases and carbohydrases is thought to be controlled by the fat body and the transforming growth factor- β (TGF-

β) ligand Dawdle (Sleiman, Schüpfer and Lemaitre, 2014), as well as carbohydrase expression additionally being promoted in males by the testis (Hudry *et al.*, 2019).

EE cells express a diverse range of hormone peptides, and co-staining with R2 EE markers like NPF, AstC or Tk would help determine which subpopulation it is expressed in (Guo *et al.*, 2019). Other male-biased maltase Mal-A3 is exclusively expressed in EE (Hudry *et al.*, 2019). EE cells are known to be important in sugar detection. After mating, glucose is converted to fructose via the polyol pathway, which is then sensed by EE cells via the gustatory reporter Gr43a. These then secrete NPF to increase ovarian GSC and egg production (Hoshino *et al.*, 2023). Food intake may be a factor that controls tumour growth, and disrupted sugar metabolism and sensing may impact feeding behaviour. It was recently reported that some R2 EE cells can dedifferentiate back into ISC in the midguts of freshly eclosed flies, but this is suppressed by starvation (Nagai *et al.*, 2023). This can also be suppressed by downregulation of sugar genes Pgi and Glut1, suggesting EE cells may directly sense glucose and metabolise it to revert to ISC. It would be interesting to test if the absence of EE cells in the *yki*^{3SA} tumours is because they have dedifferentiated into ISC (**Section 6.2.6**).

I have also shown that this set of sugar metabolism genes decreases after mating in females. This has also been noted by White and colleagues, specifically noting glucose and galactose metabolism, glucose transporters, the Mal-A1 family and other glucosidases like *tobi* (White *et al.*, 2021). This is hypothesised to be linked to the shift in the mated female midgut to lipid and protein metabolism. As they also have a preference for protein rich food, they may have less use for digestive enzymes and sugar transporters (Ribeiro and Dickson, 2010; Vargas *et al.*, 2010). However, because this sugar metabolism has such a strong inverse correlation with proliferation, I am interested in pursuing if this male-biased metabolic profile is also linked to suppressing proliferation. Although it has been shown that EC downregulation of several male-biased sugar genes (*Pdp*, *PCB*, *Indy*) did not promote proliferation in males, this was only examined in homeostatic conditions, not in a tumour context (Hudry *et al.*, 2019).

6.3.3 Male-biased sugar metabolism and its role in tumourigenesis

JAK-STAT signalling from the apical tip of the testis increases sugar metabolism in the midgut EC. However, when my colleague Pedro Gaspar and I checked for the R4 band of JAK-STAT activity with 10XStat92E::GFP reporter we did not find male-biased expression. Instead it is expressed across the midgut ISCs and EBs, which has been previously reported, as well as in some EE cells (Nagai *et al.*, 2023). Several factors could have caused the absence of the EC R4 band, including different fly rearing conditions, density and diet. Clear functional experiments have shown that EC specific downregulation of JAK-STAT signalling (*dome^{RNAi}*, *hop^{RNAi}* and *Stat92E^{RNAi}*) ablated male-specific sugar gene expression. Conversely, EC-specific upregulation of JAK-STAT signalling (*upd3^{OE}* and *hop^{TUM}*) increased female sugar gene expression. Equally, testes CySC driver *traffic-jam (tj)*-driven downregulation of *upd1* halves male-specific midgut sugar gene expression (Hudry *et al.*, 2019). As the midgut reduced in length in some tumours, they may no longer be adjacent to the testis, resulting in a loss of male-biased sugar gene expression (**Section 3.3.4**).

Regardless, there may be other inputs into sugar gene expression, especially as one of the most prominent changes in the female midgut after mating is a reduction in carbohydrate metabolic process. Previously some sugar metabolism genes have been shown to be under the control of transcription factor *GATAe*, where EC specific *GATAe* downregulation decreases the expression of *Amy-d* and *Mal-A7* (Buchon *et al.*, 2013). *GATAe* is required for ISC maintenance and ability to proliferation (Okumura *et al.*, 2016). Interestingly, there is enrichment of the binding motif for *GATAe* in genes differentially expressed between virgin and mated females (White *et al.*, 2021). Thus, this may also provide a potential link integrating sugar metabolism, proliferation and reproductive remodelling.

All midgut expressed carbohydrases and lipases are repressed by glucose, hence amylase and maltase expression are associated with reduced feeding or starvation. This is mediated by Daw released from the fat body when glucose is sensed (Sleiman, Schüpfer and Lemaitre, 2014). Daw interacts with its receptor Baboon (Babo) to activate Smad2, the primary transcription factor that mediates Activin signalling, resulting in repression of carbohydrases and lipases. Hence when this canonical TGF- β /Activin pathway is downregulated, Mal-A1 and Amy-p expression is increased. This

may offer another explanation why sugar gene expression (particularly amylase and maltase expression) is reduced after mating in females, as these flies increase their food intake (Reiff *et al.*, 2015). This may also be dysregulated in tumours, especially if there are changes in food intake.

However, there are other hints that some carbohydrate enzymes may have functions outside of digestion. The *Mal* family genes, *Amy-p*, *Amy-d* and *tobi* are all highly enriched specifically in adult trachea, and not larval trachea, but the functional significance of this is not known (Bossen *et al.*, 2023). *Mal-A1* has previously been identified as a potential tumour suppressor. In a screen of *Ras*^{G12V} *Apc*^{Q8} *Apc2*^{N175K} midgut MARCM clones, downregulation of *Mal-A1* increases the number of circulating tumour cells (Parisi *et al.*, 2023).

Chapter 7: Discussion

7.1 Summary and significance

This thesis explores the role of sex and reproductive status on genetic tumours generated in the *Drosophila* midgut. It builds on our existing knowledge and contributes some new observations on how the midgut changes with sex, by comparing virgin males and virgin females, and reproductive status by comparing virgin females and mated females.

In Chapter 3 (Results I), I initially quantified the proliferation of several genetic tumours induced in the ISC and EB using *esg^{ts}* of virgin male, virgin female and mated female flies. I showed that *Ras^{G12V} Apc^{RNAi}* tumours proliferate as expected, with virgin males having the least proliferation, and mated females the most. However, the mating proliferation difference was only found at the 6 d timepoint, as at later timepoints the reproductive difference was ablated. I also examined several Hippo pathway tumours. I found *yki^{3SA}* tumours also caused proliferation in all conditions, but that there was no reproductive difference in females. To follow this further, I quantified the proliferation of another Hippo pathway tumour with *wt^{sRNAi}* and found males did not respond to tumourigenesis. Interestingly, I also showed virgin females proliferated more than mated females. Virgin female guts continued to proliferate more than mated female guts at later timepoints.

Thus, the sex and reproductive status of the gut affects the proliferation of different genetic tumour models, with *Ras^{G12V} Apc^{RNAi}* having a clear reproductive difference, and Hippo pathway tumours losing their proliferation differences, with the *wt^{sRNAi}* virgin females proliferating more than the mated females.

In Chapter 4 (Results II) I investigated further the unusual behaviour of Hippo pathway tumours. I showed that, transcriptionally, there is a slight downregulation of the Hippo pathway in control mated females, but this is not significant. The Hippo target gene *bantam microRNA* was also strongly upregulated in mated females, especially in the anterior R2 region of the gut, implying a reduction in Hippo pathway activity post-mating. As the two Hippo pathway tumours *wt^{sRNAi}* and *yki^{3SA}* tumours behave differently, I tested if *wt^{sRNAi}* can cause proliferation in virgin male and virgin female midguts in the absence of *yki*, and found that it could not, similar to most reported

literature. As we know that *Notch^{RNAi}* tumours can be masculinised by ISC and EB specific downregulation of female-specific splicing factors, I tested this in *wt^{sRNAi}* virgin males and virgin females, and found that *wt^{sRNAi}* virgin females continue to proliferate with masculinised ISC/EB. This is unexpected but requires further experiments to prove it. I also tested feminisation with overexpression of the female-specific splicing factor *tra^F* in *wt^{sRNAi}* virgin males and virgin females and did induce an increase in proliferation in virgin males.

Thus, I showed that the Hippo pathway may be downregulated after mating in females, and that virgin male and virgin female *wt^{sRNAi}* tumours require *yki* in the midgut. Finally, I showed *wt^{sRNAi}* tumours responded to feminisation but not masculinisation.

In Chapter 5 (Results III) I performed a bulk RNAseq experiment on whole midguts with *esg^{ts}* driving *Ras^{G12V} Apc^{RNAi}*, *yki^{3SA}* and *wt^{sRNAi}* tumours in virgin males, virgin females and mated females. I then compared many Gene Ontology (GO) terms commonly different between virgin males and virgin females, and between virgin females and mated females. I found that *Ras^{G12V} Apc^{RNAi}* tumours maintain many reproductive differences, including mating-induced reduction in sugar and glutathione metabolism, but lose the mating-induced increases in lipid metabolism, peptidases and amino acid transporters. I found *yki^{3SA}* tumours maintain some reproductive differences, like an increase in peptidases after mating, but have lost most other differences. Finally, *wt^{sRNAi}* virgin and mated females are transcriptionally almost identical, with very few differentially expressed genes and GO terms enriched.

I also found that for sex differences, for *Ras^{G12V} Apc^{RNAi}* and *yki^{3SA}*, virgin males no longer have reduced enrichment in GO terms glutathione metabolism and sugar metabolism. The *wt^{sRNAi}* males, that are resistant to tumour formation, maintain high sugar metabolism. Interestingly, mated females also have a further reduction in these male-biased sugar metabolism genes.

In Chapter 6 (Results IV) I explored further the role of sugar gene metabolism in tumourigenesis. Sugar gene expression should be higher in males because of the testis relationship with the posterior midgut (Hudry *et al.*, 2019). I found that midgut sugar gene expression is lost in males with tumours, but that regardless of this the

testis are intact. I found that the amylase Amy-p, known to be sexually dimorphic and expressed in the R4 of virgin males, and not virgin females, is reduced in males and females with tumours, and expressed highly in *wts^{RNAi}* virgin males. I also found the maltase Mal-A1 follows the same pattern, but in female midguts or midguts with tumours, it becomes very highly expressed in some ECs near proliferating cells. In normal conditions, Mal-A1 also becomes concentrated in EEs, but not in tumours. I finally showed that under my conditions JAK-STAT signalling does not form a band in the R4 of males as previously shown, and that it is high in both virgin male and virgin females *yki^{3SA}* tumours.

Overall, this thesis shows that both sex and reproductive status affects tumour proliferation, and that this effect is different depending on the genetic tumour model. It highlights the importance of always declaring the sex and reproductive status in experimental animals, as this can greatly affect the phenotype. It shows the power of the *Drosophila* model, where the whole animal and the relationship between its different cell types, tissues and organs can be studied. As males lose their high sugar metabolism when they develop tumours, it hints there may be a role in tumour proliferation. Finally, as Hippo pathway tumours lose their reproductive differences in females after tumour formation, it hints the Hippo pathway may be important in post-reproduction midgut remodelling.

7.2 Outstanding questions

Several questions remain outstanding:

1. What is the role of the Hippo pathway in post-mating intestinal remodelling in females?
2. Is the role of the Hippo pathway in post-mating remodelling linked to the function of Msn and mechanosensing of food intake?
3. Is there a role for food intake in tumour formation and does this explain some of the sex and reproductive differences?
4. Why do *wts^{RNAi}* tumours not respond to masculinisation?
5. Why is male-biased sugar metabolism lost in males with tumours?

6. Is there a role for male-biased sugar metabolism in preventing excess proliferation in male midguts?

7.3 Avenues for future work

7.3.1 Transgene expression in other tissues

Many of these experiments warrant repeating using a driver line that is only expressed in ISC, and not EB. Whilst *esg-Gal4* is used for expression in both the ISC and EB, *Su(H)-Gal4* drives the expression of transgenes in the EB, and *Su(H)-Gal80* prevents transgene expression in the EB. Previously it was shown that downregulation of sex splicing factor *Sxl* only prevented female gut proliferation if downregulated with *esg-Gal4* (ISC and EB) or *esg-Gal4*, *Su(H)-Gal80* (ISC), and not with *Su(H)-Gal4* (EB). Thus, the contribution of intrinsic cell sex to gut proliferation is from the ISC, not the EB. It would be interesting to repeat some key experiments (for example Hippo tumours caused by *yki*^{3SA} or *wts*^{RNAi}) with a ISC or EB only driver, to disentangle the role of the EBs in the sexually dimorphic and mating-induced phenotypes.

Several interesting papers have described EB specific phenotypes. For example, EGFR-Ras signalling drives EB-specific mitosis during regeneration after bacterial infection, with generation of new ISC directly from the EB cells (Tian *et al.*, 2022). EBs can also be pushed into proliferation by depleting cell adhesion protein vinculin, which under physiological conditions should maintain the EB in a quiescent state until further division is necessary (Bohere, Eldridge-Thomas and Kolahgar, 2022). Thus, EBs have their own contribution to mitosis which may vary with sex and reproductive status.

One limitation of my research is the *esg-Gal4* driver is expressed not only in the midgut ISC and EB, but also in low levels in the male reproductive tract (**Section 6.1.4**), in the some ovarian cells (Reiff *et al.*, 2015; Ahmed *et al.*, 2020) and in some suboesophageal ganglion cells in the brain (Nagai *et al.*, 2023). Thus, some of these experiments may warrant testing using an alternative driver that is only expressed in the midgut progenitors. For example, the newly designed *I-KCKT-Gal4* system uses a KD recombinase under the control of an intestinal enhancer with a KDR target (KDRT) site flanked stop cassette separating a progenitor enhancer and Gal4. Hence only in intestinal cells can the stop cassette be excised, resulting in intestinal progenitor

specific Gal4 expression (and not expression in progenitor cells not in the intestine). This could be a useful tool in ensuring that interesting tumour phenotypes are not caused by the testis, ovary or brain misexpression of tumour transgenes (Buddika *et al.*, 2021).

7.3.2 The role of the Hippo pathway in post-mating remodelling

The Hippo pathway may be important in post-mating remodelling of the midgut in females (**Section 3.3**, **Section 5.4**). Finding the link between post-mating remodelling and the Hippo pathway is the next step. Initially it could be tested if JH or 20HE exposure activate the Hippo pathway in the midgut. The ecdysone transcriptional co-activator Taiman (Tai) has been shown to interact with Yki in the larval wing disc to modulate the expression of some non-canonical Yki target genes usually expressed in germline stem cells. Further investigation into these targets (*piwi*, *nanos*, *dilp8*, *Eig71Ee*) in the midgut in virgin and mated flies may explain the function of Yki in the midgut post-mating. Additionally, Tai can heterodimerise with JH receptor Met, as they are both bHLH-PAS proteins. Together they can mediate the expression of JH target genes in *Drosophila* S2 cells and other insects (Dubrovsky *et al.*, 2011; Li, Mead and Zhu, 2011; Bernardo and Dubrovsky, 2012). Thus, Tai could provide a link between 20HE, JH and the Hippo pathway.

The neuronal transcription factor Lola has been shown to interact with Wts in the midgut to prevent midgut proliferation in a Yki-independent way. Downregulation of Lola causes a similar phenotype to Hippo pathway downregulation, including an increase in midgut proliferation and an increase in JAK-STAT, EGFR and *bantam* signalling (Hao *et al.*, 2020). Although my results suggest that Wts does act through Yki to induce proliferation in virgin male and virgin female midguts (**Section 4.3**), there still may be an interesting function for Lola in the midgut related to sex or reproduction. White and colleagues identified Lola as one of the transcription factors important in post-mating remodelling (White *et al.*, 2021). It is possible that post-mating in females, Wts and Lola interact to mediate some transcriptional changes caused by the Hippo pathway. In this case, *wts* downregulated-tumours may prevent Lola-mediated post reproductive remodelling. This could be tested by downregulating *lola* in virgin female flies, then mating them, to see if changes such as increase in gut length and width can

occur, or if Lola is essential to this remodelling. Care must be taken when modulating Lola, as it has been shown to have over 20 isoforms, that function differently depending on the tissue they are expressed in (Davies *et al.*, 2013).

Another Hippo pathway input in reproduction could be via the Hippo pathway kinases Tao-1 and Msn. Midgut stretching induced by consuming yeast particles causes Yki activation. Stretching causes a change in the localisation of Msn, which can then no longer act via Wts to inhibit Yki (Li *et al.*, 2018). It would be interesting to investigate if this process occurs because of the increase of food intake post-mating (Carvalho *et al.*, 2006; Hadjieconomou *et al.*, 2020). Thus, rather than the Hippo pathway being directly stimulated by Sex peptide, JH or 20HE, it could be downregulated as a secondary effect, due to increased feeding. This could then contribute to the increased ISC proliferation post-mating. Research from the Miguel-Aliaga lab has shown that just feeding virgin female flies methyl-cellulose food to stretch the gut is not enough to mimic all post-mating remodelling, but it does increase the size of the midgut to a mating-like size (Alessandro Mineo, unpublished). This could mean that, in mated females, the Hippo pathway may already be downregulated because of Msn mechanosensing, thus further downregulation of the Hippo pathway by *wts^{RNAi}* or *yki* overexpression has no further effect on proliferation in mated females but does have an effect in virgin females. This could be tested, for example, by checking Msn localisation in virgin vs mated females, and by seeing if some post-mated changes like midgut length and proliferation can still occur if Msn or Tao-1 are overexpressed in the midgut before mating.

