Regulation of *Drosophila* intestinal regeneration by the Hippo pathway

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Declaration

I, Rachael Shaw, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Intestinal stem cells (ISCs) in the adult *Drosophila* midgut proliferate in order to self-renew and produce differentiating daughter cells that replace those lost as part of normal gut function. Intestinal stress induces the activation of Upd/Jak/Stat signalling, which promotes intestinal regeneration by inducing rapid SC proliferation and faster tissue turnover.

I have investigated the role of the Hippo (Hpo) pathway in the *Drosophila* midgut. The Hpo pathway regulates tissue size via the control of both apoptosis and proliferation during *Drosophila* development. In the midgut, Hpo pathway inactivation in either the SCs or their differentiated daughter cells, the enterocytes (ECs), induces a phenotype similar to that observed under stress situations, including increased proliferation and expression of Upd cytokines.

Hpo pathway targets are induced by stresses such as bacterial infection, suggesting that the pathway functions as a sensor of cellular stress in the ECs. In addition, Yki, the pro-growth transcription factor target of the pathway, is required in ISCs to drive the proliferative response to stress. Yki inactivation has no obvious effect on baseline homeostasis, while survival upon infection is affected by loss of Yki in either the ISCs or ECs. My findings suggest that the Hpo pathway is a mediator of the regenerative response in the midgut.

In the final part of the project, I have addressed possible mechanisms of Yki activation, with a view to gaining further insight into Yki function in the ECs. The data suggest a possible link between the generation of reactive oxygen species, JNK signalling and Yki activation. Several explanations could account for the requirement of Yki expression in the ECs. Yki expression itself might induce stress in the ECs, leading to *upd* expression and the regenerative response. Alternatively, the Hpo pathway might function as a stress sensor, triggering Upd release in response to noxious stimuli.

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Abbreviations

A.E.D	After Egg Deposition
AJ	Adherens Junction
AMOT	Angiomotin
AMP	Adult midgut progenitor
APC	Adenomatous polyposis coli
App	Approximated
aPKC	Atypical Protein Kinase C
AMP	Antimicrobial Peptide
Arm	Armadillo
Bam	Bag-of-marbles
Ban	Bantam
Baz	Bazooka
bHLH	basic helix-loop-helix
BM	BM
BMP	Bone morphogenetic protein
Bp	Base pair
BrdU	Bromodeoxyuridine
Bsk	Basket
Cas3	Caspase 3
CBC	Crypt Base Columnar cell
CGNPs	Cerebellar granule neural precursor cells
Crb	Crumbs
CRC	Colorectal Cancer
CSC	Cancer Stem Cells
CTGF	Connective tissue growth factor
Cyc	Cyclin
DAPI	4',6-diamidino-2-phenylindole
Dco	Discs overgrown
DE-cad	*Drosophila* E.cadherin
Diap	*Drosophila* Inhibitor of Apoptosis

Dilps	*Drosophila* insulin-like peptides
Dl	Delta
Dlg	Discs large
DNA	Deoxyribonucleic acid
Dome	Domeless
Dpp	Decapentaplegic
Dredd	Death related ced-3/Nedd2-like protein
Ds	Dachsous
DSS	Dextran Sulphate Sodium
Duox	Dual Oxidase
EB	Enteroblast
EC	Enterocyte
Ecc15	*Erwinia carotovora*
Ed	Echinoid
EE	Enteroendocrine Cell
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eIF4E-BP	Eukaryotic translation initiation factor 4E-binding protein
ES cell	Embryonic stem cell
Esg	Escargot
E(spl)	Enhancer of Split
Ex	Expanded
Fadd	Fas-Associated Death Domain
FERM	4.1 Ezrin Radixin Moesin
Fj	Four-jointed
FLP	FLPase
FRT	FLPase Recombinant Target
Ft	Fat
GaSC	Gastric stem cells
Gbe	Grainy head protein binding element
GFP	Green Fluorescent protein
GI	Gastrointestinal

GMC	Ganglion mother cell
GSC	Germline Stem Cell
GSK-3β	Glycogen synthase kinase 3β
Hep	Hemipterous
Hes1	Hairy and Enhancer of split 1
Hh	Hedgehog
Hop	Hopscotch
Hpo	Hippo
hsFLP	Heat-shock FLPase
Hth	Homothorax
H₂O₂	Hydrogen Peroxide
IGF	Insulin-like growth factor signalling
IIS	Insulin/insulin-like growth factor signalling
IMD	Immune Deficiency Pathway
InR	Insulin receptor
iPS	Induced pluripotent stem cell
IRC	Immune-regulated catalase
ISC	Intestinal Stem Cell
ITR	Inverted Terminal Repeats
Jak	Janus Kinase
Jub	Ajuba
JNK	Jun N-terminal Kinase
KEM	Kibra-Expanded-Merlin
Kib	Kibra
Kibra	Kidney and Brain
Lats	Large Tumour Suppressor
LB	Lysogeny Broth
Lgl	Lethal Giant Larvae
LOF	Loss Of Function
MAPK	Mitogen Activated Protein Kinase
MARCM	Mosaic analysis with a repressible cell marker
Mats	Mob as a tumour suppressor

Mer	Merlin
Mop	Myopic
mRNA	Messenger RNA
Mst	Mammalian Sterile20-like protein kinase
MT	Malpighian Tubules
NADPH	Nicotinamide adenine dinucleotide phosphate
NDR	Nuclear DBF2-related
NICD	Notch intracellular domain
NF2	Neurofibromin 2
NGS	Normal Goat Serum
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
OC	Oval cell
P-	Phospho-
PAR	Partition defective
PBS	Phosphate Buffer Saline
PC	Peripheral Cell
PCP	Planar cell polarity
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
Pdm-1	POU domain 1
P.e	*Pseudomonas entomophila*
PFA	Paraformaldehyde
PFC	Posterior follicle cell
PG	Peptidoglycan
pH	Potential Hydrogen
PHD	Peroxidase domain
PH3	Phospho-histone H3
PI3-K	Phosphatidylinositol 3-kinases
PLC-β	Phospholipase C-β
PP2A	Protein phosphatase 2A
Pros	Prospero
PTP	Protein tyrosine phosphatase

Puc	Puckered
qRT-PCR	Quantitative Reverse-Transcription PCR
RASSF	Ras-Association domain Family
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
RNAi	RNA interference
RNSCs	Renal and nephric stem cells
ROS	Reactive Oxygen Species
RT	Room Temperature
S-	Serine
SARAH	Salvador RASSF Hippo
Sav	Salvador
SC	Stem Cell
Scrib	Scribble
Sd	Scalloped
Stat	Signal Transducer and Activator of Transcription
STRIPAK	*Drosophila* Striatin-interacting phosphatase and kinase
Su(H)	Suppressor of Hairless
TA	Transit amplifying
Tak1	TGF-β activated kinase 1
TEAD/TEF	TEA-DNA binding domain/Transcription Enhancer Factor
TF	Transcription factor
TOR	Target of rapamycin
TGFβ	Transforming growth factor-β
Tsc	Tuberous Sclerosis Complex
Tsh	Teashirt
UAS	Upstream Activating Sequence
Upd	Unpaired
UTR	Untranslated Region
VM	Visceral muscle
Vn	Vein
Wbp2	WW domain binding protein 2

Wg	Wingless
WT	Wild Type
Wts	Warts
YAP1	Yes-associated Protein 1
Yki	Yorkie
Zyx	Zyxin
β –gal	Beta- Galactosidase

Chapter 1. Introduction

Overview

The overall aim of my PhD was to address the role of the Hippo (Hpo) pathway in the adult posterior midgut. In order to highlight the main ideas and concepts behind my work, I begin by introducing *Drosophila melanogaster* as a model organism for the study of growth control. Several highly conserved pathways are known to regulate growth. Work in my PhD laboratory is focussed on one particular signalling network, the Hpo pathway. In the second part of my introduction, I describe our current understanding of Hpo pathway function in both *Drosophila* and mammals. I then go on to introduce the key concepts in stem cell (SC) biology and the SC populations previously studied in *Drosophila,* before giving a brief overview of the mammalian intestine, paying particular focus to the organisation and regulation of intestinal stem cells (ISCs). Finally, I present the adult *Drosophila* posterior midgut as a model system in which to investigate ISC biology. In this final section, I not only address midgut organisation but also highlight the range of signalling pathways shown to affect epithelial turnover.

1.1 The fruit fly as model for growth control

1.1.1 *Drosophila melanogaster* as a model organism

Drosophila melanogaster, commonly known as the fruit fly, has been utilised in biological research for over one hundred years. Studies in *Drosophila* have provided insight into areas such as development, evolution, genetics and disease. As a model organism *Drosophila* offers several advantages. From a practical aspect, fruit flies are small, easy to handle and cheap to maintain (Greenspan, 1997). Their comparatively short generation time, of just 10 days when maintained at 25°C, makes *Drosophila* an ideal candidate for genetic experiments (Greenspan, 1997).

Genetically, *Drosophila* not only benefit from having just four pairs of chromosomes (one sex chromosome and three autosomes), which facilitates genetic mapping, but also

show a high degree of genetic conservation. The sequencing of the *Drosophila* genome in 2000 enabled comparative genomics, with studies showing that over 70% of human disease related genes are conserved in the fly (Reiter et al., 2001). A host of resources and genetic techniques are now available to *Drosophila* researchers, including online databases and stock centres. The generation of transgenic flies is now a routine process, details of which can be found in section 2.1.

1.1.2 Lifecycle of *Drosophila melanogaster*

The optimal temperature for maintaining *Drosophila* is 25°C. Life cycle is temperature sensitive, 10 days at 25°C but 20 days when kept at $18\,^{\circ}$C. Embryonic development lasts around 12-15 hours with fertilised females able to lay hundreds of eggs per day (Ashburner et al., 2005). Embryos hatch as first instar larvae (Figure 1.1). The larval phase involves exponential growth, with an approximately 200-fold increase in mass. The larval period of development can be divided into three moult-separated stages or instars and lasts four days in total, with moulting occurring at 24 and 48 hours. Proliferation occurs mainly in the nervous system and imaginal discs. Imaginal discs are sac-like epithelial structures that give rise to the adult external organs. At the end of the third instar the larvae stop feeding, wander out of the food and pupate (Ashburner et al., 2005).

During pupariation, the imaginal discs are subject to morphological changes and give rise to adult structures such as the wings and legs (Ashburner et al., 2005). The majority of other larval tissues degenerate by a process called histolysis. Some groups of cells, known as histoblasts, do however remain and go on to form internal structures like the digestive tract. Overall, metamorphosis lasts four to five days before adult flies emerge from the pupal case (Ashburner et al., 2005). Adults are sexually mature six to eight hours after eclosion.
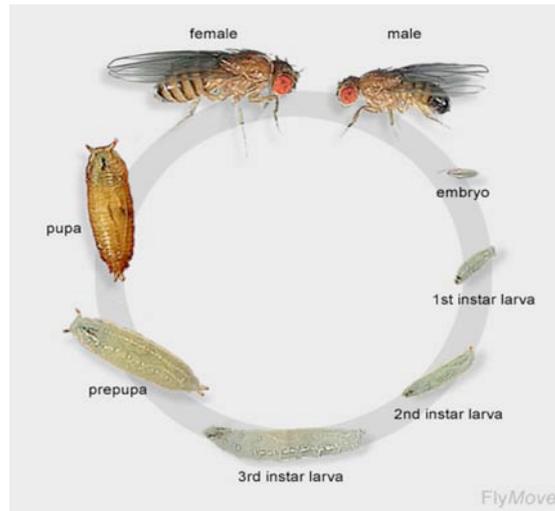
Figure 1.1: Lifecycle of *Drosophila melanogaster*

Image available on Flymove website – http://flymove.uni.muenster.de

1.1.3 Growth regulatory processes in *Drosophila*

A fundamental question in biology is how growing tissues sense when they have reached the correct size (Thompson, 2010). Understanding growth control during development can also offer vital insights into the processes underlying diseases affecting cell number, such as cancer and degeneration.

1.1.3.1 Cell growth and division

Cell number is increased via cell division, the process by which one cell replicates its DNA before dividing to form two daughter cells. Before a cell can undergo division, it is subject to a growth phase, in order that overall cell size is maintained. This process of cell growth, duplication and division is known as the cell cycle (Figure 1.2A). Cell proliferation refers to progression through the cell cycle followed by division (i.e. cell growth in combination with division) (Thomspon, 2010).

The cell cycle consists of four main phases. DNA is replicated during S phase and division occurs during the M phase. These two phases are separated by G1 and G2, gap phases, that allow time for cell growth. The cell cycle is subject to tight regulation in order to ensure that division only occurs when necessary. For example, the cell cycle
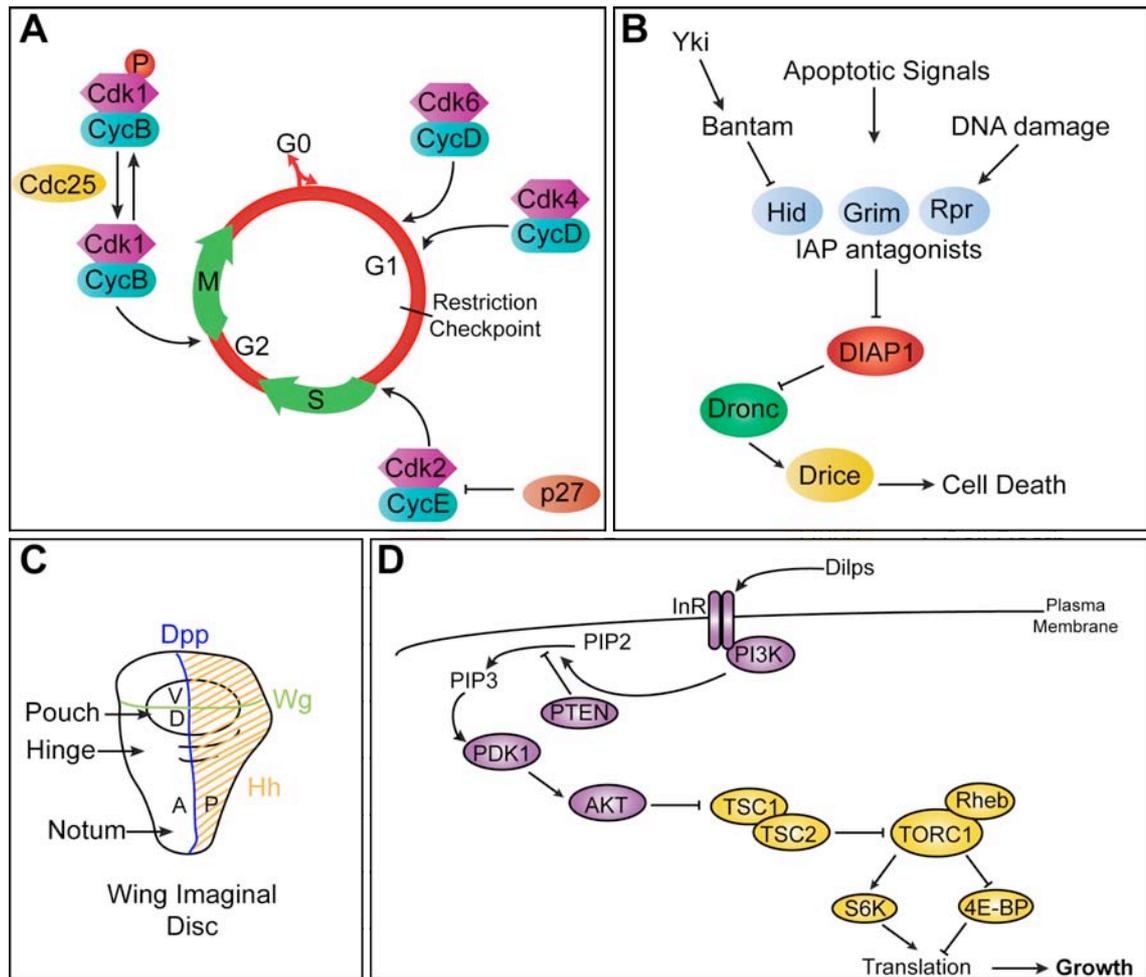
Figure 1.2: *Drosophila* **as a model organism for the study of growth control**

(A) Simplified schematic of the eukaryotic cell cycle machinery. Two of the major regulatory steps include CycE/Cdk2 regulation of G1/S transition (Knoblich et al., 1994) and CycB/Cdk1 regulation of G2/M transition (Lehner and O'Farrell, 1990). CycB/Cdk1 is positively regulated by the Cdc25 phosphatase (String in *Drosophila*).

(B) Apoptosis pathway in *Drosophila*. Note that the Hpo pathway target *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the pro-apoptotic gene *hid*. **(C)** Wing imaginal disc. Cell proliferation during the larval stages results in a tissue consisting of around 50,000 cells. Posterior cells express Hedgehog (Hh), while anterior cells respond to Hh. Signalling initiated at the anterior/posterior boundary establishes a Dpp morphogen gradient, which is involved in disc patterning and proliferation. A Wingless (Wg) gradient at the dorsal/ventral boundary is also important to wing patterning and development. The wing disc goes on to form the adult wing, hinge region and notum. **(D)** The Insulin/PI3K pathway regulates cell growth via TOR signalling

will arrest if DNA damage is detected following replication (Malumbres and Barbacid, 2005). Cyclin-dependent kinases (Cdks) and their cyclin binding partners regulate progression through the cell cycle via phosphorylation of targets involved in crucial cell cycle steps. Extracellular signals known as mitogens also regulate G1/S transition, these include EGF (epidermal growth factor), PDGF (platelet-derived growth factor) and MAPK (mitogen activated protein kinase) (Malumbres and Barbacid, 2005).

Cell growth can occur in the absence of cell division. Many cells, including the ECs of the posterior midgut, undergo endocycles. These cells are said to be polyploid and continue to increase in size and ploidy through variant cell cycles, where the DNA replicates but division does not occur (Edgar and Orr-Weaver, 2001).

1.1.3.2 Cell death

Apoptosis is a type of programmed cell death. Other types of cell death include autophagy and necrosis. Apoptosis not only regulates organ size during development but also maintains tissue integrity in the adult by providing protection against damaged or infected cells (as reviewed by (Hay et al., 2004)). Cells undergoing apoptosis undergo several morphological changes including chromatin condensation, cell shrinkage, and loss of cell-contacts. Apoptosis involves caspase (protease) activation (Figure 1.2B). The apical caspase Dronc cleaves and activates an effector caspase such as Drice. Caspase activity is regulated by *Drosophila* IAPs (inhibitor of apoptosis proteins) (Meier et al., 2000). Diap1 can bind Dronc leading to Dronc ubiquitination and inactivation. Reaper, Grim and Hid induce apoptosis by binding and destabilising Diap1 (Goyal et al., 2000).

In tissues with high turnover rates, apoptosis can be regulated by anoikis (Frisch and Screaton, 2001). Anoikis refers to 'apoptosis induced by inadequate or inappropriate cell-matrix interactions' (Frisch and Francis, 1994). The mechanism by which a loss of integrin-mediated cellular adhesion triggers the cell-death cascade is unclear. Loss of anoikis contributes to malignancy in colon cancer (Shanmugathasan and Jothy, 2000).

1.1.3.3 Regulation and co-ordination of cell growth

Cell growth and death are regulated and co-ordinated through a number of mechanisms, including both local and systemic factors. The wing imaginal disc is a commonly used tissue in the study of the signalling events involved in coordinating cell growth and division (see Figure 1.2C) (Hariharan and Bilder, 2006).

Local/intrinsic factors

A number of intrinsic signalling pathways function in growth control, including Dpp (Decapentaplegic, *Drosophila* homolog of BMP, bone morphogenetic protein), Wg (Wingless, Wnt in mammals), PI3K (Phosphatidylinositol 3-kinases), Egfr/Ras/MAPK and Hpo. Disruption to pathways modulating more than one aspect of growth, such as the Hpo pathway, can have profound effects. Ras activation, for example, drives both cell division and growth through regulation of Myc, PI3K and MAPK signalling (Prober and Edgar, 2000). Although co-ordinated, cell growth and the cell cycle can be separated. Cell growth does not depend on cell division and can be increased in order to overcome reduced proliferation, thus maintaining overall tissue size with fewer but larger cells (Johnston et al., 1977, Neufeld et al., 1998, Weigmann et al., 1997).

Morphogens are secreted signalling proteins involved in the regulation of tissue size, shape and patterning (Figure 1.2C) (as reviewed in (Dekanty and Milan, 2011)). Produced locally, they establish a diffusible signalling gradient in which expression is highest at the source. Regulation occurs at the level of morphogen synthesis, diffusion and degradation rates.

A considerable degree of crosstalk exists between the different growth regulating pathways. Dying cells, for example, secrete Wg and Dpp morphogens and in doing so signal neighbouring cells to divide. This process is referred to as compensatory proliferation (Huh et al., 2004, Perez-Garijo et al., 2004, Ryoo et al., 2004). Conversely, faster dividing cells can induce their neighbours to undergo apoptosis (Moreno et al., 2002, Li and Baker, 2007). This ability of fitter cells to eliminate their neighbours is known as cell competition (Morata and Ripoll, 1975).

Systemic/extrinsic factors

Cell growth is also regulated by systemic factors. Nutritional status at both a cellular and systemic level can regulate tissue growth (as reviewed in (Hietakangas and Cohen, 2009)). The TOR (target of rapamycin) pathway monitors cellular levels of amino acids. TOR signalling regulates growth through protein synthesis via ribosomal S6-Kinase activation and inhibition of eIF4E-BP (eukaryotic translation initiation factor 4E-binding protein) (Wullschleger et al., 2006). The main mediator of systemic nutrient sensing is insulin-like signalling (IIS) (Figure 1.2D). The insulin/PI3K/Akt pathway coordinates growth on an organism-wide level. *Drosophila* insulin-like peptides (Dilps) bind to the insulin receptor (InR) (Brogiolo et al., 2001), which triggers the PI3K/Akt signalling cascade and ultimately TOR activation. The growth-promoting action of the Dilps is counteracted by the steroid hormone ecdysone. This balance is regulated by the fat body, the *Drosophila* equivalent to the mammalian liver and adipose tissue (Colombani et al., 2005). Ecdysone also signals the larval to pupal transition, bringing about the end of the feeding phase of development.

1.2 The Hippo growth regulation pathway

1.2.1 The Hpo pathway controls both cell cycle exit and apoptosis

The highly conserved Salvador/Warts/Hippo signalling pathway is a key regulator of organ size (Figure 1.3 and Table 1.1) (Harvey and Tapon, 2007). First discovered in *Drosophila,* the pathway promotes both cell cycle exit and apoptosis and its deregulation can lead to cancer. The first core member of the pathway to be identified was Warts (Wts), which was recovered in a genetic mosaic screen designed to identify genes involved in cell growth and proliferation (Justice et al., 1995, Xu et al., 1995). Wts is a member of the Nuclear DBF2-related (NDR) family of serine/threonine kinases. Cells mutant for *wts* overproliferate leading to severe overgrowth in wing imaginal discs and epithelial tumours in adult tissues (Justice et al., 1995, Xu et al., 1995). Subsequent genetic screens led to the identification of other genes that elicit similar phenotypes when clonally deleted in imaginal discs, such as *salvador* (*sav*), *hippo* (*hpo*)
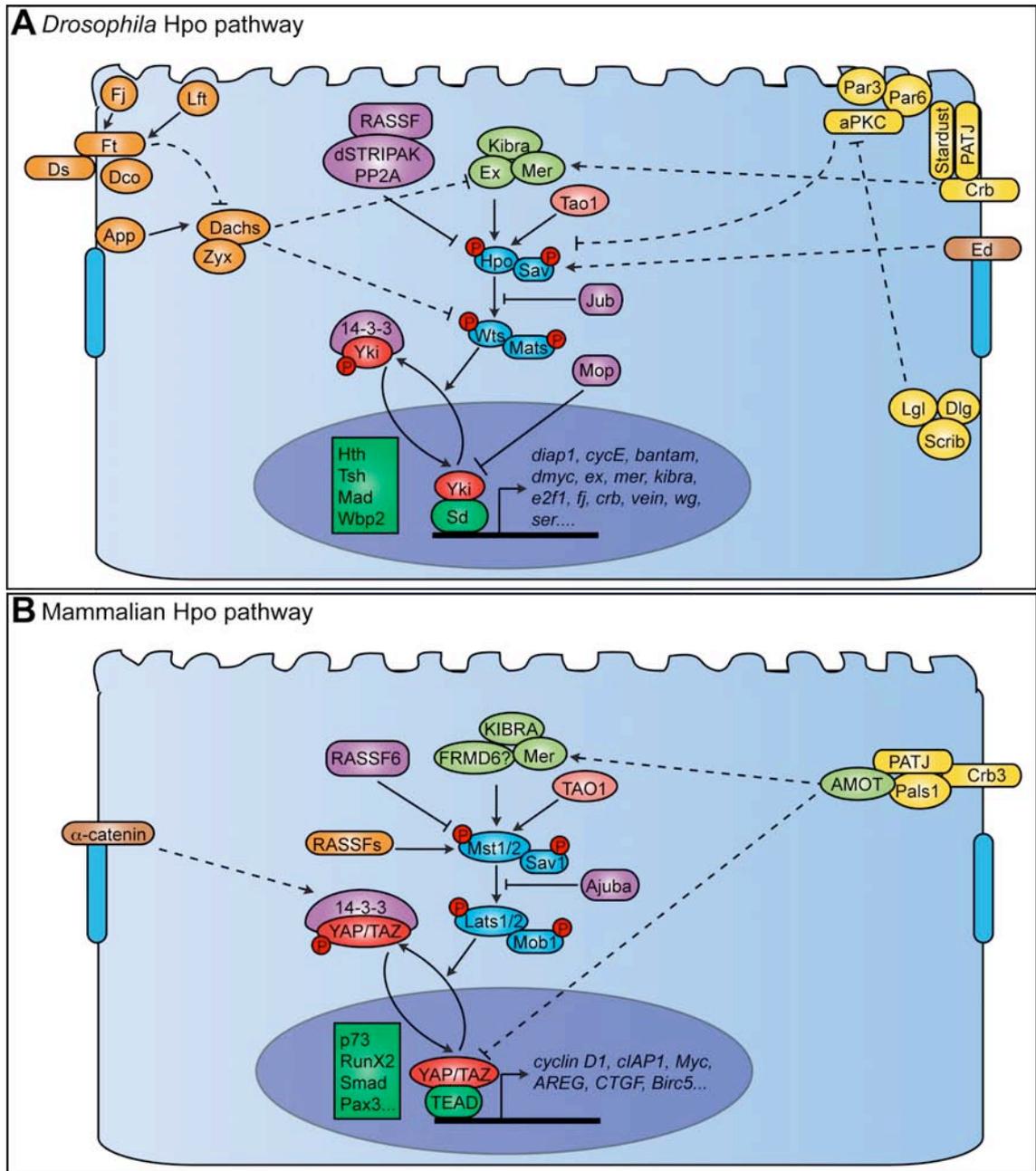
Figure 1.3: The Hippo pathway

(A) Schematic representation of the *Drosophila* Hpo pathway in an epithelial cell. **(B)** Schematic representation of the mammalian Hpo pathway in an epithelial cell. P = a phosphorylation event.

Summary of Hippo pathway components in *Drosophila*			
***Drosophila* protein**	**Vertebrate homologues**	**Protein type/motifs**	**Role**
Fat pathway			
Fat (Ft)	Fat4	Atypical cadherin	Receptor
Dachsous (Ds)	Dchs1,2	Atypical cadherin	Ligand for Fat
Lowfat (Lft)	Lix1, Lix1L	Unknown conserved domain	Regulates levels of Ft and Ds
Four-jointed (Fj)	Fjx1	Golgi-localized kinase	Phosphorylates Ft and Ds cadherin domain
Discs overgrown (Dco)	CK1δ,ε	Casein family kinase	Phosphorylates Ft cytoplasmic domain
Dachs	?	Unconventional myosin	Transduces signal from Ft to Wts
Zyx102 (Zyx)	Zyxin, Lpp, Trip6	LIM domains	Interacts with Wts
Approximated (App)	ZDHHCs	Palmitoyltransferase	Dachs membrane localisation
KEM complex			
Expanded (Ex)	FRMD6 (Willin)/AMOT(?)	FERM, PPXY domains	Regulates Hpo
Merlin (Mer)	Merlin/NF2	FERM domain	Regulates Hpo
Kibra (Kib)	KIBRA	WW domain	Regulates Hpo
Apical-basal polarity proteins			
Crumbs (Crb)	Crb1-3	Transmembrane, EGF domains	Receptor
Lethal giant larvae (Lgl)	Lgl1,2	WD40 motif	Scaffolding protein
aPKC	aPKCλ, aPKCζ	Ser/Thr kinase domain	Reduces Hpo activation
Core kinase cassette			
Hippo (Hpo)	Mst1,2	Sterile-20 family Ser/Thr kinase	Phosphorylates Wts
Salvador (Sav)	WW45/Sav1	WW, SARAH domains	Scaffolding protein
Warts (Wts)	Lats1,2	NDR family Ser/Thr kinase	Phosphorylates Yki
Mob as tumour suppressor (Mats)	MOB1A,B	NDR kinase family co-factor	Promotes Wts activity
Other regulators			
RASSF	RASSF1-6	Ras-association and SARAH domains	Scaffolding protein?
PP2A (STRIPAK)	PP2A (STRIPAK)	Phosphatase	Inhibits Hpo activity
Ajuba (Jub)	Ajuba, Limd1, Wtip	LIM domains	Inhibits Wts activiy
JNK	JNK	Kinase	Activates Yki (indirectly?)
Myopic (Mop)	Hd-PTP	Phosphatase, PPXY, Bro motifs	Binds and inhibits Yki
Tao1	TAO1	Sterile-20 family Ser/Thr kinase	Phosphorylates Hpo
Echinoid (Ed)	TSLC1 (?)	Cell adhesion molecule	Stabilises Sav at Ajs
Transcription factors			
Yorkie (Yki)	Yap, Taz	WW domains and TEAD binding	Transcriptional coactivator
Wbp2	Wbp2	PPXY motifs	Transcriptional coactivator
Scalloped (Sd)	TEAD/TEF 1-4	TEA-domain	TF, DNA binding
Mad	Smad	MH domains	TF, DNA binding
Homothorax (Htx)	Meis1-3	Homeodomain	TF, DNA binding
Teashirt (Tsh)	Tshz1-3	Zn-finger	TF, DNA binding

Table 1: Members of the Hpo pathway

and *mob as a tumour suppressor* (*mats*) (Kango-Singh et al., 2002, Tapon et al., 2002, Harvey et al., 2003, Pantalacci et al., 2003, Udan et al., 2003, Wu et al., 2003, Lai et al., 2005). Genetic and molecular interaction data examining these proteins led to the concept of the Hippo (Hpo) pathway.

1.2.2 The core kinase cascade

Hpo signalling involves a core kinase cascade. The upstream kinase Hpo activates the downstream kinase Warts (Wts), in concert with two scaffold proteins Salvador (Sav) and Mats (Figure 1.3A) (Harvey et al., 2003, Pantalacci et al., 2003, Udan et al., 2003, Tapon et al., 2002, Jia et al., 2003, Kango-Singh et al., 2002, Wu et al., 2003, Lai et al., 2005). Wts phosphorylates and inactivates Yorkie (Yki), a growth-promoting transcriptional co-activator (Huang et al., 2005). Yki promotes the expression of target genes including *Diap1* (*Drosophila* inhibitor of apoptosis protein 1) and *Cyclin E* (Dong et al., 2007, Huang et al., 2005).

1.2.2.1 Hpo and Wts kinases

Hpo is a member of the Sterile20-like family of Serine/Threonine kinases. The ability of Hpo to trans or autophosphorylate was revealed in kinase assays and a conserved T195 residue in the Hpo activation loop is crucial to its kinase activity (Pantalacci et al., 2003, Wu et al., 2003, Colombani et al., 2006). Hpo phosphorylates Wts on several serine and threonine residues and is responsible for Wts activation (Wu et al., 2003). Similarly, the mammalian orthologue of Hpo (Mst1/2) phosphorylates the Wts orthologue Lats1/2 (Chan et al., 2005, Yu et al., 2010). MST1 and 2 are pro-apoptotic kinases implicated in liver tumour formation (Song et al., 2010, Lu et al., 2010). The mammalian Wts orthologues Lats1 and 2 interact with scaffold proteins MOB1A and MOB1B and are both implicated in growth control (Praskova et al., 2008).

1.2.2.2 Sav and Mats adaptor proteins

Sav and Mats are adaptor proteins. Sav was initially identified in a genetic screen seeking mutations that induce overproliferation but do not affect differentiation (Tapon et al., 2002, Kango-Singh et al., 2002). As with Hpo and Wts inactivation, Sav LOF in

the eye leads to extra inter-ommatidial cells and reduced apoptosis but does not affect photoreceptor differentiation. In the wing disc, *sav* mutation results in cells of normal size but which exhibit faster rates of proliferation (Harvey et al., 2003, Pantalacci et al., 2003, Jia et al., 2003, Kango-Singh et al., 2002, Tapon et al., 2002, Udan et al., 2003, Wu et al., 2003). Co-expression of *hpo/sav* produces a stronger phenotype than *hpo* expression alone, while removal of either *wts* or *sav* can partially rescue the *hpo* overexpression phenotype (Jia et al., 2003, Wu et al., 2003, Udan et al., 2003). Sav has no catalytic domain but two WW domains, which can bind to Wts PPXY motifs (Tapon et al., 2002). Sav also binds to Hpo, via direct interaction between C-terminally located coiled-coiled regions, known as SARAH domains (<u>Sav</u>, <u>RA</u>SSF, <u>Hpo</u>), found in both proteins (Pantalacci et al., 2003, Udan et al., 2003, Wu et al., 2003, Harvey et al., 2003, Jia et al., 2003). Hpo-Sav binding is required for Hpo phosphorylation of Wts, and Sav itself is also phosphorylated by Hpo (Pantalacci et al., 2003, Wu et al., 2003).

Wts activity is regulated by an adaptor protein called Mats. Mats is a Hpo substrate, which binds to Wts, potentiating its kinase activity (Lai et al., 2005). Phosphorylated Mats has a higher affinity for Wts and thus increases Wts kinase activity (Wei et al., 2007). Mats is activated at the plasma membrane and it has been suggested that it may function in targeting Wts to the cell membrane for interaction with the other core pathway members (Ho et al., 2010). The mammalian orthologues of Sav and Mats, Sav1/WW45 and MOB1 are mutated in several cancer cell lines, demonstrating a high degree of functional conservation with their *Drosophila* counterparts (Tapon et al., 2002, Lai et al., 2005).

1.2.2.3 *The transcriptional co-activator Yki*

Early studies in the Hpo signalling field showed that pathway inactivation affects cell cycle progression and cell death through increased expression of both *cyclinE* and *diap1* (Harvey et al., 2003, Jia et al., 2003, Kango-Singh et al., 2002, Pantalacci et al., 2003, Tapon et al., 2002, Udan et al., 2003, Wu et al., 2003). The discovery that Wts binds to Yki, the *Drosophila* homologue of YAP (Yes-associated protein), a known transcriptional co-activator, provided a possible mechanism by which Hpo signalling

might influence gene transcription (Huang et al., 2005, Yagi et al., 1999). Yki expression is required for the overgrowth phenotype of *wts* mutant tissue and overexpression of Yki protein phenocopies Hpo pathway LOF (Huang et al., 2005). As with Sav, Yki has two WW domains, which are necessary for its interaction with Wts PPXY motifs. *In vitro* kinase assays have confirmed that Yki is a substrate for Wts and that this phosphorylation results in reduced transcriptional activity (Huang et al., 2005).

Yki harbours multiple Wts phosphorylation sites. Phosphorylation of the conserved Serine-168 is vital to Yki's growth regulatory function (Dong et al., 2007, Oh and Irvine, 2008). In Hpo mutant cells, Yki is localised to the nucleus. However, upon Wts-dependent phosphorylation of S-168, Yki binds to 14-3-3 and is sequestered in the cytoplasm, thereby reducing the expression of Yki target genes (Dong et al., 2007, Oh and Irvine, 2008, Ren et al., 2009).

While Yki is responsible for driving growth in many tissues, it remains possible that other Hpo pathway targets do also exist. Equally, Hpo signalling might modulate targets via processes other than transcriptional regulation. Hpo can, for example, phosphorylate Diap1 leading to its degradation (Harvey et al., 2003, Pantalacci et al., 2003).

Yki is an orthologue of mammalian YAP and its paralogue TAZ (Sudol, 1994, Kanai et al., 2000). Lats dependent phosphorylation of YAP S-127 corresponds to S-168 phosphorylation of Yki, leading to inactivation and exclusion from the nucleus (Dong et al., 2007, Zhao et al., 2007, Zhang et al., 2008a, Hao et al., 2008). Lats1/2 can phosphorylate YAP/TAZ on several residues (Dong et al., 2007, Zhao et al., 2007, Hao et al., 2008, Lei et al., 2008, Oka et al., 2008). Phosphorylation of S-381 primes YAP for subsequent phosphorylation by a second kinase, believed to be Casein Kinase 1. This second phosphorylation step activates a phosphorylation-dependent degradation motif (phospho-degron). Recruitment of the E3 ubiquitin ligase SCF$^{\beta\text{-TRCP}}$ by the activated phospho-degron then leads to polyubiquitination and degradation of YAP (Zhao et al., 2010). Unlike YAP and TAZ, Yki lacks a residue equivalent to S-381, suggesting that this mechanism is not conserved (Liu et al., 2010a).

1.2.2.4 Yki binding partners and targets

In order to understand Yki function, it was vital to identify its TF binding partner(s). Yki itself exhibits no DNA binding capacity. Scalloped (Sd) encodes the only TEAD/TEF (TEA DNA binding domain/Transcription enhancer factor) TF member in *Drosophila* and is required for Yki's growth regulatory function (Goulev et al., 2008, Wu et al., 2008, Zhang et al., 2008b). Yki and Sd form a complex that can bind directly to 26bp sequences, known as Hippo Response Elements (HREs), in the promoter region of target genes such as *diap1* and *cyclin E* (Zhang et al., 2008b, Wu et al., 2008). Diap1 inhibits apoptosis and Cyclin E promotes cell cycle progression. However, these two proteins alone do not account for the overgrowth phenotype induced by Yki overexpression (Tapon et al., 2002, Jia et al., 2003, Udan et al., 2003, Lai et al., 2005). Yki also targets the *bantam* miRNA and *bantam* overexpression can partially rescue the Yki mutant phenotype (Nolo et al., 2006, Thompson and Cohen, 2006). Interestingly, *bantam* is also a target of the EGFR signalling pathway (Herranz et al., 2012). Importantly, there is no mammalian homologue of Bantam and it remains to be seen whether YAP/TAZ can also regulate miRNA expression.