One factor I have not investigated is if flies with different genetic tumours eat different amounts of food, and how food intake may affect tumourigenesis. Calorie restriction has been shown to reduce risk of cancer in both animal models and human studies (Harvie and Howell, 2012). Food intake is likely to be important for fuelling tumour growth, and the higher food intake in mated females may fuel their higher tumour proliferation in *Ras^{G12V} Apc^{RNAi}* (**Section 3.3.1**) and *Notch^{RNAi}* tumours (Ahmed *et al.*, 2020). But, if in the Hippo pathway tumours reproductive remodelling cannot take place, maybe the loss of reproductive differences in proliferation in females is because both conditions eat the same. Thus, an assay like FlyPAD could be used to monitor the number of sips flies from each tumour condition take (Itskov *et al.*, 2014). My

RNAseq data shows that the proliferation differences are not the only reproductive differences lost in Hippo pathway tumours, as they also lose differences in sugar metabolism. However, this could also be explained by changes in feeding, as feeding strongly influences amylase and maltase expression (Sleiman, Schüpfer and Lemaitre, 2014).

7.3.3 The role of sex determination in Hippo pathway expression

An unexpected finding was that downregulation of the female splicing factor *tra* in ISC/EB did not masculinise the proliferation of *wt^s^{RNAi}* virgin female tumours (**Section 4.4.2**). This warrants further investigation, especially initially to test an alternative *tra^{RNAi}* construct and downregulation of *Sxl*. It would also be better answered with a MARCM clone with a *tra* mutant allele, to ensure there is absolutely no female transcriptional programme. If these experiments agree that *wt^s* downregulation mediated proliferation cannot be masculinised, then further investigation could include masculinising or feminising other cells and tissues, such as the EE cells, muscle or trachea. These experiments may also be interesting to repeat with mated females, to check if they respond differently to virgin females.

Feminisation of *wt^s^{RNAi}* tumours with *Tra^F* does induce proliferation in virgin males, indicating that the Hippo pathway may be under transcriptional control of *Tra* (**Section 4.4.1**). Generally, more needs to be done to elucidate if males and females have differences in midgut Hippo pathway activity. Although the *bantam*-sensor indicated there are no sex differences in *bantam* expression, there are other Hippo pathway readouts that may be interesting, including *diap1*, *four-jointed (fj)* and *expanded (ex)*.

7.3.4 The role of male-biased sugar metabolism in tumourigenesis

I have shown that males with tumours that proliferate (*Ras^{G12V} Apc^{RNAi}* and *yki^{3SA}*) lose some male-biased sugar gene expression, whereas males that are resistant to tumours maintain it (*wt^s^{RNAi}*; **Section 5.3, Section 6.2**). To probe the function of sugar metabolism in proliferation I am interested in using a dual driver system, with *esg^{ts}-LexA* driving ISC/EB-derived tumour generation and *mex1^{ts}-Gal4* modulating sugar metabolism in the EC. For example, in the *wt^s^{RNAi}* male tumours that do not proliferate, does depleting EC sugar digestion (*Mal-A1^{RNAi}*), citrate production (*kdn^{RNAi}*) or JAK-

STAT signalling (*dome*^{ΔCYT}) induce tumour proliferation? Conversely, in *yki*^{3SA} tumours that do proliferate, does increasing EC sugar digestion (*Mal-A1*^{OE}), citrate production (*ATPCL*^{RNAi}) or JAK-STAT signalling (*hop*^{Tum}) prevent tumour proliferation? By modulating sugar metabolism from the EC that surround the tumour, I should be able to test the non-cell autonomous effect of the EC on tumour proliferation. It is possible the reduction in sugar gene expression is because the genetic tumours I have generated are made up of ISC, EB and EB-like cells that have not fully differentiated into EC. As EC express these digestive genes, if they are lost proportionally, this would lead to a diminished carbohydrate gene expression signature in the RNAseq. However, the above experiments should distinguish between these effects, if increasing midgut EC sugar gene expression can reduce ISC-mediated genetic tumour formation.

Citrate, which is produced at high levels in the male midgut and then secreted, is also generated at high levels in the male prostate epithelium before being secreted. High levels of zinc in the prostate are essential to ensure this citrate is not oxidised, thus pausing the TCA cycle. However, in malignant cells citrate is oxidised, the TCA cycle resumes and drives tumour growth (Ahmad, Cherukuri and Choyke, 2021). We also know that zinc is important in the fly midgut, although sex differences in its function have not been fully explored (Redhai *et al.*, 2020). This may provide an explanation for how males can have high levels of citrate without fuelling cell growth.

7.3.5 Wider relevance regarding human cancer

Across all midgut tumour models, females have more proliferation than males. However in human cancer, males are more likely to develop most types of cancer, including colorectal (White *et al.*, 2018), and have higher mortality (Bray *et al.*, 2018). However, my readout is tumour proliferation, not tumour incidence or mortality, which are the male-biased factors in humans. Like humans, male flies generally live shorter lives than female flies, although this is very dependent on genetic background (Arya *et al.*, 2010). Other considerations are that female flies are larger than male flies, making them more similar to male humans, who are both larger and have a higher risk of cancer. I am inducing tumours in young flies, and these factors are likely to be influenced by age. Flies respond to one steroid hormone, 20HE, rather than both the

corticoid and gonadal steroid hormones found in humans, meaning the effect of 20HE is not necessarily the direct equivalent of oestrogen. The increased risk of midgut tumours in female flies is a result of their need for organ plasticity after mating, as they need to increase the size of their gut to eat more for energy intensive egg laying (Reiff *et al.*, 2015). Reduction, but not elimination of midgut proliferation in females will increase their lifespan (Biteau *et al.*, 2010), and female flies have specific age-related pathologies like intestinal dysplasia because of their more proliferative ISC (Regan *et al.*, 2016). Although this is the opposite trend to humans, it represents a difference in physiology influenced by the short lifespan and need to optimise egg-laying in flies. This need to optimise for reproduction is something conserved in mammals, where as a result of pregnancy and lactation, many organs are remodelled, increasing in size or altering their function to support the development of the foetus (Strange *et al.*, 1992; O'Brien *et al.*, 2010; Sanghavi and Rutherford, 2014; Kampmann *et al.*, 2019; Grattan and Ladyman, 2020; Waage *et al.*, 2022). However, as mammals live longer, they have mechanisms to reverse pregnancy and lactation-related remodelling (Faupel-Badger *et al.*, 2013; Feigman *et al.*, 2020), so when considering parity the interval between pregnancy and cancer incidence also need to be considered.

7.3.6 Sex, reproduction and Hippo in human cancer

There is only limited evidence of sexual dimorphism in the Hippo pathway in vertebrates. In the flatfish, a species with a much larger female than male, the Hippo pathway is upregulated in males and pseudomales, and it is hypothesised to contribute to this sex-biased size difference (Wang *et al.*, 2021). In medulloblastoma, the most common paediatric brain tumour, YAP and TAZ-stimulated gene expression were identified as the top biologic pathways with sex differences in methylation (Moss *et al.*, 2023).

Regarding reproduction, in mice there is some evidence of the Hippo pathway being involved in the uterine endometrium, a tissue that must be remodelled to prepare for pregnancy. In the mouse uterus the polarity gene *Scrib* is important for formation of the primary decidual zone (PDZ), a barrier that restricts the maternal circulation from the embryo after implantation. Deletion of *Scrib* in the uterine stroma causes defects in the PDZ caused by a reduction in *Lats* (Wts) and *Sav* expression (Yuan *et al.*, 2019).

In humans, analysis of Catalogue Of Somatic Mutations In Cancer (COSMIC) shows uterine corpus endometrial carcinoma had the highest rate of LATS2 and second highest rate LATS1 mutation of the different types of cancer profiled (Yu, Bachman and Lai, 2015). YAP has been shown to promote ovarian cancer and is associated with poor prognosis in human ovarian tumour samples (Xia *et al.*, 2014).

The Hippo pathway has been linked to hormonal activity in mammals before. The human YAP (Yki) target gene CYR61 is a marker of oestrogen activity in endometrial cells (Rivera-Gonzalez *et al.*, 1998; Shome *et al.*, 2020). It is elevated in endometrial carcinoma (MacLaughlan *et al.*, 2007) and breast cancer (Sampath, Winneker and Zhang, 2001), and its expression may be an interesting early biomarker, as it marks tissue that aberrantly responds to oestrogen. YAP and TAZ (another homologue of Yki) also control the expression of the angiogenesis-related genes AMOT, AMOTL1 and AMOTL2 in the epithelium where they respond to oestrogen (Chan *et al.*, 2011) and progesterone (Wang, Huang and Chen, 2011). In the mouse uterine endometrium, the expression and localisation of Yap itself is regulated by oestrogen. Yap expression fluctuates with the oestrous cycle, and it is speculated to be important in expressing factors necessary for regulation of the uterine endometrium (Moon *et al.*, 2022).

The Hippo pathway has been shown to be commonly downregulated in colorectal cancer, and its suppression correlated with disease progression and metastasis. CDX2 is an important protein in intestinal development and differentiation and is hypothesised to be a YAP target gene (Liang *et al.*, 2014). However, YAP is silenced in a subset of highly aggressive colorectal carcinomas, leading to the suggestion it can also act as a tumour suppressor in some contexts (Barry *et al.*, 2013). Regardless of these changes, the endogenous role of the Hippo pathway is poorly understood, as its function is defined by the increase in growth and apoptosis found in its repression.

Whilst there is less data on the response of colorectal and other gastrointestinal cancers to pregnancy induced organ remodelling, it should not be ignored as a potentially interesting area of research. Sex hormones like oestrogen that change during pregnancy are known to act on colon cancer (Chlebowski *et al.*, 2004; Simon *et al.*, 2012; Foster, 2013). If more studies stratify their patients by both their sex and

parity status, there may be some patterns that reveal how reproductive remodelling affects the risk of cancer and other diseases.

7.4 Closing remarks

Both sex and reproductive status are important factors to consider in the development and progression of cancer. This thesis highlights how the intrinsic and extrinsic sex differences and reproductive remodelling found in the *Drosophila* midgut can impact tumourigenesis, and how this is different depending on the genetic tumour model. It is important to always consider the tissue origin of the cancer that is being studied, and to always stratify by sex to ensure sex-specific differences in proliferation or metabolism are not missed. Additionally, the impact of parity should not be overlooked, and when looking at human cancer, factors including the recency of pregnancy should also be considered. In conclusion, this thesis aims to highlight the importance of using whole animal models like *Drosophila* to enable the study of the role of different cell types, tissues and organs in tumourigenesis and how they change with sex and reproduction.

Bibliography

- Abdel-Hafiz, H.A. *et al.* (2023) 'Y chromosome loss in cancer drives growth by evasion of adaptive immunity', *Nature*, 619(7970), pp. 624–631. Available at: <https://doi.org/10.1038/s41586-023-06234-x>.
- Ahmad, F., Cherukuri, M.K. and Choyke, P.L. (2021) 'Metabolic reprogramming in prostate cancer', *British Journal of Cancer*, 125(9), pp. 1185–1196. Available at: <https://doi.org/10.1038/s41416-021-01435-5>.
- Ahmed, S.M.H. *et al.* (2020) 'Fitness trade-offs incurred by ovary-to-gut steroid signalling in *Drosophila*', *Nature*, 584(7821), pp. 415–419. Available at: <https://doi.org/10.1038/s41586-020-2462-y>.
- Albrektsen, G. *et al.* (2005) 'Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects', *British Journal of Cancer*, 92(1), pp. 167–175. Available at: <https://doi.org/10.1038/sj.bjc.6602302>.
- Amcheslavsky, A. *et al.* (2011) 'Tuberous sclerosis complex and Myc coordinate the growth and division of *Drosophila* intestinal stem cells', *Journal of Cell Biology*, 193(4), pp. 695–710. Available at: <https://doi.org/10.1083/jcb.201103018>.
- Amcheslavsky, A. *et al.* (2014) 'Enteroendocrine Cells Support Intestinal Stem-Cell-Mediated Homeostasis in *Drosophila*', *Cell Reports*, 9(1), pp. 32–39. Available at: <https://doi.org/10.1016/j.celrep.2014.08.052>.
- Amcheslavsky, A., Jiang, J. and Ip, Y.T. (2009) 'Tissue Damage-Induced Intestinal Stem Cell Division in *Drosophila*', *Cell Stem Cell*, 4(1), pp. 49–61. Available at: <https://doi.org/10.1016/j.stem.2008.10.016>.
- Ameku, T. *et al.* (2018) 'Midgut-derived neuropeptide F controls germline stem cell proliferation in a mating-dependent manner', *PLOS Biology*, 16(9), p. e2005004. Available at: <https://doi.org/10.1371/journal.pbio.2005004>.
- Antonello, Z.A. *et al.* (2015) 'Robust intestinal homeostasis relies on cellular plasticity in enteroblasts mediated by miR-8–Escargot switch', *The EMBO Journal*, 34(15), pp. 2025–2041. Available at: <https://doi.org/10.15252/embj.201591517>.
- Apidianakis, Y. *et al.* (2009) 'Synergy between bacterial infection and genetic predisposition in intestinal dysplasia', *Proceedings of the National Academy of Sciences*, 106(49), pp. 20883–20888. Available at: <https://doi.org/10.1073/pnas.0911797106>.
- Arya, G.H. *et al.* (2010) 'Natural Variation, Functional Pleiotropy and Transcriptional Contexts of Odorant Binding Protein Genes in *Drosophila melanogaster*', *Genetics*, 186(4), pp. 1475–1485. Available at: <https://doi.org/10.1534/genetics.110.123166>.
- Aster, J.C., Pear, W.S. and Blacklow, S.C. (2017) 'The Varied Roles of Notch in Cancer', *Annual Review of Pathology: Mechanisms of Disease*, 12(1), pp. 245–275. Available at: <https://doi.org/10.1146/annurev-pathol-052016-100127>.

Ayyaz, A., Li, H. and Jasper, H. (2015) 'Haemocytes control stem cell activity in the *Drosophila* intestine', *Nature Cell Biology*, 17(6), pp. 736–748. Available at: <https://doi.org/10.1038/ncb3174>.

Bach, E.A. *et al.* (2007) 'GFP reporters detect the activation of the *Drosophila* JAK/STAT pathway in vivo', *Gene Expression Patterns*, 7(3), pp. 323–331.

Bajpai, A. *et al.* (2020) 'A *Drosophila* model of oral peptide therapeutics for adult intestinal stem cell tumors', *Disease Models & Mechanisms*, 13(7), p. dmm044420. Available at: <https://doi.org/10.1242/dmm.044420>.

Bangi, E. *et al.* (2016) 'Functional exploration of colorectal cancer genomes using *Drosophila*', *Nature Communications*, 7(1), p. 13615. Available at: <https://doi.org/10.1038/ncomms13615>.

Barker, N. *et al.* (2007) 'Identification of stem cells in small intestine and colon by marker gene *Lgr5*', *Nature*, 449(7165), pp. 1003–1007. Available at: <https://doi.org/10.1038/nature06196>.

Barry, E.R. *et al.* (2013) 'Restriction of intestinal stem cell expansion and the regenerative response by YAP', *Nature*, 493(7430), pp. 106–110. Available at: <https://doi.org/10.1038/nature11693>.

Bartlett, A.Q. *et al.* (2021) 'Immune Milieu Established by Postpartum Liver Involution Promotes Breast Cancer Liver Metastasis', *Cancers*, 13(7). Available at: <https://doi.org/10.3390/cancers13071698>.

Bashaw, G.J. and Baker, B.S. (1995) 'The *msl-2* dosage compensation gene of *Drosophila* encodes a putative DNA-binding protein whose expression is sex specifically regulated by *Sex-lethal*', *Development*, 121(10), pp. 3245–3258. Available at: <https://doi.org/10.1242/dev.121.10.3245>.

Baumgartner, R. *et al.* (2010) 'The WW Domain Protein Kibra Acts Upstream of Hippo in *Drosophila*', *Developmental Cell*, 18(2), pp. 309–316. Available at: <https://doi.org/10.1016/j.devcel.2009.12.013>.

Beagley, K.W. and Gockel, C.M. (2003) 'Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone', *FEMS Immunology & Medical Microbiology*, 38(1), pp. 13–22. Available at: [https://doi.org/10.1016/S0928-8244\(03\)00202-5](https://doi.org/10.1016/S0928-8244(03)00202-5).

Bell, L.R. *et al.* (1988) 'Sex-lethal, a *Drosophila* sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins', *Cell*, 55(6), pp. 1037–1046. Available at: [https://doi.org/10.1016/0092-8674\(88\)90248-6](https://doi.org/10.1016/0092-8674(88)90248-6).

Belote, J.M. *et al.* (1989) 'Molecular genetics of transformer, a genetic switch controlling sexual differentiation in *Drosophila*', *Developmental Genetics*, 10(3), pp. 143–154. Available at: <https://doi.org/10.1002/dvg.1020100304>.

Berghöfer, B. *et al.* (2006) 'TLR7 Ligands Induce Higher IFN- α Production in Females¹', *The Journal of Immunology*, 177(4), pp. 2088–2096. Available at: <https://doi.org/10.4049/jimmunol.177.4.2088>.

Bernardo, T.J. and Dubrovsky, E.B. (2012) 'The Drosophila juvenile hormone receptor candidates methoprene-tolerant (MET) and germ cell-expressed (GCE) utilize a conserved LIXXL motif to bind the FTZ-F1 nuclear receptor', *Journal of Biological Chemistry*, 287(10), pp. 7821–7833.

Bishop, J.G. and Corces, V.G. (1988) 'Expression of an activated ras gene causes developmental abnormalities in transgenic Drosophila melanogaster.', *Genes & Development*, 2(5), pp. 567–577. Available at: <https://doi.org/10.1101/gad.2.5.567>.