Yki is required in all imaginal cells for proliferation, while Sd is expressed at high levels in the developing wing pouch but not in dividing eye progenitor cells (Liu et al., 2000, Campbell et al., 1992, Huang et al., 2005). Yki therefore has other TF binding partners. In the eye imaginal disc, Yki regulates gene expression in conjunction with the homeodomain TF Homothorax (Hth) and the zinc-finger TF Teashirt (Tsh) (Peng et al., 2009). The expression of *bantam* is also regulated by another transcriptional complex between Yki and Mad, an effector of Dpp signalling (Oh and Irvine, 2011). Yki interaction with different binding partners might therefore be tissue-dependent and the availability of Yki partners could be a key factor in regulating Hpo pathway activity.

The Hpo pathway has been linked to cell competition through the identification of dMyc, a well-known inducer of ribosome biogenesis and cell growth, as another Yki-Sd target (Neto-Silva et al., 2010, Ziosi et al., 2010). dMyc expression induces cell competition and leads to the death of surrounding WT cells in the developing wing (de la Cova et al., 2004, Moreno and Basler, 2004). Transcriptional induction of dMyc is

required for the competitive behaviour of Yki-expressing cells and dMyc also exerts negative feedback regulation on Yki (Neto-Silva et al., 2010, Ziosi et al., 2010). Other Yki targets include: *E2F1* (Goulev et al., 2008), which may be involved in the cell-autonomous regulation of proliferation; *wg* and *vg* expression in wing discs (Cho and Irvine, 2004, Zecca and Struhl, 2010); the EGFR ligands *vein, keren and spitz* (Ren et al., 2010, Zhang et al., 2009a), which might mediate non-autonomous functions of the Hpo pathway; and the Hpo pathway genes *ex*, *kibra*, *crb* and *fj* (Hamaratoglu et al., 2006, Genevet et al., 2010, Cho et al., 2006, Genevet et al., 2009), which may constitute a pathway negative feedback loop.

TEAD/TEF family TFs are also crucial to YAP mediated growth control, with at least one TEAD isoform expressed in every adult tissue (Cao et al., 2008, Nishioka et al., 2009, Ota and Sasaki, 2008, Zhao et al., 2008, Zhao et al., 2009). Disruption of YAP/TEAD binding abolishes YAP induced proliferation and oncogenic transformation (Zhao et al., 2008). The best-characterised YAP/TEAD target is CTGF, which has an important role in proliferation and anchorage dependent growth (Zhao et al., 2008). YAP and TAZ also induce the expression of Amphiregulin and FGF1, which may account for the non cell-autonomous functions of the Hpo pathway (Zhang et al., 2009a, Hao et al., 2008).

YAP/TAZ are also known to interact with a growing list of other TFs, which include Runx2, Smads, Pax3, Tbx5 and p73 (Vassilev et al., 2001, Hong et al., 2005, Hong and Yaffe, 2006, Komuro et al., 2003, Park et al., 2004, Varelas et al., 2008). TAZ interacts with a number of binding partners, some of which do not function with YAP. This may explain the varying Hpo pathway outputs observed in different contexts. YAP/TAZ interact with TEAD1-4 via a distinct N-terminal domain, whereas interaction with other TF partners occurs via WW domains. The issue of whether other TFs (e.g. chromatin remodellers) can associate with the YAP/TEAD complex is yet to be addressed (Cao et al., 2008, Nishioka et al., 2009, Ota and Sasaki, 2008, Zhao et al., 2008, Zhao et al., 2009).

1.2.2.5 Additional regulators of Yki activity

Yki is not only regulated by Wts-mediated phosphorylation but can also bind to upstream members of the Hpo pathway, including Ex, Hpo and Wts, increasing its cytoplasmic retention (Badouel et al., 2009, Oh and Irvine, 2008). Yki binding is mediated through WW domains, which often associate with proline-rich PPXY motifs. Wbp2 and Myopic (Mop) can directly interact with Yki through its WW domains and, in doing so, modulate Hpo signalling (Zhang et al., 2011c, Gilbert et al., 2011). Wbp2 was initially identified as a YAP interacting protein and can contribute to both YAP and TAZ transcriptional activation (Chen et al., 1997, Dhananjayan et al., 2006, Chan et al., 2010). In *Drosophila*, Wbp2 can act as an enhancer for Yki transcriptional activity (Zhang et al., 2011c). Mop is the *Drosophila* homologue of His-domain protein tyrosine phosphatase (HD-PTP). Mop expression represses Yki activity by acting as a cytoplasmic anchor (Gilbert et al., 2011).

1.2.3 The Hpo pathway is regulated by multiple inputs

The Hpo pathway has multiple functions in response to a variety of developmental cues and stress signals and, as a result, upstream regulation of the pathway is equally dynamic. Multiple inputs influence Hpo signalling, acting in both a coordinated and independent manner. Inputs act at various levels within the signalling cascade. Many of the upstream regulators of Hpo signalling are associated with the plasma membrane and increasing evidence suggests that these upstream components are involved in cell communication, providing information on the surrounding extracellular environment. Unlike many other signalling pathways, a soluble ligand for Hpo signalling has not yet been identified.

1.2.3.1 Regulation of Hpo kinase activity

<u>RASSF</u>

The Ras association domain family (RASSF) oncogene was the first Hpo pathway inhibitor to be identified (Polesello et al., 2006). RASSF mutants are viable but smaller in size due to a reduction in cell number. Removal of one copy of *hpo* significantly

rescues the reduced size observed in RASSF mutants (Polesello et al., 2006). Co-immunoprecipitation and binding assays have demonstrated that RASSF and Hpo interact via C-terminal SARAH domains, with RASSF competing with Sav for Hpo binding. RASSF associates with the dSTRIPAK (*Drosophila* Striatin-interacting phosphatase and kinase) PP2A (protein phosphatase 2A) phosphatase complex (Ribeiro et al., 2010). The kinase activity of Hpo is inhibited by the dSTRIPAK complex (Ribeiro et al., 2010). dSTRIPAK depletion results in increased Hpo phosphorylation and repression of Yki target genes *in vivo*. The exact mechanism by which RASSF inhibits Hpo is unknown but may involve binding to Hpo and thus enabling dephosphorylation by the dSTRIPAK complex. Interaction between RASSF family proteins and the Hpo orthologue MST is also observed in mammals and the fact that PP2A can control MST1/2 phosphorylation in tissue culture cells suggests that dSTRIPAK function may also be conserved (Avruch et al., 2006, Ikeda et al., 2009, Praskova et al., 2004, Guo et al., 2011).

Tao1

Tao1 is a Sterile20-like kinase, which is reported to regulate Hpo signalling (Poon et al., 2011, Boggiano et al., 2011). Like Hpo, Tao1 activation restricts cell proliferation in developing wing imaginal discs. Tao1 phosphorylates T195 in the Hpo activation loop, a mechanism that is conserved in mammals and leads to Hpo activation.

1.2.3.2 The Fat branch of the Hpo pathway

Fat (Ft), a cell surface molecule with multiple cadherin repeats, is another regulator of Hpo signalling activity (Cho et al., 2006, Bennett and Harvey, 2006, Silva et al., 2006, Willecke et al., 2006, Tyler and Baker, 2007). Ft signalling is also a known mediator of planar cell polarity (PCP), a process by which cells sense their position in relation to other tissue axes and orient themselves accordingly, this modulation is independent of Hpo signalling (for review see (Sopko and McNeill, 2009). Ft forms transheterodimers with Dachsous (Ds) another cadherin expressed on neighbouring cells (Clark et al., 1995). The golgi-kinase Four-jointed (Fj) modulates the Ft-Ds interaction by phosphorylating both the Ft and Ds extracellular domains (Villano and Katz, 1995,

Ishikawa et al., 2008, Brittle et al., 2010, Simon et al., 2010). Fj and Ds are expressed in opposite proximal-distal gradients, generating a gradient of active Ds-bound Ft, whereby each cell has more ligand bound Ft on its proximal side (Ma et al., 2003, Yang et al., 2002). Ft signalling requires the atypical myosin Dachs, which accumulates at the membrane when Ft is inactive (Mao et al., 2006). Dachs requires the palmitoyltransferase Approximated (App) for its membrane localisation (Matakatsu and Blair, 2008). Discs overgrown (Dco) phosphorylates ligand bound Ft in its intracellular domain and is required to polarise Dachs (Sopko et al., 2009, Feng and Irvine, 2009, Cho and Irvine, 2004).

Ft activity influences the Hpo pathway partly by regulating the apical localisation and expression of Ex and also by preventing Dachs-dependent Wts degradation (Cho and Irvine, 2004, Bennett and Harvey, 2006, Willecke et al., 2006, Silva et al., 2006, Tyler and Baker, 2007). Fat mutants exhibit mild overgrowth reminiscent of Ex mutants. The mechanisms surrounding Ft mediated Hpo pathway regulation are relatively unclear and represent a topic of significant debate in the field. In the wing, Fj and Ds expression patterns are influenced by wing patterning signals (morphogen gradients) including Dpp, Wg and Notch. The "steepness hypothesis" suggests that the Ft-Ds module senses these morphogen gradients and translates them into growth regulation via the Hpo pathway (Lawrence et al., 2008). Evidence in favour of this proposal includes the fact that disruption of Dpp (BMP/TGFβ orthologue) alters Fj and Ds expression, while uniform Fj and Ds expression leads to reduced growth (Rogulja et al., 2008, Willecke et al., 2008). An alternative view is that Ft signalling and the Dpp gradient act in a complementary but independent manner to regulate wing growth (Schwank et al., 2011).

The relationship between Ft, Dpp and Hpo is somewhat complex. Not only is Ft-Yki possibly acting as a growth control effector of Dpp signalling (as described above) but the Hpo and Dpp/BMP/TGFβ pathways are also seemingly intertwined at the level of downstream TFs. YAP/Yki interact with the BMP/Dpp TF effector Smad1/Mad to transcriptionally regulate common target genes, while YAP/TAZ can also regulate Smad2/3 nuclear localisation (Alarcon et al., 2009, Oh and Irvine, 2011, Varelas et al., 2010).

Mammals possess four Fat-related atypical cadherins (Fat 1-4). Fat4 is the orthologue of *Drosophila* Fat. There are also two Ds (Dchs1-2) and one Fj homologue (Fjx1) in mammals (Ashery-Padan et al., 1999, Nakajima et al., 2001, Rock et al., 2005, Tanoue and Takeichi, 2005). A connection between mammalian Fat- or Ds-related cadherins and core members of the Hpo pathway is yet to be elucidated.

1.2.3.3 The Kibra-Expanded-Merlin (KEM) complex

The Kibra-Expanded-Merlin (KEM) complex, comprised of three proteins found predominantly at the sub-apical region of epithelial cells, also regulates Hpo pathway activity. Ex and Mer are partially redundant FERM (4.1/ezrin/radixin/moesin) domain proteins, with loss of either protein leading to overproliferation (Hamaratoglu et al., 2006, LaJeunesse et al., 1998, Boedigheimer et al., 1997). FERM domain proteins are believed to act as membrane-cytoskeleton linkers. Ex and Mer can both bind to the WW domain containing protein Kibra (Baumgartner et al., 2010, Genevet et al., 2010, Yu et al., 2010). Members of the complex can potentiate each other's binding, with Kibra promoting Mer-Ex interaction and Ex promoting Kibra-Mer binding (Genevet et al., 2010, Yu et al., 2010).

The KEM complex is believed to induce Hpo pathway activity by recruiting core pathway members to the apical membrane for activation. Multiple interactions exist between the KEM complex and core pathway members, including Mer binding to Sav, Kibra binding to Hpo, Sav, Wts and Yki, and Ex interacting with both Hpo and Yki (Yu et al., 2010, Genevet et al., 2010, Baumgartner et al., 2010, Badouel et al., 2009, Oh et al., 2009a, McCartney et al., 2000). These various interactions possibly enable the integration of multiple upstream inputs or perhaps safeguard against the loss of a single component. KEM complex members can partially compensate for one another's loss (Yu et al., 2010, Hamaratoglu et al., 2006, Baumgartner et al., 2010, Genevet et al., 2010). The mechanisms controlling KEM activation remain unclear and may include responding to a receptor or physical property of the cell. In *Drosophila* S2 cells, Kibra affects Wts activity but does not disrupt Wts-Yki binding, suggesting that Wts and Yki

might bind before being recruited to the KEM complex for kinase activation (Genevet et al., 2010). Interestingly, Kibra itself is also known to bind to Yki (Genevet and Tapon, 2011).

Ex also participates in Yki inhibition, sequestering Yki out of the nucleus. This mode of regulation is also conserved in mammals where Angiomotin (AMOT) and AMOT-like proteins sequester YAP/TAZ in the cytoplasm (Zhao et al., 2011, Wang et al., 2011, Chan et al., 2011). AMOT seemingly behaves as the functional equivalent to Ex, antagonising YAP/TAZ through membrane tethering, but exhibits little sequence homology with Ex. The FERM domain containing FRMD6 is the mammalian protein most obviously related to Ex, in terms of sequence homology, but lacks the PPXY motif needed to bind to the YAP/TAZ WW domain. FRMD6 expression can activate mammalian Hpo signalling, raising the possibility that the domains and function of Ex have been evolutionarily split between AMOT and FRMD6 (Angus et al., 2012).

The human orthologue of Kibra (KIBRA) interacts with the Mer orthologue NF2 (Neurofibromin 2) but not FRMD6 (Yu et al., 2010). KIBRA associates with Lats1/2 in mammalian cells, with overexpression resulting in YAP phosphorylation (Xiao et al., 2011).

1.2.3.4 Apical-basal polarity complexes

Apical-basal polarity divides cells into two membrane domains, which are separated by cell-cell junctions. The separation of these two domains is achieved by the antagonistic action of the polarity complexes. The apical domain is specified by the Crumbs (Crb) and atypical protein kinase C (aPKC) complexes, while the basolateral domain is regulated by the Scribble (Scrib) complex, comprised of Scrib and Discs-large (Dlg), and Lethal giant larvae (Lgl) modules.

Crumbs links Hpo signalling with polarity

Several studies suggest that the apical transmembrane protein Crb can regulate Hpo signalling (Grzeschik et al., 2010, Ling et al., 2010, Robinson et al., 2010, Chen et al.,

2010). Crb binds to and is required for Ex apical localisation. Interestingly, both Crb depletion and overexpression lead to Ex mislocalisation and overproliferation (Grzeschik et al., 2010, Ling et al., 2010, Robinson et al., 2010, Chen et al., 2010). The binding of Crb not only promotes Ex apical localisation but also leads to the phosphorylation and eventual degradation of Ex protein. This suggests a dual role for Crb, promoting both Ex activity and degradation, and thus fine-tuning Hpo pathway activity. AMOT, the apparent functional equivalent of Ex in mammals, also associates with junctional components, in particular the mammalian orthologues of aPKC and Crb (Wells et al., 2006).

The Lethal giant larvae polarity complex

Lgl can also promote Hpo pathway activation by antagonising aPKC (Grzeschik et al., 2010). Overexpression of aPKC or loss of Lgl leads to mislocalisation of Hpo and increased colocalisation with its negative regulator RASSF. The mechanism of this regulation remains unclear. Human KIBRA is a known aPKC substrate, suggesting that aPKC might reduce Hpo activation by phosphorylation of scaffold proteins, such as Kibra and Ex, thereby modifying their binding affinities (Buther et al., 2004). Whether mammalian Lgl orthologues regulate Hpo signalling is yet to be elucidated. Another basolateral protein, Scrib, has also been linked to Hpo signalling in flies, zebrafish and human cells (Chen et al., 2012, Skouloudaki et al., 2009, Cordenonsi et al., 2011). In mammary epithelial cells, Scrib has been proposed to inhibit TAZ by scaffolding an MST/LATS/TAZ complex (Cordenonsi et al., 2011).

1.2.3.5 The importance of cell-cell contact and apical junctions

In recent years, a common theme has emerged in which many of the regulators of Hpo pathway activity are localised apically and at cell-cell junctions, placing them in an optimal position to inform the cell about changes in polarity, cell density and the surrounding environment. It is feasible that the apical domain, and more specifically the AJs, acts not only as a membrane anchor for components of the pathway but also has a role in sensing signals from neighbouring cells. Evidence in mammals supports a role for the Hpo pathway in cell-contact inhibition of growth, a property commonly lost in

cancer cells. Alterations in Hpo pathway activity are increasingly recognised as being associated with cancer development. In cells cultured at high density, YAP is phosphorylated by Lats1/2 and restricted to the cytoplasm. Upon wounding in a dense cell culture, YAP resumes an active nuclear location enabling the induction of cell proliferation. Hence, cell-contact, density and tension-sensing lead to Hpo pathway induced proliferation (Ota and Sasaki, 2008, Zhao et al., 2007). Furthermore, α-catenin, which binds YAP at the AJs in skin cells, has also been implicated in the regulation of contact inhibition (Schlegelmilch et al., 2011). Likewise, in addition to its role in cell-cell adhesion, E-cad is proposed as being a direct mediator of contact inhibition of proliferation through Hpo signalling (Kim et al., 2011)

In flies, Echinoid (Ed), an immunoglobulin domain-containing cell adhesion molecule, has been proposed to link cell-cell contact to Hpo signalling (Yue et al., 2012). Ed was found to physically interact with and stabilize Sav at AJs. This interaction is promoted by cell-contact and loss of Ed leads to elevated Yki activity.

1.2.3.6 F-actin and the importance of the cytoskeleton

Accumulation of F-actin due to mutation or downregulation of either of the two sub-units of capping protein or expression of active formin leads to Yki activation and tissue overgrowth (Sansores-Garcia et al., 2011, Fernandez et al., 2011). Regulation of the Hpo pathway by the cytoskeleton is conserved in mammals, where in response to cell detachment or plating on a soft substrate, Lats1/2 is activated leading to YAP/TAZ phosphorylation, which results in anoikis or changes in cell fate (Dupont et al., 2011, Wada et al., 2011, Zhao et al., 2012). In addition, YAP/TAZ have been suggested to sense substrate stiffness in a Lats1/2-independent manner (Dupont et al., 2011). This raises the possibility that the Hpo pathway is a mediator of cell shape and mechanical stress in the regulation of cell physiology. Micro-environmental physical and mechanical cues, such as membrane stiffness, are being increasingly recognised as regulators of cell behaviour. Cells translate these external stimuli into intracellular biochemical signals via mechanotransduction. Since the rigidity of the tumour microenvironment has been suggested to participate in tumour growth, the ability of

YAP/TAZ to respond to physical forces may represent an important oncogenic mechanism.

1.2.3.7 Lim domain proteins

Two LIM domain-containing proteins, Ajuba (Jub) and Zyxin (Zyx), are negative regulators of Wts (Das Thakur et al., 2010, Rauskolb et al., 2011). Jub interacts with both Sav and Wts and epistasis experiments have placed its activity between Hpo and Wts (Das Thakur et al., 2010). Zyx, on the other hand, was identified in an RNAi screen and appears to function specifically in the Fat branch of the Hpo pathway (Rauskolb et al., 2011). Hpo signalling has been linked to contact inhibition in mammalian cells (Zhao et al., 2007). In vertebrates, upon cell-cell contact, Jub proteins are recruited to the AJs (Marie et al., 2003). It is plausible that recruitment of Jub to the AJs enhances Wts activation by preventing Jub from interacting with Wts and Sav. Zyx is known to function in linking mechanical stress to cell behaviour (Hirata et al., 2008) and may therefore be a mechanosensitive element of the Hpo pathway. The issue of whether the vertebrate homologues of Zyx and Wts associate with one another is still to be addressed.

1.2.3.8 Tissue Specific differences in upstream regulation

The identity and relative importance of upstream inputs differs between tissues and developmental timing. While mutation of core pathway members induces a strong phenotype in both imaginal discs and the posterior follicle cells (PFCs), loss of function mutations for upstream regulators induce varying phenotypes. For example, loss of Mer and Kibra in the PFCs induces a phenotype akin to Hpo inactivation, whereas mutation in wing imaginal discs produces a phenotype much weaker than that observed in Hpo mutant discs (Baumgartner et al., 2010, Genevet et al., 2010, Hamaratoglu et al., 2006, MacDougall et al., 2001, Meignin et al., 2007, Milton et al., 2010, Pellock et al., 2007, Polesello and Tapon, 2007, Yu et al., 2008, Yu et al., 2010). On the other hand, Fat and Ex mutations both cause strong effects in imaginal discs, whereas Fat appears entirely dispensable in the ovary and Ex mutation induces only mild effects (Hamaratoglu et al.,

2006, Meignin et al., 2007, Milton et al., 2010, Pellock et al., 2007, Polesello and Tapon, 2007, Yu et al., 2008, Tyler and Baker, 2007). Upstream members of the pathway can also act partially redundantly in different tissues at different developmental stages, adding yet another level of complexity to pathway regulation. The basis for these differences remains unclear.

1.2.4 A highly conserved signalling pathway

The Hpo pathway is highly conserved throughout evolution. All members of the core cassette are conserved from yeast to humans. The yeast orthologues of Hpo, Mats and Wts are central components of the Mitotic Exit Network in budding yeast and the Septation Initiation Network in fission yeast (for review see (Hergovich and Hemmings, 2012)). Hence, Ste20 kinases regulate NDR kinase activity throughout evolution via similar genetic cascades.

The extent of functional conservation across species is evident from the fact that human YAP, Lats1, Mst2 and Mob1 can all be utilised *in vivo* to rescue the phenotype of their corresponding *Drosophila* mutation (Tao et al., 1999, Huang et al., 2005, Lai et al., 2005, Wu et al., 2003). The phosphorylation cascade observed in fruit flies is also present in mammals. Mammalian Sav (Callus et al., 2006), Lats (Chan et al., 2005) and Mob1 (Hirabayashi et al., 2008, Praskova et al., 2008) are all Mst substrates and Mst auto-phosphorylation is crucial to its activity (Figure 1.3B and Table 1.1) (Glantschnig et al., 2002). Sav is required for phosphorylation of Lats by Mst (Lee et al., 2008), while Lats itself interacts with Mob1 in order to potentiate its own kinase activity (Praskova et al., 2008, Hergovich et al., 2006). Depending on the cellular context, Lats1/2 phosphorylates YAP or TAZ (Zhang et al., 2008a). Phosphorylation inhibits YAP/TAZ function, promoting 14-3-3 binding and nuclear exit. Loss of Mst1/2 results in YAP-dependent proliferation, resistance to apoptosis and organ overgrowth (Dong et al., 2007, Lee et al., 2010, Zhao et al., 2008, Zhao et al., 2007, Song et al., 2010).

1.2.5 Cross-talk with other pathways

A wealth of connections between the Hpo pathway and other signalling pathways have been identified. The incorporation of various signals enables cells to integrate information such as nutritional state, developmental stage and position. A significant amount of Hpo's growth regulatory function involves the regulation of secreted factors, which activate other pathways. Examples of this form of growth control include regulation of the Wnt ligand Wg in the proximal part of the developing wing (Cho and Irvine, 2004, Cho et al., 2006, Neumann and Cohen, 1996), upregulation of the Notch ligand Serrate to promote leg growth (Mao et al., 2006, Buckles et al., 2001) and the activation of the secreted ligands EGFR (Zhang et al., 2009a, Dong et al., 2011) and CTGF in mammalian cells (Zhao et al., 2008, Zhang et al., 2008a, Shi-Wen et al., 2008). YAP has been linked to a number of oncogenic pathways including TGF-β, Wnt and EGF (Varelas and Wrana, 2011, Varelas et al., 2008, Imajo et al., 2012, Heallen et al., 2011, Zhang et al., 2009a). Most recently, a degree of crosstalk has been identified between the IIS/TOR pathway and Hpo signalling, which also appears to be conserved in mammalian cells (James et al., 2009, Lopez-Lago et al., 2009, Xin et al., 2011, Strassburger et al., 2012).

1.2.6 Multiple functions of the Hpo pathway

Hpo pathway function has been implicated in processes other than growth control. Hpo and Wts participate in the refinement of sensory neuron dendritic arborisations, demonstrating a role in terminal differentiation (Emoto et al., 2006, Parrish et al., 2007). The Hpo pathway has also been implicated in cell fate decisions in the developing eye (Mikeladze-Dvali et al., 2005, Zhang et al., 2011b) as well as in the regulation of maturation of the posterior follicle epithelium (Polesello and Tapon, 2007) (Meignin et al., 2007, Yu et al., 2008). PFCs mutant for *hpo*, *wts* or *sav*, exhibit defects in apicobasal polarity and overproliferate, leading to multi-layering of the follicular epithelium. This overproliferation is due to an inability to respond to Notch maturation signals in Hpo pathway mutant PFCs. In wing imaginal discs, in addition to growth, Hpo signalling also regulates the size of the apical domain (Genevet et al., 2009,

Hamaratoglu et al., 2009). Finally, upon exposure to ionising radiation, Hpo signalling is activated by the p53-dependent DNA damage checkpoint, leading to an apoptotic response (Colombani et al., 2006).

The mammalian Hpo pathway also performs multiple functions. Hpo mediated restriction of CDX2 expression to the outer cells of the mouse embryo is crucial for lineage specification of the trophectoderm and inner cell mass (Nishioka et al., 2009), again demonstrating a role for the pathway in cell fate decisions. Lats-dependent inhibition of the cyclin-dependent kinase cdc2 and potential roles in centrosome duplication and the mitotic exit network have also implicated the pathway in cell cycle progression (Turenchalk et al., 1999, Tao et al., 1999) Bothos et al., 2005, Hergovich et al., 2009, Toji et al., 2004, Oh et al., 2010).

Hence, depending on the context, Hpo signalling can have different functions including growth suppression, mediating stress-induced apoptosis and regulation of cell-fate decisions. Two of the main mechanisms enabling these different outcomes are the fact that Yki and YAP/TAZ have multiple binding partners and transcriptional targets (see section 1.2.2.4).

1.2.7 Hpo signalling in stem cells and progenitor populations

Alongside differentiated cell types, organs also harbour stem or progenitor cells (see section 1.3). Initial studies suggested that Hpo pathway function was restricted to the regulation of proliferation and apoptosis but recent evidence has implicated the Hpo pathway in the regulation of tissue-specific SC compartments. As discussed below, in several biological contexts, the Hpo pathway limits the expansion of tissue specific progenitor cells, providing a link between SC activity and the control of tissue size. One of the earliest findings in this area was that intestinal specific YAP expression or Mst1/2 knockout leads to expansion of the SC compartment (Camargo et al., 2007, Zhou et al., 2011). An in-depth discussion of the role of the Hpo pathway in the mammalian intestine can be found in Chapter 6.

1.2.7.1 Embryonic stem (ES) cells

Hpo signalling modulation has been linked to maintenance of pluripotency in mammalian ES cells in culture (Lian et al., 2010, Varelas et al., 2008). YAP and TEAD2 are expressed at high levels in ES cells (Ramalho-Santos et al., 2002). Modulation of TEAD4, Lats2 and YAP expression has been linked to cell fate determination in early mouse embryos (Nishioka et al., 2009). Increased YAP expression in cultured ES cells increases pluripotency, while downregulation is associated with differentiation (Lian et al., 2010). YAP levels are reduced during ES cell differentiation and phosphorylation of S127 is increased, leading to reduced nuclear YAP. The Hpo pathway interplays with several important pathways linked to ES cell pluripotency, such as TGFβ/BMP signalling or LIF pathways, both in culture and *in vivo* (Varelas et al., 2010, Varelas et al., 2008, Alarcon et al., 2009, Tamm et al., 2011). YAP/TAZ are believed to maintain pluripotency by promoting the transcriptional activity of these pathways and inducing the expression of stemness genes.

1.2.7.2 Liver

Knockdown of Mst1/2, Sav1 or Mer in the liver, or YAP overexpression, leads to an increase in liver size (Camargo et al., 2007, Zhang et al., 2010, Benhamouche et al., 2010, Lee et al., 2010, Lu et al., 2010, Song et al., 2010, Zhou et al., 2009). Hpo inactivation results in an increase in the number of small cells with liver SC features, known as oval cells (OCs). One of the main drawbacks of these studies is that, in all cases, where an increase in OC numbers was observed, Hpo signalling had been genetically manipulated in the entire organ. As a result, it is unclear whether OC expansion is due to cell-autonomous effects in the OC population or non cell-autonomous effects triggered in the hepatocyte lineage. In order to truly elucidate Hpo pathway function in the various liver cell types, cell-specific genetic manipulation is required. The data to date suggest that the Hpo pathway is required to restrict OC expansion, therefore maintaining quiescence of the liver SC pool and restricting the number of adult hepatocytes. The role of the Hpo pathway in liver repair and interactions with other signalling pathways are still to be investigated.

1.2.7.3 Skin

Hpo pathway function has been associated with the continuous regeneration cycles, which occur in the skin. Embryos null for Sav1 exhibit a thickening of the epidermal layer, which coincides with increased proliferation, progenitor expansion, reduced apoptosis and an absence of differentiation (Lee et al., 2008). Equally, skin progenitor-specific expression of YAP led to progenitor amplification and reduced differentiation, while YAP deletion impairs formation of the epidermis (Zhang et al., 2011a, Schlegelmilch et al., 2011). Skin-specific deletion of Mst1/2 surprisingly has no effect in mice up to five months, suggesting that the Hpo pathway can display tissue/context specific effects (Schlegelmilch et al., 2011). A critical upstream negative regulator of YAP in the context of the skin is the AJ component and tumour suppressor α-catenin (Schlegelmilch et al., 2011, Silvis et al., 2011). Evidence in the skin therefore supports the concept that AJs can act as molecular sensors of cell density and positioning. Increased cellular density leads to a higher number of AJs, which limits SC expansion by inactivating YAP. Lower cell numbers, such as in the growing embryo or upon wounding, would increase YAP levels in turn inducing proliferation. The role of the Hpo pathway in regeneration will be addressed in Chapter 6.

1.2.7.4 Nervous system

Neural progenitors generate the mature cells of the CNS. YAP expression colocalises with Sox-2, a marker for neural progenitor cells, in the progenitor zone of mouse, frog and chick neural tubes (Cao et al., 2008, Gee et al., 2011). Loss of either Mst1/2 or Lats1/2 results in progenitor expansion, partially due to upregulation of cell-cycle re-entry and stemness genes along with the suppression of pro-differentiation genes (Cao et al., 2008). Conversely, YAP LOF leads to cell death and precocious neural differentiation (Cao et al., 2008). YAP expression is also high in cerebellar granule neural precursor cells (CGNPs), which are found in increased number in medulloblastomas (Fernandez-L et al., 2009). Shh induces YAP nuclear localisation, thereby stimulating YAP-driven proliferation of the CGNP population. YAP thus

induces proliferation of neural progenitors, possibly by serving as a link between different pathways.

1.2.7.5 Heart

Cardiac-specific YAP overexpression or deletion of the upstream kinases generates embryos with increased proliferation and cardiomyocyte number (Oh et al., 2009b, Matsui et al., 2008), while YAP deletion results in myocardial hypoplasia (Xin et al., 2011). Genetic studies have demonstrated that YAP interacts with β-catenin to promote Wnt signalling (see section 1.4.4.1), which is itself a pro-proliferation and stemness signal in the heart (Heallen et al., 2011). Other studies have shown that YAP can activate the insulin-like growth factor signalling (IGF) pathway, which leads to inactivation of GSK-3β (Glycogen synthase kinase 3β), thereby blocking β– catenin degradation and promoting cardiomyocyte proliferation (Xin et al., 2011, Shiojima and Walsh, 2006). YAP therefore promotes cardiomyocyte proliferation by both indirectly promoting Wnt signalling via the IGF pathway and directly binding to β-catenin.

1.2.7.6 The Hpo pathway can regulate SC proliferation, self-renewal and differentiation

In summary, Hpo activation promotes SC quiescence and differentiation at the expense of proliferation in a number of embryonic and adult tissues. Interestingly, YAP overexpression has no effect on hematopoietic SC proliferation (Jansson and Larsson, 2012). In order to uncover whether Hpo pathway activity can be modulated with a view to increasing the regenerative potential of terminally differentiated organs, a better understanding of the interplay between Hpo activity and other signalling pathways is required, as well as an improved understanding of the molecular mechanisms involved. Therapies involving the manipulation of the Hpo pathway will have to overcome the challenge represented by the risk of malignant transformation associated with prolonged YAP expression. Amplification of the YAP1 gene is found in several cancer types, including hepatocellular carcinoma, breast cancer, oral squamous cell carcinomas,

medulloblastomas, and oesophageal squamous cell carcinomas (Overholtzer et al., 2006, Fernandez-L et al., 2009, Snijders et al., 2005, Zender et al., 2006, Muramatsu et al., 2011). Likewise, given the sensitivity of YAP/Yki to the mechanical environment, studies investigating the manipulation of the Hpo pathway in a SC therapy-oriented manner will require the generation of models, which truly recapitulate the physical properties of tissue-specific niches.

1.3 Stem cells

1.3.1 Definition of a stem cell

Adult stem cells (SCs) have two essential characteristics. Firstly, they ensure their continued existence by generating more SCs (self-renewal). Secondly, they are able to produce all the differentiated cell lineages of their respective tissue (tissue-renewal).

SCs are present in both the embryo and adult. Adult SCs are said to be multipotent, with the range of cell types they can give rise to being limited by the tissue in which they are located. These SCs play a vital role in tissue homeostasis, serving as a reservoir for cell replacement following cell death or injury. Tissue homeostasis is the process by which tissues maintain a relatively constant internal environment, regardless of conditions in the external environment.

1.3.2 The stem cell niche

SCs are often located in specific tissue microenvironments, known as niches. A SC niche can be defined as 'a specific location in a tissue where SCs can reside for an indefinite period of time and produce progeny cells whilst self-renewing' (Ohlstein et al., 2004). Niches have been identified in numerous tissues. Examples of tissues harbouring SC niches include the germline, skin, hair follicle, mammary gland, central and peripheral nervous systems and the digestive and respiratory tracts (Wagers, 2012). Some SC niches rely on surrounding non-epithelial cells (stromal), while others involve contact with the basement membrane (Morrison and Spradling, 2008) or neighbouring

epithelial cells (Sato et al., 2011). The *Drosophila* ovary provided the first characterisation of a SC niche at both a cellular and functional level (Xie and Spradling, 2000). Both SC intrinsic factors and signals from the surrounding microenvironment regulate SC activity and niche size.

1.3.3 Stem cell division and dynamics

SCs can undergo both symmetric and asymmetric divisions. Symmetric divisions produce two daughter cells of the same fate, generally two SCs or two progenitor cells. Asymmetric divisions generate one SC and one less primitive daughter cell and are thus a conserved mechanism for establishing different cell fates (Horvitz and Herskowitz, 1992). The majority of progress in our understanding of asymmetric division has come from studies in the *Drosophila* neuroblast (see section 1.3.4.3) (as reviewed in (Knoblich, 2010)). Asymmetric divisions ensure that the SC pool is maintained alongside the generation of differentiated cells. Long-term homeostasis can be achieved either by asymmetric cell division or population asymmetry (Watt and Hogan, 2000). Studies in systems including both the mammalian intestine and hair follicle have revealed that SCs can follow stochastic behavioural patterns, in which regular SC loss is compensated for by proliferation of neighbouring SCs (population asymmetry). This process is referred to as neutral drift and results in progressive expansion of some clones, while others become extinct (Lopez-Garcia et al., 2010, Snippert et al., 2010, Zhang et al., 2009b, Klein et al., 2010, Clayton et al., 2007).

1.3.4 Stem cell populations in *Drosophila*

SC niches function via common mechanisms. Using both the male and female *Drosophila* germline niches as a starting point, I will introduce some of the key concepts in SC regulation. I will also briefly discuss other known *Drosophila* SC populations**.** A detailed description of current knowledge surrounding the murine intestinal SC population can be found in section 1.4.3.

1.3.4.1 Drosophila female germline

The *Drosophila* ovaries consist of 16-18 ovarioles, each of which have a germline SC (GSC) niche at the tip, supporting two to three GSCs throughout pupal and adult life. Three types of somatic cell establish the GSC niche. The Cap cells ensure that the GSCs remain in contact with the niche via AJs (Song and Xie, 2002), adherence to the niche being a common feature of SC regulation. Quiescent escort cells are intermingled with the GSCs. The third somatic niche cell type is the terminal filament cell, which produces Upd and related cytokines. Upd secretion activates Jak/Stat signalling in the cap and escort cells leading to BMP activation (Decotto and Spradling, 2005, Wang et al., 2008, Lopez-Onieva et al., 2008). Regulation by local signalling is another common feature of SC niches. In the case of female GSCs, BMP signalling is sufficient for GSC self-renewal. BMP ligand expression by the cap cells leads to GSC BMP activity, which inhibits expression of the master differentiation gene *bag-of-marbles* (*bam*) (Chen and McKearin, 2003, Song et al., 2004).

GSC divisions are asymmetric, giving rise to GSC daughter cells called cystoblasts. Asymmetry is achieved not through any inherent difference between daughter cells but because only one proximal daughter retains adhesive contact with the SC niche, while the more distal daughter is displaced activating *bam* transcription and differentiation (Chen and McKearin, 2003). Cystoblasts go on to form cystocytes, which eventually give rise to the oocyte and supporting nurse cells.

The anteriorly localised germarium is also home to two somatic follicle SCs (FSCs), whose progeny surround the female germline (oocyte and nurse cells). FSC maintenance requires Hh, Wg and Dpp signalling, with adhesion through E.cadherin and β-catenin again crucial to niche function (Song and Xie, 2002).

1.3.4.2 Drosophila male germline

The testis is home to a large GSC niche regulating sperm production. 10-15 support cells form the hub, which is in contact with 7-12 GSCs. Each GSC is surrounded by two

somatic cyst SCs (CySCs), which also play a role in GSC maintenance. GSCs and CySCs undergo asymmetric divisions. GSCs give rise to gonialblasts, eventually leading to sperm production, while CySCs produce cyst cells, which exit the cell cycle, increase in size and encapsulate the developing spermatogonia and spermocytes.

Similar to the case in the female germline, hub cells secrete Upd ligands, which trigger Jak/Stat activity in both the GSCs and CySCs. Stat is required for both CySC self-renewal and GSC adhesion to the hub (Tulina and Matunis, 2001, Leatherman and Dinardo, 2008, Leatherman and Dinardo, 2010). GSC self-renewal is dependent upon BMP signalling from the somatic cells, as is the case in females, which inhibits *bam* transcription (McKearin and Ohlstein, 1995). SCs not only employ local signals in their regulation but are also subject to regulation by systemic factors. Maintenance of GSCs in both male and female *Drosophila* is directly affected by insulin signalling (IIS) originating from insulin-producing cells in the CNS (LaFever and Drummond-Barbosa, 2005, Ueishi et al., 2009, Wang and Jones, 2011).

GSCs divide asymmetrically via oriented divisions. In males, the division plane is oriented perpendicular to the hub (Yamashita et al., 2003). In females the GSC spindle is oriented perpendicular to the cap cells. One daughter cell therefore maintains contact with the niche and the other is displaced. Epigenetic mechanisms are also utilised in SC regulation. GSCs are subject to regulation of chromatin structure and transcriptional activity by chromatin remodellers. In females, systemic secretion of the steroid hormone, ecdysone, interacts with chromatin remodellers in order to positively regulate BMP signalling (Ables and Drummond-Barbosa, 2010).

1.3.4.3 *Drosophila* neuroblast

The neurons and glia of the *Drosophila* central nervous system (CNS) are generated by neural SC-like progenitors, termed neuroblasts. These are specified from the neuroectoderm via a process involving Notch signalling and proneural genes during embryogenesis. Once specified, neuroblasts delaminate before generating intermediate progenitor cells called ganglion mother cells (GMCs) via asymmetric divisions.

Differential cell fate is achieved via asymmetric segregation of fate determinants. The self-renewed neuroblast inherits apical protein complexes, while the GMC inherits basal protein complexes, which contain fate determinants such as the homeodomain protein Prospero (Knoblich et al., 1995, Spana and Doe, 1995). The GMC then divides a further time giving rise to neurons or glia. Similarly, in the *Drosophila* peripheral nervous system (PNS), sensory organ precursor (SOP) cells undergo several rounds of asymmetric division. Numb, a negative regulator of Notch signalling localises basally to the cortical crescent during both neuroblast and SOP mitosis (Uemura et al., 1989, Rhyu et al., 1994).

1.3.4.4 Drosophila hindgut

The identification of an adult ISC population in the *Drosophila* midgut (see section 1.5) (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006), was followed by a study reporting that the adult hindgut also harbours an actively dividing ISC population (Takashima et al., 2008). Takashima and colleagues claim that a "hindgut proliferation zone" replaces spent cells. Wg signalling in the ISCs reportedly leads to proliferation, while Hh is required for differentiation, raising exciting parallels to the mammalian intestine (van den Brink et al., 2004, Pinto et al., 2003). Work published subsequently by Fox and Spradling argues against the existence of active hindgut ISCs (Fox and Spradling, 2009). Their study suggests that although rarely dividing cells do exist, they only divide when adjacent to dying cells, suggesting the presence of a quiescent hindgut ISC population. Interestingly, a relatively quiescent ISC population has also been shown to divide in response to intestinal stress in the mammalian intestine (see section 1.4.3) (Yan et al., 2012).

1.3.4.5 Drosophila malpighian tubules

Drosophila have two pairs of malpighian tubules, which are functionally equivalent to the mammalian kidney. Multipotent renal and nephric SCs (RNSCs) identified in the MTs provide a further model system in which to study adult SCs (Singh et al., 2007). Autocrine Jak/Stat signalling, originating from the RNSCs themselves, regulates RNSC

self-renewal, seemingly independently of any external niche (Singh et al., 2007). Sav LOF in the RNSCs results in tumour formation via oncogenic Ras activation, suggesting a possible role for the Hpo pathway (Zeng et al., 2010). Most recently, work from the same lab, again utilising clonal analysis, identified a SC pool regulated by Jak/Stat signalling at the foregut/midgut junction, which they termed gastric stem cells (GaSCs) (Singh et al., 2011). A quiescent SC population is also believed to reside in the midgut copper cell region (Strand and Micchelli, 2011).

1.3.5 Stem cells and ageing

Tissue homeostasis and regenerative capacity decline with age (reviewed in (Jones and Rando, 2011)). Ageing is not solely due to SC dysfunction but also related to local and systemic changes. It is hoped that developing approaches to delay, prevent or even reverse SC ageing, will yield improved results in the treatment of a number of age-related diseases. Likewise, understanding the mechanisms, which regulate ageing is vital to regenerative medicine.

Several factors reduce SC function and number in ageing organisms. Firstly, there is a failure of self-renewal in aged SCs. In flies, for example, spermatogenesis and oogenesis are decreased with age resulting from the presence of fewer GSCs and a reduced ability to self-renew (Boyle et al., 2007, Pan et al., 2007, Wallenfang et al., 2006). These changes coincide with a reduction in the number of self-renewal factors and cell-cell adhesion molecules being expressed by the supporting niche. Dietary restriction is known to extend lifespan via effects on both IIS and TOR signalling (Clancy et al., 2002, Kenyon, 2001, Bjedov et al., 2010). Dilps are among the systemic factors known to affect GSC proliferation and maintenance in fruit flies (LaFever and Drummond-Barbosa, 2005, Ueishi et al., 2009, Wang and Jones, 2011). Reduced IIS has been documented in the ovaries of ageing fruit flies (Hsu and Drummond-Barbosa, 2009).

Cell-intrinsic changes such as genomic instability, changes in transcriptional/epigenetic regulators and changes in DNA damage responses also affect aged SC function. The

relative contribution of increased cellular senescence and apoptosis to tissue ageing is less clear. Senescence refers to irreversible cell cycle arrest, which can result in the secretion of factors, which might have negative effects on neighbouring cells (Campisi, 2005).

Ageing tissues not only demonstrate defects in SC self-renewal but progenitor differentiation. This can range from skewed fate distributions, to the generation of abnormal cell fates. One of the best examples of this is in the ageing *Drosophila* midgut (see section 1.5.8.1), where an accumulation of mis-differentiated daughter cells expressing both SC and differentiated cell markers coincides with a loss of homeostasis (Choi et al., 2008, Biteau et al., 2008, Park et al., 2009).

1.3.6 Cancer stem cells

The majority of tumours are monoclonal in origin (Vogelstein et al., 1985, Fialkow, 1976). Thus, cells of origin must be able to generate the various cell types, which comprise the tumour. The cancer stem cell theory postulates that tumours are generated and maintained by a small subset of cells, which are able to self-renew generating the rest of the tumour population, these being termed cancer stem cells (as reviewed in (Reya et al., 2001)). This theory has existed for over 150 years. Despite growing support, it still remains possible that genetic changes could enable a differentiated cell to gain tumour-initiating properties. Colonic SCs, for example, can divide symmetrically or asymmetrically. The progeny of one SC will go on to dominate the niche and ultimately the entire crypt (monoclonal conversion), facilitating the spread of mutations within a crypt. Colorectal cancer (CRC) develops when a single cell is mutant for APC (Adenomatous polyposis coli), whether this initiating mutation has to occur in a SC remains unclear (van der Flier and Clevers, 2008).

1.4 The mammalian intestine

The mammalian digestive tract begins with food passing down the oesophagus to the stomach. The gastrointestinal (GI) tract then divides into the nutrient absorbing small

intestine followed by the large intestine (or colon) where further nutrient, water and electrolyte absorption occurs. The absorption and processing of nutrients is performed by the intestinal epithelium, termed mucosa.

1.4.1   Organisation of the intestinal epithelium

The small intestinal epithelium is composed of crypts and villi (Figure 1.4A). The crypts of Lieberkühn are a result of epithelial invaginations into the gut mucosa (Crosnier et al., 2006). Cell proliferation occurs in the crypts, while the villi extend into the intestinal lumen and are lined by a layer of columnar cells. On the basal side of the epithelium is the basement membrane (BM), a collagenous extra-cellular matrix (Sengupta and MacDonald, 2007). The space between the BM and outer musculature is divided into three layers: a dense layer of connective tissue termed the submucosa, an additional muscle layer called the muscularis mucosae and the lamina propria. The lamina propria contains connective tissue, lymph nodes (termed Peyer's patches), immune cells, blood vessels and myofibroblasts. Cellular organisation in the colon is essentially similar to the small intestine except for the absence of villi and Paneth cells (Scoville et al., 2008).

The adult posterior midgut, on which my study is focussed, is the *Drosophila* functional equivalent to the small intestine. In this section, I will therefore focus mainly on the small intestine.
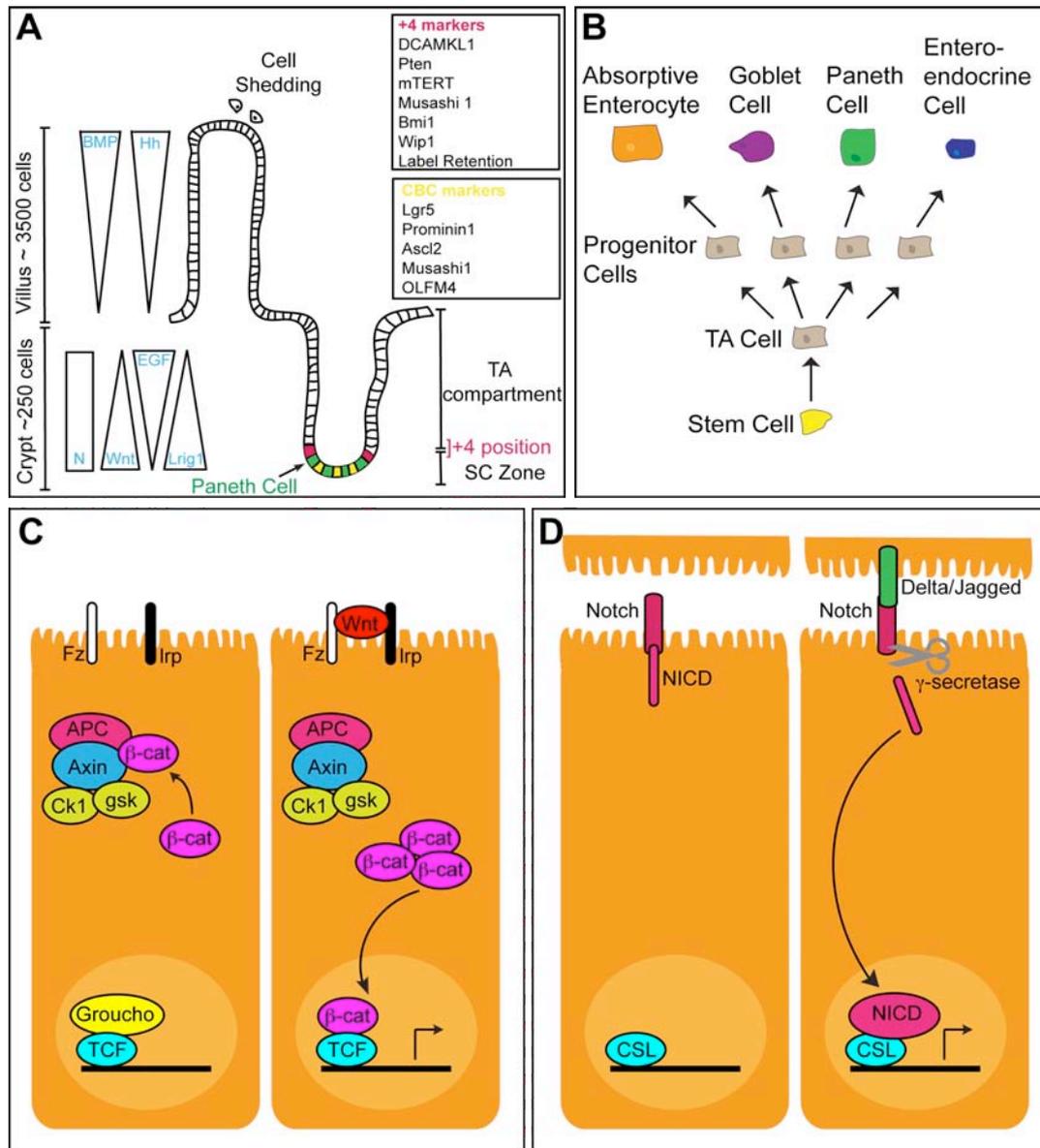
Figure 1.4: Organisation of the mammalian small intestine

(A) Organisation of the small intestine and reported ISC markers. Epithelial-derived Hh and Wnt ligands trigger stromal BMP production, which signals back to the epithelium to restrict proliferation. Lrig1 dampens EGF triggered SC expansion (Wong et al., 2012, Powell et al., 2012). **(B)** Cell lineages of the small intestine. **(C-D)** Role of Wnt and Notch signalling pathways adapted from (Radtke and Clevers, 2005). (C) Wnt-responsive cells have a receptor complex consisting of a frizzled seven-transmembrane receptor (Fz) and Lrp5 or Lrp6. In the absence of secreted Wnt factor, the destruction complex (APC, axin, and the kinases CK1 and GSK3) induces degradation of β-catenin. Tcf is bound by co-repressors, such as Groucho, leading to Wnt target gene repression. Receptor activation blocks the destruction complex allowing β-catenin to bind to nuclear Tcf and activate target gene transcription. (D) Cell bound Jagged or Delta (Notch ligands) bind the Notch receptor leading to sequential proteolytic steps. This results in release of the NICD, which travels to the nucleus to complex with the TF CSL thereby activating target gene transcription.

1.4.2 Epithelial renewal

Effective tissue homeostasis requires a fine balance between the removal of dead cells and production of new ones. The GI tract is dependent on constant self-renewal, as ingested food, pathogens and toxins can damage the epithelium. The entire population of differentiated intestinal cells is replaced every few days, meaning that the intestinal epithelium renews itself more rapidly than any other tissue in the vertebrate body. Adult ISCs give rise to all the mature cell types of the intestinal epithelium and any imbalance in this process can lead to diseases, such as cancer (Radtke and Clevers, 2005). In the adult mammalian small intestine, the ISC population is located near the base of the crypts of Lieberkühn. Each crypt gives rise to approximately 300 cells per day (Marshman et al., 2002). This process is supported by four to six ISCs per crypt (Bjerknes and Cheng, 1999), whose progeny undergo transit-amplifying divisions as they migrate upwards (Figure 1.4A). The transit-amplifying cells (TA cells) have limited self-renewal capacity, dividing three to four times before their offspring become the differentiated cells, which occupy the villi. Upon reaching the villus tips, cells undergo apoptosis and are shed into the lumen (Figure 1.4A) (Heath, 1996).

The differentiated intestinal cells can be divided into two main subgroups: the absorptive enterocyte (EC) lineage and the secretory lineage (Figure 1.4B) (Radtke and Clevers, 2005). The role of the absorptive cells is nutrient absorption and hydrolase secretion. The secretory cell lineage can be divided into three cell-types. Goblet cells secrete protective mucins, Enteroendocrine cells secrete hormones such as serotonin, substance P and secretin, and the Paneth cells, located at the bottom of the crypt, secrete antimicrobial agents.

1.4.3 Adult intestinal stem cells

The presence of a SC population in the adult crypts was first proven in experiments tracking the inheritance patterns of genetic marks randomly introduced to single crypt cells via somatic mutation (Bjerknes and Cheng, 1999, Bjerknes and Cheng, 2002).

Clones comprising marked cells of all intestinal lineages were generated, confirming that the original mutation had been induced in a single multipotent SC (Barker et al., 2010). These early experiments did not however uncover the identity of the ISCs, since the mutations were induced at random.

A number of assays have been utilised in the identification of SC populations and markers. Approaches include label retention, *in vitro* culture, transplantation and *in vivo* lineage tracing (as reviewed in (Snippert and Clevers, 2011)). Culture and transplantation strategies focus on stem cell potential, whereas lineage tracing measures the actual "stemness" of cells *in vivo* (Snippert and Clevers, 2011). Key to the use of lineage tracing strategies is the genetic marking of SCs, enabling the tracing of daughter populations. Some SC markers are still being proposed based on position within the crypt alone, indicating a need for further improvement in *in vivo* SC identification techniques (Barker et al., 2010).

Two models of SC identity have dominated the ISC field. The +4 model, developed following early cell tracking experiments, suggests that a common cell origin exists at position +4 (Figure 1.4A) (Cairnie et al., 1965). The SC zone model arose in the 1970s when immature cycling cells, known as crypt base columnar cells (CBCs), were shown to be located between the Paneth cells (Cheng and Leblond, 1974). SC markers have been proposed for both CBCs and +4 SCs (Barker et al., 2010). The most reliable +4 marker to date is Bmi-1, a component of the Polycomb transcriptional repressor complex (Sangiorgi and Capecchi, 2008). Lineage tracing experiments identified the Wnt target gene, Leucine-rich G protein-coupled receptor 5 (*Lgr5*) as a CBC marker (Barker et al., 2007, Barker and Clevers, 2010). Isolated Lgr5+ cells are capable of generating self-renewing intestinal organoids *in vitro* (Sato et al., 2009). The fact that Paneth cell ablation leads to a loss of the Lgr5+ SC compartment (Sato et al., 2011) and that isolated Lgr5+ cells can generate intestinal organoids in the absence of mesenchyme components, suggests that Paneth cell secretions could constitute the Lgr5+ niche (Sato et al., 2011). Work from Kim and colleagues, however argues against this, claiming that Paneth cells are also dispensable to CBC function (Kim et al., 2012).

Traditionally adult SCs were said to divide slowly in order to avoid exhaustion of the SC pool (Orford and Scadden, 2008). Recently, the notion that a rapidly dividing SC population might coexist with a more quiescent 'reserve' SC population has evolved (Li and Clevers, 2010). Bmi1 expressing +4 cells and Lgr5+ CBCs can both regenerate the intestinal epithelium (Barker et al., 2007, Sangiorgi and Capecchi, 2008). A hierarchy exists between these two lineages, whereby Lgr5+ CBCs are in fact dispensable for villus homeostasis. Bmi1+ cells represent a more quiescent SC population capable of replenishing the Lgr5 population when under high regenerative demand (Tian et al., 2011, Yan et al., 2012).

1.4.4 Key signalling pathways controlling intestinal crypt homeostasis

Studies in mice have provided insight into the signalling pathways controlling various aspects of intestinal development and homeostasis (Scoville et al., 2008). The gut mesenchyme tissue provides some of these signals. A number of pathways including Notch, BMP, Wnt and JNK play key roles (Sancho et al., 2004, Sancho et al., 2009).

1.4.4.1 Wnt

Wnt genes encode secreted molecules. The three Wnt ligands responsible for canonical Wnt signalling in the intestine are Wnts 3, 6 and 9B, all of which are only expressed in crypt epithelial cells (Gregorieff et al., 2005). β-catenin (Armadillo in flies) is a key molecule in the Wnt cascade. When the pathway is inactive, the scaffolding proteins APC and axin bind to β-catenin, targeting it for proteasomal degradation (Figure 1.4C). Upon Wnt ligand binding, β-catenin accumulates and binds to nuclear DNA binding proteins of the TCF/LEF family (Giles et al., 2003). Mutations in the APC tumour suppressor gene are associated with a large number of CRCs, establishing the Wnt pathway as a key regulator of SC and TA cell proliferation (Bienz and Clevers, 2000, Nakamura et al., 2007). Wnt signalling maintains crypt progenitor compartments and is expressed at higher levels in the ISCs compared to the TA cells (Figure 1.4A) (Fevr et al., 2007).

1.4.4.2 Notch

Notch signalling regulates fate decisions and differentiation processes in various contexts, including the intestine. Upon ligand binding (Delta or Jagged in mammals) Notch proteins are cleaved by γ-secretase allowing the Notch intracellular domain (NICD) to translocate to the nucleus, resulting in an active transcriptional complex with CSL (RBP-J in vertebrates, Su(H) in flies) (Figure 1.4D) (Artavanis-Tsakonas et al., 1999). Notch inactivation, for example by deletion of the Notch target Hes1, expands the secretory cell population and reduces the proliferative compartment (van Es et al., 2005). Notch overactivation by NICD expression inhibits secretory lineages and amplifies the progenitor pool (Fre et al., 2005). Hence, Notch signalling is required for maintaining cells in a proliferative state and for lineage specification. It is believed that progenitors require both Notch and Wnt signals for continued proliferation, thus a complicated degree of crosstalk exists between these two pathways.

1.5 The adult *Drosophila* posterior midgut

My PhD project utilised the adult *Drosophila* posterior midgut as a model system in which to investigate ISC biology. In this section, I will give a general overview of the *Drosophila* GI tract before focussing on the adult posterior midgut.

1.5.1 Structure of the *Drosophila* intestinal tract

The adult *Drosophila* GI tract can be divided into three sections, namely the foregut, midgut and hindgut, each of which has a distinct function and cell composition (Figure 1.5) (Royet, 2011). The foregut is a tube-like structure, which connects to the saliva-secreting salivary glands and a temporary food storage pouch called the crop. Absorption begins in the midgut (Edgecomb et al., 1994). The proventriculus, the outermost extremity of the midgut, initiates the mechanical breakdown of food. A further role of the proventriculus is synthesis of the peritrophic matrix. The peritrophic matrix is a chitin-containing membrane, which lines the midgut. In a role analogous to mucous secretions in the mammalian intestine, it is important for gut structure,

facilitates digestion and is a line of defence against bacterial infection (Kuraishi et al., 2011).

The midgut is equivalent to the mammalian small intestine. The midgut lumen exhibits gradients of pH. First, is the near neutral anterior midgut, followed by a short, narrow, strongly acidic region and then a wider, increasingly alkaline section (Dubreuil, 2004). Iron and copper cells are essential to maintaining acidity in the middle "stomach-like" region (Shanbhag and Tripathi, 2009). The midgut-hindgut boundary is home to the renal-like Malpighian Tubules (see section 1.3.4.5) responsible for absorbing solutes, water and waste from the surrounding hemolymph and releasing them into the gut as solid nitrogenous compounds. The hindgut absorbs water and ions before waste expulsion via the rectum. Neurons from the CNS innervate all three sections of the GI tract (Spiess et al., 2008). The stomatogastric CNS is responsible for feeding and transition of food into the midgut (Schoofs et al., 2009). Distinct neurons innervate specific gut regions regulating functions such as diuresis control and pH (Cognigni et al., 2011).
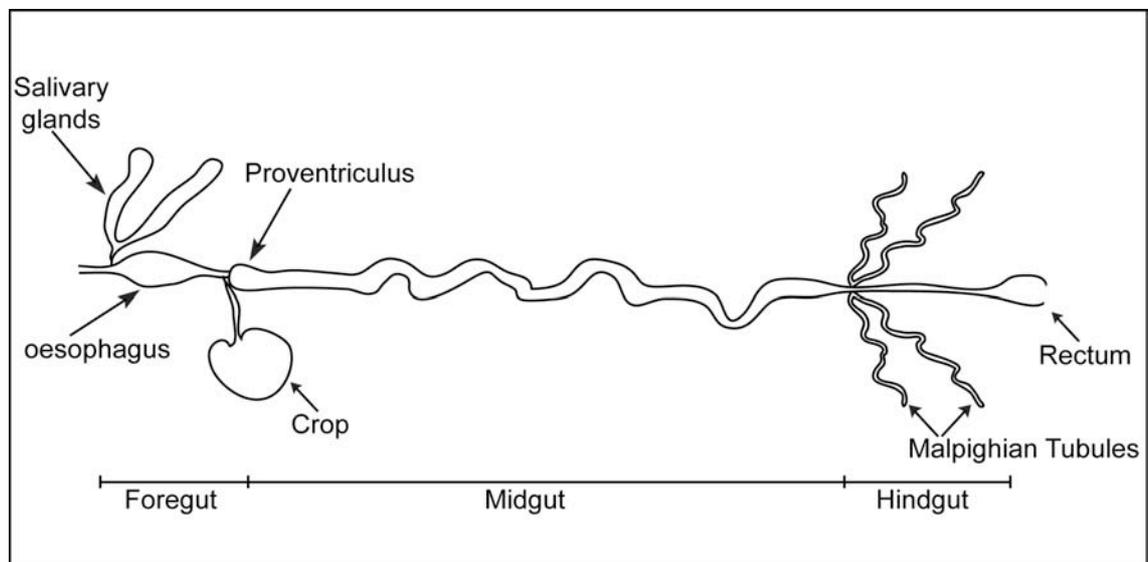
Figure 1.5: Structure of the *Drosophila* gastrointestinal tract

1.5.2 Development of the adult midgut

The *Drosophila* midgut, like the mammalian small intestine, derives from the endoderm. The hindgut and foregut are ectodermal in origin. The embryonic endoderm begins as anterior and posterior clusters of proliferating mesenchymal cells (Micchelli, 2011). During embyryogenesis, the endodermal midgut precursors undergo a mesenchymal to epithelial transition forming the larval midgut epithelium (Tepass and Hartenstein, 1994). Larval midgut cells increase in size by endoreplication. The larval gut ultimately degenerates completely and is replaced during the late larval/early pupal stages.

A pool of adult midgut progenitor cells (AMPs) is responsible for generating all epithelial cells of the adult gut (Jiang and Edgar, 2009). During larval development, AMPs proliferate in two distinct phases. At first, symmetrically dividing AMPs disperse, before later, by mid third instar, proliferating in distinct islands and eventually fusing during metamorphosis to form the adult midgut. Mitogenic signalling via EGFR/Ras/MAPK signalling is required for AMP proliferation. The EGFR ligand Vein is expressed by the visceral muscle (VM), while Spitz and Keren are expressed by the AMPs themselves (Jiang and Edgar, 2009). Ecdysone signalling also coordinates events during morphogenesis and acts directly on AMPs to regulate their expansion (Micchelli et al., 2010).

In young larvae, AMPs are identifiable as small, diploid cells, which express both *escargot* (*esg+*), a member of the snail/slug superfamily of TFs and a marker for adult ISCs (see section 1.5.4), and the Notch ligand Delta (D1) (Jiang and Edgar, 2009, Mathur et al., 2010, Micchelli et al., 2010). The AMP division preceding island formation is asymmetric, giving rise to both a D1 positive AMP and a Notch reporter (*Su(H)Gbe-LacZ*) positive peripheral cell (PC) (Mathur et al., 2010).

PCs were described previously as cells extending processes, which wrap around clusters of AMPs, but their function was unknown. The PC acts as a cell niche, instructing the AMPs to self-renew, repressing their differentiation and preventing the islands from merging. Similar to the *Drosophila* ovary and testes (see section 1.3.4), the PCs prevent premature AMP differentiation via BMP signalling. By late third instar, 20% of the

AMP islands have a second PC (Mathur et al., 2010). Upon metamorphosis, PCs undergo apoptosis. The AMPs are thus free to differentiate and the various AMP nests merge to form a continuous layer, which overgrows the former larval gut. One AMP per island does not differentiate and presumably becomes the future adult ISC.

A number of questions are yet to be addressed with regards to gut development. For example, what triggers the initial asymmetric AMP division and later PC apoptosis? It has been suggested that the AMP, which initiates island formation could be the future adult ISC, but this is yet to be addressed experimentally.

1.5.3 Cellular organisation of the posterior midgut

The fly midgut and mammalian intestine share considerable similarities with regards to tissue organisation. The midgut epithelial monolayer is composed of large, absorptive enterocytes (ECs) aligned basally by the BM, under which are both longitudinal and circular muscles (the VM) (Figure 1.6A) (Jiang and Edgar, 2009). As is the case in the mammalian intestine, the midgut is home to a population of basally located adult ISCs (Figure 1.6B-B'). Unlike the mammalian crypts, extensive folding does not occur in the posterior midgut. The ECs exhibit apicobasal polarity with a distinct striated border on their apical surface (Figure 1.6C) (Micchelli and Perrimon, 2006). Microvilli on the apical surface of the ECs increase the surface area facing the lumen (Shanbhag and Tripathi, 2009). The peritrophic membrane, which helps prevent microbes from accessing the midgut epithelium, is located above the actin-rich apical brush border (Baumann, 2001, Gartner, 1970).

Interspersed with the EC monolayer are the hormone-secreting enteroendocrine (EE) cells (Figure 1.6A). EE cells represent around 10% of epithelial cells in the gut. All known EE cells express Prospero (Pros) and different EE sub-populations are identifiable by their expression of different peptides such as allatostatin and tachykinin (Ohlstein and Spradling, 2006, Yoon and Stay, 1995, Siviter et al., 2000). The hormones produced by EE cells are involved in a range of functions such as nutrient utilisation, gastric motility and feeding behaviour.
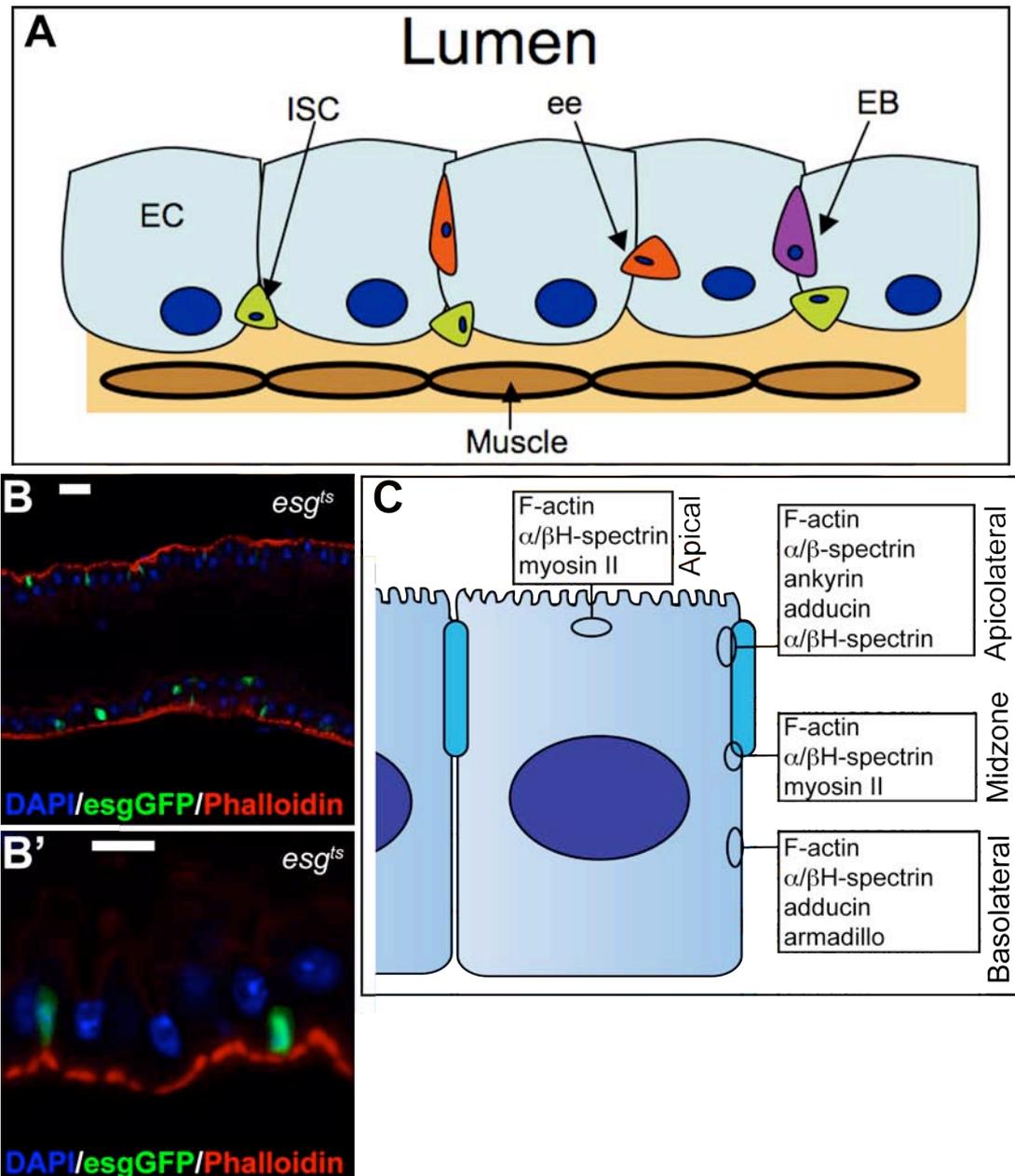
Figure 1.6: Organisation of the *Drosophila* adult posterior midgut

(A) Diagram of the adult midgut. **(B-B')** Confocal micrographs of adult posterior midguts demonstrating the basal location of the small *esg+* ISCs. Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green) and Phalloidin is in red. Scale bars = 20μm. **(C)** Schematic outline of posterior midgut ECs illustrating the composition of the membrane skeleton on the various membrane domains. Blue bars represent septate junctions (adapted from (Baumann, 2001)).

1.5.4 The adult posterior midgut intestinal stem cell population

The discovery of somatic SCs in the midgut has established *Drosophila* as a model in which to study SC-mediated tissue homeostasis. The Spradling and Perrimon groups first described an actively proliferating adult midgut ISC population in 2006 (Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006). Focussing on the posterior midgut due to its cellular simplicity and using genetic mosaic analysis, both groups carried out lineage tracing experiments showing that the differentiated cells of the midgut arise from a common lineage. The intestinal epithelium includes both large polyploid nuclei and small, basally located diploid nuclei (Figure 1.6B-B'). The Perrimon group showed that, while both these populations are able to synthesise DNA (BrdU, Bromodeoxyuridine incorporation) only a small subset of the diploid cells actually undergo cell division, as assayed by Phospho-histone H3 (PH3) staining. These small, dividing cells are the adult posterior midgut ISC population.

The basally located ISCs can give rise to both enterocytes (ECs) and small secretory EE cells, both of which undergo weekly turnover. ISCs can be identified by their small nuclear size and expression of the Notch ligand Delta (Dl). ISC self-renewal produces an identical daughter ISC along with an immature diploid daughter (progenitor) cell, termed the enteroblast (EB) (Figure 1.7A). ISCs and EBs both express the Snail/Slug family transcription factor *escargot (esg+)* (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). *esg* expression has previously been shown to be required for maintenance of diploidy (Fuse et al., 1994). These two *esg+* cell populations are often found in pairs and can be distinguished based on expression of Dl in the ISC and *Su(H)Gbe-LacZ* (a transcriptional reporter of Notch signalling) in the EB. While the ISC is in contact with the BM, the EB is located more apically.

In contrast to the mammalian TA cells, EBs undergo no transit divisions. 90% of EBs become absorptive ECs, identifiable by their large endoreplicating nuclei and Pdm1 (Nubbin) expression (Figure 1.7A) (Lee et al., 2009, Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006). The remaining 10% of cells produced are small, Pros+, EE cells.

Clonal expansion occurs from the basal ISC upwards. Individual SC clones have been shown to support approximately 15–20 cells in total. Dividing the total number of cells in the midgut by this figure gives an estimate of approximately 800–1,000 ISCs in the posterior midgut (Ohlstein and Spradling, 2006). Gut turnover rate varies greatly, even in healthy animals, and is strongly affected by age and diet (Jiang et al., 2009).

1.5.4.1 Delta/Notch signalling regulates differentiation and cell-fate specification

Notch signalling is a short-range communication pathway involved in the regulation of processes including proliferation, SC maintenance, fate specification, differentiation and cell death (see section 1.4.4.2 and Figure 1.4) (Artavanis-Tsakonas et al., 1999). Generally, the pathway links the cell fate decisions of neighbouring cells in a process termed lateral inhibition. In most Notch-dependent processes, a signal-sending cell, expressing Notch ligand (the transmembrane proteins Dl or Serrate in flies) activates Notch activity in a neighbouring cell via the Notch receptor (Figure 1.7B).

Notch is an essential regulator of commitment and terminal differentiation in ISCs (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2007, Bardin et al., 2010, Perdigoto et al., 2011). In contrast to the mammalian intestine, where Notch activation induces proliferation and progenitor cell expansion (Fre et al., 2005, van Es et al., 2005), Notch signalling in the midgut drives cells into a post-mitotic state (Figure 1.7A). The fact that there are no TA cells in the midgut could explain this apparent difference in Notch function.

In both *Drosophila* and mammals, Notch activation favours absorptive differentiation at the expense of secretory cells (Bardin et al., 2010, Fre et al., 2005, Ohlstein and Spradling, 2007, van Es et al., 2005). Notch inactivation in the midgut blocks EC cell fate whilst increasing ISCs, EE cells and proliferation. Whilst commitment requires high levels of Notch signalling, presumably to prevent SC loss through differentiation, terminal differentiation to a particular cell fate can be achieved via lower levels of
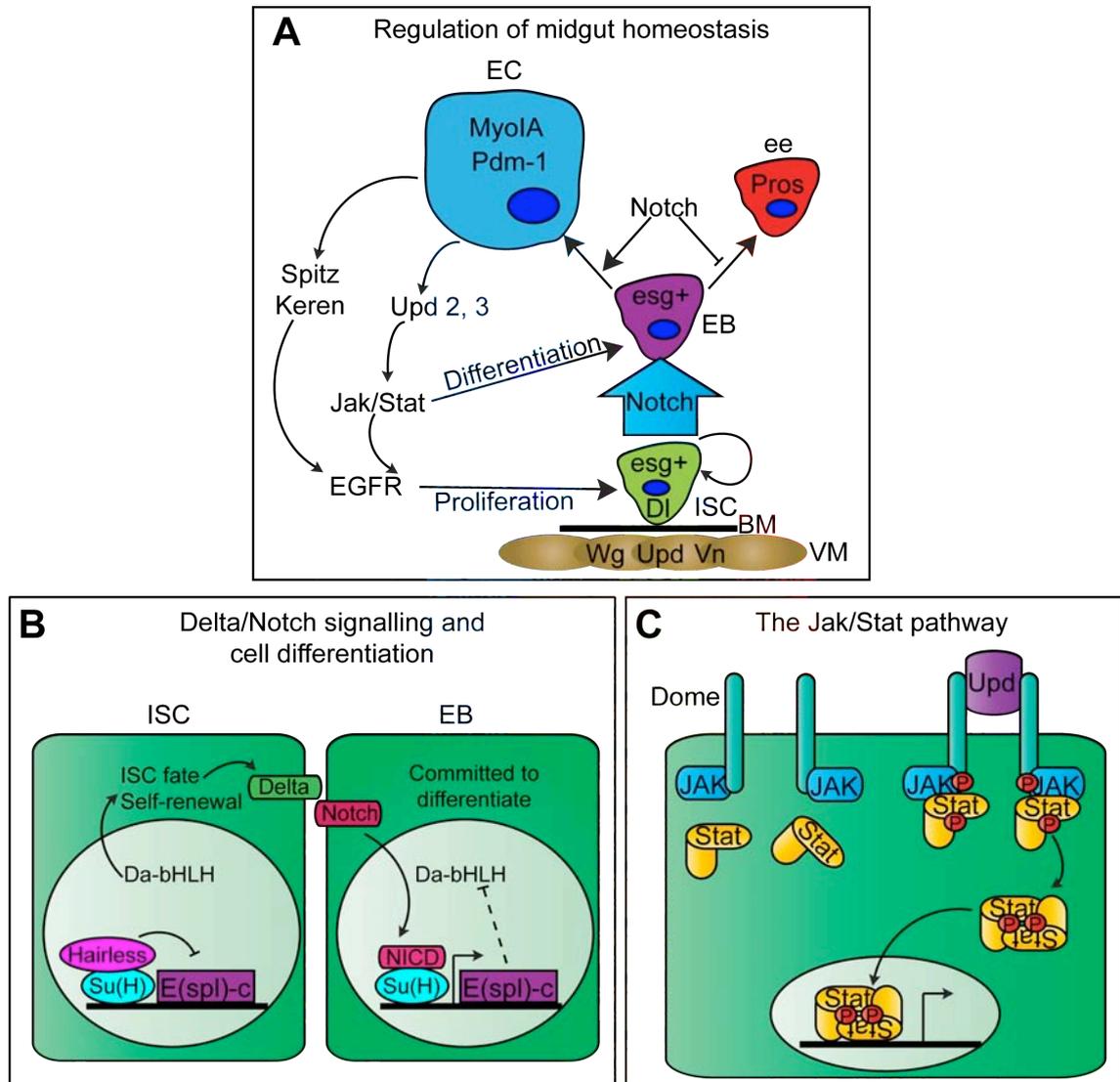
Figure 1.7: Regulation of midgut homeostasis

(A) Signalling pathways regulating midgut homeostasis (see text for details). **(B)** Dl/Notch signalling and ISC division (adapted from (Hou, 2010)). Dl is expressed on a newly emerged ISC, switching on Notch in the neighbouring EB. The NICD competes with Hairless for Su(H) and in doing so turns on Notch target genes, which inhibit Da-dependent bHLH activity. ISC identity is therefore blocked in favour of differentiation. **(C)** The Jak/Stat signalling pathway. Binding of Upd ligands to the Dome receptor leads to activation of a receptor-associated kinase called Hopscotch (JAK). Cytoplasmic Stat92E can bind to phosphorylated Dome/Hop complexes via SH2 domains, resulting in Stat phosphorylation and dimerisation. Stat dimers can translocate to the nucleus and activate transcriptional targets (Beebe et al., 2010).