Biteau, B. *et al.* (2010) 'Lifespan Extension by Preserving Proliferative Homeostasis in Drosophila', *PLOS Genetics*, 6(10), p. e1001159. Available at: <https://doi.org/10.1371/journal.pgen.1001159>.

Biteau, B., Hochmuth, C.E. and Jasper, H. (2008) 'JNK Activity in Somatic Stem Cells Causes Loss of Tissue Homeostasis in the Aging Drosophila Gut', *Cell Stem Cell*, 3(4), pp. 442–455. Available at: <https://doi.org/10.1016/j.stem.2008.07.024>.

Biteau, B. and Jasper, H. (2011) 'EGF signaling regulates the proliferation of intestinal stem cells in Drosophila', *Development*, 138(6), pp. 1045–1055. Available at: <https://doi.org/10.1242/dev.056671>.

Biteau, B. and Jasper, H. (2014) 'Slit/Robo Signaling Regulates Cell Fate Decisions in the Intestinal Stem Cell Lineage of Drosophila', *Cell Reports*, 7(6), pp. 1867–1875. Available at: <https://doi.org/10.1016/j.celrep.2014.05.024>.

Boggs, R.T. *et al.* (1987) 'Regulation of sexual differentiation in D. melanogaster via alternative splicing of RNA from the transformer gene', *Cell*, 50(5), pp. 739–747. Available at: [https://doi.org/10.1016/0092-8674\(87\)90332-1](https://doi.org/10.1016/0092-8674(87)90332-1).

Bohere, J., Eldridge-Thomas, B.L. and Kolahgar, G. (2022) 'Vinculin recruitment to α -catenin halts the differentiation and maturation of enterocyte progenitors to maintain homeostasis of the Drosophila intestine', *eLife*. Edited by K. VijayRaghavan, M. Zaidi, and J. de Navascues, 11, p. e72836. Available at: <https://doi.org/10.7554/eLife.72836>.

Bonfini, A. *et al.* (2021) 'Multiscale analysis reveals that diet-dependent midgut plasticity emerges from alterations in both stem cell niche coupling and enterocyte size', *eLife*. Edited by L.E. O'Brien, U. Banerjee, and L.E. O'Brien, 10, p. e64125. Available at: <https://doi.org/10.7554/eLife.64125>.

Borrás, C. *et al.* (2003) 'Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males', *Free Radical Biology and Medicine*, 34(5), pp. 546–552. Available at: [https://doi.org/10.1016/S0891-5849\(02\)01356-4](https://doi.org/10.1016/S0891-5849(02)01356-4).

Borrás, C. *et al.* (2005) '17 β -oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NF κ B cascade', *Aging Cell*, 4(3), pp. 113–118. Available at: <https://doi.org/10.1111/j.1474-9726.2005.00151.x>.

Bossen, J. *et al.* (2023) 'Adult and Larval Tracheal Systems Exhibit Different Molecular Architectures in *Drosophila*', *International Journal of Molecular Sciences*, 24(6). Available at: <https://doi.org/10.3390/ijms24065628>.

Brand, A.H. and Perrimon, N. (1993) 'Targeted gene expression as a means of altering cell fates and generating dominant phenotypes', *Development*, 118(2), pp. 401–415. Available at: <https://doi.org/10.1242/dev.118.2.401>.

Bray, F. *et al.* (2018) 'Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries', *CA: A Cancer Journal for Clinicians*, 68(6), pp. 394–424. Available at: <https://doi.org/10.3322/caac.21492>.

Bray, S.J. (2006) 'Notch signalling: a simple pathway becomes complex', *Nature Reviews Molecular Cell Biology*, 7(9), pp. 678–689. Available at: <https://doi.org/10.1038/nrm2009>.

Brennecke, J. *et al.* (2003) 'bantam Encodes a Developmentally Regulated microRNA that Controls Cell Proliferation and Regulates the Proapoptotic Gene *hid* in *Drosophila*', *Cell*, 113(1), pp. 25–36. Available at: [https://doi.org/10.1016/S0092-8674\(03\)00231-9](https://doi.org/10.1016/S0092-8674(03)00231-9).

Brenner, R.M. *et al.* (2003) 'Immunocytochemical assessment of mitotic activity with an antibody to phosphorylated histone H3 in the macaque and human endometrium', *Human Reproduction*, 18(6), pp. 1185–1193. Available at: <https://doi.org/10.1093/humrep/deg255>.

Broderick, N., A., Buchon, N. and Lemaitre, B. (2014) 'Microbiota-Induced Changes in *Drosophila melanogaster* Host Gene Expression and Gut Morphology', *mBio*, 5(3), p. 10.1128/mbio.01117-14. Available at: <https://doi.org/10.1128/mbio.01117-14>.

Brumby, A.M. and Richardson, H.E. (2003) 'scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in *Drosophila*', *EMBO Journal*, 22(21), pp. 5769–5779. Available at: <https://doi.org/10.1093/emboj/cdg548>.

Buchon, N. *et al.* (2009) 'Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation', *Cell Host & Microbe*, 5(2), pp. 200–211. Available at: <https://doi.org/10.1016/j.chom.2009.01.003>.

Buchon, N. *et al.* (2013) 'Morphological and Molecular Characterization of Adult Midgut Compartmentalization in *Drosophila*', *Cell Reports*, 3(5), pp. 1725–1738. Available at: <https://doi.org/10.1016/j.celrep.2013.04.001>.

Buddika, K. *et al.* (2021) 'l-KCKT allows dissection-free RNA profiling of adult *Drosophila* intestinal progenitor cells', *Development*, 148(1), p. dev196568. Available at: <https://doi.org/10.1242/dev.196568>.

Burtis, K.C. and Baker, B.S. (1989) 'Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides', *Cell*, 56(6), pp. 997–1010. Available at: [https://doi.org/10.1016/0092-8674\(89\)90633-8](https://doi.org/10.1016/0092-8674(89)90633-8).

Cachero, S. *et al.* (2010) 'Sexual Dimorphism in the Fly Brain', *Current Biology*, 20(18), pp. 1589–1601. Available at: <https://doi.org/10.1016/j.cub.2010.07.045>.

Callihan, E.B. *et al.* (2013) 'Postpartum diagnosis demonstrates a high risk for metastasis and merits an expanded definition of pregnancy-associated breast cancer', *Breast cancer research and treatment*, 138, pp. 549–559.

Cancer Research UK (2020) 'Cancer incidence for common cancers', 31 December. Available at: <https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence> (Accessed: 6 December 2023).

Carrel, L. and Willard, H.F. (2005) 'X-inactivation profile reveals extensive variability in X-linked gene expression in females', *Nature*, 434(7031), pp. 400–404.

Carvalho, G.B. *et al.* (2006) 'Allokrine Modulation of Feeding Behavior by the Sex Peptide of *Drosophila*', *Current Biology*, 16(7), pp. 692–696. Available at: <https://doi.org/10.1016/j.cub.2006.02.064>.

Caussinus, E. and Gonzalez, C. (2005) 'Induction of tumor growth by altered stem-cell asymmetric division in *Drosophila melanogaster*', *Nature genetics*, 37(10), pp. 1125–1129.

Chacko, L., Macaron, C. and Burke, C.A. (2015) 'Colorectal cancer screening and prevention in women', *Digestive diseases and sciences*, 60, pp. 698–710.

Chakrabarti, S. *et al.* (2016) 'Remote Control of Intestinal Stem Cell Activity by Haemocytes in *Drosophila*', *PLOS Genetics*, 12(5), p. e1006089. Available at: <https://doi.org/10.1371/journal.pgen.1006089>.

Chan, S.W. *et al.* (2011) 'Hippo pathway-independent restriction of TAZ and YAP by angiomin', *Journal of Biological Chemistry*, 286(9), pp. 7018–7026.

Chang, L.-C. *et al.* (2014) 'Metabolic syndrome and smoking may justify earlier colorectal cancer screening in men', *Gastrointestinal endoscopy*, 79(6), pp. 961–969.

Chapman, T. *et al.* (1995) 'Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products', *Nature*, 373(6511), pp. 241–244. Available at: <https://doi.org/10.1038/373241a0>.

Chatterjee, S.S. *et al.* (2011) 'The female-specific Doublesex isoform regulates pleiotropic transcription factors to pattern genital development in *Drosophila*', *Development*, 138(6), pp. 1099–1109. Available at: <https://doi.org/10.1242/dev.055731>.

Chen, J. *et al.* (2018) 'Transient Scute activation via a self-stimulatory loop directs enteroendocrine cell pair specification from self-renewing intestinal stem cells', *Nature Cell Biology*, 20(2), pp. 152–161. Available at: <https://doi.org/10.1038/s41556-017-0020-0>.

Chlebowski, R.T. *et al.* (2004) 'Estrogen plus Progestin and Colorectal Cancer in Postmenopausal Women', *New England Journal of Medicine*, 350(10), pp. 991–1004. Available at: <https://doi.org/10.1056/NEJMoa032071>.

Cho, S., Yeon *et al.* (2018) 'Expression of Hippo pathway genes and their clinical significance in colon adenocarcinoma', *Oncology Letters*, 15(4), pp. 4926–4936. Available at: <https://doi.org/10.3892/ol.2018.7911>.

Choi, N.H., Lucchetta, E. and Ohlstein, B. (2011) 'Nonautonomous regulation of Drosophila midgut stem cell proliferation by the insulin-signaling pathway', *Proceedings of the National Academy of Sciences*, 108(46), pp. 18702–18707. Available at: <https://doi.org/10.1073/pnas.1109348108>.

Chopra, G., Kaushik, S. and Kain, P. (2022) 'Nutrient Sensing via Gut in Drosophila melanogaster', *International Journal of Molecular Sciences*, 23(5). Available at: <https://doi.org/10.3390/ijms23052694>.

Ciresi, D.L. and Scholten, D.J. (1995) 'The continuing clinical dilemma of primary tumors of the small intestine.', *The American surgeon*, 61(8), pp. 698–702; discussion 702.

Cline, T.W. (1988) 'Evidence that sisterless-a and sisterless-b are two of several discrete "numerator elements" of the X/A sex determination signal in Drosophila that switch Sxl between two alternative stable expression states.', *Genetics*, 119(4), pp. 829–862. Available at: <https://doi.org/10.1093/genetics/119.4.829>.

Cline, T.W. (1993) 'The Drosophila sex determination signal: how do flies count to two?', *Trends in Genetics*, 9(11), pp. 385–390. Available at: [https://doi.org/10.1016/0168-9525\(93\)90138-8](https://doi.org/10.1016/0168-9525(93)90138-8).

Cohen, E. *et al.* (2020) 'Physiology, Development, and Disease Modeling in the Drosophila Excretory System', *Genetics*, 214(2), pp. 235–264. Available at: <https://doi.org/10.1534/genetics.119.302289>.

Colditz, G.A. *et al.* (2004) 'Risk Factors for Breast Cancer According to Estrogen and Progesterone Receptor Status', *JNCI: Journal of the National Cancer Institute*, 96(3), pp. 218–228. Available at: <https://doi.org/10.1093/jnci/djh025>.

Cordero, J.B. *et al.* (2012) 'Inducible progenitor-derived Wingless regulates adult midgut regeneration in Drosophila', *The EMBO Journal*, 31(19), pp. 3901–3917. Available at: <https://doi.org/10.1038/emboj.2012.248>.

Cramer, D.W. and Welch, W.R. (1983) 'Determinants of Ovarian Cancer Risk. II. Inferences Regarding Pathogenesis2', *JNCI: Journal of the National Cancer Institute*, 71(4), pp. 717–721. Available at: <https://doi.org/10.1093/jnci/71.4.717>.

Dai, G. *et al.* (2011) 'Maternal hepatic growth response to pregnancy in the mouse', *Experimental biology and medicine*, 236(11), pp. 1322–1332.

Davegårdh, C. *et al.* (2019) 'Sex influences DNA methylation and gene expression in human skeletal muscle myoblasts and myotubes', *Stem Cell Research & Therapy*, 10(1), p. 26. Available at: <https://doi.org/10.1186/s13287-018-1118-4>.

Davies, E.L. *et al.* (2013) 'The transcriptional regulator lola is required for stem cell maintenance and germ cell differentiation in the Drosophila testis', *Developmental*

Biology, 373(2), pp. 310–321. Available at:
<https://doi.org/10.1016/j.ydbio.2012.11.004>.

Davis, S.M. *et al.* (2019) 'Sex differences in infant body composition emerge in the first 5 months of life', 32(11), pp. 1235–1239. Available at:
<https://doi.org/10.1515/jpem-2019-0243>.

Demir, E. and Dickson, B.J. (2005) 'fruitless Splicing Specifies Male Courtship Behavior in *Drosophila*', *Cell*, 121(5), pp. 785–794. Available at:
<https://doi.org/10.1016/j.cell.2005.04.027>.

Dewing, P. *et al.* (2006) 'Direct Regulation of Adult Brain Function by the Male-Specific Factor SRY', *Current Biology*, 16(4), pp. 415–420. Available at:
<https://doi.org/10.1016/j.cub.2006.01.017>.

Diaz, A.V. *et al.* (2023) 'Spenito-dependent metabolic sexual dimorphism intrinsic to fat storage cells', *Genetics*, p. iyad164. Available at:
<https://doi.org/10.1093/genetics/iyad164>.

Dietzl, G. *et al.* (2007) 'A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*', *Nature*, 448(7150), pp. 151–156. Available at:
<https://doi.org/10.1038/nature05954>.

DiNardo, S. *et al.* (2011) 'lines and bowl affect the specification of cyst stem cells and niche cells in the *Drosophila* testis', *Development*, 138(9), pp. 1687–1696.

Ding, G. *et al.* (2021) 'Coordination of tumor growth and host wasting by tumor-derived Upd3', *Cell Reports*, 36(7), p. 109553. Available at:
<https://doi.org/10.1016/j.celrep.2021.109553>.

DiSario, J.A. *et al.* (1994) 'Small bowel cancer: epidemiological and clinical characteristics from a population-based registry.', *American Journal of Gastroenterology (Springer Nature)*, 89(5).

Doggett, K. *et al.* (2015) 'BTB-Zinc Finger Oncogenes Are Required for Ras and Notch-Driven Tumorigenesis in *Drosophila*', *PLOS ONE*, 10(7), p. e0132987. Available at: <https://doi.org/10.1371/journal.pone.0132987>.

Dong, D.-J. *et al.* (2015) 'The Steroid Hormone 20-Hydroxyecdysone Up-regulates Ste-20 Family Serine/Threonine Kinase Hippo to Induce Programmed Cell Death*', *Journal of Biological Chemistry*, 290(41), pp. 24738–24746. Available at:
<https://doi.org/10.1074/jbc.M115.643783>.

Dossus, L. *et al.* (2010) 'Reproductive risk factors and endometrial cancer: the European Prospective Investigation into Cancer and Nutrition', *International Journal of Cancer*, 127(2), pp. 442–451. Available at: <https://doi.org/10.1002/ijc.25050>.

Dow, J.A.T., Simons, M. and Romero, M.F. (2022) '*Drosophila melanogaster*: a simple genetic model of kidney structure, function and disease', *Nature Reviews Nephrology*, 18(7), pp. 417–434. Available at: <https://doi.org/10.1038/s41581-022-00561-4>.

Dubrovsky, E.B. *et al.* (2011) 'The Drosophila FTZ-F1 Nuclear Receptor Mediates Juvenile Hormone Activation of E75A Gene Expression through an Intracellular Pathway', *Journal of Biological Chemistry*, 286(38), pp. 33689–33700. Available at: <https://doi.org/10.1074/jbc.M111.273458>.

Duffy, J.B. and Gergen, J.P. (1991) 'The Drosophila segmentation gene runt acts as a position-specific numerator element necessary for the uniform expression of the sex-determining gene Sex-lethal.', *Genes & Development*, 5(12a), pp. 2176–2187. Available at: <https://doi.org/10.1101/gad.5.12a.2176>.

Dunford, A. *et al.* (2017) 'Tumor-suppressor genes that escape from X-inactivation contribute to cancer sex bias', *Nature Genetics*, 49(1), pp. 10–16. Available at: <https://doi.org/10.1038/ng.3726>.

Dutta, D. *et al.* (2015) 'Regional Cell-Specific Transcriptome Mapping Reveals Regulatory Complexity in the Adult Drosophila Midgut', *Cell Reports*, 12(2), pp. 346–358. Available at: <https://doi.org/10.1016/j.celrep.2015.06.009>.

Edgren, G. *et al.* (2012) 'Enigmatic sex disparities in cancer incidence', *European Journal of Epidemiology*, 27(3), pp. 187–196. Available at: <https://doi.org/10.1007/s10654-011-9647-5>.

Endogenous Hormones Breast Cancer Collaborative Group (2003) 'Body Mass Index, Serum Sex Hormones, and Breast Cancer Risk in Postmenopausal Women', *JNCI: Journal of the National Cancer Institute*, 95(16), pp. 1218–1226. Available at: <https://doi.org/10.1093/jnci/djg022>.

Enomoto, M. and Igaki, T. (2011) 'Deciphering tumor-suppressor signaling in flies: Genetic link between Scribble/Dlg/Lgl and the Hippo pathways', *Drosophila Models for Studying Cancer and Tumorigenesis*, 38(10), pp. 461–470. Available at: <https://doi.org/10.1016/j.jgg.2011.09.005>.

Fathalla, M.F. (1971) 'INCESSANT OVULATION—A FACTOR IN OVARIAN NEOPLASIA?', *Originally published as Volume 2, Issue 7716*, 298(7716), p. 163. Available at: [https://doi.org/10.1016/S0140-6736\(71\)92335-X](https://doi.org/10.1016/S0140-6736(71)92335-X).

Faupel-Badger, J.M. *et al.* (2013) 'Postpartum Remodeling, Lactation, and Breast Cancer Risk: Summary of a National Cancer Institute–Sponsored Workshop', *JNCI: Journal of the National Cancer Institute*, 105(3), pp. 166–174. Available at: <https://doi.org/10.1093/jnci/djs505>.

Fearnhead, N.S., Britton, M.P. and Bodmer, W.F. (2001) 'The ABC of APC', *Human Molecular Genetics*, 10(7), pp. 721–733. Available at: <https://doi.org/10.1093/hmg/10.7.721>.

Fedorka, K.M. *et al.* (2007) 'Post-mating disparity between potential and realized immune response in Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 274(1614), pp. 1211–1217. Available at: <https://doi.org/10.1098/rspb.2006.0394>.

Feigman, M.J. *et al.* (2020) 'Pregnancy reprograms the epigenome of mammary epithelial cells and blocks the development of premalignant lesions', *Nature*

Communications, 11(1), p. 2649. Available at: <https://doi.org/10.1038/s41467-020-16479-z>.

Fell, B., Smith, K.A. and Campbell, R.M. (1963) 'Hypertrophic and hyperplastic changes in the alimentary canal of the lactating rat.', *Journal of Pathology and Bacteriology*, 85, pp. 179–188.

Ferlay, J. *et al.* (2019) 'Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods', *International Journal of Cancer*, 144(8), pp. 1941–1953. Available at: <https://doi.org/10.1002/ijc.31937>.

Ferres-Marco, D. *et al.* (2006) 'Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing', *Nature*, 439(7075), pp. 430–436.

Ferveur, J.-F. *et al.* (1995) 'Genetic Feminization of Brain Structures and Changed Sexual Orientation in Male *Drosophila*', *Science*, 267(5199), pp. 902–905. Available at: <https://doi.org/10.1126/science.7846534>.

Figuerola-Clavevega, A. and Bilder, D. (2015) 'Malignant *Drosophila* tumors interrupt insulin signaling to induce cachexia-like wasting', *Developmental cell*, 33(1), pp. 47–55.

Fletcher, G.C. *et al.* (2018) 'Mechanical strain regulates the Hippo pathway in *Drosophila*', *Development*, 145(5), p. dev159467. Available at: <https://doi.org/10.1242/dev.159467>.

Foster, P.A. (2013) 'Oestrogen and colorectal cancer: mechanisms and controversies', *International Journal of Colorectal Disease*, 28(6), pp. 737–749. Available at: <https://doi.org/10.1007/s00384-012-1628-y>.

Furriols, M. and Bray, S. (2001) 'A model Notch response element detects Suppressor of Hairless–dependent molecular switch', *Current Biology*, 11(1), pp. 60–64. Available at: [https://doi.org/10.1016/S0960-9822\(00\)00044-0](https://doi.org/10.1016/S0960-9822(00)00044-0).

Gateff, E. and Schneiderman, H. (1969) 'Neoplasms in mutant and cultured wild-type tissues of *Drosophila*', *Natl Cancer Inst Monogr*, pp. 365–97.

Gateff, E. and Schneiderman, H.A. (1967) 'Developmental studies of a new mutant of *Drosophila melanogaster*-lethal malignant brain tumor (1 (2) GL4)', in *American Zoologist*. SOC INTEGRATIVE COMPARATIVE BIOLOGY 1313 DOLLEY MADISON BLVD, NO 402, MCLEAN ..., pp. 760-+.

Gateff, E. and Schneiderman, H.A. (1974) 'Developmental capacities of benign and malignant neoplasms of *Drosophila*', *Wilhelm Roux'Archiv für Entwicklungsmechanik der Organismen*, 176, pp. 23–65.

Gilboa, L. *et al.* (2003) 'Germ line stem cell differentiation in *Drosophila* requires gap junctions and proceeds via an intermediate state', *Development*, 130(26), pp. 6625–6634. Available at: <https://doi.org/10.1242/dev.00853>.

GILLOTT, C. (1996) 'Male insect accessory glands: Functions and control of secretory activity', *Invertebrate Reproduction & Development*, 30(1–3), pp. 199–205.

- Gladstone, M. and Su, T.T. (2011) 'Chemical genetics and drug screening in *Drosophila* cancer models', *Journal of Genetics and Genomics*, 38(10), pp. 497–504.
- Goddard, E.T. *et al.* (2017) 'The rodent liver undergoes weaning-induced involution and supports breast cancer metastasis', *Cancer discovery*, 7(2), pp. 177–187.
- Golic, K.G. and Lindquist, S. (1989) 'The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome', *Cell*, 59(3), pp. 499–509.
- Gonzalez, C. (2013) '*Drosophila melanogaster*: a model and a tool to investigate malignancy and identify new therapeutics', *Nature Reviews Cancer*, 13(3), pp. 172–183.
- Goodwin, S.F. *et al.* (2000) 'Aberrant Splicing and Altered Spatial Expression Patterns in fruitless Mutants of *Drosophila melanogaster*', *Genetics*, 154(2), pp. 725–745. Available at: <https://doi.org/10.1093/genetics/154.2.725>.
- Gordon, K.E., Wolfner, M.F. and Lazzaro, B.P. (2022) 'A single mating is sufficient to induce persistent reduction of immune defense in mated female *Drosophila melanogaster*', *Journal of Insect Physiology*, 140, p. 104414. Available at: <https://doi.org/10.1016/j.jinsphys.2022.104414>.
- Goto, H. *et al.* (1999) 'Identification of a Novel Phosphorylation Site on Histone H3 Coupled with Mitotic Chromosome Condensation*', *Journal of Biological Chemistry*, 274(36), pp. 25543–25549. Available at: <https://doi.org/10.1074/jbc.274.36.25543>.
- Goulev, Y. *et al.* (2008) 'SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in *Drosophila*', *Current Biology*, 18(6), pp. 435–441. Available at: <https://doi.org/10.1016/j.cub.2008.02.034>.
- Goyal, L. *et al.* (2000) 'Induction of apoptosis by *Drosophila* reaper, hid and grim through inhibition of IAP function', *The EMBO journal*, 19(4), pp. 589–597.
- GraphPad Prism (2023) 'GraphPad Prism for Mac OS'. Boston, Massachusetts USA: GraphPad Software.
- Grattan, D.R. and Ladyman, S.R. (2020) 'Chapter 2 - Neurophysiological and cognitive changes in pregnancy', in E.A.P. Steegers, M.J. Cipolla, and E.C. Miller (eds) *Handbook of Clinical Neurology*. Elsevier, pp. 25–55. Available at: <https://doi.org/10.1016/B978-0-444-64239-4.00002-3>.
- Greenspan, L.J., De Cuevas, M. and Matunis, E. (2015) 'Genetics of gonadal stem cell renewal', *Annual review of cell and developmental biology*, 31, pp. 291–315.
- Gregoire, E.P. *et al.* (2023) 'The -KTS splice variant of WT1 is essential for ovarian determination in mice', *Science*, 382(6670), pp. 600–606. Available at: <https://doi.org/10.1126/science.add8831>.
- Griesbeck, M. *et al.* (2015) 'Sex Differences in Plasmacytoid Dendritic Cell Levels of IRF5 Drive Higher IFN- α Production in Women', *The Journal of Immunology*, 195(11), pp. 5327–5336. Available at: <https://doi.org/10.4049/jimmunol.1501684>.

- Grimm, S.A. *et al.* (2019) 'DNA methylation in mice is influenced by genetics as well as sex and life experience', *Nature Communications*, 10(1), p. 305. Available at: <https://doi.org/10.1038/s41467-018-08067-z>.
- Grmai, L. *et al.* (2018) 'Chinmo prevents transformer alternative splicing to maintain male sex identity', *PLOS Genetics*, 14(2), p. e1007203. Available at: <https://doi.org/10.1371/journal.pgen.1007203>.
- Grusche, F.A., Richardson, H.E. and Harvey, K.F. (2010) 'Upstream Regulation of the Hippo Size Control Pathway', *Current Biology*, 20(13), pp. R574–R582. Available at: <https://doi.org/10.1016/j.cub.2010.05.023>.
- Gubbay, J. *et al.* (1990) 'A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes', *Nature*, 346(6281), pp. 245–250. Available at: <https://doi.org/10.1038/346245a0>.
- Guo, L. *et al.* (2014) 'PGRP-SC2 Promotes Gut Immune Homeostasis to Limit Commensal Dysbiosis and Extend Lifespan', *Cell*, 156(1), pp. 109–122. Available at: <https://doi.org/10.1016/j.cell.2013.12.018>.
- Guo, X. *et al.* (2019) 'The Cellular Diversity and Transcription Factor Code of Drosophila Enteroendocrine Cells', *Cell Reports*, 29(12), pp. 4172–4185.e5. Available at: <https://doi.org/10.1016/j.celrep.2019.11.048>.
- Gupta, V. *et al.* (2022) 'Inherent constraints on a polyfunctional tissue lead to a reproduction-immunity tradeoff', *BMC Biology*, 20(1), p. 127. Available at: <https://doi.org/10.1186/s12915-022-01328-w>.
- GURLEY, L.R. *et al.* (1978) 'Histone Phosphorylation and Chromatin Structure during Mitosis in Chinese Hamster Cells', *European Journal of Biochemistry*, 84(1), pp. 1–15. Available at: <https://doi.org/10.1111/j.1432-1033.1978.tb12135.x>.
- Gutiérrez-Adán, A. *et al.* (1997) 'Early transcription of the SRY gene by bovine preimplantation embryos', *Molecular Reproduction and Development*, 48(2), pp. 246–250. Available at: [https://doi.org/10.1002/\(SICI\)1098-2795\(199710\)48:2<246::AID-MRD12>3.0.CO;2-W](https://doi.org/10.1002/(SICI)1098-2795(199710)48:2<246::AID-MRD12>3.0.CO;2-W).
- Hadjieconomou, D. *et al.* (2020) 'Enteric neurons increase maternal food intake during reproduction', *Nature*, 587(7834), pp. 455–459. Available at: <https://doi.org/10.1038/s41586-020-2866-8>.
- Hamaratoglu, F. *et al.* (2006) 'The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis', *Nature Cell Biology*, 8(1), pp. 27–36. Available at: <https://doi.org/10.1038/ncb1339>.
- Hammond, K.A. (1997) 'Adaptation of the maternal intestine during lactation', *Journal of mammary gland biology and neoplasia*, 2, pp. 243–252.
- Han, C. *et al.* (2022) 'The doublesex gene regulates dimorphic sexual and aggressive behaviors in Drosophila', *Proceedings of the National Academy of Sciences*, 119(37), p. e2201513119. Available at: <https://doi.org/10.1073/pnas.2201513119>.

Hanahan, D. and Weinberg, R.A. (2011) 'Hallmarks of Cancer: The Next Generation', *Cell*, 144(5), pp. 646–674. Available at: <https://doi.org/10.1016/j.cell.2011.02.013>.

Hao, X. *et al.* (2020) 'Lola regulates Drosophila adult midgut homeostasis via non-canonical hippo signaling', *eLife*. Edited by K.S.E. Cheah, U. Banerjee, and Y. Yang, 9, p. e47542. Available at: <https://doi.org/10.7554/eLife.47542>.

Hao, Y. *et al.* (2008) 'Tumor Suppressor LATS1 Is a Negative Regulator of Oncogene YAP*', *Journal of Biological Chemistry*, 283(9), pp. 5496–5509. Available at: <https://doi.org/10.1074/jbc.M709037200>.

Hardy, R. *et al.* (1979) 'The germinal proliferation center in the testis of Drosophila melanogaster', *Journal of ultrastructure research*, 69(2), pp. 180–190.

Harvey, K. and Tapon, N. (2007) 'The Salvador–Warts–Hippo pathway—an emerging tumour-suppressor network', *Nature Reviews Cancer*, 7(3), pp. 182–191.

Harvey, K.F., Pfleger, C.M. and Hariharan, I.K. (2003) 'The Drosophila Mst Ortholog, hippo, Restricts Growth and Cell Proliferation and Promotes Apoptosis', *Cell*, 114(4), pp. 457–467. Available at: [https://doi.org/10.1016/S0092-8674\(03\)00557-9](https://doi.org/10.1016/S0092-8674(03)00557-9).

Harvie, M. and Howell, A. (2012) 'Energy restriction and the prevention of breast cancer', *Proceedings of the Nutrition Society*. 2012/03/14 edn, 71(2), pp. 263–275. Available at: <https://doi.org/10.1017/S0029665112000195>.

Hatzaras, I. *et al.* (2007) 'Small-Bowel Tumors: Epidemiologic and Clinical Characteristics of 1260 Cases From the Connecticut Tumor Registry', *Archives of Surgery*, 142(3), pp. 229–235. Available at: <https://doi.org/10.1001/archsurg.142.3.229>.

Hayashi, S. *et al.* (2002) 'GETDB, a database compiling expression patterns and molecular locations of a collection of gal4 enhancer traps', *genesis*, 34(1–2), pp. 58–61. Available at: <https://doi.org/10.1002/gene.10137>.

Hayashi, S. and Kondo, T. (2018) 'Development and Function of the Drosophila Tracheal System', *Genetics*, 209(2), pp. 367–380. Available at: <https://doi.org/10.1534/genetics.117.300167>.

He, L. *et al.* (2018) 'Mechanical regulation of stem-cell differentiation by the stretch-activated Piezo channel', *Nature*, 555(7694), pp. 103–106. Available at: <https://doi.org/10.1038/nature25744>.

Hedrington, M.S. and Davis, S.N. (2015) 'Sexual Dimorphism in Glucose and Lipid Metabolism during Fasting, Hypoglycemia, and Exercise', *Frontiers in Endocrinology*, 6. Available at: <https://www.frontiersin.org/articles/10.3389/fendo.2015.00061>.

Heinrichs, V., Ryner, L.C. and Baker, B.S. (1998) 'Regulation of Sex-Specific Selection of fruitless 5' Splice Sites by transformer and transformer-2', *Molecular and Cellular Biology*, 18(1), pp. 450–458. Available at: <https://doi.org/10.1128/MCB.18.1.450>.

Hendzel, M.J. *et al.* (1997) 'Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation', *Chromosoma*, 106(6), pp. 348–360. Available at: <https://doi.org/10.1007/s004120050256>.

Hollister, A. *et al.* (1987) 'Reproduction in mice: liver enlargement in mice during pregnancy and lactation', *Life sciences*, 40(1), pp. 11–18.

Hoshino, R. *et al.* (no date) 'Circulating fructose regulates a germline stem cell increase via gustatory receptor-mediated gut hormone secretion in mated *Drosophila*', *Science Advances*, 9(8), p. eadd5551. Available at: <https://doi.org/10.1126/sciadv.add5551>.

Huang, H. *et al.* (2014) 'Bantam is essential for *Drosophila* intestinal stem cell proliferation in response to Hippo signaling', *Developmental Biology*, 385(2), pp. 211–219. Available at: <https://doi.org/10.1016/j.ydbio.2013.11.008>.

Hudry, B. *et al.* (2019) 'Sex Differences in Intestinal Carbohydrate Metabolism Promote Food Intake and Sperm Maturation', *Cell*, 178(4), pp. 901–918.e16. Available at: <https://doi.org/10.1016/j.cell.2019.07.029>.

Hudry, B., Khadayate, S. and Miguel-Aliaga, I. (2016) 'The sexual identity of adult intestinal stem cells controls organ size and plasticity', *Nature*, 530(7590), pp. 344–348. Available at: <https://doi.org/10.1038/nature16953>.

Huh, J., Moon, H. and Chung, W.S. (2007) 'Incidence and Clinical Significance of Sex Chromosome Losses in Bone Marrow of Patients with Hematologic Diseases', *Annals of Laboratory Medicine*. 2007/02/01 edn, 27(1), pp. 56–61. Available at: <https://doi.org/10.3343/kjlm.2007.27.1.56>.

Hyatt, H.W. *et al.* (2019) 'Changes in metabolism, mitochondrial function, and oxidative stress between female rats under nonreproductive and 3 reproductive conditions', *Reproductive Sciences*, 26, pp. 114–127.

Ide, T. *et al.* (2002) 'Greater Oxidative Stress in Healthy Young Men Compared With Premenopausal Women', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 22(3), pp. 438–442. Available at: <https://doi.org/10.1161/hq0302.104515>.

Ippolito, J.E. *et al.* (2017) 'Sexual dimorphism in glioma glycolysis underlies sex differences in survival', *JCI Insight*, 2(15). Available at: <https://doi.org/10.1172/jci.insight.92142>.

Itskov, P.M. *et al.* (2014) 'Automated monitoring and quantitative analysis of feeding behaviour in *Drosophila*', *Nature communications*, 5(1), p. 4560.

Janerich, D.T. (1980) 'The influence of pregnancy on breast cancer risk: Is it endocrinological or immunological?', *Medical Hypotheses*, 6(11), pp. 1149–1155. Available at: [https://doi.org/10.1016/0306-9877\(80\)90137-1](https://doi.org/10.1016/0306-9877(80)90137-1).

Janerich, D.T. (1994) 'The fetal antigen hypothesis for breast cancer, revisited', *Medical Hypotheses*, 43(2), pp. 105–110. Available at: [https://doi.org/10.1016/0306-9877\(94\)90059-0](https://doi.org/10.1016/0306-9877(94)90059-0).

Jensen, B.A.H. *et al.* (2023) 'Small intestine vs. colon ecology and physiology: Why it matters in probiotic administration', *Cell Reports Medicine*, 4(9), p. 101190. Available at: <https://doi.org/10.1016/j.xcrm.2023.101190>.