Notch activity (Perdigoto et al., 2011).

Asymmetric Dl expression from ISC to EB is functionally required for differential Notch activation (Ohlstein and Spradling, 2007). During mitosis, Dl is expressed in both daughter cells. Immediately following cell division, one cell retains punctate D1 expression, while the other loses Dl and activates Notch targets and reporters (Ohlstein and Spradling, 2007, Bardin et al., 2010). Stable attachment between the two cells requires E-cadherin; when E-cadherin is not expressed, Notch is not activated and cell differentiation is blocked (Maeda et al., 2008). In ISCs, Notch targets are inhibited by Hairless and Suppressor of Hairless complexes (Su(H)) and chromatin modifications via the histone ubiquitin protease Scrawny (Buszczak et al., 2009). In EBs, activated Notch ICD binds *Su(H)*, relieving the suppression of Notch target genes, this results in the suppression of bHLH TFs, such as *daughterless,* which normally inactivate genes required for cell fate (Figure 1.7B) (Bardin et al., 2010). This cascade of bHLH TF activation is conserved in mammalian ISC maintenance where *daughterless* homologues are expressed in Lgr5+ ISCs (Figure 1.7B) (van der Flier et al., 2009).

1.5.4.2 Asymmetric division outcome versus population asymmetry

Homeostasis requires a balance between SC proliferation and differentiation. As discussed in section 1.5.4.1, ISCs were initially suggested to follow a pattern of single SC asymmetry, with ISC division resulting in production of a daughter ISC and an EB. (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). To date, no asymmetric segregation of intracellular signalling components prior to division has been reported, suggesting that the division itself is symmetric, whilst the overall outcome of ISC divisions in young, healthy guts can be asymmetric. A slight tilt in division angle with respect to the BM has been described in ISC division, whereby the ISC maintains a greater degree of contact with the BM than its daughter cell, there is, however, no indication that this affects daughter cell fate (Ohlstein and Spradling, 2007). Likewise, no correlation has been found between spindle orientation and daughter cell fate.

Lineage tracing experiments have however shown that a clone derived from a single ISC can contain more than one Dl+ cell, suggesting the occurrence of symmetric divisions, which result in two daughter ISCs. Given that ISCs comprise approximately 18% of the total cell population, one would expect each ISC to support a unit of around five to six cells. Clones of 10-20 cells have been described in several publications (Ohlstein and Spradling, 2006, Simons and Clevers, 2011) and the number of surviving clones declines rapidly following initial induction. Most recently, de Navascues *et al* have shown, using a combination of lineage analysis and mathematical simulation, that ISCs follow a pattern of population asymmetry (see section 1.3.3), with a proportion of the population undergoing symmetric self-renewal or differentiation of both daughters (de Navascues et al., 2012).

It is plausible that a switch from a consistently asymmetric division outcome to symmetric divisions occurs in response to environmental challenges or intestinal stress. An increased number of ISCs is observed following starvation and refeeding, with work in the Bilder lab showing that ISCs drive tissue growth in response to food abundance by increasing division rate and driving symmetrical self-renewal divisions (McLeod et al., 2010, O'Brien et al., 2011). Likewise, the number of ectopic Dl expressing cells is increased following infection, oxidative stress or ageing and this could be due to ISC symmetric divisions in order to cope with the requirements of the tissue. It remains possible that the ISC population could be heterogeneous, whereby some ISCs are primarily involved in maintaining homeostatic growth and others are predominant during regeneration.

1.5.5 Regulation of homeostatic proliferation in the posterior midgut

The signalling events regulating homeostatic proliferation of ISCs have been characterised in detail, thanks to the availability of lineage-tracing techniques for lineages derived from mutant ISCs. Midgut ISCs are slow proliferating at baseline, only becoming highly proliferative when challenged (see section 1.5.8). In healthy guts, BrdU incorporation is only observed in 5-10% of all ISCs in a 48-hour window (Hochmuth et al., 2011).

1.5.5.1 Wingless signalling as a regulator of proliferation

The Wnt/Wingless (Wg) signal transduction pathway regulates various developmental processes, including SC behaviour (see section 1.4.4.1 and Figure 1.4) (Cadigan and Nusse, 1997, Clevers, 2006). Wg is the fly homologue of Wnt1 (Rijsewijk et al., 1987) and controls segment polarity during larval development (Nusslein-Volhard and Wieschaus, 1980). The role of Wg in the *Drosophila* midgut is somewhat disputed (Lin et al., 2008, Lee et al., 2009, Cordero et al., 2009, Hou, 2010, Jiang and Edgar, 2011). Compared to other mitogenic signals, the effects of Wg signalling activity are relatively mild. Reduced levels of Wg are associated with ISC loss, whereas activation leads to hyperplasia.

Initial reports from Lin and colleagues suggested that Wg secreted from the underlying VM acts upstream of Notch in order to regulate the balance between ISC self-renewal and differentiation (Lin et al., 2008). Work from the Micchelli lab argues in favour of a slightly different role for Wg signalling. As expected, *APC* loss results in an increase in the number of dividing cells. However, the presence of differentiated cells following APC inactivation would suggest that this increased proliferation cannot be explained solely by a Notch-dependent effect on self-renewal and cell fate, as suggested by Lin and colleagues (Lee et al., 2009, Cordero et al., 2009). Thus, Wnt activation is not sufficient to convert all ISC progeny to SCs and does not alter ISC self-renewal. It would therefore appear that APC is required specifically in the ISCs in order to regulate proliferation but is not required for ISC self-renewal or Notch-dependent cell-fate specification, as was suggested by Lin et al.

In a separate study, Takashima et al showed that Wg is detected in the epithelial cells at the midgut-hindgut junction and not in the VM, arguing against Wg secretion by the VM as an ISC niche signal (Takashima et al., 2008). Hence, further studies are required in order to truly identify the source and function of midgut Wg activity.

1.5.5.2 The Jak/Stat pathway regulates ISC proliferation and EB differentiation

Jak/Stat signalling has been implicated in the regulation of stem cells (SCs) in multiple tissues (Arbouzova and Zeidler, 2006) and is proposed to be a common regulator of SC proliferation, also promoting SC self-renewal efficiency in mouse embryonic SCs (Gregory et al., 2008). The *Drosophila* Jak/Stat pathway consists of three leptin-like (IL-6 family) Unpaired (Upd) cytokines (Harrison et al., 1998, Gilbert et al., 2005, Hombria et al., 2005). The Upd cytokines bind to the Domeless (*dome*, Il-6R-like) receptor (Brown et al., 2001, Chen et al., 2002), thereby activating Hopscotch (*hop*), the fly Janus kinase (Jak) (Binari and Perrimon, 1994), which in turn regulates gene transcription through nuclear translocation of STAT92E, a STAT3-like TF (Figure 1.7C) (Hou et al., 1996, Yan et al., 1996). Transcriptional targets of Stat92E include the receptor Dome and *socs36E*, a repressor of receptor-Jak complexes.

Jak/Stat signalling is a major mitogenic signal for the midgut ISCs in both baseline homeostasis and regenerative conditions (Jiang et al., 2009, Lin et al., 2010, Beebe et al., 2010, Liu et al., 2010b, Xu et al., 2011, Buchon et al., 2009b). Upd expression has been reported in epithelial cells, including ISCs (Jiang et al., 2009, Liu et al., 2010b). Upd3 expression is mainly restricted to the ECs, while Upd 1 and 2 are commonly expressed in small progenitor cells. Reports of Upd expression in the VM suggest a possible Jak/Stat function as a niche signal in the regulation of ISC proliferation and maintenance (Lin et al., 2010). Upd activation leads to Jak/Stat signalling activity in the ISCs and EBs, which results in ISC proliferation and EB differentiation in order to replace lost/damaged cells (Figure 1.7A). In the midgut, Stat reporters are active in both ISCs and EBs, but not in terminally differentiated cells (Jiang et al., 2009, Beebe et al., 2010, Liu et al., 2010b).

Clones mutant for Jak/Stat exhibit a reduction in mature cell types due to a failure to differentiate. The majority of cells are EB-like progenitors, while the number of Dl+ cells remains normal or slightly reduced, suggesting that in healthy animals Jak/Stat is essential for differentiation but not ISC maintenance (Lin et al., 2010, Jiang et al., 2009,

Beebe et al., 2010). Epistasis data suggest that Jak/Stat signalling acts downstream or in parallel to Notch in regulating EB differentiation (Beebe et al., 2010, Jiang et al., 2009). Under conditions of adaptive homeostasis, Upd ligands, produced by epithelial cells, trigger Dl expression in the ISCs, suggesting that Jak/Stat signalling can act both upstream and downstream of Dl/Notch activity (Jiang et al., 2009, Buchon et al., 2009a).

1.5.5.3 EGFR signalling regulates ISC proliferation

The EGFR/Ras/MAPK signalling pathway is also required for midgut homeostasis (Figure 1.7A). Under baseline conditions, high levels of di-phospho-Erk (activated MAPK) can be found in ISCs and EBs. Silencing components of the EGFR/Ras/MAPK pathway results in reduced ISC division and compromises ISC survival (Buchon et al., 2010, Jiang et al., 2011, Biteau and Jasper, 2011, Ren et al., 2010). The EGFR ligands, Spitz and Keren, are expressed in midgut epithelial cells. A third EGFR ligand, Vein, is expressed in the VM. Levels of all three ligands are increased upon infection (see section 1.5.8.2). The three EGFR ligands function redundantly to regulate EGFR pathway activity in the ISCs, which leads to their division. The MAPK p38 was also shown to be required for ISC proliferation downstream of PVR (PDGF/VEGF Receptor) (Park et al., 2009, Park et al., 2010). Unlike Jak/Stat, EGFR seemingly functions solely as a growth/proliferation factor, with no effect on fate specification or differentiation. ISC proliferation induced by the Jak/Stat pathway is reported to be EGFR-dependent (Jiang et al., 2011, Buchon et al., 2010). Importantly, EGFR signalling is known to play a similar role in the mammalian intestine (Roberts et al., 2002). Several therapies targeting EGFR are clinically approved to treat colorectal cancer, which is commonly associated with gain-of-function K-Ras mutations.

1.5.5.4 Insulin signalling regulates ISC proliferation in response to nutrient availability

Systemic and midgut IIS levels (see section 1.1.3.3 and Figure 1.2D) regulate ISC proliferation (Amcheslavsky et al., 2009, Amcheslavsky et al., 2011, Biteau et al., 2010, McLeod et al., 2010, O'Brien et al., 2011, Choi et al., 2011). Neurosecretory cells in the

CNS express Dilps, which can influence ISC proliferation. Downstream mediators of the pathway such as the InR, PI3K and AKT are all essential to ISC growth and division following epithelial damage (Amcheslavsky et al., 2009, Amcheslavsky et al., 2011). Reduced IIS or overexpression of the downstream TF Foxo limits ISC proliferation and extend lifespan (Biteau et al., 2010).

Nutritional state, oxidative stress and DNA damage all significantly affect Dilp expression (Slaidina et al., 2009, Wang et al., 2005, Karpac et al., 2011). Nutrition affects Tsc/TOR signalling. The Tsc protein complex, consisting of Tsc1 and 2, negatively regulates TOR to control translation and cell growth. Loss of Tsc leads to excess ISC growth and division defects, indicating that the complex is an essential regulator of midgut ISC growth (Amcheslavsky et al., 2011). Nutrient deprivation or reduced IIS also reduces EC growth (Choi et al., 2011). When the InR is mutated, higher levels of DE-cadherin are observed between ISCs and EBs leading to prolonged cell-contact and inhibition of proliferation. This work suggests that ISCs might indirectly sense changes in nutritional status through contact with their daughters, potentially linking tissue growth to ISC proliferation (Choi et al., 2011).

Recently, using the Twin spot MARCM system to mark diving ISCs in different colours, O'Brien and colleagues showed that increased symmetric ISC division is a key mechanism for feeding-induced growth of the midgut (O'Brien et al., 2011). Well-fed flies have a higher number of ISCs and larger guts, but the relative fraction of ISCs in relation to total gut cell number remains constant. Upon starvation, this increased gut size and ISC number is reverted. Dilp3 expression in the VM is required and sufficient for this feeding-induced ISC activation. O'Brien et al suggest that local Dilp production in the VM synergises with systemic Dilp production in order to fine tune SC activity during adaptive growth. One possibility is that signalling from the fat body, which is a key sensor of nutrient availability, might trigger VM Dilp production, as is the case in glial cells (Sousa-Nunes et al., 2011).

1.5.6 The midgut ISC niche

Whether midgut ISCs are located in niches similar to those observed in other *Drosophila* tissues is still to be fully elucidated. The ECs and surrounding muscle may both have a niche-like function, since both secrete mitogens important in controlling SC proliferation (Buchon et al., 2009a, Jiang and Edgar, 2009, Jiang et al., 2009, Lin et al., 2008, Lin et al., 2010). Wnt, EGF and Upds have all been proposed as being secreted from the VM (Lin et al., 2008, Jiang et al., 2011, Biteau and Jasper, 2011, Lin et al., 2010, Xu et al., 2011). Simultaneous disruption of Wg, Jak/Stat and EGFR signalling in triple mutant clones leads to rapid and complete elimination of ISCs (Xu et al., 2011). This not only supports the notion that the VM could be a component of the ISC niche but also suggests that ISCs are governed by a robust mechanism, whereby signalling pathways compensate for one another in order to maintain the ISC population.

Several lines of evidence argue against the importance of the VM in ISC regulation. Firstly, ISC self-renewal and differentiation are regulated primarily by Notch signalling, seemingly independent of any signal from the VM. Whilst the EGFR ligand Vein is upregulated during regeneration, this is not required in order for compensatory proliferation to occur (Jiang and Edgar, 2009). Induction of two other EGFR ligands, Spitz and Keren, is also observed in the ECs during regeneration, suggesting a significant amount of EGFR signalling comes from the epithelium itself. Similarly the epithelial cells are the primary source of Upd cytokines (Jiang et al., 2009, Buchon et al., 2009b). The most important component of the midgut niche might therefore be the epithelial cells themselves.

1.5.7 The gut microbiome and immune system

The intestine not only encounters pathogenic bacteria but is also home to a number of resident commensal microorganisms. The intestinal immune response must therefore discriminate between these different populations. Numerous microbiome studies have begun to examine the *Drosophila* and human microbiota in both health and disease demonstrating that intestinal homeostasis is disrupted when the indigenous microbiota

are altered (Corby-Harris et al., 2007, Ren et al., 2007). Intestinal homeostasis therefore requires maintenance of commensals, elimination of pathogenic bacteria and regulation of inflammation and regeneration.

The *Drosophila* midgut combines physical defence to bacteria, in the form of the peritrophic membrane, with a molecular response. The gut immune response has two main components. An initial oxidative burst of reactive oxygen species (ROS) generated by the NADPH oxidase enzyme Dual Oxidase (Duox) and the synthesis of antimicrobial peptides (AMPs) controlled by the Immune deficiency (Imd) pathway (reviewed in (Lemaitre and Hoffmann, 2007)). If bacteria do traverse the gut epithelium and enter the hemolymph, specialised hemocytes are responsible for the phagocytosis and encapsulation of these invading pathogens. The main tissue involved in the humoral and systemic response to infection is the fat body, which acts as both a storage organ and a site of AMP and growth factor production. Interaction between the gut, hemocytes and fat body has to date been minimally explored. Recently, however, Wu and colleagues demonstrated that larval midgut infection induces an innate immune response in the fat body. Midgut ROS production, as well as the presence of hemocytes, was shown to be critical to communication between the midgut and fat body (Wu et al., 2012).

1.5.7.1 The Dual oxidase enzyme regulates midgut ROS generation

Intestinal redox homeostasis, mediated by the Duox enzyme and subsequent ROS elimination by immune-regulated catalase (IRC), is essential to host survival upon pathogenic infection (Ha, 2005, Ha et al., 2005, Ha et al., 2009). Gut–microbe interactions induce different amounts of Duox-dependent ROS production and a balance between the ROS levels required to eliminate bacteria and ROS-induced damage to the epithelium must be maintained.

Duox enzymes are members of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX) family. Humans have five NOXs along with Duox 1 and 2.

Duox 2 is primarily localised to the apical membrane of ECs. In *Drosophila,* there are two NADPH oxidase homologues, Nox and Duox, only Duox is present in the midgut. NOX enzymes catalyse the reduction of oxygen, generating superoxide anion, which can lead to hydrogen peroxide (H_2O_2) production. Duox proteins form a conserved family of molecules, which contain, in addition to the NADPH domain, an N-terminal extracellular peroxidase domain (PHD). Enzymatic assays have demonstrated that the PHD of *Drosophila* Duox can use H_2O_2 as a substrate to generate the highly microbicidal HOCl. The PHD of Duox is therefore vital to microbial clearance and host survival (Ha, 2005).

Duox enzymatic activity is regulated by the 'Duox activity' pathway, which involves a Gαq-PLCβ-Ca^{2+} linear cascade (Figure 1.8A). The Duox activity pathway is basally activated by small amounts of non-peptidoglycan (PG) ligands. Most pathogenic bacteria are gram-positive, meaning that they express high levels of PG in their cell wall. The exact identity of the non-PG ligand(s) responsible for Duox activation is unknown, while PG is the only known agonist for the IMD pathway. Low levels of ROS generated by the Duox activity pathway control microbiota proliferation. The Duox activity pathway negatively regulates the 'Duox expression' pathway (Figure 1.8A). The large amounts of microbial ligands, both PG and non-PG, present in the gut following pathogenic infection trigger the 'Duox expression' pathway, which maximizes ROS production in order to fight infection (Ha et al., 2009, Ha, 2005). Such mechanisms of Duox regulation are highly conserved in humans (Ha et al., 2009). Duox knockdown flies are unable to control bacteria in the gut and are therefore highly susceptible to infection.

1.5.7.2 The IMD pathway regulates midgut AMP production

NFκB activation is a common innate immune response to intestinal infection. *Drosophila* have two NFκB signalling pathways, IMD and Toll, but lack an adaptive immune system. Both pathways function in the fat body to regulate the systemic immune response. The IMD pathway has homology to the Tumour Necrosis Factor-α pathway in mammals and regulates most of the genes whose transcription is altered by
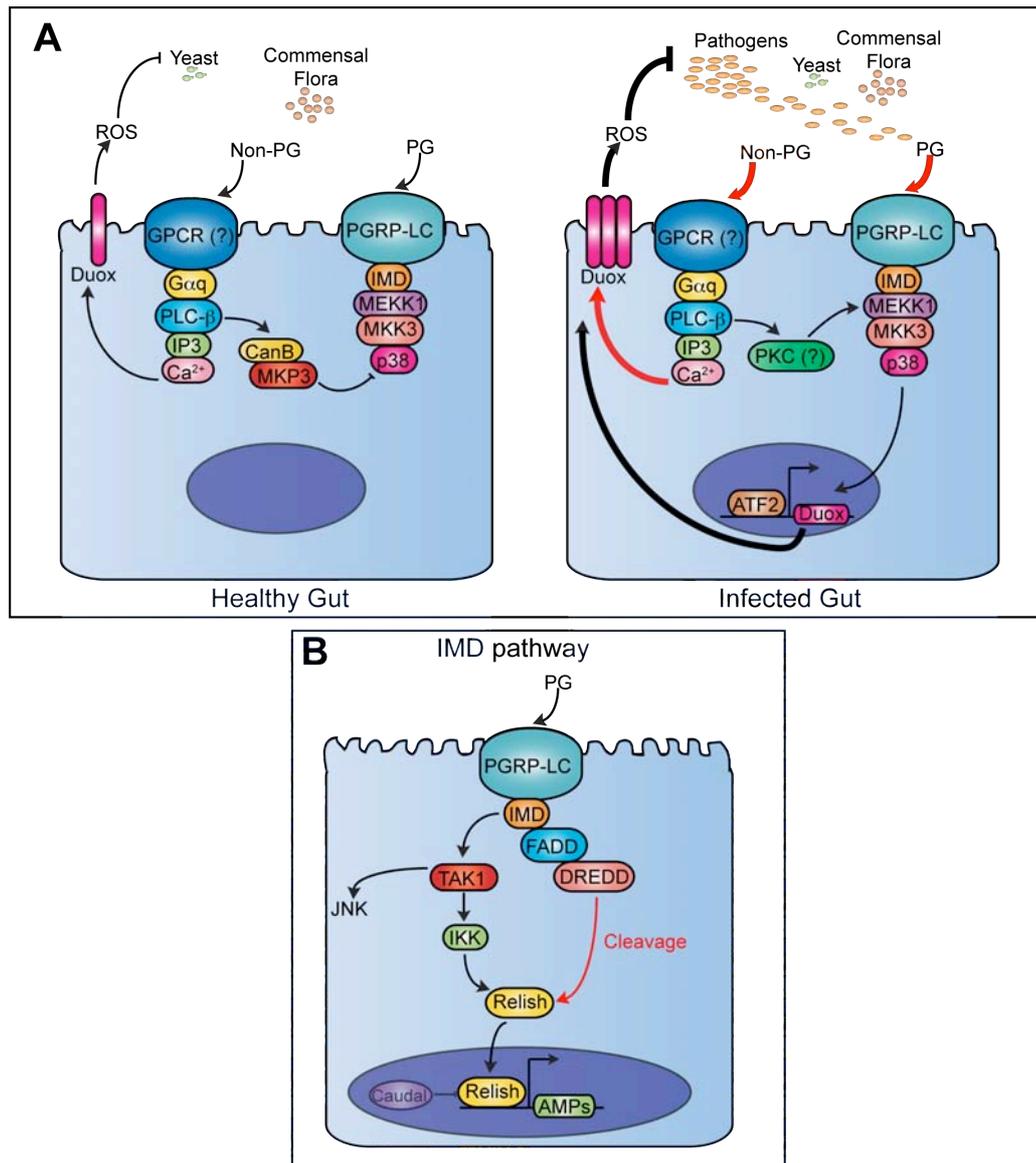
Figure 1.8: Midgut response to bacterial infection

(A) The DUOX-activity pathway is basally activated by small amounts of non-PG ligands to produce ROS, which is sufficient to maintain healthy gut–microbe interactions. The basal DUOX-activity pathway negatively controls the DUOX-expression pathway. Upon infection, infectious bacteria dominate, leading to the presence of large amounts of non-PG and PG ligands. Under such conditions, the DUOX-activity pathway is strongly activated to enhance DUOX activity. Strong PLC-β activation triggers the DUOX-expression pathway, possibly through PKC. Large amounts of PG also activate the DUOX-expression pathway. The DUOX-expression pathway is hence activated in both a PG-dependent and independent manner. Adapted from (Bae et al., 2010). **(B)** The IMD pathway. Upon direct interaction with bacteria, PGRP-LC recruits the adaptor IMD. IMD interacts with FADD, which binds to the apical caspase Dredd. Relish is phosphorylated by the IKK signaling complex, which is activated by TAK1. Once phosphorylated, Relish is cleaved by Dredd and its N-terminal transactivating domain translocates to the nucleus in order to drive AMP production, the Relish inhibitory domain remains in the cytoplasm.

midgut infection (Buchon et al., 2009a). AMPs produced by the IMD pathway during infection eliminate bacteria. Prolonged AMP production can, however, have detrimental effects to the host. In the absence of infection, small amounts of PG from commensal bacteria activate the IMD pathway leading to nuclear translocation of the NFkB homolog Relish. In these steady-state conditions, Caudal, a homeobox TF, represses transcription of many of the Relish-dependent AMPs, thus maintaining appropriate AMP levels for preservation of the normal flora community structure. Upon pathogenic infection, Caudal no longer suppresses AMP production (Figure 1.8B) (Ryu et al., 2008).

1.5.8 The intestinal response to stress or injury

A substantial amount of attention following the discovery of adult ISCs in *Drosophila* has focussed on the midgut response to injury, infection and aging and how these processes influence gut physiology and overall fitness. As discussed in section 1.5.5, ISC regulation involves systemic, muscle-derived, local and cell-intrinsic signals, all of which are integrated in order to adapt ISC function in response to physiological or environmental challenges (Figure 1.9). ISCs respond to stress or injury by increasing their proliferative activity. This occurs in response to infection (Apidianakis et al., 2009, Buchon et al., 2009a, Buchon et al., 2009b, Chatterjee and Ip, 2009, Cronin et al., 2009, Jiang et al., 2009), oxidative stress (Biteau et al., 2008, Buchon et al., 2009b, Choi et al., 2008), DNA damage (Amcheslavsky et al., 2009) and other factors that induce tissue damage or apoptosis (Amcheslavsky et al., 2009, Jiang et al., 2009).

1.5.8.1 Intestinal epithelial regeneration

<u>The JNK signalling pathway</u>

The Jun-N-terminal kinase (JNK) signalling pathway increases stress tolerance and lifespan in both flies and worms (Oh et al., 2005, Wang et al., 2003, Wang et al., 2005). JNK is an evolutionarily conserved stress-activated protein kinase and is activated by a number of intrinsic and environmental challenges, including oxidative stress and DNA

damage. In mammals, these stimuli activate members of the JNK kinase kinase family, which go on to phosphorylate and activate MKK family kinases resulting in JNK phosphorylation on Serine/Threonine and Tyrosine residues. JNK has both nuclear and cytoplasmic targets, most of which are TFs, including the AP-1 family members Jun and Fos (for a review of JNK signalling see (Johnson and Nakamura, 2007, Weston and Davis, 2007)).

Flies have only one JNK, Basket (bsk) and two JNK kinases. The MKK7 homolog Hemipterous (Hep) mediates the majority of JNK activity in flies, while dMKK4 has been less studied. The JNK-specific phosphatase, puckered (puc), is an important AP-1 target in flies, restricting JNK activity in a negative feedback loop (McEwen and Peifer, 2005, Martin-Blanco et al., 1998). The diverse effects of JNK activation are context-dependent and frequently involve interaction with other signalling pathways.

The ageing *Drosophila* midgut

The ageing *Drosophila* midgut is characterised by an increase in proliferation accompanied by the presence of misdifferentiated daughter cells expressing ISC markers as well as markers of differentiated cell types (Choi et al., 2008, Biteau et al., 2008, Park et al., 2009). These polyploid misdifferentiated cells are seemingly EBs unable to terminally differentiate into fully functional ECs. The accumulation of these misdifferentiated cells, many of which retain *esg* expression, contributes to age-related deterioration of the epithelium and midgut dysplasia (Biteau et al., 2008). Increased JNK signalling activity is observed in ISCs and EBs of old flies. JNK activation induces ISC proliferation and leads to ectopic Dl activation in the ISC lineage (Biteau et al., 2008). Activation of JNK signalling in ISCs causes age-related epithelial deterioration, while reducing JNK activity can prevent age-associated changes in gut morphology. Expression of the JNK phosphatase, Puc, is required to restrain proliferation in healthy animals, suggesting that regulation of JNK signalling activity is crucial to maintaining normal midgut homeostasis (Biteau et al., 2008). Incidentally, a strong reduction in JNK activity reduces lifespan due to complete inhibition of ISC proliferation (Biteau et al., 2008)

p38 and PVF2 are required for ISC regulation in ageing flies (Biteau et al., 2008, Choi et al., 2008, Park et al., 2009). PVF2, a PVR ligand, is upregulated in midgut progenitors in ageing flies leading to ISC proliferation (Choi et al., 2008).

The midgut stress response

Midgut regeneration is also induced by tissue damage. Whereas ageing is seemingly linked to disruption of intestinal homeostasis due to progenitor misdifferentiation, cell-damaging agents induce a regenerative response to tissue damage. Several approaches have been utilised in the study of the midgut response to tissue damage, most of which involve feeding the flies different tissue-damage inducing agents. Approaches have included: the induction of oxidative stress using Hydrogen peroxide or Paraquat (Biteau et al., 2008, Chatterjee and Ip, 2009, Choi et al., 2008), disruption to the basement membrane using Dextran Sulphate Sodium (DSS) (Amcheslavsky et al., 2009), damage to ECs using bleomycin (Amcheslavsky et al., 2009) and the expression of pro-apoptotic genes leading to EC cell death (Jiang et al., 2009).

JNK signalling is required for ISC activation following damage due to oxidative stress (Biteau et al., 2008). Activation of JNK in midgut ECs presumably promotes their elimination, which in turn induces compensatory proliferation via the secretion of Upd and EGFR ligands. In the ISCs, JNK activation not only protects cells from oxidative damage but also induces proliferation (Biteau et al., 2008, Buchon et al., 2009b). JNK is also responsible for the misdifferentiation, by inducing higher levels of Dl expression in progenitor cells, as is seen in ageing guts, but any beneficial function of this in regeneration is unclear.

EGFR signalling is also required for ISC proliferation induced by JNK activity (Biteau and Jasper, 2011). EC apoptosis or JNK activation leads to Vein expression in the VM (Jiang et al., 2011). The AP-1 TF Fos is phosphorylated by JNK and the EGFR responsive MAPK, Erk on distinct sites (Ciapponi et al., 2001). Fos phosphorylation by JNK activates ISC proliferation. Hence, Fos is required for both homeostatic and stress-induced proliferation by integrating JNK and EGFR signalling (Figure 1.9).

While JNK signalling has been implicated in the cellular stress response of multiple *Drosophila* tissues, a mitogenic role for JNK in ISCs is more surprising (Goberdhan and Wilson, 1998). However, work in the mouse intestine has also reported a role for JNK signalling in intestinal crypt proliferation and enhancement of tumour development in the colon (Sancho et al., 2009).

Cellular redox state and ISC proliferation

A low concentration of intracellular ROS is increasingly understood to be necessary for stemness and pluripotency (Ito et al., 2004, Liu et al., 2009, Smith et al., 2000, Tothova et al., 2007). ISC intracellular redox state is regulated by Nrf2 and its negative regulator Keap1 (Hochmuth et al., 2011). Nrf2 maintains a low redox state in quiescent ISCs. Gut damage results in inhibition of Nrf2 by Keap1 leading to increased ISC ROS levels and ISC proliferation (Figure 1.9). Loss of redox management accelerates age-related degeneration of the midgut epithelium (Hochmuth et al., 2011). Hence, while transiently high ROS expression induces proliferation, persistently high levels disrupt midgut homeostasis. Age-dependent gut dysplasia can be delayed by progenitor expression of Jafrac1, a peroxiredoxin, which detoxifies ROS (Biteau et al., 2010). Interestingly, Jafrac1 expression also extended lifespan by 20-25%. Redox state must therefore be carefully balanced between protecting ISCs from oxidative stress and activating ISCs in response to damage.
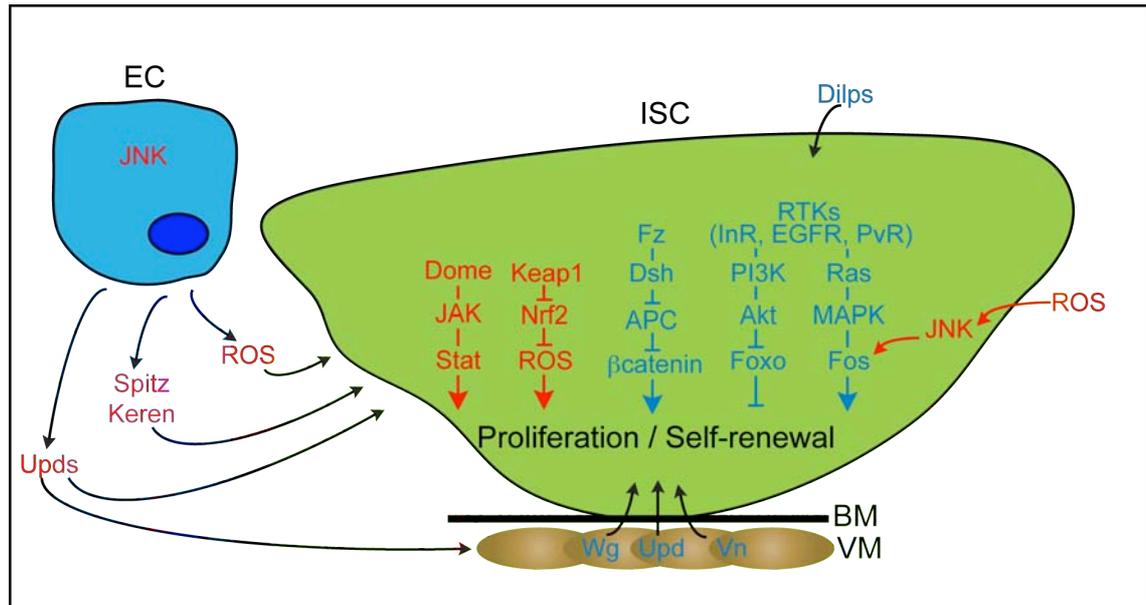
Figure 1.9: Key signalling pathways in the midgut

Local and systemic cues must be integrated with ISC intrinsic signals in order to adjust proliferation rate in response to tissue demand. The pathways required for stress/injury-induced ISC proliferation are shown in red. Figure adapted from (Biteau et al., 2011).

1.5.8.2 The intestinal response to infection

Several groups have examined the midgut response to infection using different bacterial species including *Erwinia carotovora* (*Ecc15,* non-lethal gram-negative), *Serratia marcescens* (lethal), and *Pseudomonas* (Buchon et al., 2009a, Cronin et al., 2009, Chatterjee and Ip, 2009, Jiang et al., 2009). Oral infection stimulates ISC division and midgut turnover, similar to that observed following other forms of intestinal damage (Buchon et al., 2009a, Jiang et al., 2009, Cronin et al., 2009). Studies examining changes in gene expression upon infection showed that infection triggers a combination of immune, stress and developmental signalling pathways, providing an unexpected link between infection and epithelial renewal (Cronin et al., 2009, Buchon et al., 2009a). mRNA expression-profiling experiments using guts from infected flies showed that, as expected, the majority of the genes affected by midgut infection are regulated by the IMD pathway, with levels unchanged in an IMD mutant background (Buchon et al., 2009a). Surprisingly, however, developmental pathways including Notch, Jak/Stat, and EGFR were also activated, suggesting that the gut response to infection involves diverse aspects of gut physiology.

IMD pathway

Bacterial recognition and subsequent activation of the IMD pathway were found to be dispensable for ISC division and EB differentiation in response to infection, demonstrating that PG is not a trigger for ISC activation (Buchon et al., 2009b). Ingestion of bacteria induces widespread apoptosis. Given the intestinal renewal observed following infection is reminiscent of that induced by tissue damaging agents (see section 1.5.8.1), it is plausible to suggest that bacteria induced apoptosis in ECs is responsible for ISC proliferation (Buchon et al., 2009b, Jiang et al., 2009, Amcheslavsky et al., 2009, Chatterjee and Ip, 2009).

Jak/Stat signalling

Following infection, increased expression of Upd3 in the ECs activates Jak/Stat signalling in the ISCs leading to regenerative proliferation (Figure 1.9). Stat activation in the ISCs also induces immune effectors such as Drosomycin 3 (an AMP) in the ECs (Buchon et al., 2009a). Loss of Jak/Stat signalling is sufficient to block midgut proliferative regeneration (Buchon et al., 2009b). Gut injury induced using forceps also results in localised Upd3 expression (Buchon et al., 2009b). The cellular and molecular mechanisms leading to Upd3 upregulation by ECs are currently unknown, although it has been suggested that cell damage and ROS production are likely factors. Interestingly, the cytokine interleukin-6 and Stat3 are involved in the induction of mammalian intestinal inflammation and cancer (Atreya and Neurath, 2008).

JNK signalling

The role of JNK signalling in intestinal infection varies depending on the type of pathogen used. Pathogens such as *P.entomophila* or *P.aeruginosa,* which directly damage mature ECs, primarily induce JNK activity in the mature EC population, presumably promoting their elimination (Jiang et al., 2009, Apidianakis et al., 2009). By contrast, infection utilising non-pathogenic bacteria, such as *Ecc15*, which damages the gut via an oxidative burst, activate JNK in both mature ECs and progenitor cells, similar to the effects observed with oxidative damaging agents (Buchon et al., 2009b). JNK signalling is required for ISC survival following *Ecc15* infection, seemingly due to

activation of stress response genes, which protect the ISCs from oxidative damage (Buchon et al., 2009b). As is the case for EC ablation or apoptosis, JNK signalling is not required in ECs for the proliferative response to infection (Buchon et al., 2009b, Jiang et al., 2009). Thus, JNK activity is not required in the ECs for Jak/Stat or EGFR ligand production.

Non-microbicidal effects of Duox activation

In the absence of Duox expression, Jak/Stat signalling is reduced and delayed indicating that a Duox-dependent oxidative burst is essential to Upd3 production following infection (Buchon et al., 2009b). In mammals, ROS have been shown to activate growth and proliferation via Jak2 and Stat3 (Jay et al., 2008). ROS can regulate many signalling pathways, acting as intracellular second messengers, but whether ROS are direct inducers of Jak/Stat signalling in this particular context is still unclear (Bae et al., 2000). It remains to be elucidated whether microbe/damage-induced ROS act as direct inducers of SC signalling or simply cause tissue damage, which in turn signals to SCs via other means.

Role of indigenous bacteria

The signalling pathways regulating regenerative proliferation following infection are essentially the same as those involved in steady-state homeostasis, indicating that during infection bacteria activate a molecular and cellular response, which occurs naturally in uninfected guts. Consistent with this, flies lacking commensal bacteria exhibit reduced ISC division and epithelial renewal, suggesting that under normal conditions, gut microbiota stimulate these pathways, promoting a basal level of epithelium renewal (Buchon et al., 2009b). The Jak/Stat and JNK pathways maintain gut homeostasis in response to infection. Old flies exhibit increased numbers of gut microbes leading to chronic Jak/Stat and JNK activation, which is thought to cause the disorganisation and overproliferation observed in aged guts. Flies lacking Jak/Stat or JNK succumb to bacterial infection much earlier and are generally EC depleted, highlighting the physiological importance of intestinal renewal (Buchon et al., 2009b). The host is also more susceptible to infection when Notch is depleted, indicating the importance of ISC differentiation to survival upon infection (Jiang et al., 2009).