Jiang, H. *et al.* (2009) 'Cytokine/Jak/Stat Signaling Mediates Regeneration and Homeostasis in the Drosophila Midgut', *Cell*, 137(7), pp. 1343–1355. Available at: <https://doi.org/10.1016/j.cell.2009.05.014>.

Jiang, H. and Edgar, B.A. (2009) 'EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors', *Development*, 136(3), pp. 483–493. Available at: <https://doi.org/10.1242/dev.026955>.

Jiang, Y. *et al.* (2018) 'An intrinsic tumour eviction mechanism in Drosophila mediated by steroid hormone signalling', *Nature Communications*, 9(1), p. 3293. Available at: <https://doi.org/10.1038/s41467-018-05794-1>.

Jindra, M. *et al.* (2015) 'Genetic Evidence for Function of the bHLH-PAS Protein Gce/Met As a Juvenile Hormone Receptor', *PLOS Genetics*, 11(7), p. e1005394. Available at: <https://doi.org/10.1371/journal.pgen.1005394>.

Jindra, M., Palli, S.R. and Riddiford, L.M. (2013) 'The Juvenile Hormone Signaling Pathway in Insect Development', *Annual Review of Entomology*, 58(1), pp. 181–204. Available at: <https://doi.org/10.1146/annurev-ento-120811-153700>.

Jinks, T.M. *et al.* (2000) 'The JAK/STAT Signaling Pathway Is Required for the Initial Choice of Sexual Identity in Drosophila melanogaster', *Molecular Cell*, 5(3), pp. 581–587. Available at: [https://doi.org/10.1016/S1097-2765\(00\)80451-7](https://doi.org/10.1016/S1097-2765(00)80451-7).

JOLICOEUR, L., ASSENLIN, J. and MORISSET, J. (1980) 'Trophic effects of gestation and lactation on rat pancreas', *Biomedical Research*, 1(6), pp. 482–488.

Justice, R.W. *et al.* (1995) 'The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation.', *Genes & development*, 9(5), pp. 534–546.

Kampmann, U. *et al.* (2019) 'Determinants of Maternal Insulin Resistance during Pregnancy: An Updated Overview', *Journal of Diabetes Research*. Edited by B. Portha, 2019, p. 5320156. Available at: <https://doi.org/10.1155/2019/5320156>.

Kapuria, S. *et al.* (2012) 'Notch-Mediated Suppression of TSC2 Expression Regulates Cell Differentiation in the Drosophila Intestinal Stem Cell Lineage', *PLOS Genetics*, 8(11), p. e1003045. Available at: <https://doi.org/10.1371/journal.pgen.1003045>.

Karageorgi, S. *et al.* (2010) 'Reproductive factors and postmenopausal hormone use in relation to endometrial cancer risk in the Nurses' Health Study cohort 1976–2004', *International Journal of Cancer*, 126(1), pp. 208–216. Available at: <https://doi.org/10.1002/ijc.24672>.

Karim, F.D. and Rubin, G.M. (1998) 'Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues', *Development*, 125(1), pp. 1–9. Available at: <https://doi.org/10.1242/dev.125.1.1>.

- Karpowicz, P. *et al.* (2013) 'The Circadian Clock Gates the Intestinal Stem Cell Regenerative State', *Cell Reports*, 3(4), pp. 996–1004. Available at: <https://doi.org/10.1016/j.celrep.2013.03.016>.
- Karpowicz, P., Perez, J. and Perrimon, N. (2010) 'The Hippo tumor suppressor pathway regulates intestinal stem cell regeneration', *Development*, 137(24), pp. 4135–4145. Available at: <https://doi.org/10.1242/dev.060483>.
- Kasai, Y. and Cagan, R. (2010) 'Drosophila as a tool for personalized medicine: a primer', *Personalized medicine*, 7(6), pp. 621–632.
- Katsanis, W.A. *et al.* (1998) 'Immune Recognition of Endometrial Tumor Antigens Induced by Multiparity', *Gynecologic Oncology*, 70(1), pp. 33–39. Available at: <https://doi.org/10.1006/gyno.1998.4988>.
- KELSEY, J.L. *et al.* (1982) 'A CASE-CONTROL STUDY OF CANCER OF THE ENDOMETRIUM', *American Journal of Epidemiology*, 116(2), pp. 333–342. Available at: <https://doi.org/10.1093/oxfordjournals.aje.a113417>.
- Kennedy, G., Pearce, W.M. and PARROTT, D.M. (1958) 'Liver growth in the lactating rat', *Journal of Endocrinology*, 17(2), pp. 158–160.
- Khalifa, A.R.M. *et al.* (2017) 'Sex-specific differences in mitochondria biogenesis, morphology, respiratory function, and ROS homeostasis in young mouse heart and brain', *Physiological Reports*, 5(6), p. e13125. Available at: <https://doi.org/10.14814/phy2.13125>.
- Kiger, A.A., White-Cooper, H. and Fuller, M.T. (2000) 'Somatic support cells restrict germline stem cell self-renewal and promote differentiation', *Nature*, 407(6805), pp. 750–754. Available at: <https://doi.org/10.1038/35037606>.
- Kim, H.-B. and Myung, S.-J. (2018) 'Clinical implications of the Hippo-YAP pathway in multiple cancer contexts', *BMB Reports*. 2018/03/31 edn, 51(3), pp. 119–125. Available at: <https://doi.org/10.5483/BMBRep.2018.51.3.018>.
- Kimura, K., Iwata, H. and Thompson, J.G. (2008) 'The effect of glucosamine concentration on the development and sex ratio of bovine embryos', *Animal Reproduction Science*, 103(3), pp. 228–238. Available at: <https://doi.org/10.1016/j.anireprosci.2006.12.014>.
- King, D.G. (1988) 'Cellular organization and peritrophic membrane formation in the cardia (Proventriculus) of *Drosophila melanogaster*', *Journal of Morphology*, 196(3), pp. 253–282. Available at: <https://doi.org/10.1002/jmor.1051960302>.
- Kinnunen, T.I. *et al.* (2004) 'Pregnancy weight gain and breast cancer risk', *BMC Women's Health*, 4(1), p. 7. Available at: <https://doi.org/10.1186/1472-6874-4-7>.
- Kitadate, Y. and Kobayashi, S. (2010) 'Notch and Egfr signaling act antagonistically to regulate germ-line stem cell niche formation in *Drosophila* male embryonic gonads', *Proceedings of the National Academy of Sciences*, 107(32), pp. 14241–14246.

Koopman, P. *et al.* (1990) 'Expression of a candidate sex-determining gene during mouse testis differentiation', *Nature*, 348(6300), pp. 450–452. Available at: <https://doi.org/10.1038/348450a0>.

Korzelius, J. *et al.* (2014) 'Escargot maintains stemness and suppresses differentiation in Drosophila intestinal stem cells', *The EMBO Journal*, 33(24), pp. 2967–2982. Available at: <https://doi.org/10.15252/embj.201489072>.

Kozlova, T. and Thummel, C.S. (2000) 'Steroid Regulation of Postembryonic Development and Reproduction in Drosophila', *Trends in Endocrinology & Metabolism*, 11(7), pp. 276–280. Available at: [https://doi.org/10.1016/S1043-2760\(00\)00282-4](https://doi.org/10.1016/S1043-2760(00)00282-4).

Krumsiek, J. *et al.* (2015) 'Gender-specific pathway differences in the human serum metabolome', *Metabolomics*, 11(6), pp. 1815–1833. Available at: <https://doi.org/10.1007/s11306-015-0829-0>.

Kumar, K. *et al.* (2021) 'A Superfamily-wide Activity Atlas of Serine Hydrolases in Drosophila melanogaster', *Biochemistry*, 60(16), pp. 1312–1324. Available at: <https://doi.org/10.1021/acs.biochem.1c00171>.

Kuraishi, T. *et al.* (2011) 'Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in Drosophila melanogaster', *Proceedings of the National Academy of Sciences*, 108(38), pp. 15966–15971. Available at: <https://doi.org/10.1073/pnas.1105994108>.

Kurkulos, M. *et al.* (1991) 'Polyadenylation in Copia Requires Unusually Distant Upstream Sequences', *Proceedings of the National Academy of Sciences of the United States of America*, 88(8), pp. 3038–3042.

Kwon, Y. *et al.* (2015) 'Systemic Organ Wasting Induced by Localized Expression of the Secreted Insulin/IGF Antagonist ImpL2', *Developmental Cell*, 33(1), pp. 36–46. Available at: <https://doi.org/10.1016/j.devcel.2015.02.012>.

LaJeunesse, D.R. *et al.* (2010) 'Peristalsis in the junction region of the Drosophila larval midgut is modulated by DH31 expressing enteroendocrine cells', *BMC physiology*, 10, pp. 1–14.

Lawrence, P.A. and Casal, J. (2013) 'The mechanisms of planar cell polarity, growth and the Hippo pathway: Some known unknowns', *Developmental Biology*, 377(1), pp. 1–8. Available at: <https://doi.org/10.1016/j.ydbio.2013.01.030>.

Le Bras, S. and Van Doren, M. (2006) 'Development of the male germline stem cell niche in Drosophila', *Developmental Biology*, 294(1), pp. 92–103. Available at: <https://doi.org/10.1016/j.ydbio.2006.02.030>.

Lee, B.-W. *et al.* (1996) 'Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: From birth to adulthood', *Cytometry*, 26(1), pp. 8–15. Available at: [https://doi.org/10.1002/\(SICI\)1097-0320\(19960315\)26:1<8::AID-CYTO2>3.0.CO;2-E](https://doi.org/10.1002/(SICI)1097-0320(19960315)26:1<8::AID-CYTO2>3.0.CO;2-E).

- Lee, G. *et al.* (2000) 'Spatial, temporal, and sexually dimorphic expression patterns of the fruitless gene in the Drosophila central nervous system', *Journal of Neurobiology*, 43(4), pp. 404–426. Available at: [https://doi.org/10.1002/1097-4695\(20000615\)43:4<404::AID-NEU8>3.0.CO;2-D](https://doi.org/10.1002/1097-4695(20000615)43:4<404::AID-NEU8>3.0.CO;2-D).
- Lee, G., Hall, J.C. and Park, J.H. (2002) 'Doublesex gene expression in the central nervous system of Drosophila Melanogaster', *Journal of Neurogenetics*, 16(4), pp. 229–248. Available at: <https://doi.org/10.1080/01677060216292>.
- Lee, J. *et al.* (2020) 'Dissemination of RasV12-transformed cells requires the mechanosensitive channel Piezo', *Nature Communications*, 11(1), p. 3568. Available at: <https://doi.org/10.1038/s41467-020-17341-y>.
- Lee, J. *et al.* (2021) 'Tumors overcome the action of the wasting factor Impl2 by locally elevating Wnt/Wingless', *Proceedings of the National Academy of Sciences*, 118(23), p. e2020120118. Available at: <https://doi.org/10.1073/pnas.2020120118>.
- Lee, T. and Luo, L. (1999) 'Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis', *Neuron*, 22(3), pp. 451–461.
- Lee, W.-C. *et al.* (2009) 'Adenomatous polyposis coli regulates Drosophila intestinal stem cell proliferation', *Development*, 136(13), pp. 2255–2264. Available at: <https://doi.org/10.1242/dev.035196>.
- Li, M., Mead, E.A. and Zhu, J. (2011) 'Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression', *Proceedings of the National Academy of Sciences*, 108(2), pp. 638–643. Available at: <https://doi.org/10.1073/pnas.1013914108>.
- Li, Q. *et al.* (2014) 'The Conserved Misshapen-Warts-Yorkie Pathway Acts in Enteroblasts to Regulate Intestinal Stem Cells in Drosophila', *Developmental Cell*, 31(3), pp. 291–304. Available at: <https://doi.org/10.1016/j.devcel.2014.09.012>.
- Li, Q. *et al.* (2018) 'Ingestion of Food Particles Regulates the Mechanosensing Misshapen-Yorkie Pathway in Drosophila Intestinal Growth', *Developmental Cell*, 45(4), pp. 433–449.e6. Available at: <https://doi.org/10.1016/j.devcel.2018.04.014>.
- Li, Z. *et al.* (2013) 'Trachea-Derived Dpp Controls Adult Midgut Homeostasis in Drosophila', *Developmental Cell*, 24(2), pp. 133–143. Available at: <https://doi.org/10.1016/j.devcel.2012.12.010>.
- Liang, K. *et al.* (2014) 'Expression of Hippo Pathway in Colorectal Cancer', *Saudi Journal of Gastroenterology*, 20(3). Available at: https://journals.lww.com/sjga/fulltext/2014/20030/expression_of_hippo_pathway_in_colorectal_cancer.8.aspx.
- Lin, G., Xu, N. and Xi, R. (2008) 'Paracrine Wingless signalling controls self-renewal of Drosophila intestinal stem cells', *Nature*, 455(7216), pp. 1119–1123. Available at: <https://doi.org/10.1038/nature07329>.

Lindsley, D.L. and Grell, E.H. (1967) *Genetic Variations of Drosophila Melanogaster*. Carnegie Institution of Washington. Available at: <https://books.google.co.uk/books?id=LWzwAAAAMAAJ>.

Lisse, I.M. *et al.* (1997) 'T-lymphocyte subsets in West African children: Impact of age, sex, and season', *The Journal of Pediatrics*, 130(1), pp. 77–85. Available at: [https://doi.org/10.1016/S0022-3476\(97\)70313-5](https://doi.org/10.1016/S0022-3476(97)70313-5).

Liu, Q., Liu, X. and Song, G. (2021) 'The Hippo Pathway: A Master Regulatory Network Important in Cancer', *Cells*, 10(6). Available at: <https://doi.org/10.3390/cells10061416>.

Losick, V.P., Fox, D.T. and Spradling, A.C. (2013) 'Polyploidization and Cell Fusion Contribute to Wound Healing in the Adult Drosophila Epithelium', *Current Biology*, 23(22), pp. 2224–2232. Available at: <https://doi.org/10.1016/j.cub.2013.09.029>.

Love, M.I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology*, 15(12), p. 550. Available at: <https://doi.org/10.1186/s13059-014-0550-8>.

Loza-Coll, M.A. *et al.* (2014) 'Regulation of Drosophila intestinal stem cell maintenance and differentiation by the transcription factor Escargot', *The EMBO Journal*, 33(24), pp. 2983–2996. Available at: <https://doi.org/10.15252/embj.201489050>.

Loza-Coll, M.A. *et al.* (2019) 'Heterochromatin Protein 1 (HP1) inhibits stem cell proliferation induced by ectopic activation of the Jak/STAT pathway in the Drosophila testis', *Experimental Cell Research*, 377(1), pp. 1–9. Available at: <https://doi.org/10.1016/j.yexcr.2019.02.024>.

Lucchesi, J.C. and Kuroda, M.I. (2015) 'Dosage Compensation in Drosophila', *Cold Spring Harbor Perspectives in Biology*, 7(5). Available at: <https://doi.org/10.1101/cshperspect.a019398>.

Lund, L.R. *et al.* (1996) 'Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways', *Development*, 122(1), pp. 181–193. Available at: <https://doi.org/10.1242/dev.122.1.181>.

Ma, Q., Wawersik, M. and Matunis, E.L. (2014) 'The Jak-STAT Target Chinmo Prevents Sex Transformation of Adult Stem Cells in the Drosophila Testis Niche', *Developmental Cell*, 31(4), pp. 474–486. Available at: <https://doi.org/10.1016/j.devcel.2014.10.004>.

MacDonald, B.T., Tamai, K. and He, X. (2009) 'Wnt/ β -Catenin Signaling: Components, Mechanisms, and Diseases', *Developmental Cell*, 17(1), pp. 9–26. Available at: <https://doi.org/10.1016/j.devcel.2009.06.016>.

MacDougall, N. *et al.* (2001) 'Merlin, the Drosophila homologue of neurofibromatosis-2, is specifically required in posterior follicle cells for axis formation in the oocyte', *Development*, 128(5), pp. 665–673. Available at: <https://doi.org/10.1242/dev.128.5.665>.

MacLaughlan, S.D. *et al.* (2007) 'Endometrial Expression of Cyr61: A Marker of Estrogenic Activity in Normal and Abnormal Endometrium', *Obstetrics & Gynecology*, 110(1). Available at: [10.1097/01.AOG.0000269047.46078.28](https://doi.org/10.1097/01.AOG.0000269047.46078.28).

MacMahon, B. *et al.* (1970) 'Age at first birth and breast cancer risk*', *Bulletin of the World Health Organization*, 43(2), pp. 209–221.

Main, C. *et al.* (2022) 'Understanding How Pregnancy Protects Against Ovarian and Endometrial Cancer Development: Fetal Antigens May Be Involved', *Endocrinology*, 163(11), p. bqac141. Available at: <https://doi.org/10.1210/endocr/bqac141>.

Mansilla, A. *et al.* (2016) 'Ligand-independent requirements of steroid receptors EcR and USP for cell survival', *Cell Death & Differentiation*, 23(3), pp. 405–416. Available at: <https://doi.org/10.1038/cdd.2015.108>.

Marianes, A. and Spradling, A.C. (2013) 'Physiological and stem cell compartmentalization within the Drosophila midgut', *eLife*. Edited by A. Brand, 2, p. e00886. Available at: <https://doi.org/10.7554/eLife.00886>.

Martorell, Ò. *et al.* (2014) 'Conserved Mechanisms of Tumorigenesis in the Drosophila Adult Midgut', *PLOS ONE*, 9(2), p. e88413. Available at: <https://doi.org/10.1371/journal.pone.0088413>.

Masuyama, H. *et al.* (2012) 'Dmrt1 mutation causes a male-to-female sex reversal after the sex determination by Dmy in the medaka', *Chromosome Research*, 20(1), pp. 163–176. Available at: <https://doi.org/10.1007/s10577-011-9264-x>.

Matson, C.K. *et al.* (2011) 'DMRT1 prevents female reprogramming in the postnatal mammalian testis', *Nature*, 476(7358), pp. 101–104. Available at: <https://doi.org/10.1038/nature10239>.

Mauvais-Jarvis, F. (2015) 'Sex differences in metabolic homeostasis, diabetes, and obesity', *Biology of Sex Differences*, 6(1), p. 14. Available at: <https://doi.org/10.1186/s13293-015-0033-y>.

McCormick, H. *et al.* (2017) 'Isogenic mice exhibit sexually-dimorphic DNA methylation patterns across multiple tissues', *BMC Genomics*, 18(1), p. 966. Available at: <https://doi.org/10.1186/s12864-017-4350-x>.

McCullough, E.L. *et al.* (2022) 'The life history of Drosophila sperm involves molecular continuity between male and female reproductive tracts', *Proceedings of the National Academy of Sciences*, 119(11), p. e2119899119. Available at: <https://doi.org/10.1073/pnas.2119899119>.

McGuire, S.E., Mao, Z. and Davis, R.L. (2004) 'Spatiotemporal Gene Expression Targeting with the TARGET and Gene-Switch Systems in Drosophila', *Science's STKE*, 2004(220), pp. pl6–pl6. Available at: <https://doi.org/10.1126/stke.2202004pl6>.

McKeown, M., Belote, J.M. and Baker, B.S. (1987) 'A molecular analysis of transformer, a gene in drosophila melanogaster that controls female sexual differentiation', *Cell*, 48(3), pp. 489–499. Available at: [https://doi.org/10.1016/0092-8674\(87\)90199-1](https://doi.org/10.1016/0092-8674(87)90199-1).

McKeown, M., Belote, J.M. and Boggs, R.T. (1988) 'Ectopic expression of the female transformer gene product leads to female differentiation of chromosomally male drosophila', *Cell*, 53(6), pp. 887–895. Available at: [https://doi.org/10.1016/S0092-8674\(88\)90369-8](https://doi.org/10.1016/S0092-8674(88)90369-8).

Meng, Z., Moroishi, T. and Guan, K.-L. (2016) 'Mechanisms of Hippo pathway regulation', *Genes & Development*, 30(1), pp. 1–17. Available at: <https://doi.org/10.1101/gad.274027.115>.

Micchelli, C.A. and Perrimon, N. (2006) 'Evidence that stem cells reside in the adult Drosophila midgut epithelium', *Nature*, 439(7075), pp. 475–479. Available at: <https://doi.org/10.1038/nature04371>.

Miguel-Aliaga, I., Jasper, H. and Lemaitre, B. (2018) 'Anatomy and Physiology of the Digestive Tract of Drosophila melanogaster', *Genetics*, 210(2), pp. 357–396. Available at: <https://doi.org/10.1534/genetics.118.300224>.

Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) 'A simple salting out procedure for extracting DNA from human nucleated cells', *Nucleic Acids Research*, 16(3), pp. 1215–1215. Available at: <https://doi.org/10.1093/nar/16.3.1215>.

Moeller, M.E. *et al.* (2017) 'Warts Signaling Controls Organ and Body Growth through Regulation of Ecdysone', *Current Biology*, 27(11), pp. 1652–1659.e4. Available at: <https://doi.org/10.1016/j.cub.2017.04.048>.

Mohr, O.L. (1919) 'CHARACTER CHANGES CAUSED BY MUTATION OF AN ENTIRE REGION OF A CHROMOSOME IN DROSOPHILA', *Genetics*, 4(3), pp. 275–282. Available at: <https://doi.org/10.1093/genetics/4.3.275>.

Molina, J.R. and Adjei, A.A. (2006) 'The Ras/Raf/MAPK Pathway', *Journal of Thoracic Oncology*, 1(1), pp. 7–9. Available at: [https://doi.org/10.1016/S1556-0864\(15\)31506-9](https://doi.org/10.1016/S1556-0864(15)31506-9).

Molnar, C. *et al.* (2019) 'The histone code reader PHD finger protein 7 controls sex-linked disparities in gene expression and malignancy in Drosophila', *Science Advances*, 5(8), p. eaaw7965. Available at: <https://doi.org/10.1126/sciadv.aaw7965>.

Moon, S. *et al.* (2022) 'Estrogen Regulates the Expression and Localization of YAP in the Uterus of Mice', *International Journal of Molecular Sciences*, 23(17). Available at: <https://doi.org/10.3390/ijms23179772>.

Mor, G. and Cardenas, I. (2010) 'REVIEW ARTICLE: The Immune System in Pregnancy: A Unique Complexity', *American Journal of Reproductive Immunology*, 63(6), pp. 425–433. Available at: <https://doi.org/10.1111/j.1600-0897.2010.00836.x>.

Moshitzky, P. *et al.* (1996) 'Sex-peptide activates juvenile hormone biosynthesis in the Drosophila melanogaster corpus allatum', *Archives of Insect Biochemistry and Physiology*, 32(3–4), pp. 363–374. Available at: [https://doi.org/10.1002/\(SICI\)1520-6327\(1996\)32:3/4<363::AID-ARCH9>3.0.CO;2-T](https://doi.org/10.1002/(SICI)1520-6327(1996)32:3/4<363::AID-ARCH9>3.0.CO;2-T).

- Moss, R.M. *et al.* (2023) 'Sex differences in methylation profiles are apparent in medulloblastoma, particularly among SHH tumors', *Frontiers in Oncology*, 13. Available at: <https://www.frontiersin.org/articles/10.3389/fonc.2023.1113121>.
- Most, J. *et al.* (2019) 'Energy Intake Requirements in Pregnancy', *Nutrients*, 11(8). Available at: <https://doi.org/10.3390/nu11081812>.
- Murakami, R. *et al.* (1994) 'Novel tissue units of regional differentiation in the gut epithelium of *Drosophila*, as revealed by P-element-mediated detection of enhancer', *Roux's archives of developmental biology*, 203(5), pp. 243–249. Available at: <https://doi.org/10.1007/BF00360519>.
- Nagai, H. *et al.* (2023) 'Nutrient-driven dedifferentiation of enteroendocrine cells promotes adaptive intestinal growth in *Drosophila*', *Developmental Cell*, 58(18), pp. 1764–1781.e10. Available at: <https://doi.org/10.1016/j.devcel.2023.08.022>.
- Nagoshi, R.N. *et al.* (1988) 'The control of alternative splicing at genes regulating sexual differentiation in *D. melanogaster*', *Cell*, 53(2), pp. 229–236. Available at: [https://doi.org/10.1016/0092-8674\(88\)90384-4](https://doi.org/10.1016/0092-8674(88)90384-4).
- Neumüller, R.A. *et al.* (2012) 'Stringent Analysis of Gene Function and Protein–Protein Interactions Using Fluorescently Tagged Genes', *Genetics*, 190(3), pp. 931–940. Available at: <https://doi.org/10.1534/genetics.111.136465>.
- Newcomb, P.A., Pocobelli, G. and Chia, V. (2008) 'Why hormones protect against large bowel cancer: old ideas, new evidence', *Hormonal carcinogenesis V*, pp. 259–269.
- Ngo, S. *et al.* (2020) 'Disruption of EGF Feedback by Intestinal Tumors and Neighboring Cells in *Drosophila*', *Current Biology*, 30(8), pp. 1537–1546.e3. Available at: <https://doi.org/10.1016/j.cub.2020.01.082>.
- Nieto, M.A. (2002) 'The snail superfamily of zinc-finger transcription factors', *Nature reviews Molecular cell biology*, 3(3), pp. 155–166.
- Nolo, R. *et al.* (2006) 'The bantam MicroRNA Is a Target of the Hippo Tumor-Suppressor Pathway', *Current Biology*, 16(19), pp. 1895–1904. Available at: <https://doi.org/10.1016/j.cub.2006.08.057>.
- O'Brien, J. *et al.* (2010) 'Alternatively Activated Macrophages and Collagen Remodeling Characterize the Postpartum Involuting Mammary Gland across Species', *The American Journal of Pathology*, 176(3), pp. 1241–1255. Available at: <https://doi.org/10.2353/ajpath.2010.090735>.
- O'Brien, L.E. *et al.* (2011) 'Altered Modes of Stem Cell Division Drive Adaptive Intestinal Growth', *Cell*, 147(3), pp. 603–614. Available at: <https://doi.org/10.1016/j.cell.2011.08.048>.
- Oh, H. and Irvine, K.D. (2009) 'In vivo analysis of Yorkie phosphorylation sites', *Oncogene*, 28(17), pp. 1916–1927. Available at: <https://doi.org/10.1038/onc.2009.43>.

- Ohlstein, B. and Spradling, A. (2006) 'The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells', *Nature*, 439(7075), pp. 470–474. Available at: <https://doi.org/10.1038/nature04333>.
- Ohlstein, B. and Spradling, A. (2007) 'Multipotent *Drosophila* Intestinal Stem Cells Specify Daughter Cell Fates by Differential Notch Signaling', *Science*, 315(5814), pp. 988–992. Available at: <https://doi.org/10.1126/science.1136606>.
- Ohsawa, S. *et al.* (2012) 'Mitochondrial defect drives non-autonomous tumour progression through Hippo signalling in *Drosophila*', *Nature*, 490(7421), pp. 547–551.
- Okumura, T. *et al.* (2016) 'GATAe regulates intestinal stem cell maintenance and differentiation in *Drosophila* adult midgut', *Developmental Biology*, 410(1), pp. 24–35. Available at: <https://doi.org/10.1016/j.ydbio.2015.12.017>.
- Olson, S.H. *et al.* (1998) 'Relation of Time since Last Birth and Parity to Survival of Young Women with Breast Cancer', *Epidemiology*, 9(6). Available at: https://journals.lww.com/epidem/fulltext/1998/11000/relation_of_time_since_last_birth_and_parity_to.19.aspx.
- Onland-Moret, N.C. *et al.* (2003) 'Urinary endogenous sex hormone levels and the risk of postmenopausal breast cancer', *British Journal of Cancer*, 88(9), pp. 1394–1399. Available at: <https://doi.org/10.1038/sj.bjc.6600890>.
- Onuma, T. *et al.* (2023) 'Recognition of commensal bacterial peptidoglycans defines *Drosophila* gut homeostasis and lifespan', *PLOS Genetics*, 19(4), p. e1010709. Available at: <https://doi.org/10.1371/journal.pgen.1010709>.
- Ostroverkhova, D., Przytycka, T.M. and Panchenko, A.R. (2023) 'Cancer driver mutations: predictions and reality', *Trends in Molecular Medicine*, 29(7), pp. 554–566. Available at: <https://doi.org/10.1016/j.molmed.2023.03.007>.
- Page, P. *et al.* (2016) 'National Diet and Nutrition Survey results from years 5 and 6 (combined) of the Rolling Programme (2012/2013–2013/2014)'.
- Palmer, A.K. and Jensen, M.D. (2022) 'Metabolic changes in aging humans: current evidence and therapeutic strategies', *The Journal of Clinical Investigation*, 132(16). Available at: <https://doi.org/10.1172/JCI158451>.
- Palmer, J.R. *et al.* (2014) 'Parity, Lactation, and Breast Cancer Subtypes in African American Women: Results from the AMBER Consortium', *JNCI: Journal of the National Cancer Institute*, 106(10), p. dju237. Available at: <https://doi.org/10.1093/jnci/dju237>.
- Pan, D. (2010) 'The Hippo Signaling Pathway in Development and Cancer', *Developmental Cell*, 19(4), pp. 491–505. Available at: <https://doi.org/10.1016/j.devcel.2010.09.011>.
- Panning, B. (2008) 'X-chromosome inactivation: the molecular basis of silencing', *Journal of Biology*, 7(8), p. 30. Available at: <https://doi.org/10.1186/jbiol95>.

- Parisi, E. *et al.* (2023) 'PLA2G12A as a Novel Biomarker for Colorectal Cancer with Prognostic Relevance', *International Journal of Molecular Sciences*, 24(13). Available at: <https://doi.org/10.3390/ijms241310889>.
- Patel, P.H., Dutta, D. and Edgar, B.A. (2015) 'Niche appropriation by *Drosophila* intestinal stem cell tumours', *Nature Cell Biology*, 17(9), pp. 1182–1192. Available at: <https://doi.org/10.1038/ncb3214>.
- Paulson, J.R. and Taylor, S.S. (1982) 'Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes.', *Journal of Biological Chemistry*, 257(11), pp. 6064–6072. Available at: [https://doi.org/10.1016/S0021-9258\(20\)65105-5](https://doi.org/10.1016/S0021-9258(20)65105-5).
- Pavlou, H.J. *et al.* (2016) 'Neural circuitry coordinating male copulation', *eLife*. Edited by M. Ramaswami, 5, p. e20713. Available at: <https://doi.org/10.7554/eLife.20713>.
- Pearce, C.L. *et al.* (2013) 'Combined and Interactive Effects of Environmental and GWAS-Identified Risk Factors in Ovarian Cancer', *Cancer Epidemiology, Biomarkers & Prevention*, 22(5), pp. 880–890. Available at: <https://doi.org/10.1158/1055-9965.EPI-12-1030-T>.
- Perochon, J. *et al.* (2021) 'Dynamic adult tracheal plasticity drives stem cell adaptation to changes in intestinal homeostasis in *Drosophila*', *Nature Cell Biology*, 23(5), pp. 485–496. Available at: <https://doi.org/10.1038/s41556-021-00676-z>.
- Petsakou, A. *et al.* (2023) 'Cholinergic neurons trigger epithelial Ca²⁺ currents to heal the gut', *Nature* [Preprint]. Available at: <https://doi.org/10.1038/s41586-023-06627-y>.
- Phillips, K.-A. *et al.* (2004) 'Prognosis of Premenopausal Breast Cancer and Childbirth Prior to Diagnosis', *Journal of Clinical Oncology*, 22(4), pp. 699–705. Available at: <https://doi.org/10.1200/JCO.2004.07.062>.
- Polesello, C. and Tapon, N. (2007) 'Salvador-warts-hippo signaling promotes *Drosophila* posterior follicle cell maturation downstream of notch', *Current Biology*, 17(21), pp. 1864–1870.
- Pranoto, I.K.A., Lee, J. and Kwon, Y.V. (2023) 'The roles of the native cell differentiation program aberrantly recapitulated in *Drosophila* intestinal tumors', *Cell Reports*, 42(10), p. 113245. Available at: <https://doi.org/10.1016/j.celrep.2023.113245>.
- Prior, I.A., Lewis, P.D. and Mattos, C. (2012) 'A Comprehensive Survey of Ras Mutations in Cancer', *Cancer Research*, 72(10), pp. 2457–2467. Available at: <https://doi.org/10.1158/0008-5472.CAN-11-2612>.
- Quan, Z. *et al.* (2013) 'TSC1/2 regulates intestinal stem cell maintenance and lineage differentiation through Rheb–TORC1–S6K but independently of nutritional status or Notch regulation', *Journal of Cell Science*, 126(17), pp. 3884–3892. Available at: <https://doi.org/10.1242/jcs.125294>.

R Core Team (2021) 'R: A Language and Environment for Statistical Computing'. Vienna, Austria: R Foundation for Statistical Computing. Available at: <https://www.R-project.org>.

Rahman, M.M. *et al.* (2017) 'Local Juvenile Hormone activity regulates gut homeostasis and tumor growth in adult *Drosophila*', *Scientific Reports*, 7(1), p. 11677. Available at: <https://doi.org/10.1038/s41598-017-11199-9>.

Ray, P.F. *et al.* (1995) 'Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization', *Reproduction*, 104(1), pp. 165–171. Available at: <https://doi.org/10.1530/jrf.0.1040165>.

Read, R.D., Bach, E.A. and Cagan, R.L. (2004) 'Drosophila C-terminal Src kinase negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun N-terminal kinase, and STAT pathways', *Molecular and Cellular Biology*, 24(15), pp. 6676–6689.

Reczek, C.R. and Chandel, N.S. (2017) 'The Two Faces of Reactive Oxygen Species in Cancer', *Annual Review of Cancer Biology*, 1(1), pp. 79–98. Available at: <https://doi.org/10.1146/annurev-cancerbio-041916-065808>.

Reddy, B., Rauskolb, C. and Irvine, K.D. (2010) 'Influence of fat-hippo and notch signaling on the proliferation and differentiation of *Drosophila* optic neuroepithelia', *Development*, 137(14), pp. 2397–2408.

Redhai, S. *et al.* (2020) 'An intestinal zinc sensor regulates food intake and developmental growth', *Nature*, 580(7802), pp. 263–268. Available at: <https://doi.org/10.1038/s41586-020-2111-5>.

Regan, J.C. *et al.* (2016) 'Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction', *eLife*. Edited by A. Dillin, 5, p. e10956. Available at: <https://doi.org/10.7554/eLife.10956>.

Regan, J.C. *et al.* (2022) 'Sexual identity of enterocytes regulates autophagy to determine intestinal health, lifespan and responses to rapamycin', *Nature Aging*, 2(12), pp. 1145–1158. Available at: <https://doi.org/10.1038/s43587-022-00308-7>.

Reiff, T. *et al.* (2015) 'Endocrine remodelling of the adult intestine sustains reproduction in *Drosophila*', *eLife*. Edited by M. Freeman, 4, p. e06930. Available at: <https://doi.org/10.7554/eLife.06930>.