EGFR pathway and midgut cell death

The EGFR pathway is implicated in several stages of epithelial renewal. ISC proliferation, the generation and morphogenesis of new ECs and the elimination of damaged cells are all EGFR dependent (Jiang et al., 2011, Buchon et al., 2010, Biteau and Jasper, 2011). Buchon and colleagues have characterised the different stages of the regenerative response to infection showing an initial 40% decrease in midgut length due to EC cell death (Buchon et al., 2010). Cell death only occurs once cells have been expelled from the epithelium into the lumen, similar to anoikis in mammalian guts (Gilmore, 2005). Cell loss is initially buffered by EB differentiation followed by ISC proliferation, with gut repair complete within two days. Infection increases EGFR ligand expression in both the ISCs and ECs. Reduced EGFR signalling not only prevents proliferation but ECs no longer delaminate resulting in apoptosis within the epithelium (Buchon et al., 2010). EGFR therefore seemingly mediates the disassembly of EC cell-cell junctions enabling anoikis to occur.

EGFR signals via the Ras/MAPK pathway. Interestingly, Ragab and colleagues have shown that Ras/MAPK signalling negatively regulates the IMD pathway in the gut (Ragab et al., 2011). This negative regulation is dependent upon the PDGF/VEGF receptor (PVR). The PVR ligand PVF2 is activated in ISCs and EBs following oxidative stress (Choi et al., 2008). This could suggest that PVR expression activates Ras/MAPK signalling in the ISCs, which in turn leads to inhibition of the IMD pathway, thus preventing excessive AMP production and therefore enabling ISC function to be maintained.

1.6 Aim of PhD project: A role for the Hippo pathway in the adult posterior midgut?

The identification of ISCs in the adult *Drosophila* posterior midgut has provided a new model system in which to investigate ISC biology (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). A better understanding of ISC biology should ultimately assist in the diagnosis and treatment of a range of diseases affecting the GI

tract. Similarities between the mammalian and fly GI tracts are not limited to structure but also include physiology and turnover. The mammalian small intestine and fly midgut are both comprised of absorptive and secretory epithelial cells with tissue turnover supported by a population of adult ISCs. In both systems tissue turnover takes approximately one week. The high degree of conservation with respect to the signalling pathways controlling intestinal development and regeneration combined with the genetic amenability of the fruit fly make the *Drosophila* posterior midgut an excellent candidate for studying ISC physiology.

When I first began my PhD, the *Drosophila* ISC field was still very much in its infancy. Many of the signalling pathways involved in regulating midgut homeostasis were still to be identified, including a full characterisation of any possible role for the Hpo pathway. The highly conserved Hpo signalling pathway is a known regulator of organ size (Harvey and Tapon, 2007) but has seldom been studied in *Drosophila* adult homeostasis. My initial aim was therefore to examine Hpo pathway function in the context of the gut, with a view to gaining a better understanding of the regulation of midgut homeostasis.

Chapter 2.   Materials & Methods

2.1 *Drosophila* manipulations and genetic techniques

Drosophila have one X chromosome and three pairs of autosomes. Chromosome four is mostly heterochromatic. Meiotic recombination only occurs in females.

2.1.1 Techniques for generating fly stocks

2.1.1.1 Balancer chromosomes

Balancer chromosomes (or "balancers") are a valuable resource in *Drosophila* research. Balancers have multiple inversions, which suppress the occurrence of recombination between two homologous chromosomes at meiosis. The use of balancers enables stocks carrying a homozygous lethal mutation to be stably maintained across generations. Balancers usually carry a dominant visible marker and are homozygous lethal, allowing the generation of stocks in which flies carry one copy of a mutation of interest and one copy of the balancer chromosome. The presence of visible markers, such as changes in eye colour or bristle size, enables geneticists to track their mutation of interest throughout a cross scheme. Balancers are available for the X, second and third chromosomes and include *FM7a, CyO* and *TM3*. Given the X chromosome must exist in a hemizygous state in males, most X chromosome balancers do not carry homozygous lethal mutations but instead carry recessive mutations, which result in female sterility.

2.1.1.2 Recombination

Meiotic recombination in females allows two loci of interest, located on the same chromosome, to be put in *cis*. As an example, the cross scheme used to create a stock carrying both the *puc^{e69}* (*puc-lacZ*) reporter and *UAS-yki* transgene on the third chromosome (as used in Figure 5.9) is shown below. When crossed to an EC specific Gal4 driver*,* this stock allowed *puckered* expression to be visualized upon Yki overexpression:

♀♀   *w ; UAS-yki / TM3*   X   ♂♂   *w ; puc^{e69} / TM3*

↓

F1:   ♀♀   *w ; UAS-yki / puc^{e69}*   X   ♂♂   *w ; TM3 / TM6B*

↓

F2:   Single ♂   *w ; UAS-yki , puc^{e69} / TM3*   X   ♀♀   *w ; TM3 / TM6B*

↓

F3:   Stock establishment: ♀♀   X   ♂♂   *w ; UAS-yki , puc^{e69} / TM3*
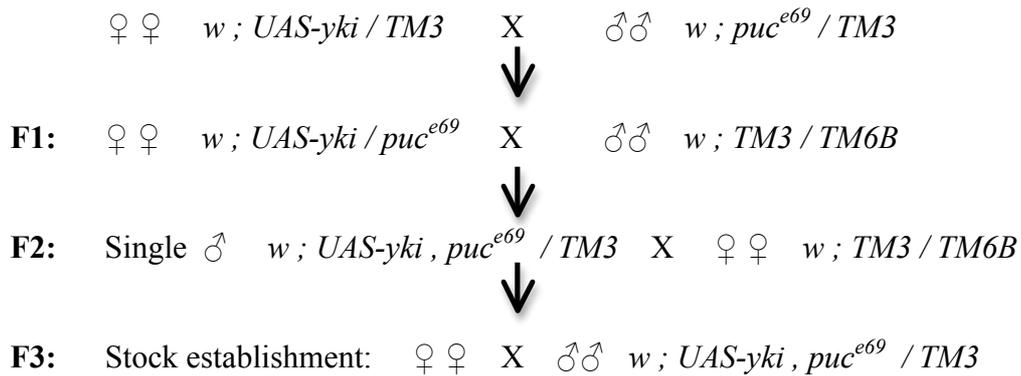
Initially, flies carrying the *UAS-yki* transgene were crossed to *puc^{e69}* males. At the F1 stage, virgins carrying both *UAS-yki* and *puc^{e69}* (identified by a lack of the *TM3* marker *Sb*) were crossed to males carrying balancers for the appropriate chromosome, in this case the third. Recombination occurs in the germline of these females, resulting in the generation of recombinant males carrying both *UAS-yki* and *puc^{e69}* on the same chromosome. The further the distance between the two loci, the greater the frequency of recombination events. Individual recombinant males are then crossed to balancer virgins. In order to check flies of the correct genotype have been established, one can either use PCR or check for the presence of a visible marker, such as eye colour. Stocks are only established from F2 males shown to be carrying both loci of interest.

2.1.1.3 Use of double balancer stocks

Often two loci of interest are located on different autosomes. Double balancers can be used to generate and maintain stocks carrying both loci of interest. I used the second and third chromosome balancer *T(2;3)SM6a-TM6B,Tb* (from here on referred to as *SM66B*) on several occasions during my PhD. *SM6a* and *TM6B* cannot be segregated in this context because of translocation. The cross scheme used to create a stock carrying both the *UAS-bsk-RNAi* (2nd) and *UAS-yki* (3rd) transgenes, generated for use in epistasis experiments (Figure 5.9), is shown below:

♀♀   *w ; UAS-bsk-RNAi* (2nd)   X   ♂♂   *w ; UAS-yki / TM3*

↓

F1:   ♀♀   *w ; Sp / SM66B*   X   ♂♂   *w ; UAS-bsk-RNAi / +; UAS-yki / +*

↓

F2: Single ♂   *w ; UAS-bsk-RNAi ; UAS-yki / SM66B*   X   ♀♀   *w ; Sp / SM66B*

↓

F3: Stock establishment: ♀♀   X   ♂♂   *w ; UAS-bsk-RNAi ; UAS-yki / SM66B*
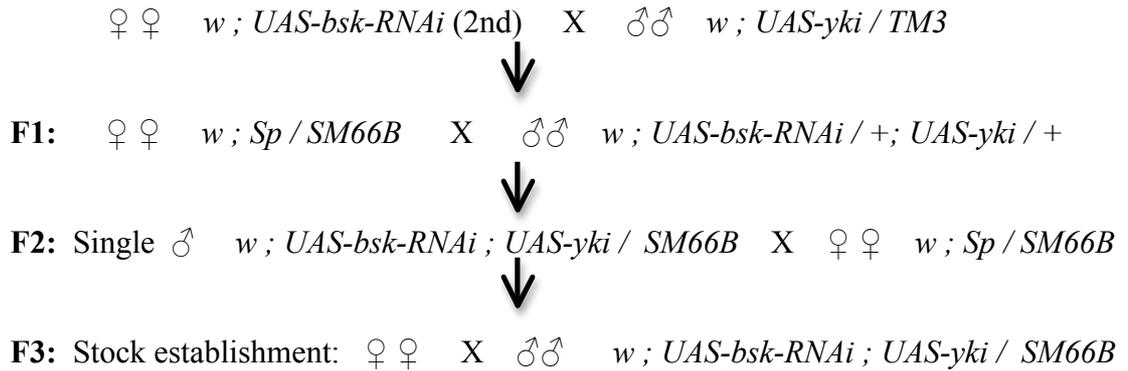
2.1.2 The use of P-elements in *Drosophila*

Transposable elements (or transposons) are thought to constitute 10-20% of the *Drosophila* genome. Transposable elements are parasitic DNA fragments, which can integrate into genomic DNA and 'jump' to different chromosomal locations (Engels, 1992). P-elements are a class of transposon found in *Drosophila*. Roughly 2.9kb in length, P-elements contain a four exon-coded transposase enzyme and 31bp perfect inverted terminal repeats (ITR). A range of P-element based genetic techniques are commonly used in *Drosophila*, such as the generation of transgenics, mutagenesis, analysis of gene expression patterns, gene replacement and clonal analysis. Many of the stocks used in this study were initially generated using such techniques.

2.1.2.1 Generation of transgenics

P-elements can be used as vectors in the generation of transgenic animals (Rubin and Spradling, 1982, Spradling and Rubin, 1982). The gene of interest is inserted into a P-element based plasmid where the transposase gene is replaced with a marker gene (such as eye colour). The plasmid is then microinjected into pre-blastoderm embryos in the presence of a functional transposase. When the P-element randomly inserts into the genome of the germline, transformant flies will emerge, which can be selected and maintained by screening for the marker gene present in the P-element sequence.

2.1.2.2 Mutagenesis by P-element insertion into the Drosophila genome

There are four general techniques for inducing mutagenesis in *Drosophila* - chemical, irradiation, insertional mutagenesis and homologous recombination. Chemical mutagenesis yields mostly point mutations. Radiation primarily results in rearrangements (i.e. translocations, duplications, deletions and inversions) (Greenspan, 1997). Insertional mutagenesis gives the best leverage for rapid molecular cloning of the mutated gene.

The insertion of a P-element into the region of a gene can result in a loss of function (LOF) phenotype. A large number of single insertion P-element lines have now been generated and are readily available from *Drosophila* stock centres (Bellen et al., 2004). Since P-elements harbour a degree of insertion point bias, other transposons, such as Piggyback and Minos, have been used to improve overall coverage.

Exogenous DNA sequences can also be inserted into the *Drosophila* genome using P-element based techniques. The insertion of a P-element containing the cDNA of a given gene downstream of a UAS sequence, for example, enables overexpression of a protein of interest (see section 2.1.3). Gene silencing can be induced through integration of P-element vectors coding for dsRNA hairpin constructs, which activate the RNA interference (RNAi) machinery. RNAi fly lines are now available for most *Drosophila* transcripts. It is also possible to create transgenic fly lines controlling either overexpression or downregulation of a protein of interest in a spatially and/or temporally controlled manner.

2.1.2.3 Mutagenesis by imprecise P-element excision

P-element mobilisation by the transposase creates a double-stranded DNA break at the original insertion site. The break is sometimes repaired aberrantly, giving rise to loss of DNA around the insertion site (imprecise excision). Imprecise excisions can be used to generate LOF alleles in genes located close to the insertion site.

2.1.2.4 Visualising expression patterns by enhancer trapping

Enhancer trapping involves the use of a P-element containing the *LacZ* gene (or another reporter) under the control of a minimal promoter, and the selection of re-insertion lines. Enhancer elements present in the chromatin near the reintegration site affect *LacZ* gene expression. This technique has been used to identify genes with interesting expression patterns during development (O'Kane and Gehring, 1987). An example of an enhancer trap line used in this study is *ex-LacZ (P[lacW]ex⁶⁹⁷)* (Hamaratoglu et al., 2006).

2.1.3 Generation of mosaic tissues in *Drosophila*

Genetic mosaic techniques allow genetic manipulations to be induced in a subset of cells or tissues (clones) (Blair, 2003), providing a way of examining genetic changes, which would be lethal if applied to the entire organism (Perrimon, 1998). Clonal analysis allows mutant cells to be studied alongside control cells within the same tissue. Genetic mosaic techniques have also been used to trace cell lineage and in the study of cell-autonomy of gene function (Blair, 2003).

2.1.3.1 The FLP-FRT system (and loss of function clones)

The FLP-FRT system is adapted from yeast, with its use in *Drosophila* first demonstrated in 1989 (Golic and Lindquist, 1989). This technique relies on site-specific recombination between two FRT (FLPase recombination target) sites catalysed by the yeast FLP recombinase (FLPase). Clonal analysis of approximately 95% of *Drosophila* genes is now possible thanks to the generation of stable fly lines containing FRT sites inserted at the base of each major chromosomal arm. P-element based plasmids were used to insert the FRT sequences (Xu and Rubin, 1993).

In order to generate mosaics, one must firstly recombine the mutation of interest onto a chromosome with an FRT site at the base of the chromosome arm. These flies are then crossed to a line containing a WT homologous chromosome with the same FRT site, a distal cell marker (usually *GFP* alternatively *LacZ*) and the FLPase (see Figure 2.1A).

The presence of a transgene expressing FLPase under the control of a heat shock promoter ensures that when the next generation of developing flies are subjected to a heat shock (37°C), mitotic recombination occurs between the two FRT sites. Recombination between the two FRT sites, resulting in the exchange of chromatids between two homologous chromosomes, occurs at random and only in a small percentage of cells. Following FLP-induced recombination and chromosome segregation, daughter cells of different genotypes can be produced. One daughter cell will be homozygous for the mutation (GFP negative) and the other will carry two copies of the cell marker (WT, GFP positive). Surrounding cells, which did not undergo recombination will also be GFP-positive (heterozygous for the mutation and marker). The mutant cell will continue to divide, giving rise to a mutant clone; likewise, the WT sister cell will divide giving rise to a WT clone, known as the twinspot (cells carrying two copies of GFP).

2.1.3.2 The Gal4-UAS system for loss or gain of function analysis

The Gal4-UAS system has been adapted from yeast and enables the study of both overexpression and LOF phenotypes (Brand and Perrimon, 1993). The yeast TF Gal4 binds to UAS (Upstream Activating Sequence) sites located in the 5' UTR of genes (see Figure 2.1B). In flies, one transgene will express the Gal4 TF under the control of a specific promoter, while another transgene will harbour the UAS sequences upstream of a gene of interest. When the two transgenes are expressed together, Gal4 binds to the UAS sequences to drive expression of a gene of interest. Use of a UAS-GFP transgene enables the areas of gene expression to be labelled (Brand and Perrimon, 1993).

The use of tissue-specific promoters with known patterns of gene expression enables researchers to observe protein overexpression in a specific tissue or cell-type (as demonstrated by the midgut progenitor-specific *esg-Gal4* and EC-specific *MyoIA-Gal4* drivers used in this study). The Gal4-UAS system also allows LOF function analysis by expressing dominant negative forms of a protein of interest or RNAi constructs (Dietzl et al., 2007).
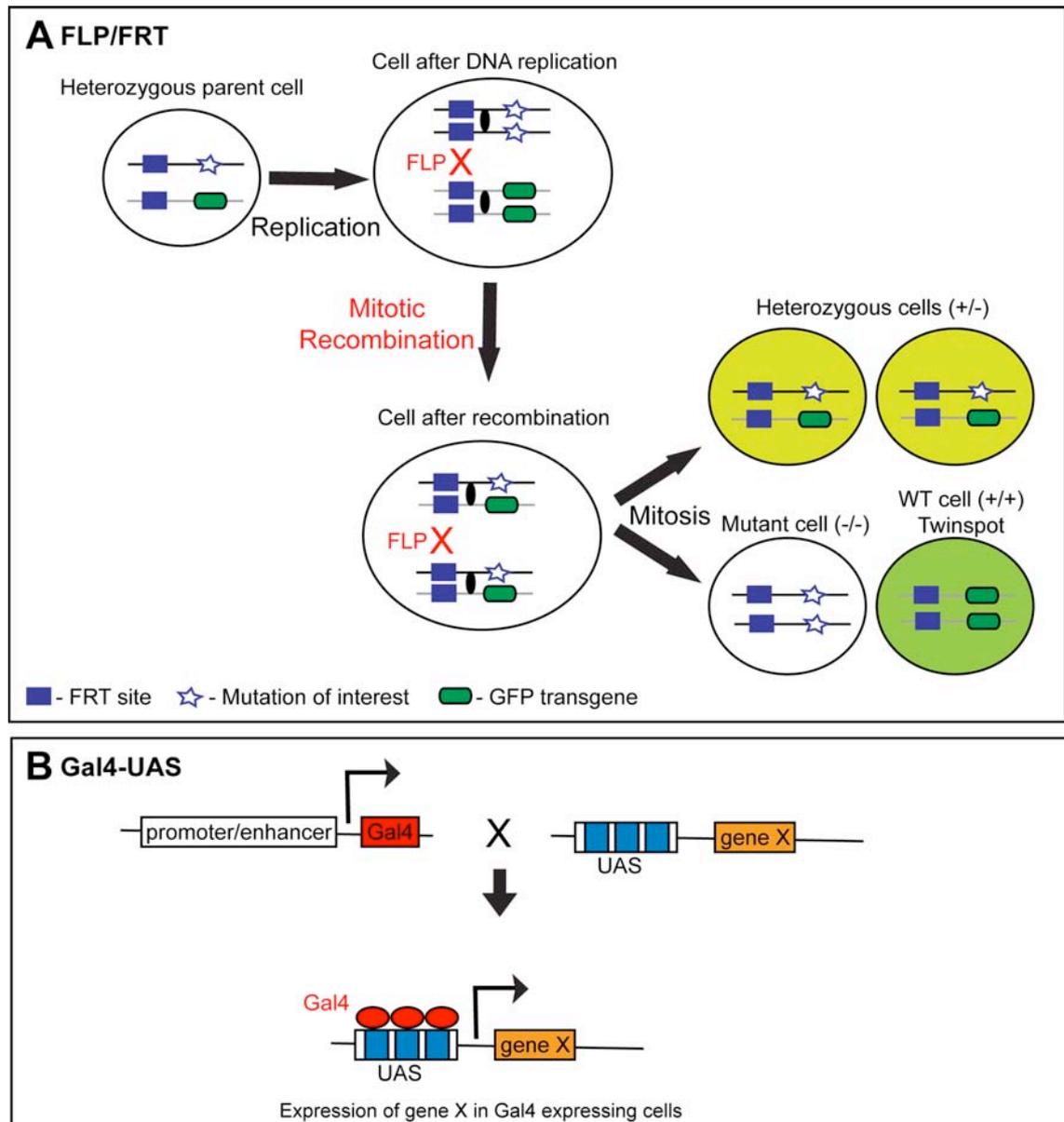
Figure 2.1: Generation of mosaic tissues in *Drosophila* – FLP/FRT and Gal4-UAS

(A) Schematic representation of the FLP/FRT technique. (B) Schematic representation of the Gal4-UAS system

The timing of Gal4 expression can be regulated in a number of ways, including incorporation of drug or hormone sensitivity into the Gal4. In this study, I have used a temperature sensitive version of the Gal4 inhibitor Gal80 (Gal80^{ts}) (Lee and Luo, 1999) as a means of controlling the Gal4-UAS system. Tubulin-driven Gal80 expression can inhibit Gal4 activity. Fly lines combining the temperature sensitive Gal80 with a Gal4 construct include the two main drivers used in this study, *esg^{ts}* and *MyoIA^{ts}*. At the permissive temperature (18°C), Gal80^{ts} blocks Gal4 activity, but when shifted to a higher restrictive temperature (29 ^{o}C), Gal80^{ts} no longer blocks Gal4.

2.1.3.3 The FLPout technique

The FLP/FRT and Gal4-UAS systems are combined to generate positively marked clones in what is known as the FLPout technique (see Figure 2.2A)(Pignoni and Zipursky, 1997, Ito et al., 1997, de Celis and Bray, 1997). These positively marked mutant clones are easier to spot in dense tissue. In this technique, the Gal4 gene is separated from a constitutive promoter by a "stuffer", comprising two FRT sites and a transcription termination site and, as a result, is silenced at baseline. FLP activity, generally under the control of the heat-shock promoter, is used to induce random recombination between the FRT sites leading to the removal of the "stuffer". Removal of the "stuffer" results in constitutive Gal4 expression in that sub-set of cells and their descendants. This technique, combined with a *UAS-GFP* present on the same chromosome as the FLPout construct, in order to visualise the cells in which recombination had taken place (FLPout clones), was for example used in Figure 3.12.

2.1.3.4 The MARCM (mosaic analysis with a repressible cell marker) system

The MARCM system combines the FLP-FRT and Gal4-UAS techniques allowing the generation of positively marked LOF clones (see Figure 2.2B) (Lee and Luo, 1999). At least six transgenes are required for MARCM analysis: two homologous FRT sites, one FLP recombinase, one UAS-marker, one Gal4 driver and the *tub-Gal80* transgene
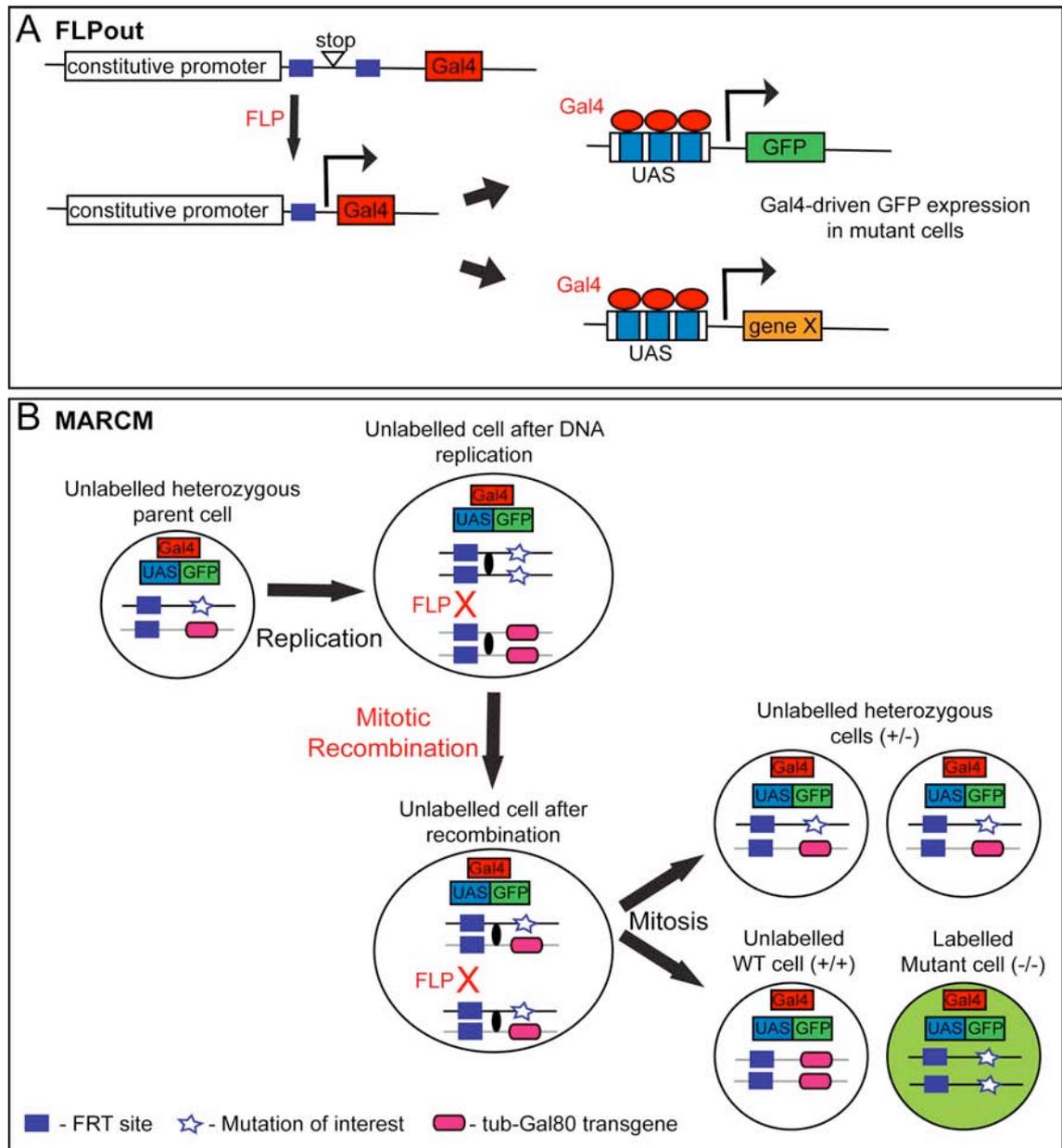
Figure 2.2 Generation of mosaic tissues in *Drosophila* – FLPout and MARCM

(A) Schematic representation of the FLPout technique. (B) Schematic representation of the MARCM system.

(which is placed downstream of the FRT site and in trans to the gene of interest) (Lee and Luo, 1999). Upon heat-shock induced FLP expression, random recombination events can result in one daughter cell carrying no *tubGal80* expression (homozygous mutant), thus enabling Gal4 to drive *UAS-GFP* expression, leading to the appearance of GFP positive clones. Mutant clones are generated when a mutation in a gene of interest is provided on an FRT site identical to the FRT site in the *tub-Gal80* transgene (as performed in Figure 3.8D-D" for example). Using this technique, it is also possible to induce clones, which simultaneously overexpress one gene of interest whilst being homozygous mutant for another. This is useful for epistasis experiments but was not used in this study. Since labelled cells are the progeny of one cell and the production of marked clones after mitotic recombination depends upon subsequent cell division, the MARCM technique has been used in midgut lineage tracing experiments and as a direct means to assay proliferation (Micchelli and Perrimon, 2006).

2.2 Tissue sample preparation

2.2.1 General fly husbandry

Flies were maintained in vials of standard fly food media (see recipe below) on a 12 hour light-dark cycle. On average, genetic crosses were set up using 13 female and 5-6 male flies.

Fly Food (50L)

50L H_2O
360g Agar
3600g Maize
3600g Malt
1200ml Molasses
440g Soya
732g Yeast
280ml of acid mix (500ml Propionic acid + 32ml Orthophosphoric acid)

2.2.2 Temperature shift experiments

Two gut specific drivers were used in this study. To restrict transgene expression to progenitor cells, expression was placed under the control of the ISC/EB driver *esg-Gal4*, and temporally restricted using temperature-sensitive *Gal80* under the control of the *tubulin* promoter (*esg-Gal4;tub-Gal80^{ts},UAS-GFP* referred to as *esg^{ts}*) (Micchelli and Perrimon, 2006, McGuire et al., 2003). *MyoIAGal4* is an enhancer trap inserted in the gut-specific brush border *myosin IA* gene (Morgan et al., 1994). The inducible *Myo1A-Gal4, tub-Gal80^{ts}* system (referred to as *MyoIA^{ts}*) drives UAS expression in midgut ECs (Jiang et al., 2009).

Crosses using the *Gal80^{ts}* system were set up and maintained at 18°C, the permissive temperature, until adulthood. Crosses were transferred to a fresh vial every seven days. Adults were maintained at 18°C for 1-2 days post-eclosion before being shifted to 29°C routinely for six days prior to dissection. Adult flies were transferred to fresh food vials every three days. Flies were shifted to the restrictive temperature for shorter periods in time-course experiments (days indicated in Figure 3.2).

2.2.3 FLPout clones in the gut

For adult gut FLPout clones, crosses were set up and cultured at room temperature (RT). Crosses were transferred to a fresh vial every five days. Flies were heat-shocked for 30 minutes five days after eclosion and dissected five days later. For *pucRNAi* analysis flies were analysed 0, 1 and 2 days after heat shock (Figure 5.8).

2.2.4 FLPout clones in wing imaginal discs

For wing imaginal disc FLPout clones (Figure 4.4), crosses were established at 25°C. Crosses were transferred to a fresh vial every day. Larvae were heat shocked for 10 minutes 72 hours A.E.D (after egg deposition) and dissected 120 hours A.E.D (Pignoni et al., 1997).

2.2.5 MARCM clones

MARCM clones were generated as previously described (Lee and Luo, 1999). Crosses were raised at RT, adult female flies were heat shocked for 45 minutes five days after eclosion and dissected 5-10 days after clone induction. Analysis of BrdU+ cells per clone was performed by simultaneous co-staining in flies fed BrdU for 10 hours on day seven of clone growth (Figures 3.14). Epithelial architecture of *hpo* mutant clones was analysed in 14-day old clones to allow continued overproliferation (Figure 3.3H-H'). Clones were analysed seven days after induction for Figure 3.5F. Clones were analysed 10 days after induction for Figures 3.8D-E" and 3.13.

2.2.6 List of genotypes used in this study

Figure	Genotype
Figure 3.1	
(A-A")	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B-B")	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C-C")	*yw;esgGal4,UAS-wts-RNAi;tubGal80tsUAS-GFP*
Figure 3.2	
(A)	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
Figure 3.3	
(A, F)	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B)	*yw;UAS-Notch-RNAi,esgGal4;tubGal80tsUAS-GFP*
(C, G)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(D-D")	*y,w,hsflp;esg-LacZ;Act>CD2>Gal4,UAS-GFP*
(E-E")	*y,w,hsflp;esg-LacZ;Act>CD2>Gal4,UAS-GFP/UAS-yki*
(H-H')	*w;FRT42Dhpo42-47/y,w,hsFlp,UASGFP-NLS;FRT42DTubGal80,TubGal4*
Figure 3.4	
(A-A', D)	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B-B', D)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C-C')	*yw;esgGal4,UAS-wts-RNAi;tubGal80tsUAS-GFP*
Figure 3.5	
(A, E)	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B, E)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C, E)	*yw;esgGal4,UAS-wts-RNAi;tubGal80tsUAS-GFP*

(D)	*yw; esgGal4; tubGal80ts UAS-GFP, UAS-yki*
(F)	*w;P[ry+hs-neoRFRT]42DP[mini-w+,hs-pmyc]45F/y,w,hsFlp,UASGFP-NLS; FRT42DTubGal80,TubGal4*
(F)	*FRT42Dhpo42-47/y,w,hsFlp,UASGFP-NLS;FRT42DTubGal80, tubGal4*
(F)	*y,w;eyFLP;FRT82Bsavshrp1/y,w,hsFlp,UAS-GFP-CD8;+;TubGal4,FRT82B, tubGal80*
Figure 3.6	
(A-A')	*yw, Su(H)Gbe-LacZ; esgGal4; tubGal80ts UAS-GFP*
(B-B')	*yw , Su(H)Gbe-LacZ; esgGal4; tubGal80ts UAS-GFP, UAS-yki*
(C-C'')	*w,Su(H)Gbe-LacZ; MyoIAGal4;tubGal80tsUAS-GFP,UAS-yki*
Figure 3.7	
(A, D)	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B, D)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C, D)	*yw;esgGal4,UAS-wts-RNAi;tubGal80tsUAS-GFP*
Figure 3.8	
(A-A')	*yw;esgGal4;tubGal80ts,UAS-GFP*
(B-B')	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C-C')	*yw;esgGal4,UAS-wts-RNAi;tubGal80tsUAS-GFP*
(D-D'')	*yw;+;FRT82b,wtslatsX1/ywTubGAL4 hsFLP 122 UAS-nucGFPmyc;;FRT82B CD21 y+ TubG80.LL3.*
(E-E'')	*ywTubGAL4 hsFLP 122 UAS-nucGFPmyc;;FRT82B CD21 y+ TubG80.LL3*
Figure 3.9	
(A-A'', D)	*w;MyoIAGal4;tubGal80ts,UAS-GFP*
(B-B'', D)	*w;MyoIAGal4;tubGal80tsUAS-GFP,UAS-yki*
(C-C'', D)	*w;MyoIAGal4,UAS-wtsRNAi;tubGal80tsUAS-GFP*
Figure 3.10	
(A-B'')	*w;MyoIAGal4, esg-LacZ;tubGal80ts,UAS-GFP*
(C-E)	*w;MyoIAGal4, esg-LacZ;tubGal80tsUAS-GFP,UAS-yki*
Figure 3.11	
(A, D, E)	*w;MyoIAGal4;tubGal80ts,UAS-GFP*
(B, D, E)	*w;MyoIAGal4;tubGal80tsUAS-GFP,UAS-yki*
(C, D)	*w;MyoIAGal4,UAS-wtsRNAi;tubGal80tsUAS-GFP*
Figure 3.12	
(A-A'')	*y,w,hsflp,Upd-LacZ;+;Act>CD2>Gal4,UAS-GFP*
(B-B'')	*y,w,hsflp,Upd-LacZ;+;Act>CD2>Gal4,UAS-GFP/UAS-yki*
(C-C'')	*y,w, Su(H)Gbe-LacZ;+;Act>CD2>Gal4,UAS-GFP*
(D-D'')	*y,w, Su(H)Gbe-LacZ;+;Act>CD2>Gal4,UAS-GFP/UAS-yki*
Figure 3.13	

(A-A'',C-C'')	*yw;+;FRT82b,wtslatsX1/ywTubGAL4 hsFLP122 UASnuc GFPmyc; ; FRT82B CD21 y+ TubG80.LL3.*
(B-B", D-D'')	*ywTubGAL4 hsFLP 122 UAS-nucGFPmyc; ;FRT82B CD21 y+ TubG80.LL3*
Figure 3.14	
(A-B',D,E)	*y,w;eyFLP;FRT82Bsavshrp1/y,w,hsFlp,UAS-GFP-CD8;+;TubGal4,FRT82B, tubGal80*
(C-E)	*y,w,hsFlp,UAS-GFP-CD8;+;TubGal4,FRT82B,tubGal80/FRT82B*
Figure 4.1	
(A-A'')	*y,w,hsflp,Upd-LacZ;+;Act>CD2>Gal4,UAS-GFP*
(B-B'')	*y,w,hsflp,Upd-LacZ;+;Act>CD2>Gal4,UAS-GFP/UAS-yki*
Figure 4.2	
(A-A")	*Upd-lacZ;+;FRT82b,wtslatsX1/ywTubGAL4hsFLP122UAS-nucGFPmyc;;FRT82B CD21 y+ TubG80.LL3.*
(B-B")	*Upd-lacZ;+;FRT82b/ ywTubGAL4 hsFLP122UAS-nucGFPmyc;;FRT82B CD21 y+ TubG80.LL3*
(C-C")	*Upd-lacZ;FRT42Dhpo42-47/y,w,hsFlp,UASGFP-NLS;FRT42DTubGal80, tubGal4*
(D-D")	*Upd-lacZ;FRT42DykiB5,hpo42-47/y,w,hsFlp,UASGFP-NLS;FRT42D TubGal80,TubGal4*
Figure 4.3	
(A-A")	*w,hsf;upd3Gal4-UASGFPtub>y+>yki*
(B)	*yw;esgGal4/statRFP;tubGal80tsUAS-GFP*
(C-C')	*yw;esgGal4/statRFP;tubGal80tsUAS-GFP,UAS-yki*
(D-D')	*w;MyoIAGal4/statRFP;tubGal80ts*
(E-E',G)	*w;MyoIAGal4/statRFP;tubGal80ts/UAS-yki*
(F-F')	*w;MyoIAGal4/UAS-wtsRNAi;tubGal80ts/statRFP*
Figure 4.4	
(A-B'')	*y,w,hsflp, Upd-LacZ; +; Act>CD2>Gal4, UAS-GFP / UAS-yki*
Figure 4.5	
(A,E,I)	*yw;esgGal4;tubGal80tsUAS-GFP*
(B,F,I)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C,G,I)	*yw;esgGal4,UAS-statRNAi;tubGal80tsUAS-GFP*
(D,H,I)	*yw;esgGal4,UAS-statRNAi;tubGal80tsUAS-GFP,UAS-yki*
Figure 4.6:	
(A,E,I)	*w;MyoIAGal4;tubGal80ts,UAS-GFP*
(B,F,I)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
(C,G,I)	*w;MyoIAGal4, UAS-statRNAi; tubGal80ts UAS-GFP*
(D,H,I) *i*	*w;MyoIAGal4,UAS-statRNAi;tubGal80tsUAS-GFP,UAS-yk*
Figure 4.7:	

(A-A')	*ywTubGAL4 hsFLP 122 UAS-nucGFPmyc; ;FRT82B CD21 y+ TubG80.LL3*
(B-B')	*yw;+;FRT82b,wtslatsX1/ywTubGAL4hsFLP122UASnuc GFPmyc;;FRT82B CD21 y+ TubG80.LL3.*
(C-D')	*FRT42DykiB5/y,w,hsFlp,UASGFP-NLS;FRT42D TubGal80,TubGal4*
(E-E', I-I''')	*yw;esgGal4;tubGal80tsUAS-GFP*
(F-F')	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(G-G')	*w;MyoIAGal4;tubGal80ts,UAS-GFP*
(H-H')	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
Figure 4.8:	
(A-C')	*yw;esgGal4;tubGal80tsUAS-GFP*
Figure 4.9:	
(A)	*w1118*
(B-C")	*y,w;ex-LacZ/cyo*
Figure 4.10:	
(A,C,E,G,I)	*w;esgGal4;tubGal80tsUAS-GFP*
(B,D,F,H,I)	*w;esgGal4,UAS-yki-RNAI;tubGal80tsUAS-GFP*
Figure 4.11:	
(A,C,E,F,H)	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(B,D,E,G) *P*	*w;MyoIAGal4,UAS-yki-RNAi;tubGal80tsUAS-GF*
(H)	*w;MyoIAGal4,UAS-hpo;tubGal80tsUAS-GFP*
Figure 4.12:	
(A,B)	*yw;esgGal4;tubGal80tsUAS-GFP*
(A,B)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(A,B)	*w;esgGal4,UAS-yki-RNAi;tubGal80tsUAS-GFP*
(C,D)	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(C,D)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
(C,D)	*w;MyoIAGal4,UAS-yki-RNAi;tubGal80tsUAS-GFP*
Figure 4.13:	
(A and C)	*yw;esgGal4;tubGal80tsUAS-GFP*
(B and D)	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(B)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
(C)	*w;esgGal4,UAS-yki-RNAi;tubGal80tsUAS-GFP*
(D)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
(D)	*w;MyoIAGal4,UAS-yki-RNAi;tubGal80tsUAS-GFP*
Figure 5.1:	
(A-A',E)	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(B-B', E)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
(C-C', E)	*w;MyoIAGal4,UAS-kib-RNAi,UAS-kib-RNAi; tubGal80tsUAS-GFP*

(D-D', E)	*w;MyoIAGal4,UAS-kib-RNAi; tubGal80tsUAS-GFP,UAS-mer-RNAi*
Figure 5.2:	
(A-B')	*fj-LacZ*
(C-D')	*yw;sp/cyo;HRE-diap1-GFP/Tm6b*
Figure 5.3:	
(A-D, G-J")	*yw;esgGal4;tubGal80tsUAS-GFP*
(E-E')	*w;MyoIAGal4; tubGal80tsUAS-GFP*
(F-F')	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
Figure 5.4:	
(A)	*w1118*
(A)	*UAS-yki/TM3*
(B)	*yw;esgGal4;tubGal80tsUAS-GFP*
(B)	*yw;esgGal4;tubGal80tsUAS-GFP,UAS-yki*
Figure 5.5:	
(A-B)	*w;MyoIAGal4; tubGal80tsUAS-GFP*
(A-B)	*w;MyoIAGal4,UAS-relish-RNAi; tubGal80tsUAS-GFP*
(A-B)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-dredd-RNAi*
(A-B)	*w;MyoIAGal4,UAS-tak1-RNAi; tubGal80tsUAS-GFP*
(A-B)	*w;MyoIAGal4,UAS-dFADD-RNAi; tubGal80tsUAS-GFP*
Figure 5.6:	
(A)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
(A)	*w;MyoIAGal4,UAS-duox-RNAi; tubGal80tsUAS-GFP,UAS-yki*
(A, B, C)	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(A, D, E)	*w;MyoIAGal4,UAS-duox-RNAi;tubGal80tsUAS-GFP*
Figure 5.7:	
(A)	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(A)	*w;MyoIAGal4,UAS-duox-RNAi;tubGal80tsUAS-GFP*
(A)	*w;MyoIAGal4; tubGal80tsUAS-GFP,UAS-yki*
(B)	*yw;esgGal4,ex-LacZ;tubGal80tsUAS-GFP*
Figure 5.8:	
(A)	*y,w,hsflp; +; Act>CD2>Gal4, UAS-GFP / UAS-pucRNAi*
Figure 5.9:	
(A-A')	*w;MyoIAGal4;tubGal80tsUAS-GFP*
(B-B')	*w;MyoIAGal4;tubGal80tsUAS-GFP/UAS-hepCA*
(C)	*w;MyoIAGal4,UAS-puc;tubGal80tsUAS-GFP*
(D)	*w;MyoIAGal4,UAS-bskRNAi;tubGal80tsUAS-GFP*
(E)	*w;MyoIAGal4,UAS-puc;tubGal80tsUAS-GFP/UAS-yki*
(F)	*w;MyoIAGal4,UAS-bskRNAi;tubGal80tsUAS-GFP/UAS-yki*

(G-G')	*w;MyoIAGal4;tubGal80tsUAS-GFP/puce69*
(H-H')	*w;MyoIAGal4;tubGal80tsUAS-GFP/puce69,UAS-yki*

Table 2: List of genotypes

2.3 Dissection and immunostaining protocols

2.3.1 Gut dissection and immunostaining

10x Fly PBS (Phosphate Buffer Solution) (1L)   PBST

76.1g NaCl   Fly PBS
18.8g Na_2HPO4 (sodium phosphate dibasic)   0.1% Triton X-100
4.1g NaH_2PO4 (sodium phosphate monobasic)
Add ddH_2O (doubly distilled) up to 1L and autoclave

Gut fixative (8ml)

2ml 16% paraformaldehyde
4ml Fly PBS
2ml H_2O

2.3.1.1 Gut immunostaining protocol

Female adult flies were dissected on a silicon pad in ice-cold 1x Fly PBS. The entire gastrointestinal (GI) tract was dissected using a light microscope and tweezers. The Malpighian Tubules were removed and discarded from the rest of the gut. The GI tract was then placed in a small glass depression well, covered with a sheet of foil to protect from the light and fixed in 0.5X Fly PBS with 4% paraformaldehyde for 30 minutes at RT. Samples were then transferred to eppendorfs containing 0.1% Triton X-100 (PBST) using tweezers. A maximum of seven guts were placed in any one eppendorf. The following steps were all carried out with agitation on a Vari-Mix platform rocker (Thermo Scientific). Samples were washed three times in 0.1% PBST, permeabilised for 30 minutes in 0.3% PBST and pre-blocked for one hour in 10% Normal Goat Serum (NGS, MP Biomedicals), before incubation with primary antibody overnight at 4°C (generally in a volume of 300µl). Samples were washed a further four times in PBST and pre-blocked for one hour before incubation with secondary antibody for 3-4 hours at RT. Samples were then washed three times in PBST followed by a single wash with

PBS. Whole guts were mounted in Vectashield (Vector) containing DAPI (4',6-diamidino-2-phenylindole).