Reiter, L.T. *et al.* (2001) 'A Systematic Analysis of Human Disease-Associated Gene Sequences In *Drosophila melanogaster*', *Genome Research*, 11(6), pp. 1114–1125. Available at: <https://doi.org/10.1101/gr.169101>.

Reizel, Y. *et al.* (2015) 'Gender-specific postnatal demethylation and establishment of epigenetic memory', *Genes & Development*, 29(9), pp. 923–933.

Ren, F. *et al.* (2010) 'Hippo signaling regulates *Drosophila* intestine stem cell proliferation through multiple pathways', *Proceedings of the National Academy of Sciences*, 107(49), pp. 21064–21069. Available at: <https://doi.org/10.1073/pnas.1012759107>.

Renault, C.H. *et al.* (2020) 'Minipuberty of human infancy – A window of opportunity to evaluate hypogonadism and differences of sex development?', *Ann Pediatr Endocrinol Metab*, 25(2), pp. 84–91. Available at: <https://doi.org/10.6065/apem.2040094.047>.

Ribalta, T. *et al.* (2004) 'The Mitosis-Specific Antibody Anti-Phosphohistone-H3 (PHH3) Facilitates Rapid Reliable Grading of Meningiomas According to WHO 2000 Criteria', *The American Journal of Surgical Pathology*, 28(11). Available at: https://journals.lww.com/ajsp/fulltext/2004/11000/the_mitosis_specific_antibody.16.aspx.

Ribeiro, C. and Dickson, B.J. (2010) 'Sex Peptide Receptor and Neuronal TOR/S6K Signaling Modulate Nutrient Balancing in *Drosophila*', *Current Biology*, 20(11), pp. 1000–1005. Available at: <https://doi.org/10.1016/j.cub.2010.03.061>.

Rideout, E.J. *et al.* (2010) 'Control of sexual differentiation and behavior by the doublesex gene in *Drosophila melanogaster*', *Nature Neuroscience*, 13(4), pp. 458–466. Available at: <https://doi.org/10.1038/nn.2515>.

Rideout, E.J., Narsaiya, M.S. and Grewal, S.S. (2016) 'The Sex Determination Gene transformer Regulates Male-Female Differences in *Drosophila* Body Size', *PLOS Genetics*, 11(12), p. e1005683. Available at: <https://doi.org/10.1371/journal.pgen.1005683>.

Rivera-Gonzalez, R. *et al.* (1998) 'Estrogen-induced genes in the uterus of ovariectomized rats and their regulation by droloxifene and tamoxifen', *The Journal of Steroid Biochemistry and Molecular Biology*, 64(1), pp. 13–24. Available at: [https://doi.org/10.1016/S0960-0760\(97\)00142-8](https://doi.org/10.1016/S0960-0760(97)00142-8).

Rojas Villa, S.E., Meng, F.W. and Biteau, B. (2019) 'zfh2 controls progenitor cell activation and differentiation in the adult *Drosophila* intestinal absorptive lineage', *PLOS Genetics*, 15(12), p. e1008553. Available at: <https://doi.org/10.1371/journal.pgen.1008553>.

Ross, J. *et al.* (2003) 'Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships', *Gene*, 304, pp. 117–131. Available at: [https://doi.org/10.1016/S0378-1119\(02\)01187-3](https://doi.org/10.1016/S0378-1119(02)01187-3).

Rubin, G.M. and Spradling, A.C. (1982) 'Genetic Transformation of *Drosophila* with Transposable Element Vectors', *Science*, 218(4570), pp. 348–353. Available at: <https://doi.org/10.1126/science.6289436>.

Ryner, L.C. *et al.* (1996) 'Control of Male Sexual Behavior and Sexual Orientation in *Drosophila* by the fruitless Gene', *Cell*, 87(6), pp. 1079–1089. Available at: [https://doi.org/10.1016/S0092-8674\(00\)81802-4](https://doi.org/10.1016/S0092-8674(00)81802-4).

Rynes, J. *et al.* (2012) 'Activating Transcription Factor 3 Regulates Immune and Metabolic Homeostasis', *Molecular and Cellular Biology*, 32(19), pp. 3949–3962. Available at: <https://doi.org/10.1128/MCB.00429-12>.

- Salk, J.J., Fox, E.J. and Loeb, L.A. (2010) 'Mutational Heterogeneity in Human Cancers: Origin and Consequences', *Annual Review of Pathology: Mechanisms of Disease*, 5(1), pp. 51–75. Available at: <https://doi.org/10.1146/annurev-pathol-121808-102113>.
- Salz, H. and Erickson, J.W. (2010) 'Sex determination in *Drosophila*: The view from the top', *Fly*, 4(1), pp. 60–70. Available at: <https://doi.org/10.4161/fly.4.1.11277>.
- Sampath, D., Winneker, R.C. and Zhang, Z. (2001) 'Cyr61, a Member of the CCN Family, Is Required for MCF-7 Cell Proliferation: Regulation by 17 β -Estradiol and Overexpression in Human Breast Cancer', *Endocrinology*, 142(6), pp. 2540–2548. Available at: <https://doi.org/10.1210/endo.142.6.8186>.
- Sánchez, L., Granadino, B. and Torres, M. (1994) 'Sex determination in *Drosophila melanogaster*: X-linked genes involved in the initial step of Sex-lethal activation', *Developmental Genetics*, 15(3), pp. 251–264. Available at: <https://doi.org/10.1002/dvg.1020150307>.
- Sanghavi, M. and Rutherford, J.D. (2014) 'Cardiovascular Physiology of Pregnancy', *Circulation*, 130(12), pp. 1003–1008. Available at: <https://doi.org/10.1161/CIRCULATIONAHA.114.009029>.
- Sawicka, A. and Seiser, C. (2012) 'Histone H3 phosphorylation – A versatile chromatin modification for different occasions', *Special Section on Epigenetics*, 94(11), pp. 2193–2201. Available at: <https://doi.org/10.1016/j.biochi.2012.04.018>.
- Sawyer, J.K., Cohen, E. and Fox, D.T. (2017) 'Interorgan regulation of *Drosophila* intestinal stem cell proliferation by a hybrid organ boundary zone', *Development*, 144(22), pp. 4091–4102. Available at: <https://doi.org/10.1242/dev.153114>.
- Schindelin, J. *et al.* (2012) 'Fiji: an open-source platform for biological-image analysis', *Nature Methods*, 9(7), pp. 676–682. Available at: <https://doi.org/10.1038/nmeth.2019>.
- Schlegelmilch, K. *et al.* (2011) 'Yap1 Acts Downstream of α -Catenin to Control Epidermal Proliferation', *Cell*, 144(5), pp. 782–795. Available at: <https://doi.org/10.1016/j.cell.2011.02.031>.
- Schmahl, J. *et al.* (2000) 'Sry induces cell proliferation in the mouse gonad', *Development*, 127(1), pp. 65–73.
- Schütze, M. *et al.* (2011) 'Alcohol attributable burden of incidence of cancer in eight European countries based on results from prospective cohort study', *Bmj*, 342.
- Schwenke, R.A. and Lazzaro, B.P. (2017) 'Juvenile Hormone Suppresses Resistance to Infection in Mated Female *Drosophila melanogaster*', *Current Biology*, 27(4), pp. 596–601. Available at: <https://doi.org/10.1016/j.cub.2017.01.004>.
- Scopelliti, A. *et al.* (2019) 'A Neuronal Relay Mediates a Nutrient Responsive Gut/Fat Body Axis Regulating Energy Homeostasis in Adult *Drosophila*', *Cell Metabolism*, 29(2), pp. 269–284.e10. Available at: <https://doi.org/10.1016/j.cmet.2018.09.021>.

- Sefton, L. *et al.* (2000) 'An extracellular activator of the *Drosophila* JAK/STAT pathway is a sex-determination signal element', *Nature*, 405(6789), pp. 970–973. Available at: <https://doi.org/10.1038/35016119>.
- Sênos Demarco, R. *et al.* (2022) 'Escargot controls somatic stem cell maintenance through the attenuation of the insulin receptor pathway in *Drosophila*', *Cell Reports*, 39(3). Available at: <https://doi.org/10.1016/j.celrep.2022.110679>.
- Sharma, R.P. and Chopra, V.L. (1976) 'Effect of the wingless (*wg1*) mutation on wing and haltere development in *Drosophila melanogaster*', *Developmental Biology*, 48(2), pp. 461–465. Available at: [https://doi.org/10.1016/0012-1606\(76\)90108-1](https://doi.org/10.1016/0012-1606(76)90108-1).
- Shaw, R.L. *et al.* (2010) 'The Hippo pathway regulates intestinal stem cell proliferation during *Drosophila* adult midgut regeneration', *Development*, 137(24), pp. 4147–4158. Available at: <https://doi.org/10.1242/dev.052506>.
- Shields, L.B.E. *et al.* (1997) 'Induction of Immune Responses to Ovarian Tumor Antigens by Multiparity', *The Journal of the Society for Gynecologic Investigation: JSGI*, 4(6), pp. 298–304. Available at: <https://doi.org/10.1177/107155769700400606>.
- Shingleton, A.W. and Veal, I.M. (2023) 'Sex-specific regulation of development, growth and metabolism', *Special Issue: Neural Crest Formation by Tatjana Sauka-Spengler / Special Issue: Metabolism in time and space by Jason M. Tennessen and Elizabeth J. Rideout*, 138, pp. 117–127. Available at: <https://doi.org/10.1016/j.semcd.2022.04.017>.
- Shome, D. *et al.* (2020) 'The HIPPO Transducer YAP and Its Targets CTGF and Cyr61 Drive a Paracrine Signalling in Cold Atmospheric Plasma-Mediated Wound Healing', *Oxidative Medicine and Cellular Longevity*. Edited by R. Matsui, 2020, p. 4910280. Available at: <https://doi.org/10.1155/2020/4910280>.
- Short, S.M. and Lazzaro, B.P. (2010) 'Female and male genetic contributions to post-mating immune defence in female *Drosophila melanogaster*', *Proceedings of the Royal Society B: Biological Sciences*, 277(1700), pp. 3649–3657. Available at: <https://doi.org/10.1098/rspb.2010.0937>.
- Short, S.M., Wolfner, M.F. and Lazzaro, B.P. (2012) 'Female *Drosophila melanogaster* suffer reduced defense against infection due to seminal fluid components', *Journal of Insect Physiology*, 58(9), pp. 1192–1201. Available at: <https://doi.org/10.1016/j.jinsphys.2012.06.002>.
- Sieber, M.H. and Spradling, A.C. (2015) 'Steroid Signaling Establishes a Female Metabolic State and Regulates SREBP to Control Oocyte Lipid Accumulation', *Current Biology*, 25(8), pp. 993–1004. Available at: <https://doi.org/10.1016/j.cub.2015.02.019>.
- Simon, M.S. *et al.* (2012) 'Estrogen Plus Progestin and Colorectal Cancer Incidence and Mortality', *Journal of Clinical Oncology*, 30(32), pp. 3983–3990. Available at: <https://doi.org/10.1200/JCO.2012.42.7732>.

Sinclair, A.H. *et al.* (1990) 'A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif', *Nature*, 346(6281), pp. 240–244. Available at: <https://doi.org/10.1038/346240a0>.

Sleiman, M.S.B., Schüpfer, F. and Lemaitre, B. (2014) 'Transforming growth factor β /activin signaling functions as a sugar-sensing feedback loop to regulate digestive enzyme expression', *Cell reports*, 9(1), pp. 336–348.

Slepicka, P.F., Cyrill, S.L. and dos Santos, C.O. (2019) 'Pregnancy and Breast Cancer: Pathways to Understand Risk and Prevention', *Trends in Molecular Medicine*, 25(10), pp. 866–881. Available at: <https://doi.org/10.1016/j.molmed.2019.06.003>.

Smendziuk, C.M. *et al.* (2015) 'Bi-directional gap junction-mediated soma-germline communication is essential for spermatogenesis', *Development*, 142(15), pp. 2598–2609. Available at: <https://doi.org/10.1242/dev.123448>.

Song, W. *et al.* (2019) 'Tumor-Derived Ligands Trigger Tumor Growth and Host Wasting via Differential MEK Activation', *Developmental Cell*, 48(2), pp. 277–286.e6. Available at: <https://doi.org/10.1016/j.devcel.2018.12.003>.

Song, W., Veenstra, J.A. and Perrimon, N. (2014) 'Control of lipid metabolism by tachykinin in *Drosophila*', *Cell reports*, 9(1), pp. 40–47.

Sonoshita, M. and Cagan, R.L. (2017) 'Modeling human cancers in *Drosophila*', *Current topics in developmental biology*, 121, pp. 287–309.

Speakman, J.R. (2007) 'The physiological costs of reproduction in small mammals', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1490), pp. 375–398. Available at: <https://doi.org/10.1098/rstb.2007.2145>.

Sponagel, J. *et al.* (2022) 'Sex differences in brain tumor glutamine metabolism reveal sex-specific vulnerabilities to treatment', *Med*, 3(11), pp. 792–811.e12. Available at: <https://doi.org/10.1016/j.medj.2022.08.005>.

Spradling, A. *et al.* (2008) 'Stem cells and their niches: integrated units that maintain *Drosophila* tissues', in: *Cold Spring Harbor symposia on quantitative biology*, Cold Spring Harbor Laboratory Press, pp. 49–57.

Spradling, A.C. and Rubin, G.M. (1982) 'Transposition of cloned P elements into *Drosophila* germ line chromosomes', *Science*, 218(4570), pp. 341–347.

Staley, B.K. and Irvine, K.D. (2010) 'Warts and Yorkie Mediate Intestinal Regeneration by Influencing Stem Cell Proliferation', *Current Biology*, 20(17), pp. 1580–1587. Available at: <https://doi.org/10.1016/j.cub.2010.07.041>.

Stark, M.B. (1918) 'An Hereditary Tumor in the Fruit Fly, *Drosophila*', *The Journal of Cancer Research*, 3(3), pp. 279–301. Available at: <https://doi.org/10.1158/jcr.1918.279>.

Stark, M.B. (1919) 'A Benign Tumor that is Hereditary in *Drosophila*', *Proceedings of the National Academy of Sciences*, 5(12), pp. 573–580. Available at: <https://doi.org/10.1073/pnas.5.12.573>.

Steinhardt, A.A. *et al.* (2008) 'Expression of Yes-associated protein in common solid tumors', *Human Pathology*, 39(11), pp. 1582–1589. Available at: <https://doi.org/10.1016/j.humpath.2008.04.012>.

Stockinger, P. *et al.* (2005) 'Neural Circuitry that Governs *Drosophila* Male Courtship Behavior', *Cell*, 121(5), pp. 795–807. Available at: <https://doi.org/10.1016/j.cell.2005.04.026>.

Stoffolano, J.G. and Haselton, A.T. (2013) 'The Adult Dipteran Crop: A Unique and Overlooked Organ', *Annual Review of Entomology*, 58(1), pp. 205–225. Available at: <https://doi.org/10.1146/annurev-ento-120811-153653>.

Strand, M. and Micchelli, C.A. (2011) 'Quiescent gastric stem cells maintain the adult *Drosophila* stomach', *Proceedings of the National Academy of Sciences*, 108(43), pp. 17696–17701. Available at: <https://doi.org/10.1073/pnas.1109794108>.

Strange, R. *et al.* (1992) 'Apoptotic cell death and tissue remodelling during mouse mammary gland involution', *Development*, 115(1), pp. 49–58. Available at: <https://doi.org/10.1242/dev.115.1.49>.

Strilbytska, O.M. *et al.* (2020) 'Insulin Signaling in Intestinal Stem and Progenitor Cells as an Important Determinant of Physiological and Metabolic Traits in *Drosophila*', *Cells*, 9(4). Available at: <https://doi.org/10.3390/cells9040803>.

Strutt, H. and Strutt, D. (2021) 'How do the Fat–Dachsous and core planar polarity pathways act together and independently to coordinate polarized cell behaviours?', *Open Biology*, 11(2), p. 200356. Available at: <https://doi.org/10.1098/rsob.200356>.

Su, T. *et al.* (2017) 'Kibra and Merlin Activate the Hippo Pathway Spatially Distinct from and Independent of Expanded', *Developmental Cell*, 40(5), pp. 478–490.e3. Available at: <https://doi.org/10.1016/j.devcel.2017.02.004>.

Su, T.T. *et al.* (1998) 'Exit from mitosis in *Drosophila* syncytial embryos requires proteolysis and cyclin degradation, and is associated with localized dephosphorylation', *Genes & Development*, 12(10), pp. 1495–1503. Available at: <https://doi.org/10.1101/gad.12.10.1495>.

Sun, Y. *et al.* (2015) 'Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis', *Journal of Receptors and Signal Transduction*, 35(6), pp. 600–604. Available at: <https://doi.org/10.3109/10799893.2015.1030412>.

Tamamouna, V. *et al.* (2021) 'Remodelling of oxygen-transporting tracheoles drives intestinal regeneration and tumorigenesis in *Drosophila*', *Nature Cell Biology*, 23(5), pp. 497–510. Available at: <https://doi.org/10.1038/s41556-021-00674-1>.