2.3.1.2 Antibody pre-absorption protocol

The majority of antibodies were pre-absorbed using larval tissue in order to improve staining quality. Ten fixed and permeabilised inverted larvae were added to an eppendorf containing 300μl of the antibody of interest (at ten times the concentration to be used experimentally) in PBST. Antibodies were incubated with the larval tissue for 3-4 hours at RT. The solution was then removed and stored at 4°C for up to four weeks.

2.3.1.3 BrdU (Bromodeoxyuridine) incorporation protocol

Flies carrying seven day old MARCM clones were transferred to food containing BrdU (100μg/ml in PBS) mixed into the upper layer and dissected ten hours later (Figure 3.14).

2.3.1.4 Table of midgut cell markers

Cell Type	Marker
Intestinal Stem Cell	Delta (cytoplasmic vesicles) *esg-GFP, esg-lacZ* (marks all ISCs and EBs) PH3 (marks actively dividing ISCs)
Enteroblast	*Su(H)Gbe-LacZ* (nuclear with some cytoplasmic bleed through) *esg-GFP, esg-lacZ* (marks all ISCs and EBs)
Enterocyte	Pdm-1 (nuclear) DAPI (large polyploid nuclei) *MyoIA-LacZ, MyoIA-GFP* (also known as *Myo31DF*)
Enteroendocrine cell	Prospero (nuclear)

Table 3: Midgut cell markers

2.3.2 Wing imaginal disc dissection and immunostaining

Wing disc fixative (8ml)

2ml 16% paraformaldehyde
6ml Fly PBS

2.3.2.1 Wing disc immunostaining protocol

Wing imaginal discs were dissected in 1x PBS and then fixed in 4% paraformaldehyde for 22 minutes. All other steps were conducted as described in section 2.3.1.1.

2.3.3 Gut sections

For cryosections, midguts were dissected, fixed and embedded in O.C.T Tissue-Tek medium (Sakura) according to standard procedures and subsequently processed for immunofluorescence staining as above. For optical sections, orthogonal representations of z-stacks spanning the midgut epithelium were presented using Nikon NIS-elements imaging software.

2.3.4 Table of Antibodies

Antibody	Species	Concentration	Source
aPKC (sc-216)	Rabbit	1 in 500	Santa Cruz
Arm (N2 7A1)	Mouse	1 in 10	DSHB
Beta-galactosidase	Rabbit	1 in 500	Cappell
Beta-galactosidase	Mouse	1 in 500	Promega
BrdU	Mouse	1 in 100	BD Biosciences
Delta	Mouse	1 in 100	DSHB
DE.Cadherin	Rat	1 in 20	DSHB
Pdm-1	Rabbit	1 in 100	W.Chia
PH3	Rabbit	1 in 1000	Upstate Biotechnology
PH3	Mouse	1 in 1000	Upstate Biotechnology

Prospero	Mouse	1 in 20	DSHB
Yki	Rabbit	1 in 500	Ken Irvine
Yki69 (aa381-395)	Rat	1 in 100	Eurogentec SA, Belgium
Rhodamine Red X (RRX)	Mouse	1 in 500	Jackson ImmunoResearch
Rhodamine Red X (RRX)	Rabbit	1 in 500	Jackson ImmunoResearch
Rhodamine Red X (RRX)	Rat	1 in 500	Jackson ImmunoResearch
Rhodamine Phalloidin		1 in 500	Molecular Probes

Table 4: Antibodies

2.4 Imaging and image analysis

2.4.1 Microscopy and Imaging

Flies were sorted and dissected using a dissection microscope (Leica MZ7s) with external light source.

Fluorescence images were generally acquired on a Zeiss LSM510 confocal microscope. Figure 3.3A-C was obtained using a Leica SP5. Figures 3.3G-H', 3.14 and 4.2C-D" were obtained using a Nikon A1Rsi. Images were generated using XY acquisitions and a 40X water objective (zoom 1x, 2x or 4x). The pinhole was set at 2.5μm, with the only exception being Figure 4.4, pinhole 1μm.

Images were processed using ImageJ Fiji and Adobe Photoshop. Figures were compiled using Adobe Illustrator.

2.4.2 Quantification

Dl positive cell numbers were quantified from square areas 115.2μm across (Figures 3.4D and 3.9D). Pros positive cell numbers were also quantified from square areas 115.2μm across (Figure 3.7). For MARCM clone analysis, number of BrdU positive

cells per clone was obtained by manual counting after confocal imaging and 3D projection of confocal slices (Figure 3.14). Total number of proliferating cells was calculated by counting PH3 positive cells for entire guts (Figures 3.5E and F, 3.11D, 4.5I, 4.6I, 4.10I, 4.11E and H, 4.13A and C, 5.1E, 5.4B, 5.5, 5.6A and 5.7A). Survival rates were calculated as a percentage of the total number of flies at day 0 (Figures 4.12 and 4.13B and D). Graphs were generated using Prism5 or Microsoft Excel (Figures 3.14, 4.3G, 4.9A and 5.8)

2.4.3 Statistical Analysis

The statistical significance of the difference between the various measured genotypes was examined using a two-tailed Mann-Whitney non-parametric test, (also called the rank sum test) using Prism. The number of samples analysed and p-values are shown in the relevant figure legends. To perform the Mann-Whitney test, Prism first ranks all the values from low to high, the smallest number gets a rank of one. The largest number gets a rank of N, where N is the total number of values in the two groups. Prism then sums the ranks in each group, and reports the two sums. If the sums of the ranks are very different, the P value will be small. The P value is the probability that the results could have occurred by chance. One can reject the null hypothesis when the P value is less than the significance level.

2.5 Feeding assays and survival experiments

2.5.1 Bacterial Infection

For gut infections, crosses and infections were essentially performed as described in (Jiang et al., 2009). The *Pseudomonas entomophila* (*P.e*) glycerol stock used was originally received from Bruce Edgar (ZMBH, Heidelberg) and stored at -80°C. *P.e* cultures were grown in conical flasks at 30°C for two x overnight in L-Broth (LB) medium with selection on 100 μg/ml rifampicin (Sigma). Cultures were spun down at 4000 rpm using an eppendorf 5810R centrifuge. The bacterial pellet was then resuspended in a one in ten volume of the initial bacterial culture volume using 5%

sucrose/PBS (Fischer Scientific). 0.5 ml of this concentrated bacterial suspension was mixed with a spatula into the upper few mm of fly food in a standard 10ml vial. 5% sucrose/PBS without bacteria was used as a control. A small piece of folded filter paper (Schleicher and Schuell) was added to the vial to prevent the flies from drowning. Flies were starved for two hours prior to oral infection. For RNAi experiments, crosses were maintained at 18°C and adult flies were shifted to 29°C for 4-5 days before infection. For rescue experiments, *P.e* infected flies were transferred to food lacking bacteria but containing 100 U/mL penicillin and 100 μg/ml streptomycin in PBS (Gibco) (Figure 4.9A).

<u>LB, 1L</u>

10g Tryptone
5g Yeast extract
5g NaCl
1ml 1M NaOH

2.5.2 Dextran Sulphate Sodium (DSS) Treatment

For DSS treatment, crosses and DSS feeding were essentially performed as described in (Amcheslavsky et al., 2009). DSS was stored at RT (MP Biochemical). A 3% DSS, 5% sucrose solution was made. A small piece of $6cm^2$ folded tissue (Kimberley-Clark) was secured to the bottom of an empty fly food vial using double sided tape. 0.5mls of 3% DSS, 5% sucrose solution was then pipetted onto the tissue. 5% sucrose solution without DSS was used as a control. For RNAi experiments, crosses were maintained at 18°C and adult flies were shifted to 29°C for 4-5 days before DSS treatment. Flies were transferred to a fresh vial of DSS after 24 hours and treated with DSS for 48 hours in total (Figure 4.13).

2.5.3 Hydrogen Peroxide (H$_2$O$_2$) Treatment

H_2O_2 treatment was essentially performed following the same method as used for DSS treatment. 30% H_2O_2 solution was stored at 4°C (Sigma). 0.5mls of 0.1% (Figure 5.7) or 0.2% (Figure 5.7B) H_2O_2 with 5% sucrose solution was pipetted onto a piece of tissue

at the bottom of an empty fly food vial. 5% sucrose solution without H_2O_2 was used as a control. Crosses were maintained at 18°C, adult flies were shifted to 29°C for 4-5 days before H_2O_2 treatment. Flies were transferred to a fresh vial of H_2O_2 after 24 hours and were treated with H_2O_2 for 48 hours in total (Figure 5.8) before dissection.

2.5.4 Axenic food preparation

Axenic fly food was prepared using our regular fly food recipe (see section 2.2.1). Food was allowed to cool to 60°C before the addition of a cocktail of antibiotics. Final antibiotic concentrations were: Tetracycline 10mg/ml (Sigma), Kanamycin 100mg/ml (Sigma), Ampicillin 100mg/ml (Sigma) and Erythromycin 50mg/ml (Sigma). All fly stocks used in the axenic feeding study were maintained on the germ-free food for two generations in order to eliminate the gut flora. Adult flies were crushed in 0.5ml PBS and the solution was spread onto LB agar plates (LB + 1% agar) in order to observe bacterial growth overnight at 37°C (Figure 5.4A). Experimental crosses and the resultant adult flies were set up and maintained on the axenic food until dissection (Figure 5.4B).

2.5.5 Survival experiments

For survival experiments, crosses were maintained at 18°C and adult flies were shifted to 29°C for 2-3 days before infection (Figure 4.12) or treatment with DSS (Figure 4.13). Flies were then transferred to a vial containing either *P.e* (Figure 4.12) or DSS (Figure 4.13) for two consecutive days (flies were transferred to a fresh vial of DSS on day two). The number of living flies was counted roughly every 12 hours in order to monitor survival. Flies maintained on control food were monitored alongside their experimental counterparts. Following the initial two-day "stress treatment", flies were transferred to fresh vials of standard fly food every three days.

2.6 Molecular Biology

2.6.1 Genomic DNA extraction

Genomic DNA was prepared from adult *Drosophila* males (1-3 males per extraction) homogenised in 200μl of PBS. DNA was extracted using the Quiagen DNeasy Blood and Tissue Kit (cultured cells protocol). 2μl of extracted DNA was then used in a 20μl PCR reaction.

2.6.2 Polymerase Chain Reaction (PCR)

PCR fragments were amplified using the Taq PCR Master-mix kit (Quiagen) and a PTC-200 Peltier thermal cycler (MJ Research). DNA fragments were amplified from the template DNA using oligonucleotide primers specifically designed to flank the region of interest. Primers were typically 20-30 nucleotides in length and manufactured by Sigma-Aldrich (see 2.6.3 Table of Primers).

A typical 20μl PCR reaction was composed of the following reagents:

10μl Taq PCR Master-mix

1μl 5' primer (10μm)

1μl 3' primer (10μm)

2μl Template DNA

6μl Distilled H_2O

A typical PCR program was as follows:

1) $95^{o}C$ for 5 minutes

2) $95^{o}C$ for 30 seconds (Denaturation)

3) $55^{o}C$ for 30 seconds (Annealing)

4) $72^{o}C$ for 1 minute per kb of product (Extension)

5) Go to step (2) – 32 cycles

6) $72^{o}C$ for 10 minutes

7) $4^{o}C$ for ever

2.6.3 Table of primers for fly genotyping

Primer Name	Primer Sequence
Flp 5'	CCTAAGGTGCTTGTTCGTCAGTTTG
Flp 3'	GTGATATTATCCCATTCCATGCGG
5' Gal4	TTCTTCTGTCGACGATGTGC
3' Gal4	GCGGTCTCGTTATTCTCAGC
5' Gal80	GTGGCCAGCCATTATGAAGT
3' Gal80	GGTAGGTTTGCCACCTTTGA
GFP Forward	GGAGTACAACTACAACAGCC
GFP Rev	CTTCGGGCATGGCGGACTTG
5' LacZ	TTCACTGGCCGTCGTTTTACAACGTCGTGA
3' LacZ	ATGTGAGCGAGTAACAACCCGTCGGATTCT
Neo2	AGAGGCGCTTCGTCTACGGAGCGACA
Hsp70	CGGCAAGCAGGCATCGCCATGGGTC
pMF3L	CCAGCAACCAAGTAAATCAAC
Stat-RNAI	CGCGAATTCCGCCCTCATCCAACGCATCT
Bsk RNAi	CGCGAATTCCGCCGCAAAGGAACTTGGAA
Notch RNAi	CGCGAATTCGCACCGAAAAGCAGGGCAAC
5' UAS hep	CCTCGTCATCATCCTCATCCGCATCC
3' UAS hep	CCACATTGCCGCTAGTCCCATTGCC
5' UAS puc	CCTCAAGTACATGCAAATACCTGCCAG
3' UAS puc	GGATTGCTAGGACTATTCAGGTGCGG
5' UAS yki	CCAGCAGCAATACAAACAGCC
3' UAS yki	CTGGGATCATTCCATGAAGTCGTTC

Table 5: Primers

2.6.4 Gel electrophoresis

PCR products were run on 0.8% agarose gels (50μl 0.5x TAE + 0.4g agarose (Invitrogen) + 1μl Ethidium Bromide (Roche). Gels were imaged using an IMAGO compact imaging system.

TAE

40mM Tris Acetate
1mM EDTA

2.6.5 Quantitative Reverse-Transcription PCR (qRT-PCR)

Ten guts of the relevant genotype were collected (as described in section 2.3.1), placed in an eppendorf, and frozen in liquid Nitrogen. Guts were later disrupted and homogenised in Trizol (Invitrogen), using a small hand-held motor driven pestle. Total RNA was isolated using Trizol and cleaned by column purification and on-column DNAse I treatment (Quiagen). 1.5 μg of RNA was reverse transcribed (SuperScript III cDNA Synthesis Kit, Invitrogen) using random hexamers to produce cDNA. To measure mRNA levels, qPCRs were carried out on reverse-transcribed mRNA. RT-qPCR was performed on cDNA corresponding to 3.75ng input RNA on a LightCycler 480 II using SYBR Green I (Roche). Experiments were performed in triplicates. Relative fold differences in expression levels of target genes were calculated as a ratio to the mean of three reference genes *dp1* (*dodeca-satellite-binding protein 1*), *rp49* (*ribosomal protein 49*) and *myo1A* for the analysis of short-term effects of PE infection (Figure 4.9), and to the mean of *dp1* and *rp49* for the analysis of long-term Yki expression (Figure 4.3 and 5.8).

2.6.6 Table of primers for qRT-PCR

Primer Name	Primer Sequence
CycE-F	ACAAATTTGGCCTGGGACTA
CycE-R	GGCCATAAGCACTTCGTCA
Diap1-F	GAAAAAGAGAAAAGCCGTCAAGT
Diap1-R	TGTTTGCCTGACTCTTAATTTCTTC
Dp1-F	CCGCAAATTCGACAGAGAC
Dp1-R	CGCAACATTTCGTTTTTCTG
Ex-F	GATGCTGGACACCGAACTC
Ex-R	CTTGCTCTCGGGATCTGC

Myo1A-F	GAAGCTGGAGTGCAGGACTT
Myo1A-R	GATGGATCCGTTTTGGAATC
Puc-F	GCCACATCAGAACATCAAGC
Puc-R	CCGTTTTCCGTGCATCTT
Rp49-F	CGGATCGATATGCTAAGCTGT
Rp49-R	CGACGCACTCTGTTGTCG
Socs36E-F	ACGCAACACAGCAGCAAG
Socs36E-R	GGACACGGATGTGGATGC
Upd1-F	CCTACTCGTCCTGCTCCTTG
Upd1-R	TGCGATAGTCGATCCAGTTG
Upd2-F	CATCGTCATCCTCATCATCG
Upd2-R	ATGTTCCGCAAGTTTTCGAG
Upd3-F	AAATTCGACAAAGTCGCCTG
Upd3-R	TTCCACTGGATTCCTGGTTC
Yki-F	GCGCCTTGCCGCCGGGATG
Yki-R	GCTGGCGATATTGGATTCTG

Table 6: Primers for qRT-PCR

Chapter 3.   Results

A role for the Hippo pathway in the adult *Drosophila* posterior midgut

3.1 Aims of this project

3.1.1 Overall Aim

At the start of my project, the majority of studies investigating the Hpo pathway had focussed on developmental contexts, particularly on its role in limiting tissue growth. Hpo pathway function in adult organisms has been relatively less explored. Growth control in the maintenance of adult homeostasis is a distinct process, whereby dying cells have to be replaced in the absence of tissue mass increase. The initial aim of my project was therefore to address whether the pathway might have a role in the adult fly.

As a key regulator of developmental organ growth, it was reasonable to hypothesise that the Hpo pathway might play a role in restricting regenerative growth in adult tissues. The Hpo pathway is known to be deregulated in various types of cancer (reviewed in (Fernandez and Kenney, 2010)). Understanding what role the pathway plays in adult homeostasis and abnormal growth could therefore be of significance to cancer therapy and regenerative medicine.

3.1.2 The adult posterior midgut

The adult population of ISCs in the *Drosophila* posterior midgut were discovered two years prior to the start of my PhD (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). This finding not only provided a new model system in which to investigate SC biology but also a new adult model system in which to utilise *Drosophila* genetic tools to develop our understanding of various signalling pathways.

Further support for an in-depth investigation into the role of the Hpo pathway in the adult posterior midgut came one year later. A study from Camargo *et al* showed that in

the mouse intestine, overexpression of YAP1, the mammalian orthologue of Yki, results in expansion of the progenitor cell compartment. It thus became clear that the Hpo pathway might play a role in regulating growth in the intestine (Camargo et al., 2007).

3.1.3 Initial approach

My initial aim was to investigate whether Hpo pathway inactivation had any effect in the adult posterior midgut, the *Drosophila* equivalent of the small intestine. To examine the effect of Hpo pathway inactivation in the midgut, I began by inducing Yki overexpression, which phenocopies *hpo* or *wts* loss in imaginal discs (Huang et al., 2005). To ensure transgene expression was restricted to progenitor cells in the adult midgut, expression was placed under the control of the ISC/EB driver *esg-Gal4*, and temporally restricted using temperature-sensitive *Gal80* under the control of the *tubulin* promoter (*esg-Gal4;tub-Gal80^{ts},UAS-GFP* from here on referred to as *esg^{ts}*) (Micchelli and Perrimon, 2006, McGuire et al., 2003). Adult flies were shifted to the restrictive temperature (29°C) in order to activate transgene expression, and later dissected (detailed timings of temperature shifts can be found in Chapter 2).

Although Yki overexpression has been shown to phenocopy *hpo* or *wts* loss in imaginal discs, throughout this study I have tried to avoid relying solely on overexpression when drawing conclusions on Hpo pathway function in the midgut. Misexpression of the Yki transgene is not necessarily representative of endogenous Yki function. In order to address this issue, where possible loss-of-function experiments using a *wts*-RNAi line or MARCM clones mutant for members of the Hpo pathway were also generated. However, since YAP amplification has been reported in a number of cancer types (Steinhardt et al., 2008, Zender et al., 2006, Fernandez and Kenney, 2010), my overexpression experiments may be analogous to intestinal tumourigenesis.

3.2 Effects of Hpo pathway inactivation in ISCs

3.2.1 Hpo pathway inactivation induces an increase in the number of *esg+* cells

esg^{ts}-driven expression of Yki led to an increase in the number of *esg+* ISCs and EBs compared to controls (Figure 3.1A-B"). An increase in the number of *esg+* cells was also apparent when the upstream kinase Wts was silenced in the ISCs and EBs (Figure 3.1C-C"). No obvious phenotype was observed upon esg^{ts}-driven expression of an *hpo*-RNAi construct (data not shown), although only one *hpo*-RNAi line was tested.

The appearance of this mutant phenotype was characterised in more detail by examining different regions of the gut at various time points post-temperature shift (Figure 3.2). As detailed in Chapter 2, crosses we set up at 18°C and adult flies were maintained at 18°C for 1-2 days post-eclosion, before shifting to the non-permissive temperature (29°C). This 1-2 day delay was to avoid transgene expression having an effect on the final stages of gut development in the young adult flies.

An increase in *esg+* cells was occasionally observed as early as 24 hours post-temperature shift (day three in Figure 3.2), with small clusters of cells beginning to form (Figure 3.2B). By day seven, these small clusters formed larger patches. The same phenotype was apparent at various locations along the midgut (Figure 3.2B). The strongest phenotype, in terms of a consistent increase in *esg+* cells, was observed in the posterior midgut (Figure 3.1) and in a small patch of tissue located in the anterior midgut. I therefore decided to focus the study on the posterior midgut since the cellular organisation and *esg* expression pattern in this region is better characterised (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2007).

It is important to note that one of the drawbacks of using the midgut as a model system is the level of variability. Gut homeostasis is a carefully controlled process and factors such as age and diet can have rapid and significant effects on homeostatic balance. Unsurprisingly, at an early stage in the project, it became apparent that flies reared at the same temperature, on the same diet and aged for the same length of time, often
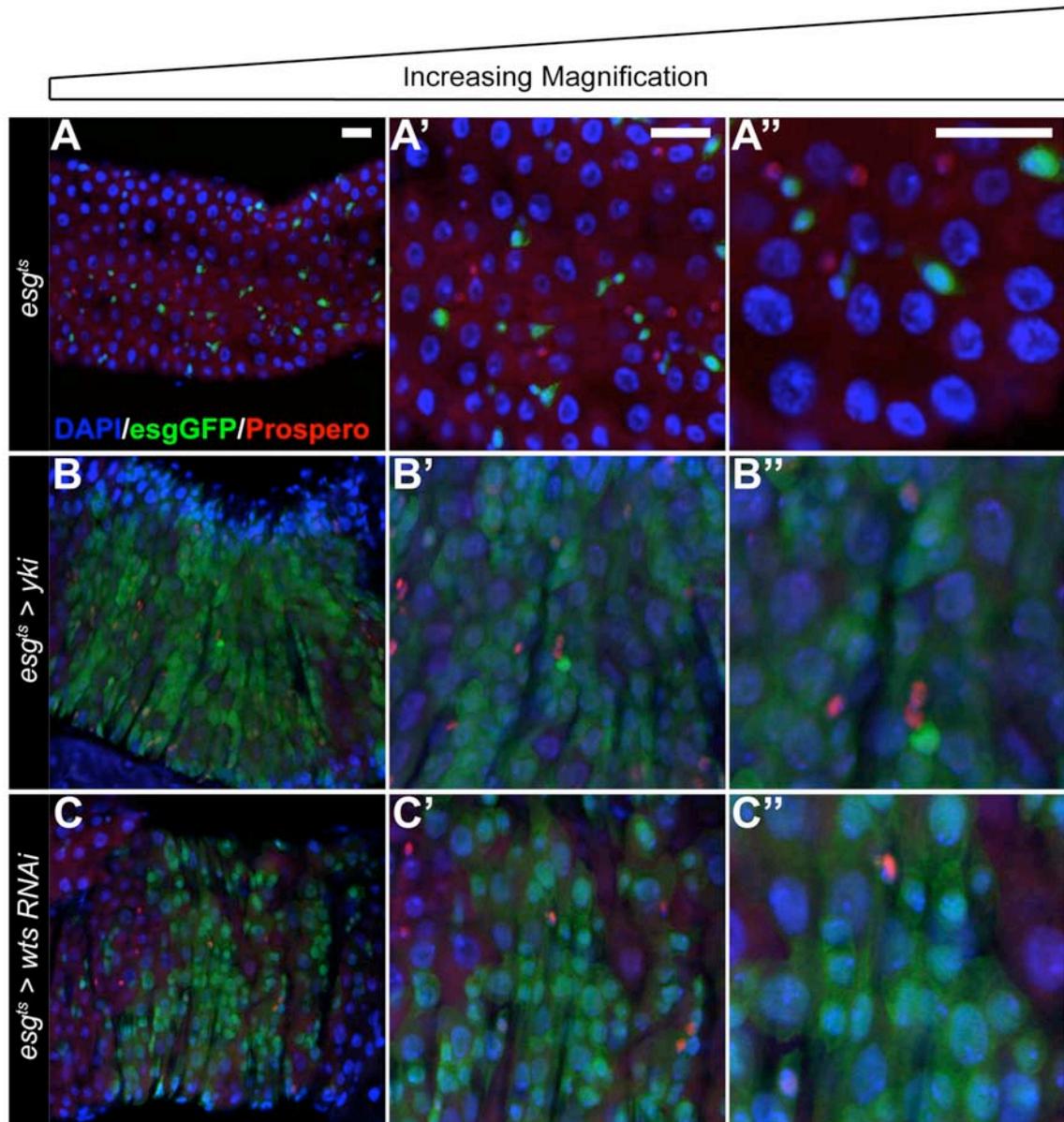
Figure 3.1: Hpo pathway inactivation in ISCs induces an increase in *esg*-positive cell number

Confocal micrographs of adult posterior midguts of increasing magnification. **(A-C'')** *esg^{ts}*-driven expression of Yki (B-B'') or *wts*-RNAi (C-C'') in ISCs and EBs leads to an increase in *esg+* cells compared to control (A-A''), but no obvious effect on *pros+* cell numbers. Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green) and Pros is in red. Scale bars = 20μm.
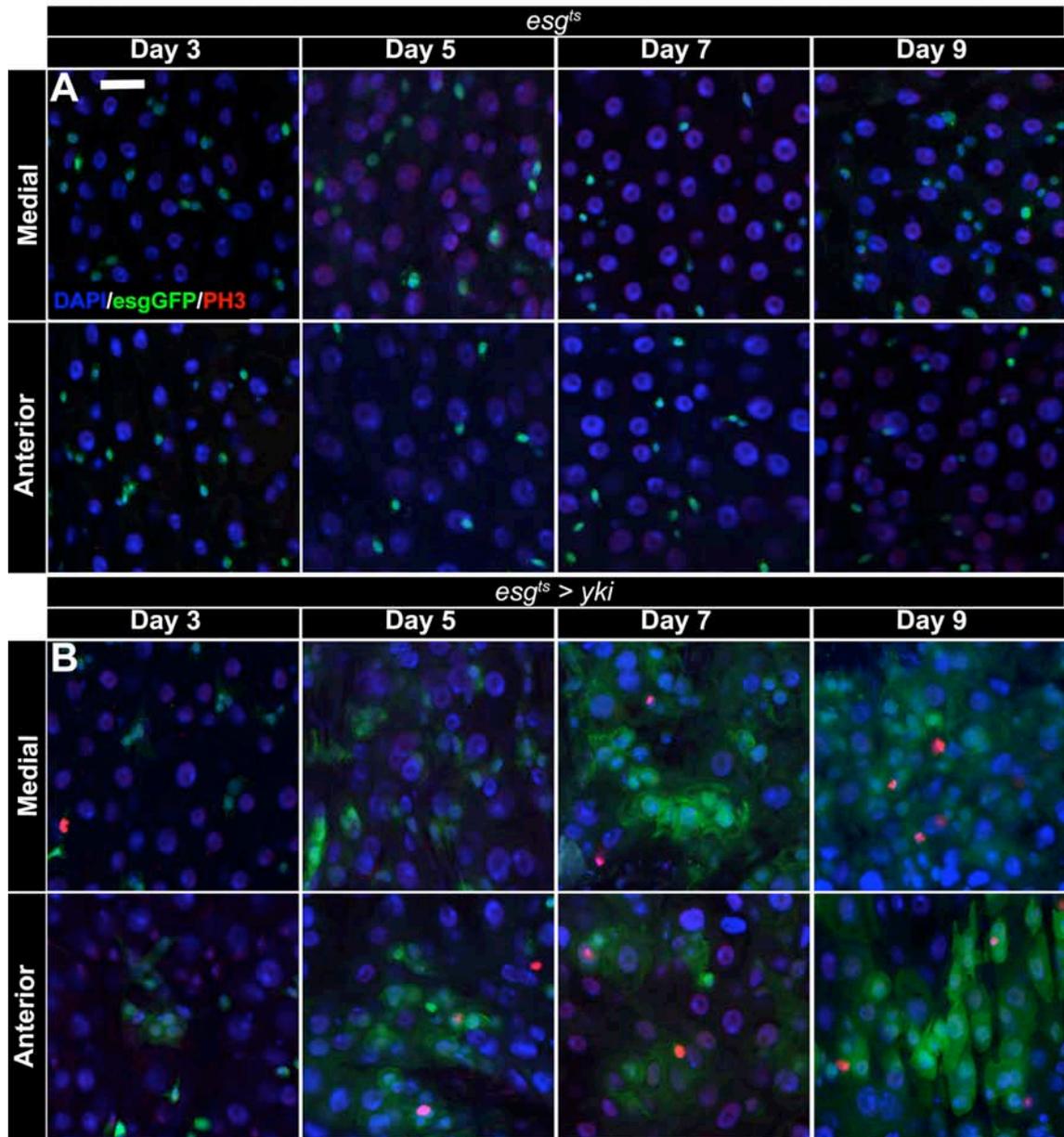
Figure 3.2: Time course of Yki expression in ISCs in different regions of the midgut

Confocal micrographs of adult midguts at different time points following shift to the non-permissive temperature in order to observe progression of the phenotype. Images from the medial and anterior midgut are shown. **(A-B)** *esg^{ts}*-driven expression of Yki (B) induces a gradual increase in numbers of *esg+* cells and PH3 marked mitoses in various regions of the midgut when compared to control (A). Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green) and PH3 is in red. Yki overexpression could induce clumping of *esg+* cells after just 24hrs (day three) at the non-permissive temperature. By day seven large patches of *esg+* cells are present. Scale bar = 20μm.

exhibited a large degree of variability in terms of *esg+* cell number and proliferation. This is also true for the Yki overexpression phenotype and generally of other mutant/overexpression phenotypes I examined. In order to overcome this, experimental timings and diet were carefully controlled (as detailed in Chapter 2) and large numbers of guts were used for all experiments in order to control for environmental variability.

3.2.2 Hpo pathway inactivation affects the size of *esg+* cells

Notch signalling has previously been shown to promote ISC differentiation (Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006). I wanted to see how the Yki overexpression phenotype compared to Notch loss of function. As expected, Notch depletion caused an increase in the number of small, *esg+* cells representing ISC-like tumours due to a blockage in differentiation (Figure 3.3B). In contrast, Yki overexpression led to overproliferation and the formation of patches of *esg+* cells which had a mixture of small nuclei typical of ISCs and EBs (Figure 3.3C, arrowheads) and much larger nuclei (Figure 3.3C, arrow). The induction of Yki overexpressing Flipout clones also caused an increase in *esg+* cell size (Figure 3.3D-E").

Transverse cryosections through the midgut showed that esg^{ts}-driven expression of Yki leads to increased numbers of progenitor cells (*esg+*), an enhanced cell density of differentiated cell types (*esg-*) and an increased thickness of the epithelium (Figure 3.3F-G). This was also apparent in optical sections through MARCM clones mutant for the upstream kinase *hpo* compared to the surrounding non-mutant epithelium (Figure 3.3H-H'). I therefore concluded that inactivation of the Hpo pathway in the posterior midgut leads to an accumulation of *esg+* cells, both smaller (ISCs/progenitors) and larger, as well as a thickening of the epithelium.

3.2.3 Hpo pathway inactivation promotes Dl expression

In order to examine whether *esg+* cells retain SC characteristics upon Yki expression, I examined the expression of Dl, currently the best-characterised ISC-specific marker
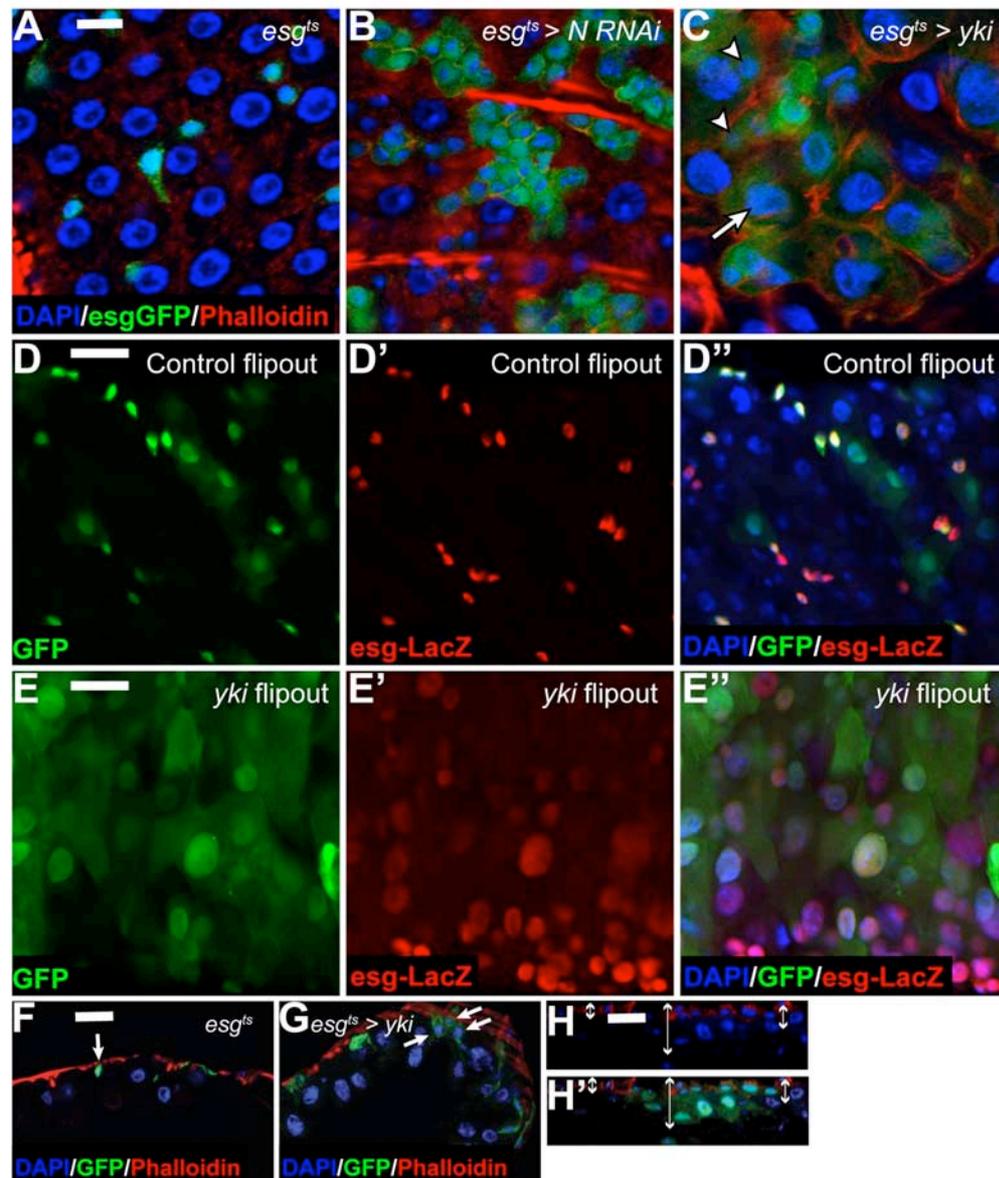
Figure 3.3: Yki overexpression increases *esg*-positive cell number and size

(A-C) *esg^ts^*-driven expression of Yki (C) or *Notch*-RNAi (B) in ISCs and EBs induces an increase in *esg*+ cell number compared to control (A). Yki overexpression induces the appearance of *esg*+ cells with large nuclei (arrow), but smaller nuclei remain (arrowheads). **(D-E")** Flipout clones overexpressing Yki (E-E'') induce an increase in size and number of esg+ cells compared to control (D-D"). **(F-G)** Orthogonal cryosections of the adult midgut epithelium showing that *esg^ts^*-driven expression of Yki (G) leads to increased nuclear density and number of basally located *esg*+ cells (arrows) compared to control (F). **(H-H')** Orthogonal section of a two-week-old MARCM *hpo* clone (H-H') shows increased epithelial thickness (arrows) compared to surrounding control tissue. Nuclei are stained with DAPI (blue), *esg*+ cells marked by GFP (green in A-C and F-G) and β-Galactosidase (red in D-E''), Phalloidin (A-C, F-G) and armadillo (β-catenin) staining (H-H') are in red. Flipout (D-E'') or MARCM (H, H') clones are marked with GFP in green. Scale bar = 10μm (A-C) Scale bars = 20μm (D-H"). Panels A-C generated by C.Polesello. F-H' are data from A.Kohlmaier.

(Ohlstein and Spradling, 2007). In WT (wild type) guts, Dl-expressing cells are not found immediately adjacent to one another, presumably since Dl can block its own expression by inducing Notch signalling in neighbouring cells (Figure 3.4A-A'). Following Yki overexpression, I observed an increase in the number of cells expressing Dl (Figure 3-4B-B'), possibly suggesting an increase in ISC number. Moreover, Dl expression was no longer restricted to small cells, with some larger *esg+* cells now being Dl+ (Figure 3.4B'). Given the apparent increase in cell size and Dl expression, it is unclear whether these larger Dl+ cells are true ISCs. From here on in I therefore refer to this population of large Dl+ cells as "ISC-like cells".

Quantification of the number of Dl+ cells upon Yki overexpression showed the number of Dl+ cells per unit area to be significantly increased (Figure 3.4D). Since the total number of midgut epithelial cells or cell density were not taken into account, it is not possible to conclude whether the proportion of Dl+ cells in relation to the total midgut cell population is increased or whether the apparent increase in Dl+ cells per unit area merely reflects a general increase in cell number and density within the midgut. Silencing the Hpo pathway using a *wts*-RNAi construct also resulted in the appearance of clusters of Dl+ cells (Figure 3.4C-C'). Interestingly, not only was the number and often size of Dl+ cells increased but the level of Dl being expressed by individual cells was also enhanced (Figure 3.4 B', C' compared to A').

3.3 Hpo pathway inactivation promotes proliferation in the midgut

Increased Dl+ cell number and thickening of the epithelium suggest an overproliferation phenotype. This issue was addressed using phospho-Histone H3 (PH3) staining, which marks condensed chromatin during mitosis and therefore dividing cells. In control flies, I observed few PH3+ cells in the gut (Figure 3.5A, E). Hpo signalling disruption by Yki overexpression or Wts depletion in progenitor cells (ISCs and EBs) caused a marked increase in PH3+ cell numbers (Figure 3.5 B-C and E). PH3 staining coincided with the increase in number of *esg+* cells but varied greatly between individual guts, and
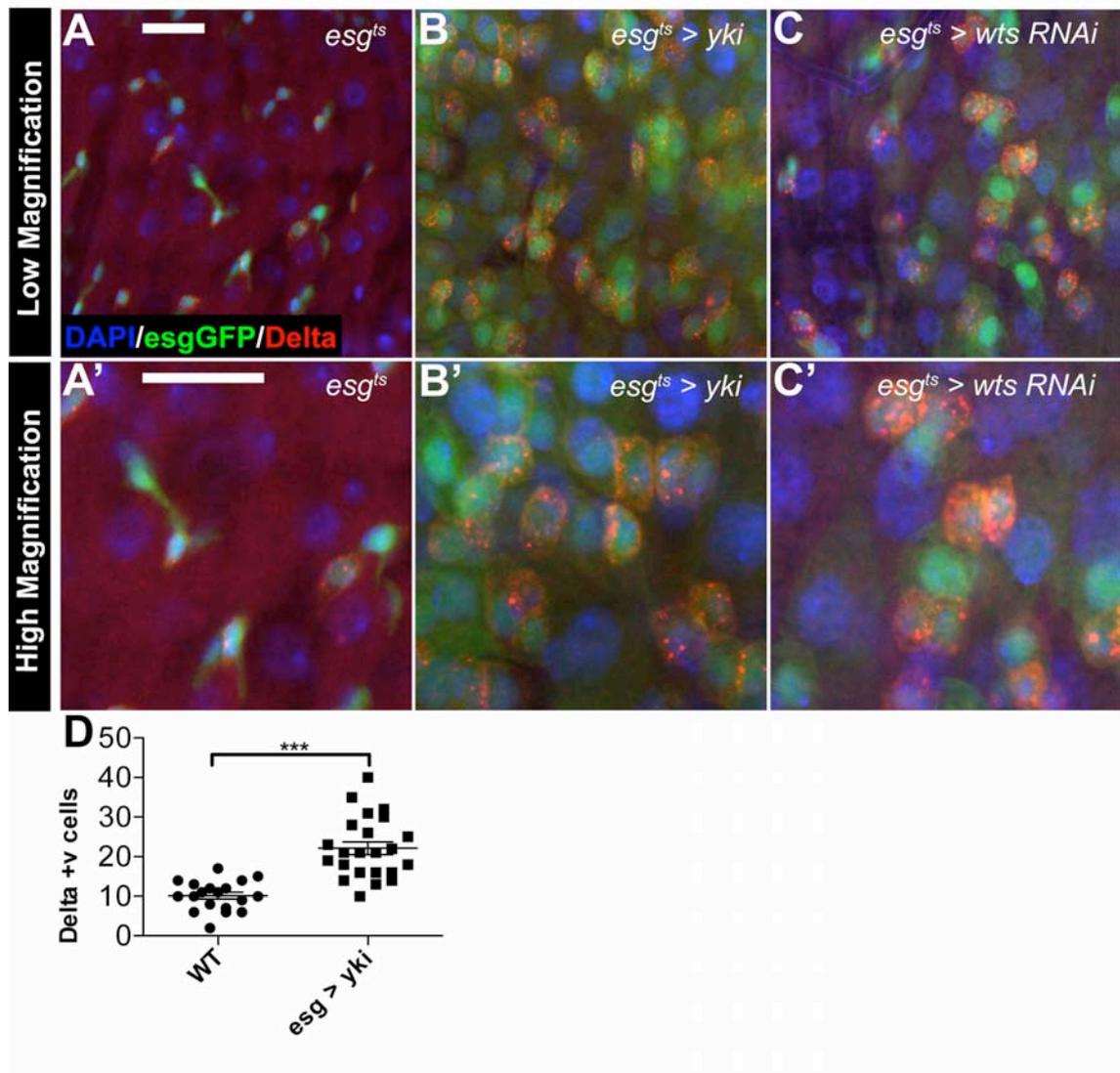
Figure 3.4: Hpo pathway inactivation promotes Delta expression

(A-C') *esg^{ts}*-driven expression of Yki (B-B') or *wts*-RNAi (C-C') induces increased numbers of Dl+ cells compared to control (A-A'). Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green) and Dl is in red. Scale bars = 20μm. **(D)** Quantification of Dl+ cells. *esg^{ts}*-driven expression of Yki significantly increases the number of Dl+ cells in a given area in adult midguts compared to control (p<0.0001, n>19).
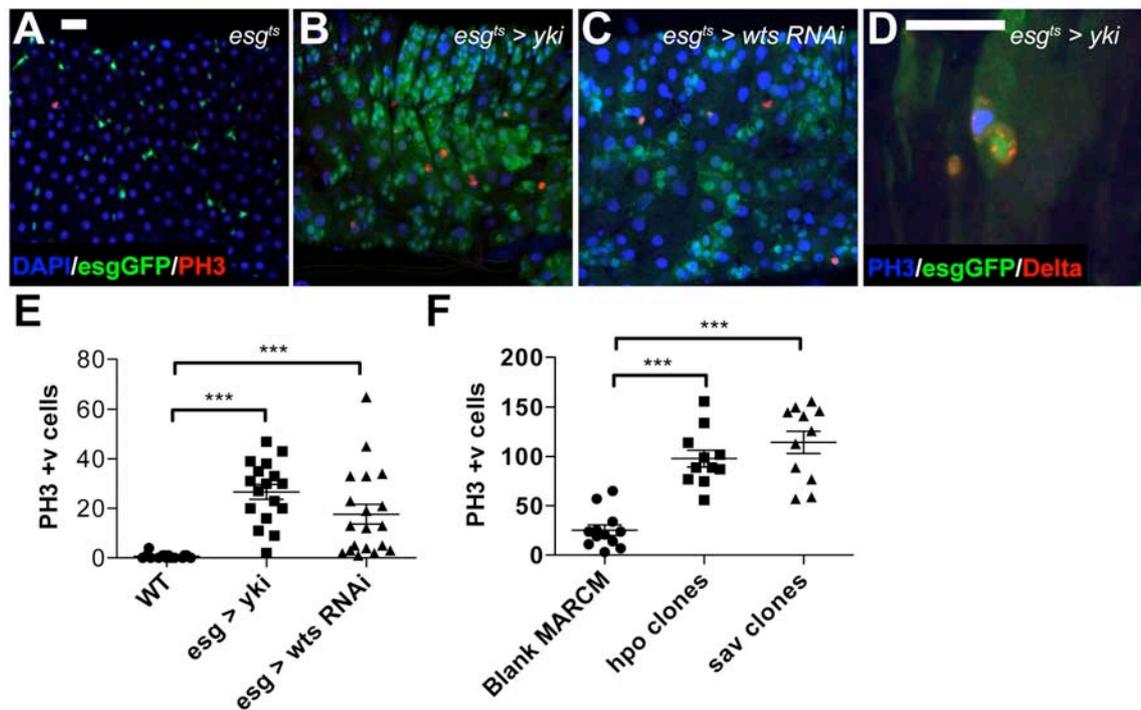
Figure 3.5: Loss of Hpo signalling increases proliferation in the midgut

(A-C) *esg^ts*-driven expression of Yki (B) or *wts*-RNAi (C) increases the number of PH3+ cells compared to control (A). **(D)** Dividing cells are Dl+ when Yki is overexpressed in ISCs and EBs, as is the case in WT guts. Nuclei stained with DAPI, *esg+* cells are marked by GFP (green) and PH3 (A-C) and Dl (D) are in red. Scale bar = 20μm. **(E)** Quantification of PH3+ cells. *esg^ts*-driven expression of Yki or *wts*-RNAi significantly increases the total number of PH3+ cells in adult midguts compared to control. In both cases $p < 0.0001$, $n > 15$. **(F)** *hpo^{42-47}* or *sav^{shrp1}* mutant MARCM clones increased mitotic rates (PH3+ cells/gut) along the entire midgut compared to control clones. $p < 0.0001$, $n = 12$. Panel F was generated by A. Kohlmaier.

according to position within the gut (Figure 3.2B). Quantification of PH3 foci in populations of guts revealed a significant increase in the number of dividing cells upon Hpo pathway inactivation (Figure 3.5E). In addition, we used the MARCM approach to generate *hpo* or *sav* mutant clones and assess their impact on global midgut proliferation rate. Loss of either *hpo* or *sav* in MARCM clones significantly increased total number of PH3+ cells (Figure 3.5F).

Quantification of the total number of PH3+ cells clearly demonstrates an increase in the number of dividing cells at a given time in the midgut. This increase in the number of PH3+ cells could suggest that midgut ISCs and ISC-like cells are dividing at a faster rate and hence more PH3+ cells can be seen at any particular moment in time. Equally, it is also possible that the increased number of PH3+ cells is merely a reflection of a general increase in the number of cells capable of dividing. Hence, given that the data in Figure 3.5 do not take into account overall cell number and density it is difficult to conclude whether the behaviour of individual dividing cells has been altered (such as proliferation rate or division outcome) or whether more cells capable of dividing are present.

The cells undergoing mitosis were ISC-like in the sense that they were labelled for Dl (Figure 3.5D). The true identity of these Dl+ ISC-like cells remains unclear. It is possible that their larger size is a result of faster rates of cell growth (mass accumulation) in order to accommodate the need to generate more differentiated progeny. A further possibility is that the appearance of large Dl+ cells is due to an alternative population of cells (i.e non-ISCs) being induced to divide. The observation that no *Su(H)Gbe-LacZ* (Notch) positive EB re-entered mitosis (Figure 3.6B'; n=10 guts) argues against the possibility that Yki activation causes EB dedifferentiation or interferes with exit from the mitotic cycle in specified EBs. Equally, cells expressing markers for the differentiated cell types of the midgut were never seen to divide. Although the majority of Dl+ cells present in the midgut following Yki overexpression are increased in size, a population of small, Dl+ cells is still apparent and may represent the "true" ISC population (data not shown).

3.3.1 Study of MARCM clones in the midgut

It must be noted that, from my observations, even blank control MARCM clones generally lead to elevated numbers of PH3+ cells in midgut tissue. Use of the MARCM technique proved difficult at various stages of my project. In particular, it was difficult to control clone number, resulting in either too few or too many clones (leading to fusion of multiple clones). As a result, I was unable to complete as many experiments as I would have liked using this technique. For example, ideally one would be able to assess the number of cells per clone in Hpo pathway mutant MARCM clones. My results suggest that this number is increased in *hpo* mutant MARCM clones but I have never been able to complete this experiment to my full satisfaction. The difficulty of generating MARCM clones, along with the level of variability seen following Yki overexpression in the ISCs, highlights further the need to complete a range of genetic analyses when drawing conclusions on studies in the gut.

3.3.2 Parallels between Hpo pathway inactivation and gut regeneration

The increase in cell size following Hpo pathway inactivation suggests that the Yki overexpression phenotype is not solely due to the increase in proliferation. Many, but not all, of the large *esg+* cells present in midguts following Hpo pathway inactivation are Dl+. The Yki overexpression phenotype bears a striking resemblance to the midgut regenerative response. Several laboratories have observed that following stress stimuli such as infection with bacteria, tissue damage or JNK signalling, the midgut responds by increasing ISC Dl levels in order to promote differentiation into functional ECs (Amcheslavsky et al., 2009, Biteau et al., 2008, Buchon et al., 2009a, Buchon et al., 2009b, Cronin et al., 2009, Jiang et al., 2009). This increase in Dl levels is accompanied by an increase in the number and size of the *esg+* cells. The presence of large *esg+* cells has been suggested to be indicative of a faster rate of cell turnover in the gut: as the production of ECs accelerates, the relatively slow rate of GFP decay results in some differentiated cells retaining the esg>GFP marker. Hence, Hpo pathway inactivation induces a phenotype akin to gut regeneration.

3.4 Effect of Hpo pathway inactivation on midgut terminal differentiation

Next, I studied whether Hpo signalling affects the terminal differentiation of midgut cells. The EBs undergo no transit amplifying divisions and give rise to one of two cell types – the secretory EE cells and absorptive ECs (Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006). Notch signalling is required to drive cells into a post-mitotic state and also functions in specifying cell fate (Ohlstein and Spradling, 2007). Notch activation favours absorptive differentiation at the expense of secretory cells (Ohlstein and Spradling, 2007, Bardin et al., 2010). Notch mutant clones contain an excess of D1+ ISCs and high numbers of *pros+* EE cells at the expense of ECs, while Notch activation leads to terminal differentiation of ECs (Micchelli and Perrimon, 2006).

3.4.1 Hpo pathway inactivation alters Notch reporter levels

To determine if the increase in Dl levels affects Notch signalling activity, the expression of the Notch reporter *Su(H)Gbe-LacZ* was examined (Furriols and Bray, 2001). *Su(H)Gbe-LacZ* is normally expressed by the EBs (Figure 3.6A-A' arrow) as a result of Dl expression in the ISCs (Figure 3.6A-A' arrowhead) (Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006, Ohlstein and Spradling, 2007). Unsurprisingly, Yki overexpression resulted in increased numbers of Notch reporter-positive cells, with diffuse *Su(H)Gbe-LacZ* levels throughout the *esg+* area, presumably reflecting the increase in the number of Dl+ cells (Figure 3.6B-B'). Despite the increase in the number of Dl+ and *Su(H)Gbe-LacZ+* cells, these markers remain expressed in separate cells, except in rare cases where cells expressing *Su(H)Gbe-LacZ* also had very low levels of Dl (Figure 3.6C-C''').

The presence of cells positive for both Dl and *Su(H)Gbe-LacZ* might reflect an increased rate of progenitor production triggered by accelerated ISC division. As the number of ISCs and EBs increases in a single location, the process of lateral inhibition,
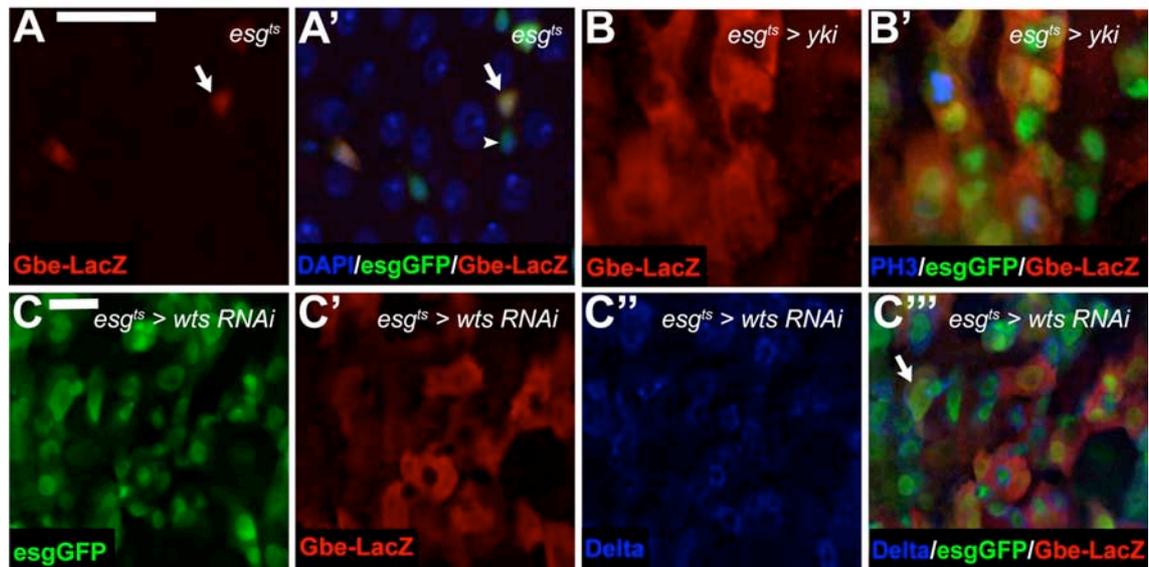
Figure 3.6: Hpo pathway inactivation alters Notch reporter levels

(A-B') Overexpression of Yki in ISCs and EBs (B-B') induces increased staining for the Notch reporter *Su(H)Gbe-LacZ* compared to control (A-A') with dividing cells remaining negative for the Notch reporter. **(C-C''')** *esg^{ts}*-driven expression of *wts*-RNAi (C-C''') induces an increase in both Dl and *Su(H)Gbe-LacZ* levels, but these markers are only very rarely found to be expressed in the same cell (arrow in C'''). Nuclei stained with DAPI (A'), PH3+ cells (B') and D1 (C-C''') are in blue, *esg+* cells are marked by GFP (green), β-Galactosidase (A-C''') is in red. Scale bar = 20μm.

which is thought to downregulate Dl expression in the EBs, may be too slow to accommodate the extra cells, resulting in the appearance of intermediates between the EB and ISC fates.

3.4.2 Hpo pathway inactivation does not prevent EE differentiation

Secretory EE cells represent ~10% of differentiated cells in the midgut and can be marked by Pros staining (Figure 3.7A). As highlighted in section 3.2, Yki overexpression leads to an increase in the number of *esg+* cells. An obvious possibility was that Yki might act via Notch signalling. If this were the case, one would expect the number of EE cells to be reduced upon Yki overexpression, as is the case in Notch mutants. I observed no apparent change in the number of EE cells per unit area in response to Hpo pathway inactivation using *esg^{ts}* (Figure 3.7B-D and 3.1). This result demonstrates that EE cell number is not increased upon Yki overexpression. The approach taken in Figure 3.7D does not however rule out the possibility that the ratio of EEs versus total cell number may in fact be reduced, since any change in cell density was not taken into account when Pros+ cells were quantified. The possibility therefore remains that Hpo pathway inactivation may have an effect on cell fate specification. This could in theory be due to alterations on cell division outcome, for example cells might be pushed to divide symmetrically to produce more ISCs/ISC-like cells rather than differentiated cells. Regardless of this uncertainty surrounding the effect of Hpo pathway inactivation on cell division outcome, one can still conclude that differentiated cells can be generated even in the absence of Hpo signalling activity.

3.4.3 Hpo pathway inactivation does not prevent EC differentiation

The class II POU domain transcription factor Pdm-1 stains the large polyploid nuclei of ECs (Figure 3.8A-A') (Lee et al., 2009). Hpo pathway inactivation does not block EC differentiation, since a dense network of Pdm-1+ cells is still observed in Yki overexpressing and *wts*-RNAi guts (Figure 3.8B-B' and C-C'). However, a close study of Pdm-1 expression in both *UAS-yki* and *wts*-RNAi backgrounds revealed that some
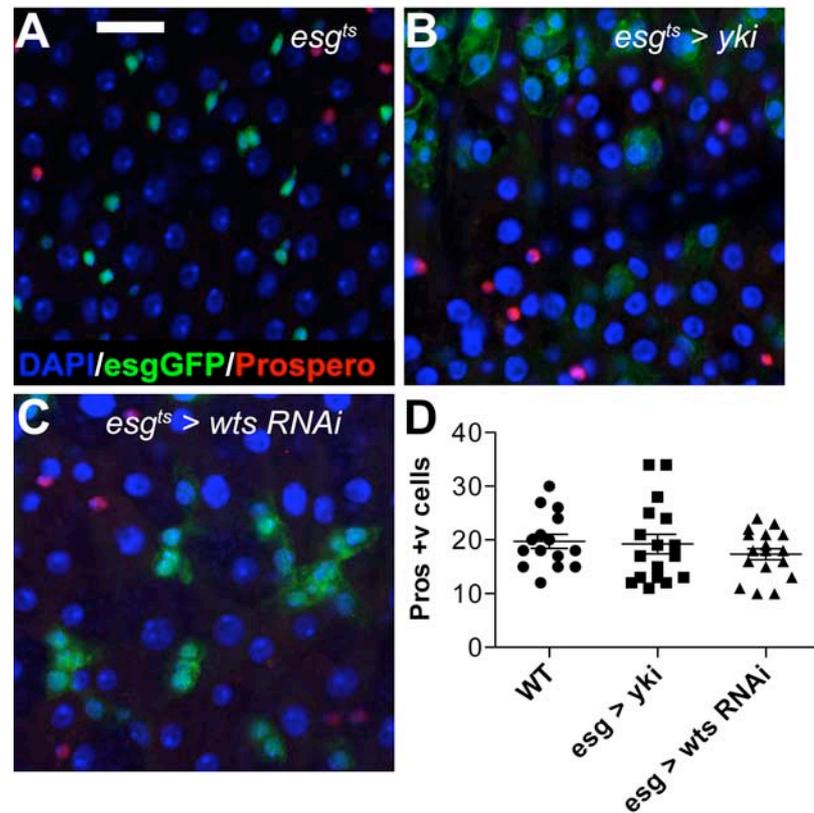
Figure 3.7: Hpo pathway inactivation does not prevent EE differentiation

(A-C) *esg^ts*-driven expression of Yki (B) or *wts*-RNAi (C) does not alter EE cell number per unit area compared to control (A). Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green) and Pros is in red. Scale bar = 20μm. **(D)** Quantification of Pros+ cells in the indicated genotypes (n>15).
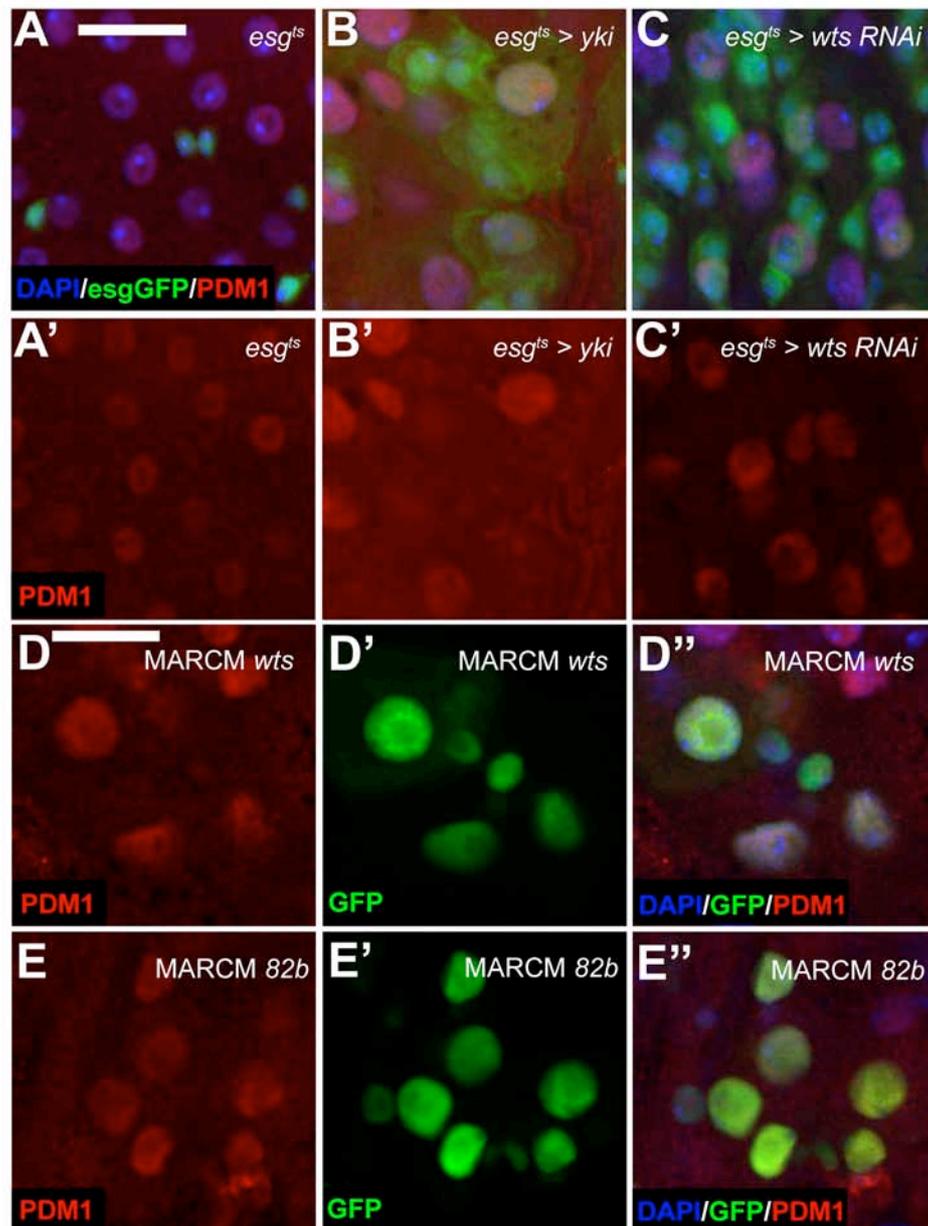
Figure 3.8: Hpo pathway inactivation does not prevent EC differentiation

(A-C') *esg^ts*-driven expression of Yki (B-B') or *wts*-RNAi (C-C') increases EC nuclear size compared to control (A-A'). **(D-E")** Pdm1 staining in 10 day old *wts* mutant MARCM clones (D-D") reveals that terminal differentiation can still occur as in WT MARCM clones (E-E"). Nuclei are stained with DAPI (blue), *esg+* cells marked by GFP (A-C') and MARCM clones marked by GFP (D-E') are in green, and Pdm1 is in red. Scale bars = 20μm.

Pdm-1+ cells retain *esg* expression (Figure 3.8B, C). In addition, many of the Pdm-1+ cells appeared to be larger than control ECs (compare Figure 3.8B-B' to Figure 3.8A-A'). It is plausible that this larger cell size is due to increased endoreplication as a result of *yki* overexpression (see section 3.6.1). Sustained *esgGFP* expression in ECs has been suggested to be indicative of an increased differentiation rate upon gut regenerative growth, which would cause a perdurance of GFP expression in differentiated cells (Jiang et al., 2009). To further demonstrate that *hpo* pathway inactivation does not abolish terminal differentiation, I generated *wts* mutant MARCM clones (figure 3.8D-E") and confirmed the presence of Pdm-1+ cells. Thus, Hpo pathway inactivation leads to increased ISC proliferation, while still allowing terminal differentiation to proceed. This is in contrast to Notch inactivation, which prevents terminal differentiation (Ohlstein and Spradling, 2007).

3.5 Effects of Hpo pathway inactivation in ECs

Expression induced by the *esg*^{ts} driver is mostly restricted to ISCs and EBs, although some transcript and protein product is likely to persist in ECs, given that there are no transit-amplifying divisions in the ISC lineage. Since Hpo pathway inactivation elicits a phenotype similar to that observed upon stress signalling activation in ECs (Amcheslavsky et al., 2009, Biteau et al., 2008, Buchon et al., 2009a, Buchon et al., 2009b, Cronin et al., 2009, Jiang et al., 2009), I decided to examine the effects of Yki overexpression specifically in this cell population. *MyoIAGal4* is an enhancer trap inserted in the gut-specific brush border *myosin IA* gene (Morgan et al., 1994). The inducible *Myo1AGal4, tubGal80*^{ts} system (from here on referred to as *MyoIA*^{ts}) drives UAS expression in midgut ECs (Figure 3.9A) (Jiang et al., 2009).

3.5.1 Hpo pathway inactivation in ECs triggers Delta expression

I used the *MyoIA*^{ts} system combined with UAS-GFP in order to drive *UAS-yki* in the polyploid ECs. When restricted to the EC population, *UAS-yki* overexpression induced a strong increase in Dl staining (Figure 3.9B-B") along with clusters of small *esg+* cells and some perduring *esg-LacZ* expression in ECs (Figure 3.10A-D"). Quantification of
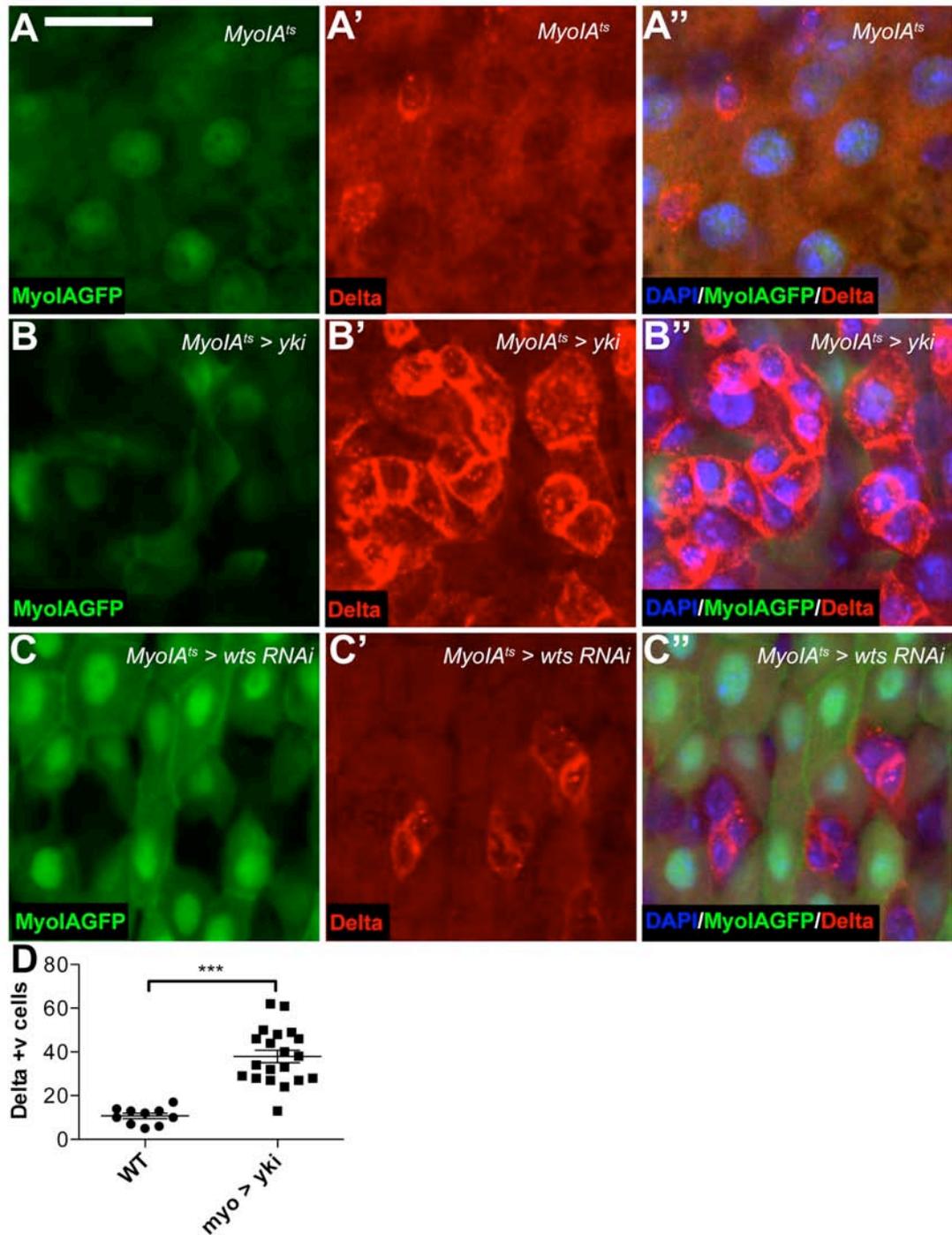
Figure 3.9: Hpo pathway inactivation in ECs triggers Delta expression

(A-C'') *MyoIA^ts*-driven expression of Yki (B-B'') or *wts*-RNAi (C-C'') in ECs induces a marked increase in the number of Dl+ cells per unit area compared to control (A-A''). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green) and Dl is in red. Scale bars = 20μm. **(F)** Quantification of Dl+ cells per unit area in the indicated genotypes (p<0.0001, n>10).
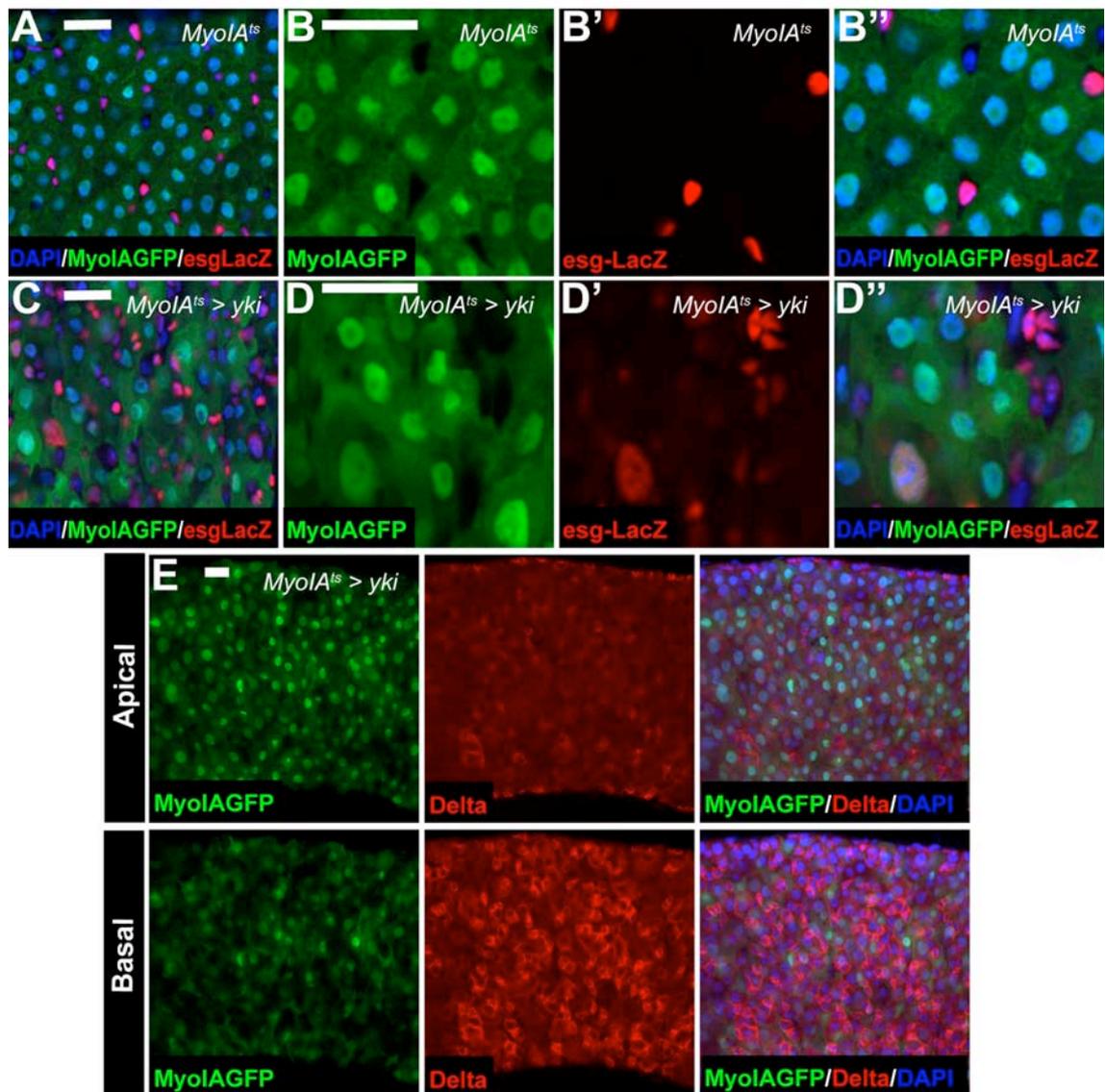
Figure 3.10: Hpo pathway inactivation in ECs increases *esg*-positive cell numbers

(A-D") *MyoIA^{ts}*-driven expression of Yki (C-D") results in increased numbers of *esg+* cells, many of which have large nuclei compared to WT (A-B''). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green) and β-Galactosidase marking *esg+* cells is in red. **(E)** *MyoIA^{ts}*-driven expression of Yki (E) results in an increase in basally located Dl positive cells. Nuclei are stained with DAPI (blue), ECs are marked by GFP (green) and Dl is in red. Scale bars = 20μm.

the number of Dl+ cells showed that the number of Dl+ cells per unit area was significantly increased when Yki was overexpressed in the ECs (Figure 3.9D). Similar results were seen when *MyoIA^ts* was used to drive *wts*-RNAi in the ECs (Figure 3.9C-C") with small clusters of Dl+ cells starting to form. Upon closer examination, it is clear that the majority of the Dl+ cells in these mutant guts remain basally located, as is the case for Dl+ ISCs in WT guts (Figure 3.10E). This observation suggests that, while some of the Dl+ cells are increased in size, they remain ISC-like in terms of location.