- Taneja, V. (2018) 'Sex Hormones Determine Immune Response', *Frontiers in Immunology*, 9. Available at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01931>.
- Tang, D. *et al.* (2023) 'SRplot: A free online platform for data visualization and graphing', *PLOS ONE*, 18(11), p. e0294236. Available at: <https://doi.org/10.1371/journal.pone.0294236>.
- Taylor, B.J. (1992) 'Differentiation of a male-specific muscle in *Drosophila melanogaster* does not require the sex-determining genes doublesex or intersex.', *Genetics*, 132(1), pp. 179–191. Available at: <https://doi.org/10.1093/genetics/132.1.179>.
- Tazuke, S.I. *et al.* (2002) 'A germline-specific gap junction protein required for survival of differentiating early germ cells', *Development*, 129(10), pp. 2529–2539. Available at: <https://doi.org/10.1242/dev.129.10.2529>.
- Thompson, B.J. and Cohen, S.M. (2006) 'The Hippo Pathway Regulates the bantam microRNA to Control Cell Proliferation and Apoptosis in *Drosophila*', *Cell*, 126(4), pp. 767–774. Available at: <https://doi.org/10.1016/j.cell.2006.07.013>.
- Tian, A. *et al.* (2022) 'Damage-induced regeneration of the intestinal stem cell pool through enteroblast mitosis in the *Drosophila* midgut', *The EMBO Journal*, 41(19), p. e110834. Available at: <https://doi.org/10.15252/embj.2022110834>.
- Troisi, R. *et al.* (2018) 'The role of pregnancy, perinatal factors and hormones in maternal cancer risk: a review of the evidence', *Journal of Internal Medicine*, 283(5), pp. 430–445. Available at: <https://doi.org/10.1111/joim.12747>.
- Truman, J.W. and Riddiford, L.M. (2019) 'The evolution of insect metamorphosis: a developmental and endocrine view', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374(1783), p. 20190070. Available at: <https://doi.org/10.1098/rstb.2019.0070>.
- Tsilidis, K.K. *et al.* (2011) 'Oral contraceptive use and reproductive factors and risk of ovarian cancer in the European Prospective Investigation into Cancer and Nutrition', *British Journal of Cancer*, 105(9), pp. 1436–1442. Available at: <https://doi.org/10.1038/bjc.2011.371>.
- Tu, C.D. and Akgül, B. (2005) 'Drosophila Glutathione S-Transferases', in H. Sies and L. Packer (eds) *Methods in Enzymology*. Academic Press, pp. 204–226. Available at: [https://doi.org/10.1016/S0076-6879\(05\)01013-X](https://doi.org/10.1016/S0076-6879(05)01013-X).
- Tzou, P. *et al.* (2000) 'Tissue-Specific Inducible Expression of Antimicrobial Peptide Genes in *Drosophila* Surface Epithelia', *Immunity*, 13(5), pp. 737–748. Available at: [https://doi.org/10.1016/S1074-7613\(00\)00072-8](https://doi.org/10.1016/S1074-7613(00)00072-8).
- Udan, R.S. *et al.* (2003) 'Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway', *Nature Cell Biology*, 5(10), pp. 914–920. Available at: <https://doi.org/10.1038/ncb1050>.

- Uhlenhaut, N.H. *et al.* (2009) 'Somatic Sex Reprogramming of Adult Ovaries to Testes by FOXL2 Ablation', *Cell*, 139(6), pp. 1130–1142. Available at: <https://doi.org/10.1016/j.cell.2009.11.021>.
- Uhlirova, M., Jasper, H. and Bohmann, D. (2005) 'Non-cell-autonomous induction of tissue overgrowth by JNK/Ras cooperation in a Drosophila tumor model', *Proceedings of the National Academy of Sciences*, 102(37), pp. 13123–13128. Available at: <https://doi.org/10.1073/pnas.0504170102>.
- Uppal, S.S., Verma, S. and Dhot, P.S. (2003) 'Normal values of CD4 and CD8 lymphocyte subsets in healthy indian adults and the effects of sex, age, ethnicity, and smoking', *Cytometry Part B: Clinical Cytometry*, 52B(1), pp. 32–36. Available at: <https://doi.org/10.1002/cyto.b.10011>.
- Valero, V., Pawlik, T.M. and Anders, R.A. (2015) 'Emerging role of Hpo signaling and YAP in hepatocellular carcinoma', *Journal of Hepatocellular Carcinoma*, 2, pp. 69–78.
- Vanderveken, M. and O'Donnell, M.J. (2014) 'Effects of diuretic hormone 31, drosokinin, and allatostatin A on transepithelial K⁺ transport and contraction frequency in the midgut and hindgut of larval Drosophila melanogaster', *Archives of insect biochemistry and physiology*, 85(2), pp. 76–93.
- Vargas, M.A. *et al.* (2010) 'A Role for S6 Kinase and Serotonin in Postmating Dietary Switch and Balance of Nutrients in D. melanogaster', *Current Biology*, 20(11), pp. 1006–1011. Available at: <https://doi.org/10.1016/j.cub.2010.04.009>.
- Vining, B. *et al.* (2021) 'Diverse Regulation but Conserved Function: SOX9 in Vertebrate Sex Determination', *Genes*, 12(4). Available at: <https://doi.org/10.3390/genes12040486>.
- de Visser, K.E. and Joyce, J.A. (2023) 'The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth', *Cancer Cell*, 41(3), pp. 374–403. Available at: <https://doi.org/10.1016/j.ccell.2023.02.016>.
- Vodovar, N. *et al.* (2005) 'Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species', *Proceedings of the National Academy of Sciences*, 102(32), pp. 11414–11419. Available at: <https://doi.org/10.1073/pnas.0502240102>.
- Voog, J. *et al.* (2014) 'Escargot Restricts Niche Cell to Stem Cell Conversion in the Drosophila Testis', *Cell Reports*, 7(3), pp. 722–734. Available at: <https://doi.org/10.1016/j.celrep.2014.04.025>.
- Waage, C.W. *et al.* (2022) 'Lipid and lipoprotein concentrations during pregnancy and associations with ethnicity', *BMC Pregnancy and Childbirth*, 22(1), p. 246. Available at: <https://doi.org/10.1186/s12884-022-04524-2>.
- Wang, C. *et al.* (2013) 'APC loss-induced intestinal tumorigenesis in Drosophila: Roles of Ras in Wnt signaling activation and tumor progression', *Developmental Biology*, 378(2), pp. 122–140. Available at: <https://doi.org/10.1016/j.ydbio.2013.03.020>.

Wang, N. *et al.* (2021) 'Integration of Transcriptome and Methylome Highlights the Roles of Cell Cycle and Hippo Signaling Pathway in Flatfish Sexual Size Dimorphism', *Frontiers in Cell and Developmental Biology*, 9. Available at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.743722>.

Wang, W., Huang, J. and Chen, J. (2011) 'Angiomotin-like proteins associate with and negatively regulate YAP1', *Journal of Biological Chemistry*, 286(6), pp. 4364–4370.

Wang, Y., Salmon, A.B. and Harshman, L.G. (2001) 'A cost of reproduction: oxidative stress susceptibility is associated with increased egg production in *Drosophila melanogaster*', *Experimental Gerontology*, 36(8), pp. 1349–1359. Available at: [https://doi.org/10.1016/S0531-5565\(01\)00095-X](https://doi.org/10.1016/S0531-5565(01)00095-X).

Wat, L.W. *et al.* (2020) 'A role for triglyceride lipase brummer in the regulation of sex differences in *Drosophila* fat storage and breakdown', *PLOS Biology*, 18(1), p. e3000595. Available at: <https://doi.org/10.1371/journal.pbio.3000595>.

Watson, ChristineJ. and Kreuzaler, PeterA. (2011) 'Remodeling mechanisms of the mammary gland during involution', *The International Journal of Developmental Biology*, 55(7–8–9), pp. 757–762. Available at: <https://doi.org/10.1387/ijdb.113414cw>.

Wei, Y. *et al.* (1999) 'Phosphorylation of Histone H3 Is Required for Proper Chromosome Condensation and Segregation', *Cell*, 97(1), pp. 99–109. Available at: [https://doi.org/10.1016/S0092-8674\(00\)80718-7](https://doi.org/10.1016/S0092-8674(00)80718-7).

Wernli, K.J. *et al.* (2009) 'The Relationship between Gravidity and Parity and Colorectal Cancer Risk', *Journal of Women's Health*, 18(7), pp. 995–1001. Available at: <https://doi.org/10.1089/jwh.2008.1068>.

White, A. *et al.* (2018) 'A review of sex-related differences in colorectal cancer incidence, screening uptake, routes to diagnosis, cancer stage and survival in the UK', *BMC Cancer*, 18(1), p. 906. Available at: <https://doi.org/10.1186/s12885-018-4786-7>.

White, M.A. *et al.* (2021) '*Drosophila melanogaster* sex peptide regulates mated female midgut morphology and physiology', *Proceedings of the National Academy of Sciences*, 118(1), p. e2018112118. Available at: <https://doi.org/10.1073/pnas.2018112118>.

White-Cooper, H. (2010) 'Molecular mechanisms of gene regulation during *Drosophila* spermatogenesis', *Reproduction*, 139(1), p. 11.

Whiteley, M. *et al.* (1992) 'The *Drosophila* gene escargot encodes a zinc finger motif found in snail-related genes', *Mechanisms of development*, 36(3), pp. 117–127.

Williams, T.J. (1986) 'A technique for sexing mouse embryos by a visual colorimetric assay of the X-linked enzyme, glucose 6-phosphate dehydrogenase', *Theriogenology*, 25(5), pp. 733–739. Available at: [https://doi.org/10.1016/0093-691X\(86\)90131-7](https://doi.org/10.1016/0093-691X(86)90131-7).

Williams, T.M. and Carroll, S.B. (2009) 'Genetic and molecular insights into the development and evolution of sexual dimorphism', *Nature Reviews Genetics*, 10(11), pp. 797–804. Available at: <https://doi.org/10.1038/nrg2687>.

Wilson, I.T. (1924) 'TWO NEW HEREDITARY TUMORS IN DROSOPHILA', *Genetics*, 9(4), pp. 343–362. Available at: <https://doi.org/10.1093/genetics/9.4.343>.

Wu, A.H. *et al.* (2017) 'Timing of births and oral contraceptive use influences ovarian cancer risk', *International Journal of Cancer*, 141(12), pp. 2392–2399. Available at: <https://doi.org/10.1002/ijc.30910>.

Wu, K. *et al.* (2021) 'Aging-related upregulation of the homeobox gene caudal represses intestinal stem cell differentiation in *Drosophila*', *PLOS Genetics*, 17(7), p. e1009649. Available at: <https://doi.org/10.1371/journal.pgen.1009649>.

Wu, S. *et al.* (2003) 'hippo Encodes a Ste-20 Family Protein Kinase that Restricts Cell Proliferation and Promotes Apoptosis in Conjunction with salvador and warts', *Cell*, 114(4), pp. 445–456. Available at: [https://doi.org/10.1016/S0092-8674\(03\)00549-X](https://doi.org/10.1016/S0092-8674(03)00549-X).

Wu, S. *et al.* (2008) 'The TEAD/TEF Family Protein Scalloped Mediates Transcriptional Output of the Hippo Growth-Regulatory Pathway', *Developmental Cell*, 14(3), pp. 388–398. Available at: <https://doi.org/10.1016/j.devcel.2008.01.007>.

Xia, Y. *et al.* (2014) 'YAP Promotes Ovarian Cancer Cell Tumorigenesis and Is Indicative of a Poor Prognosis for Ovarian Cancer Patients', *PLOS ONE*, 9(3), p. e91770. Available at: <https://doi.org/10.1371/journal.pone.0091770>.

Xiang, J. *et al.* (2017) 'EGFR-dependent TOR-independent endocycles support *Drosophila* gut epithelial regeneration', *Nature Communications*, 8(1), p. 15125. Available at: <https://doi.org/10.1038/ncomms15125>.

Xiao, Y. and Dong, J. (2021) 'The Hippo Signaling Pathway in Cancer: A Cell Cycle Perspective', *Cancers*, 13(24). Available at: <https://doi.org/10.3390/cancers13246214>.

Xu, N. *et al.* (2011) 'EGFR, Wingless and JAK/STAT signaling cooperatively maintain *Drosophila* intestinal stem cells', *Developmental Biology*, 354(1), pp. 31–43. Available at: <https://doi.org/10.1016/j.ydbio.2011.03.018>.

Xu, T. *et al.* (1995) 'Identifying tumor suppressors in genetic mosaics: the *Drosophila* lats gene encodes a putative protein kinase', *Development*, 121(4), pp. 1053–1063.

Xu, T. and Rubin, G.M. (1993) 'Analysis of genetic mosaics in developing and adult *Drosophila* tissues', *Development*, 117(4), pp. 1223–1237.

Yager, J.D. and Davidson, N.E. (2006) 'Estrogen Carcinogenesis in Breast Cancer', *New England Journal of Medicine*, 354(3), pp. 270–282. Available at: <https://doi.org/10.1056/NEJMra050776>.

- Yang, S. *et al.* (2019) 'Sugar Alcohols of Polyol Pathway Serve as Alarmins to Mediate Local-Systemic Innate Immune Communication in *Drosophila*', *Cell Host & Microbe*, 26(2), pp. 240-251.e8. Available at: <https://doi.org/10.1016/j.chom.2019.07.001>.
- Ye, B. *et al.* (2007) 'Growing Dendrites and Axons Differ in Their Reliance on the Secretory Pathway', *Cell*, 130(4), pp. 717–729. Available at: <https://doi.org/10.1016/j.cell.2007.06.032>.
- Young, M.D. *et al.* (2010) 'Gene ontology analysis for RNA-seq: accounting for selection bias', *Genome Biology*, 11(2), p. R14. Available at: <https://doi.org/10.1186/gb-2010-11-2-r14>.
- Yu, H.-H. *et al.* (2009) 'Twin-spot MARCM to reveal the developmental origin and identity of neurons', *Nature neuroscience*, 12(7), pp. 947–953.
- Yu, J. *et al.* (2008) 'The hippo pathway promotes Notch signaling in regulation of cell differentiation, proliferation, and oocyte polarity', *PLoS One*, 3(3), p. e1761.
- Yu, T., Bachman, J. and Lai, Z.-C. (2015) 'Mutation analysis of large tumor suppressor genes LATS1 and LATS2 supports a tumor suppressor role in human cancer', *Protein & Cell*, 6(1), pp. 6–11.
- Yuan, J. *et al.* (2019) 'Primary decidual zone formation requires Scribble for pregnancy success in mice', *Nature communications*, 10(1), p. 5425.
- Zeng, X. and Hou, S.X. (2015) 'Enteroendocrine cells are generated from stem cells through a distinct progenitor in the adult *Drosophila* posterior midgut', *Development*, 142(4), pp. 644–653. Available at: <https://doi.org/10.1242/dev.113357>.
- Zhang, C. *et al.* (2015) 'The Ecdysone Receptor Coactivator Taiman Links Yorkie to Transcriptional Control of Germline Stem Cell Factors in Somatic Tissue', *Developmental Cell*, 34(2), pp. 168–180. Available at: <https://doi.org/10.1016/j.devcel.2015.05.010>.
- Zhang, N. *et al.* (2010) 'The Merlin/NF2 Tumor Suppressor Functions through the YAP Oncoprotein to Regulate Tissue Homeostasis in Mammals', *Developmental Cell*, 19(1), pp. 27–38. Available at: <https://doi.org/10.1016/j.devcel.2010.06.015>.
- Zhang, S. *et al.* (2023) 'Eclosion muscles secrete ecdysteroids to initiate asymmetric intestinal stem cell division in *Drosophila*', *Developmental Cell* [Preprint]. Available at: <https://doi.org/10.1016/j.devcel.2023.11.016>.
- Zhang, Y. *et al.* (2020) 'Distinct Roles and Synergistic Function of FruM Isoforms in *Drosophila* Olfactory Receptor Neurons', *Cell Reports*, 33(11), p. 108516. Available at: <https://doi.org/10.1016/j.celrep.2020.108516>.
- Zhao, B., Tumaneng, K. and Guan, K.-L. (2011) 'The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal', *Nature Cell Biology*, 13(8), pp. 877–883. Available at: <https://doi.org/10.1038/ncb2303>.

Zhao, H. *et al.* (2022) 'Wnt signaling in colorectal cancer: pathogenic role and therapeutic target', *Molecular Cancer*, 21(1), p. 144. Available at: <https://doi.org/10.1186/s12943-022-01616-7>.

Zhao, Wenxia *et al.* (2021) 'Transcriptional co-activators YAP/TAZ: Potential therapeutic targets for metastatic breast cancer', *Biomedicine & Pharmacotherapy*, 133, p. 110956. Available at: <https://doi.org/10.1016/j.biopha.2020.110956>.

Zhao, X. and Karpac, J. (2020) 'The Drosophila midgut and the systemic coordination of lipid-dependent energy homeostasis', *Global change biology * Molecular physiology section*, 41, pp. 100–105. Available at: <https://doi.org/10.1016/j.cois.2020.07.003>.

Zhou, J. and Boutros, M. (2020) 'JNK-dependent intestinal barrier failure disrupts host–microbe homeostasis during tumorigenesis', *Proceedings of the National Academy of Sciences*, 117(17), pp. 9401–9412. Available at: <https://doi.org/10.1073/pnas.1913976117>.

Zipper, L. *et al.* (2020) 'Ecdysone steroid hormone remote controls intestinal stem cell fate decisions via the PPAR γ -homolog Eip75B in Drosophila', *eLife*. Edited by E. Knust, K. VijayRaghavan, and J. Korzelius, 9, p. e55795. Available at: <https://doi.org/10.7554/eLife.55795>.

Zipper, L. *et al.* (2022) 'The MicroRNA miR-277 Controls Physiology and Pathology of the Adult Drosophila Midgut by Regulating the Expression of Fatty Acid β -Oxidation-Related Genes in Intestinal Stem Cells', *Metabolites*, 12(4). Available at: <https://doi.org/10.3390/metabo12040315>.