3.5.2 Hpo pathway inactivation in ECs triggers increased proliferation

The observed increase in the number of *esg+* and Dl+ cells could be suggestive of a faster rate of ISC proliferation, as was the case following Hpo pathway inactivation under the *esg* promoter. Indeed, higher PH3+ cell numbers were observed in *MyoIA^ts UAS-yki* and *MyoIA^ts UAS-wts*-RNAi guts, indicating that Hpo pathway inactivation in ECs can potently trigger midgut proliferation (Figure 3.11A-D). In accordance with this increased proliferation, cell density is also increased in *MyoIA^ts UAS-yki* guts compared to controls (Figure 3.11E). As was the case in Figure 3.5, it is unclear whether this increased number of PH3+ cells is due to a change in the behaviour of individual ISC and ISC-like proliferating cells (such as increased proliferation rate) or merely represents an overall increase in the number of dividing cells present in the midgut.

3.6 Non cell-autonomous effects of Hpo pathway inactivation

The ability of Yki expression in the ECs to induce proliferation of the neighbouring ISCs suggests that Hpo pathway inactivation in the ECs triggers a non cell-autonomous proliferation signal. To further investigate the non cell-autonomous effects of Hpo pathway repression, I examined Dl expression in a clonal context. The induction of Yki overexpressing Flipout clones revealed an increase in Dl expression both within the clones and in the surrounding WT tissue (Figure 3.12A-B''). Unsurprisingly, clonal Yki expression also induced ectopic Notch activation in neighbouring cells (Figure 3.12C-D''). This is most likely due to the increase in Dl levels. In further support of a non cell-autonomous role for the Hpo pathway in the gut, *wts* mutant MARCM clones
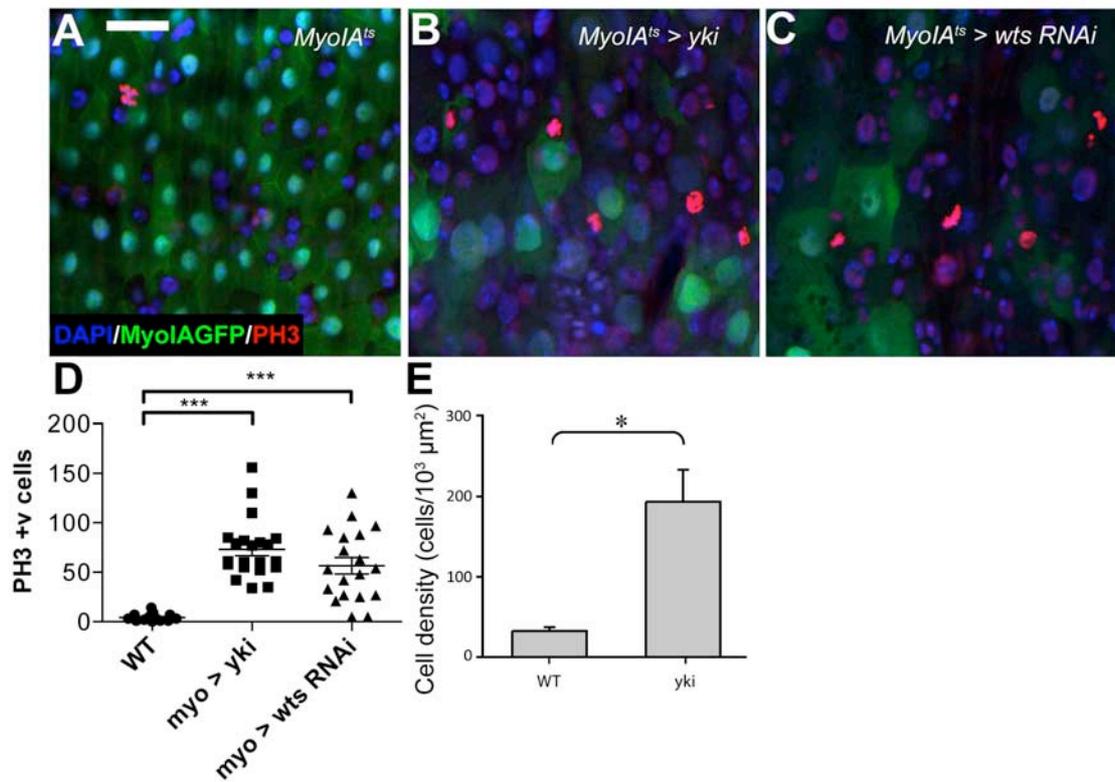
Figure 3.11: Hpo pathway inactivation in ECs triggers proliferation in the midgut

(A-C) *MyoIA^ts*-driven expression of Yki (B) or *wts*-RNAi (C) in ECs induces an increase in the number of PH3+ cells compared to control (A). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green) and PH3 is in red. Scale bar = 20μm. **(D)** Quantification of PH3+ cells in the indicated genotypes (p<0.0001, n>15). **(E)** *MyoIA^ts*-driven expression of Yki significantly increases cellularity. Cell numbers were quantified by counting all nuclei in an epithelial sheet of given surface area in the posterior midgut region after z-projection and normalization to tissue size. Data shown as mean and standard error of the mean. p<0.01, n=5 guts (>1500 nuclei). Panel E was generated by A.Kohlmaier.
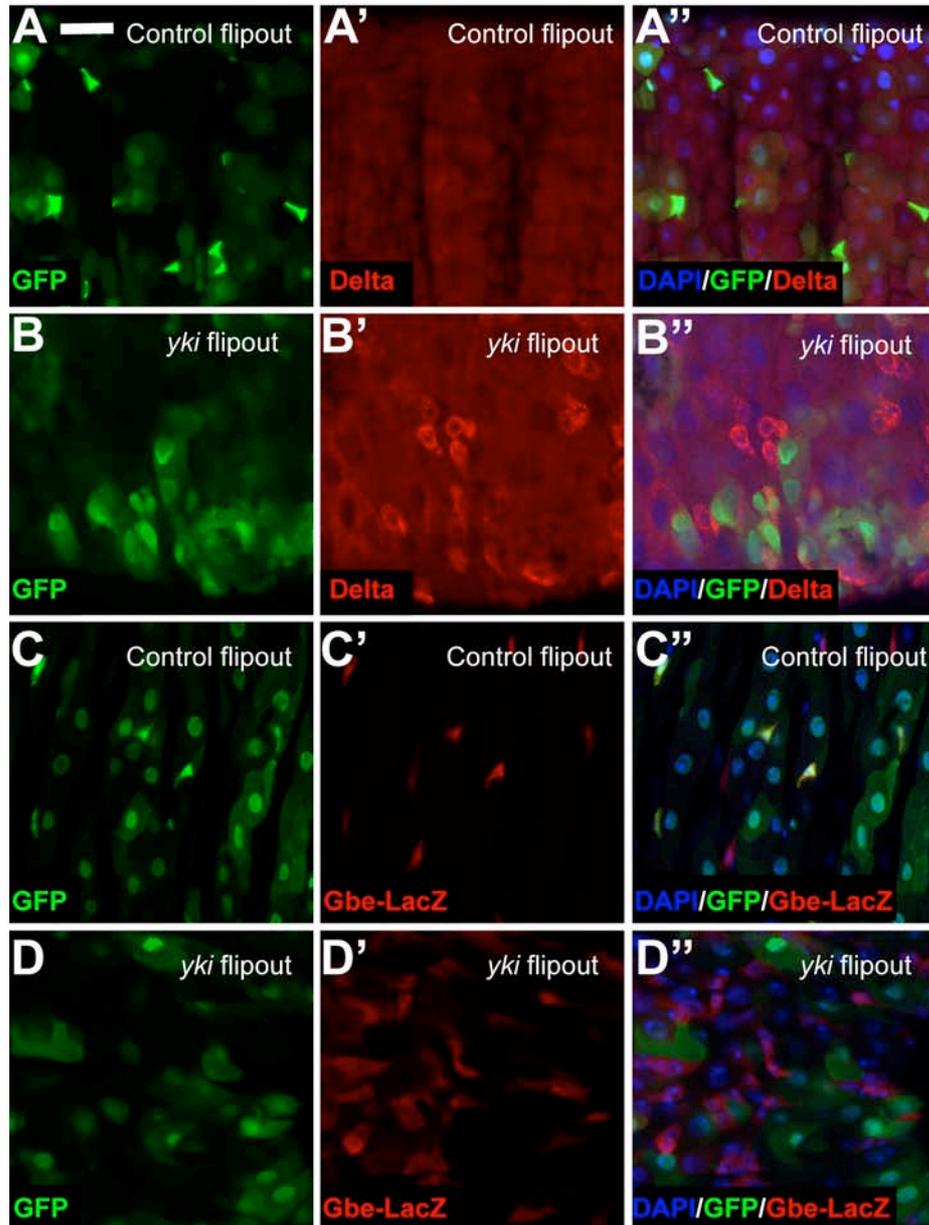
Figure 3.12: Hpo pathway inactivation induces non cell-autonomous effects

(A-B'') Flipout clones overexpressing Yki (B-B'') induce an increase in Dl staining in the surrounding WT tissue compared to control (A-A''). Nuclei are stained with DAPI (blue), GFP is green and Dl is in red. **(C-D")** Flipout clones overexpressing Yki (D-D") induce an increase in Notch reporter levels compared to control (C-C"). Nuclei are stained with DAPI (blue), GFP is green and β-Galactosidase is in red. Scale bar = 20μm.

led to the formation of large Dl+ cells in the surrounding tissue (Figure 3.13A-B"). As a control, I have also shown that *wts* mutant MARCM clones in the gut lead to increased levels of *ex* using an *ex-LacZ* enhancer trap line (Figure 3.13C-D") (Hamaratoglu et al., 2006). Ex, a known target of the Hpo pathway, was only upregulated in the mutant clone areas as expected.

3.6.1 Increased rates of DNA synthesis

In order to address the increased cell size observed upon Hpo pathway inactivation, our collaborator Alexander Kohlmaier assayed DNA synthesis rates by feeding flies food containing BrdU for 10 hours. This showed an increased frequency of replicating cells in *sav* mutant clones (Figure 3.14). Interestingly, the increase in BrdU was not restricted to the small dividing cells but was also apparent in larger nuclei. These data therefore indicate not only accelerated proliferation of ISCs but also increased endoreplication of ECs. This observation suggests that the increase in cell and nuclear size upon Hpo pathway inactivation is, at least in part, due to increased endoreplication. Again, these effects were not confined to cells within the mutant clones, confirming the non-autonomous effect of Hpo pathway inactivation (Figure 3.14B-B', E).

3.7 Conclusions and discussion

In this first part of my project, I have characterized the Hpo pathway inactivation phenotype in different cell types of the adult posterior midgut. Yki overexpression or Wts inactivation increased the number of proliferating midgut cells, though terminal differentiation was not blocked. The function of Yki in proliferation is well documented, so this first result regarding the induction of proliferation when Yki is expressed in progenitor cells was not surprising. Careful examination of the phenotype soon suggested that the effects of Yki expression are not merely restricted to cell-autonomous induction of proliferation. Pathway silencing in differentiated ECs non-autonomously induced strong cell proliferation. These observations led me to draw parallels between Hpo silencing and the regenerative response to intestinal stress, which elicits a very
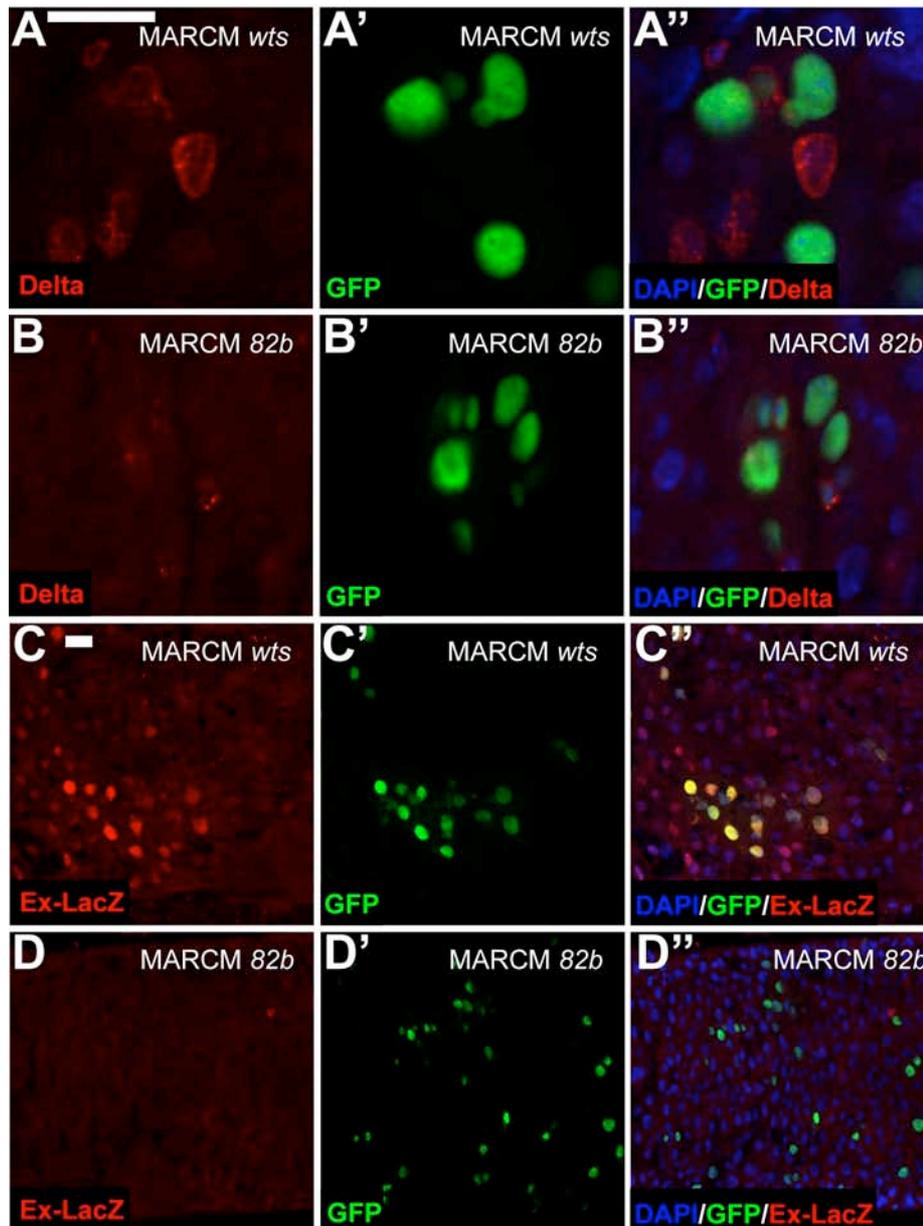
Figure 3.13: Non cell-autonomous effects of Hpo pathway inactivation in MARCM clones

(A-B'') Non cell-autonomous induction of Dl in cells neighbouring 10-day old *wts* mutant MARCM clones (A-A'') compared to control clones (B-B''). Nuclei are stained with DAPI (blue), GFP is green and Dl is in red. **(C-D'')** *wts* mutant MARCM clones (C-C'') exhibit increased levels of *ex-LacZ* compared to control (D-D''). Nuclei are stained with DAPI (blue), GFP is green and β-Galactosidase is in red. Scale bar = 20μm.
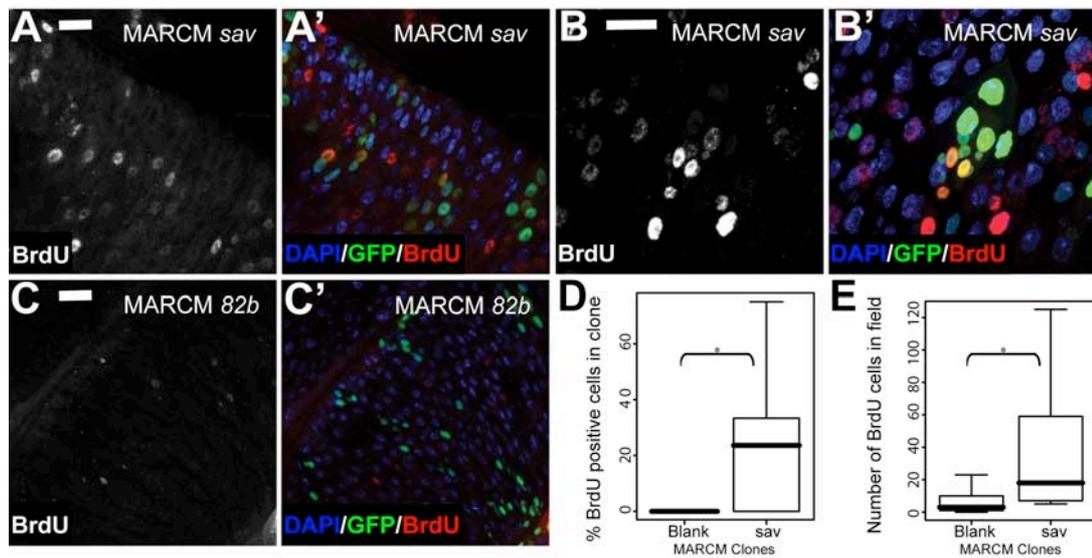
Figure 3.14: Increased BrdU incorporation rates in Sav mutant MARCM clones

(A-C') Cell-autonomous and non cell-autonomous increases in DNA replication rates (BrdU incorporation) upon induction of sav^{shrp1} mutant MARCM clones (A-B') compared to neutral clones (C-C'). Nuclei are stained with DAPI (blue), GFP is green and BrdU is in red. (D) BrdU labelling rates were quantified as the percentage of BrdU positive cells per MARCM clone (cell autonomy) in sav^{shrp1} mutant compared to neutral clones after 3D confocal imaging of the entire clone and subsequent 3D projection. P<0.0001. (E) Number of BrdU positive nuclei within all midgut cells including clone and non-clone tissue (non-cell autonomy). P<0.01. Scale bars = 20μm. Data in this figure were generated by A.Kohlmaier.

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similar effect (Amcheslavsky et al., 2009, Biteau et al., 2008, Buchon et al., 2009a, Buchon et al., 2009b, Cronin et al., 2009, Jiang et al., 2009).

The overgrowth phenotype seen following Yki expression in ECs is even more marked than that observed upon Yki expression in ISCs, potentially indicating that the esg^{ts} phenotype may in fact be caused by perduring Yki overexpression in the ECs (observations later in the project would argue against this – see section 4.5.2).

3.7.1 How does the *Drosophila* Hpo pathway inactivation phenotype compare to studies in the mouse intestine?

In the mammalian intestine, endogenous YAP1 expression is restricted to the progenitor/SC compartment with activation leading to expansion of this population (Camargo et al., 2007). Given that inhibition of Notch signalling using γ-secretase inhibitors suppresses the YAP1 phenotype, the activity of YAP1 in this respect is proposed to be via Notch, although this has not been investigated genetically. In fact, it is unclear whether this Notch dependency of YAP1-induced expansion of the proliferative compartment is due to a direct effect of YAP1 on Notch signalling, or whether the requirement for Notch in ISC self-renewal simply leads to ISC depletion in the γ-secretase inhibitor-treated animals.

In the *Drosophila* midgut, Notch signalling promotes differentiation rather than self-renewal (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). The Yki overexpression phenotype described here does not appear to be due to disruption of Notch signalling since no block in differentiation was apparent. Previous work demonstrates reduced Notch signalling in Hpo mutant clones (Polesello and Tapon, 2007, Yu et al., 2008, Genevet et al., 2009). This has been suggested to result from reduced Notch endocytosis, thereby preventing cleavage by γ-secretase. It is possible that while Yki overexpression may lead to a slight reduction in levels of Notch activity, this could be overridden by the presence of high levels of the Notch ligand Dl in neighbouring cells.

In the *Drosophila* midgut, both commitment (i.e. exit from self-renewal) and terminal differentiation are controlled by Notch signalling (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2007). It has been shown that these two roles require distinct levels of Notch activity - commitment requires high Notch activity, whereas terminal differentiation can occur with lower Notch activity (Perdigoto et al., 2011). From a cell-fate perspective, the role of Notch signalling appears conserved from flies to mammals. In the mouse intestine, Notch has been directly implicated in intestinal lineage specification, with high Notch signalling levels favouring absorptive differentiation. When Notch signalling is blocked, the whole cell population stops proliferating and becomes secretory goblet cells (van Es et al., 2005).

In the mouse intestine, Notch has been reported to promote proliferation (rather than differentiation) (Fre et al., 2005, van Es et al., 2005). Fre et al showed that Notch activation is capable of amplifying the progenitor pool while inhibiting cell differentiation (Fre et al., 2005). A more recent study has shown that Notch signalling modulates proliferation and differentiation of intestinal crypt base columnar stem cells (CBCs) (Vandussen et al., 2012). A number of CBC SC markers are now available including *Lgr5, Ascl2* and *Olfm1* and these cells are considered to be responsible for maintaining the crypt epithelium (Barker et al., 2007, van der Flier et al., 2009). VanDussen et al demonstrate an absence of the CBC marker *Lgr5-GFP* following Notch pathway inhibition with DBZ, supporting the notion that Notch signalling is required for CBC self-renewal (Vandussen et al., 2012). This recent work would suggest that the role of Notch signalling differs from flies to mammals with Notch maintaining SC proliferation in the mouse intestine but promoting progenitor cell commitment in the fruit fly midgut.

It is still somewhat unclear whether YAP1 (or Notch) induces an increase in the SC rather than committed progenitor cell population in mice. Therefore, it is too early to conclude that the *Drosophila* and mammalian intestine are different with respect to the role of the Notch and Hpo pathways. My results in flies do, however, indicate that Notch and Hpo signalling have distinct functions. Finally, whether the YAP1-induced increase in the proliferating progenitor/SC compartment is dependent on secreted

factors has not yet been explored (Camargo et al., 2007).

3.7.2 Does the Hpo pathway play a role in regulation of midgut cell death?

An interesting question I would have liked to address is whether Yki activation in either the ISCs or ECs induces apoptosis. It is feasible that expression of a pro-growth signal, like Yki in cells, which are already committed to a particular fate, could result in apoptosis.

I have tried several techniques for studying cell death in the midgut but unfortunately all have had limited success, even when tested in conditions known to induce apoptosis in the midgut (such as JNK signalling activation and bacterial infection). My approaches to date have included the use of TUNEL to label fragmented DNA (Gavrieli et al., 1992), anti-activated *Drosophila* effector caspase *drICE* and anti-activated caspase-3 staining (recently demonstrated successfully in the midgut by O'Brien *et al* (O'Brien et al., 2011)). Most recently, I have tested the Apoliner system, which is thought to be detected before other markers of apoptosis (Bardet et al., 2008). The Apoliner sensor comprises two fluorophores, mRFP and eGFP, linked by an efficient and specific caspase cleavage site. Upon caspase activation, the sensor is cleaved and eGFP translocates to the nucleus, leaving mRFP at the membranes. Although initial progress using Apoliner appeared promising, I have been unable to confirm whether this technique is truly reliable in the midgut.

I believe the difficulty in observing cell death in the midgut is most probably due to the nature of the cell death process in this system. Upon infection, it has been shown that damaged ECs delaminate from the rest of the epithelium and are forced into the lumen (Buchon et al., 2010). These delaminating cells have fragmented nuclei with multiple large vacuoles, suggesting they are undergoing cell death. This process by which apoptosis is induced by loss of cell attachment is known as anoikis (Frisch and Francis, 1994). Importantly, caspase 3 staining was shown to only be detectable once cells had detached from the epithelium. As a result, dying cells could only be visualised using

histological sections (Buchon et al., 2010). Given the small size of the *Drosophila* midgut, this is a technically challenging approach to observing apoptosis, both in terms of finding the correct location and generating sufficient data for quantification.

My results to date suggest that Yki does not induce widespread apoptosis in the midgut, supporting the notion that the proliferative response induced by Yki expression is not simply a by-product of EC apoptosis.

3.7.3   A role for the Hpo pathway in midgut regeneration?

The clear non-cell autonomous effect of Yki overexpression in the ECs on cell proliferation raises the question of how Yki expression in the ECs elicits such a strong response in the ISCs. Yki expression was seen not only to induce proliferation but also to increase the size of *esg+* cells. Large *esg+* cells are known to be a hallmark of midgut regeneration and are thought to represent faster gut turnover. The parallels between Yki-induced tissue overproliferation and damage-induced tissue growth would suggest that Yki might play a role in midgut regeneration. I will address this possibility in Chapter 4.

Chapter 4.   Results

The Hippo pathway is a mediator of the *Drosophila* midgut regenerative response

4.1 Aims of this project

In chapter 3, I characterised the Hpo pathway inactivation phenotype in the adult midgut. Hpo pathway inactivation in either progenitor cells or differentiated ECs led to a proliferative phenotype highly reminiscent of the adult midgut regenerative response. How adult ISCs respond to damage, switching from a homeostatic to a rapid proliferative state, in order to regenerate damaged tissue, is unclear. Here, I examine whether the Hpo pathway plays an important role in the intestinal response to stress. I begin by firstly addressing how Yki activation in the ECs induces ISC proliferation.

4.1.1 A role for the Jak/Stat signalling pathway

Several groups have examined how the *Drosophila* midgut responds to various forms of stress uncovering a role for Jak/Stat signalling in this process (Amcheslavsky et al., 2009, Biteau et al., 2008, Buchon et al., 2009a, Jiang et al., 2009, Cronin et al., 2009). The Jak/Stat signalling pathway has been implicated in the regulation of SCs in multiple tissues and is proposed to be a common regulator of SC proliferation, also promoting SC self-renewal efficiency in mouse embryonic SCs (Gregory et al., 2008). In the midgut, Stat reporters are active in both ISCs and EBs, but not in terminally differentiated cells (Jiang et al., 2009, Beebe et al., 2010, Liu et al., 2010b). Unpaired cytokines (Upds, the ligands for the Jak/Stat pathway in *Drosophila*) are produced by ECs in response to a wide range of stress situations, such as apoptosis, JNK signalling or bacterial infection (Jiang et al., 2009, Buchon et al., 2009b). This leads to activation of Jak/Stat signalling in ISCs and EBs, promoting their division and differentiation, thereby accelerating midgut tissue renewal. The Jak/Stat pathway therefore regulates ISC proliferation, although its precise role in baseline homeostasis remains unclear (Beebe et al., 2010).

The strong non cell-autonomous effect of Yki expression in the ECs on cell proliferation, and the parallels between Yki-induced tissue overproliferation and damage-induced tissue growth, led me to hypothesize that Yki activation may influence ISC behaviour via Jak/Stat signalling.

4.2 The Hpo pathway and Jak/Stat signalling activity

4.2.1 Induction of Upd1 expression upon Hpo pathway inactivation

Cytokines of the Upd family are upregulated by intestinal stress (Buchon et al., 2009b). In turn, the Upd ligands activate the Jak/Stat pathway, promoting ISC proliferation. *upd-lacZ* is an enhancer trap, which reports *upd1* transcription (Chao et al., 2004). Yki expression in flipout clones led to an increase in *upd-LacZ* levels compared to control clones (Figure 4.1A-B"). Occasionally, *upd-lacZ* activation in cells neighbouring large Yki-expressing clones was also observed, suggesting a small degree of non cell-autonomy (arrowheads in Figure 4.1B'). Mutating *wts* in MARCM clones revealed increased levels of *upd-lacZ* expression compared to the surrounding midgut tissue, although not all clones were equally affected (Figure 4.2A-B"). Similar effects were confirmed in *hpo*-mutant MARCM clones (Figure 4.2C-C"). *upd-lacZ* expression was no longer observed in MARCM clones deficient for both *hpo* and its downstream target *yki* (Figure 4.2D-D"). Thus, Hpo pathway inactivation leads to *upd1* expression in a *yki*-dependent manner.

4.2.2 Hpo pathway inactivation induces Upd3 expression

Upd3 is reported to be the main mediator of the midgut response to bacterial infection (Jiang et al., 2009). I used a tubulin Flipout transgenic to drive clonal Yki expression in the gut (Dong et al., 2007, Huang et al., 2005), and monitored *upd3* expression with a UAS-GFP driven by an *upd3-GAL4* enhancer trap. Clonal overexpression of Yki induced a robust induction of *upd3>GFP* in ECs (Figure 4.3A-A"). Hpo pathway downregulation therefore promotes midgut regeneration by inducing transcription of
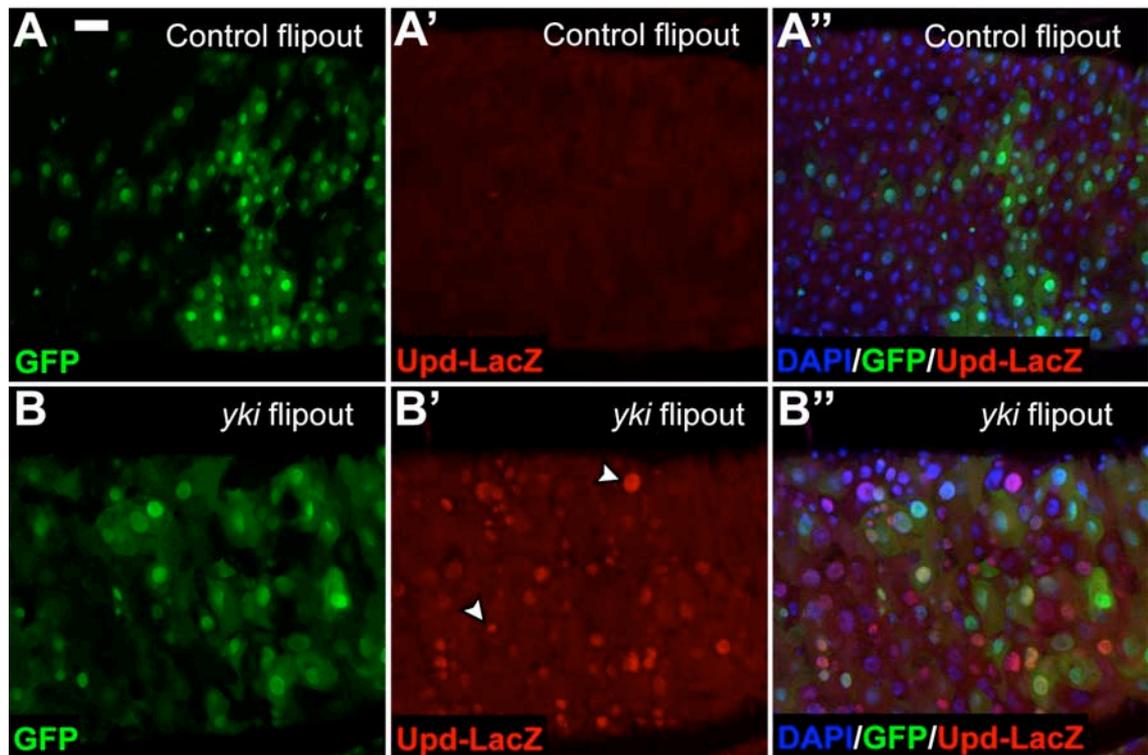
Figure 4.1: Yki activation induces expression of Upd1

(A-B") Clones overexpressing Yki (B-B'') induce an increase in levels of the Jak/Stat ligand *upd1* compared to control (A-A''). Arrowheads indicate occasional *upd-lacZ* expression in cells neighbouring mutant tissue. Nuclei are stained with DAPI (blue), mutant clones are marked by GFP (green) and β-Galactosidase is in red. Scale bar = 20μm.
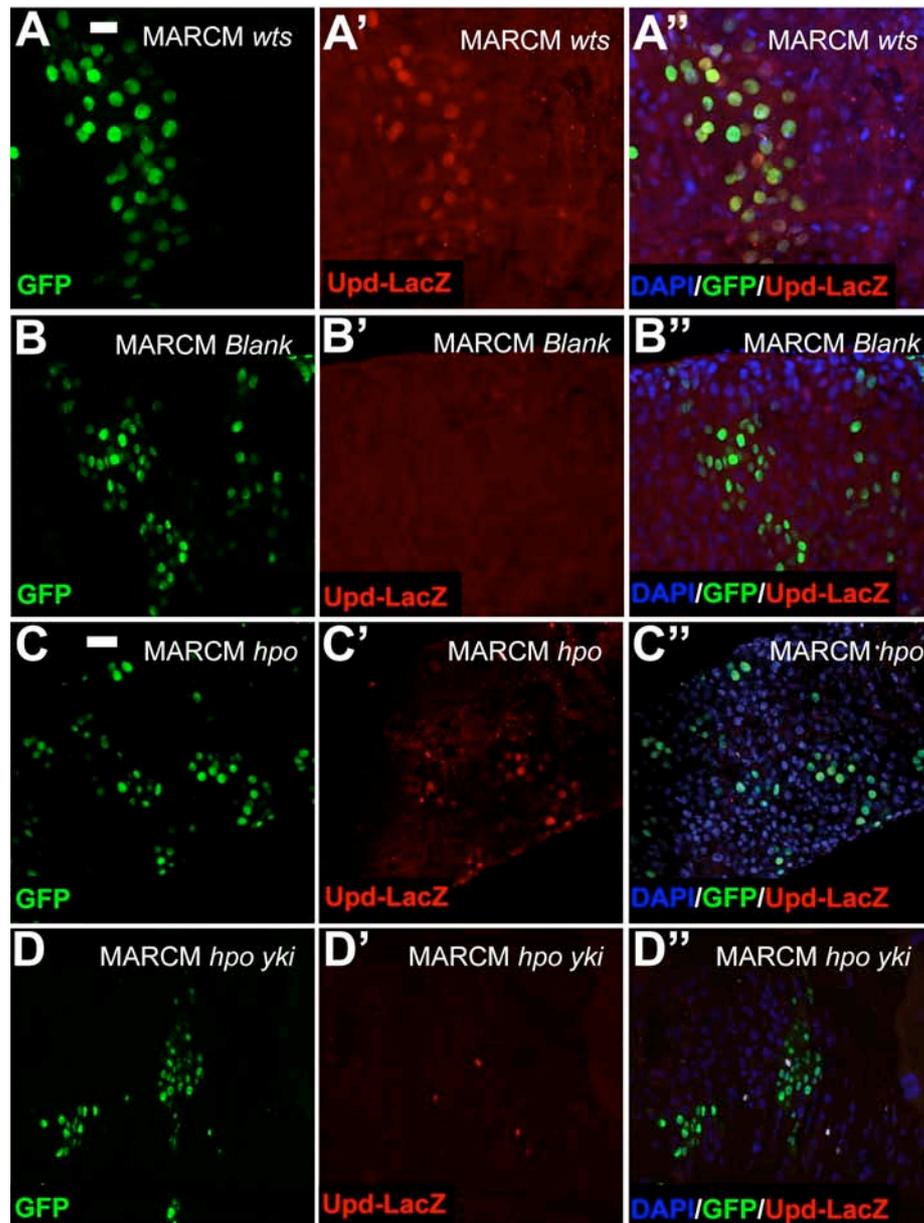
Figure 4.2: Hpo pathway inactivation induces Upd expression

(A-B'') *upd-lacZ* expression is increased in five day old *wts*-mutant MARCM clones (A-A'') compared to control clones (B-B''). **(C-C'')** *upd-lacZ* expression is increased in *hpo*-mutant MARCM clones compared to the surrounding midgut tissue. **(D-D'')** *upd-lacZ* induction is abolished in *hpo,yki* double mutant MARCM clones. Nuclei are stained with DAPI (blue), mutant clones are marked by GFP (green) and β-Galactosidase is in red. Scale bars = 20μm. Panels C-D'' were generated by A.Kohlmaier.

upd genes in a *yki*-dependent manner. This Yki-induced increase in EC Upd3 levels is presumably the signal, which stimulates ISC proliferation.

4.2.3   Hpo pathway inactivation increases Jak/Stat signalling activity

The transcriptional activity of the Jak/Stat signalling pathway can be monitored using a Stat92E reporter driving the expression of RFP (10XStat-RFP, M.Zeidler). Expression of Yki in *esg+* cells (Figure 4.3B-C') or ECs (Figure 4.3D-E') led to an increase in Jak/Stat reporter expression. This increase was mainly apparent basally, where the ISCs and EBs are located (Figure 4.3E-F' and data not shown) and was also visible following *wts*-inactivation (Figure 4.3F-F'). Our collaborator, Alexander Kohlmaier, used Reverse-Transcription quantitative Polymerase Chain Reaction (RT-qPCR) to quantitatively show that expression of all three *upd* genes and the endogenous Jak/Stat target *socs36e* are elevated when Yki is overexpressed in the ECs (Figure 4.3G). Together, these results show that Hpo pathway disruption in the ECs leads to increased abundance of Jak/Stat ligands and an increase in Stat transcriptional activity.

4.2.4   Hpo pathway inactivation in wing imaginal discs alters Upd
expression

To determine whether *upd* transcriptional regulation by Yki is a general phenomenon, I induced Yki overexpression flipout clones in wing imaginal discs. I observed that *Upd-LacZ* levels were also increased in wing discs (arrowhead in Figure 4.4A-A''). Interestingly, *upd-LacZ* upregulation was not seen in all clones; for example *upd* expression was not upregulated in Yki flipout clones located in the wing pouch (arrow in Figure 4.4A-A''). In WT 3rd instar larval wings, five separate domains are known to express *upd*, particularly in the hinge region (Bach et al., 2007). Since *upd* upregulation in Yki clones is only observed in the wing hinge, the response is only seen close to the endogenous *upd*-expressing area. This suggests that, although Yki can promote *upd* expression in certain cellular contexts, this regulation is likely to involve other transcription regulators.
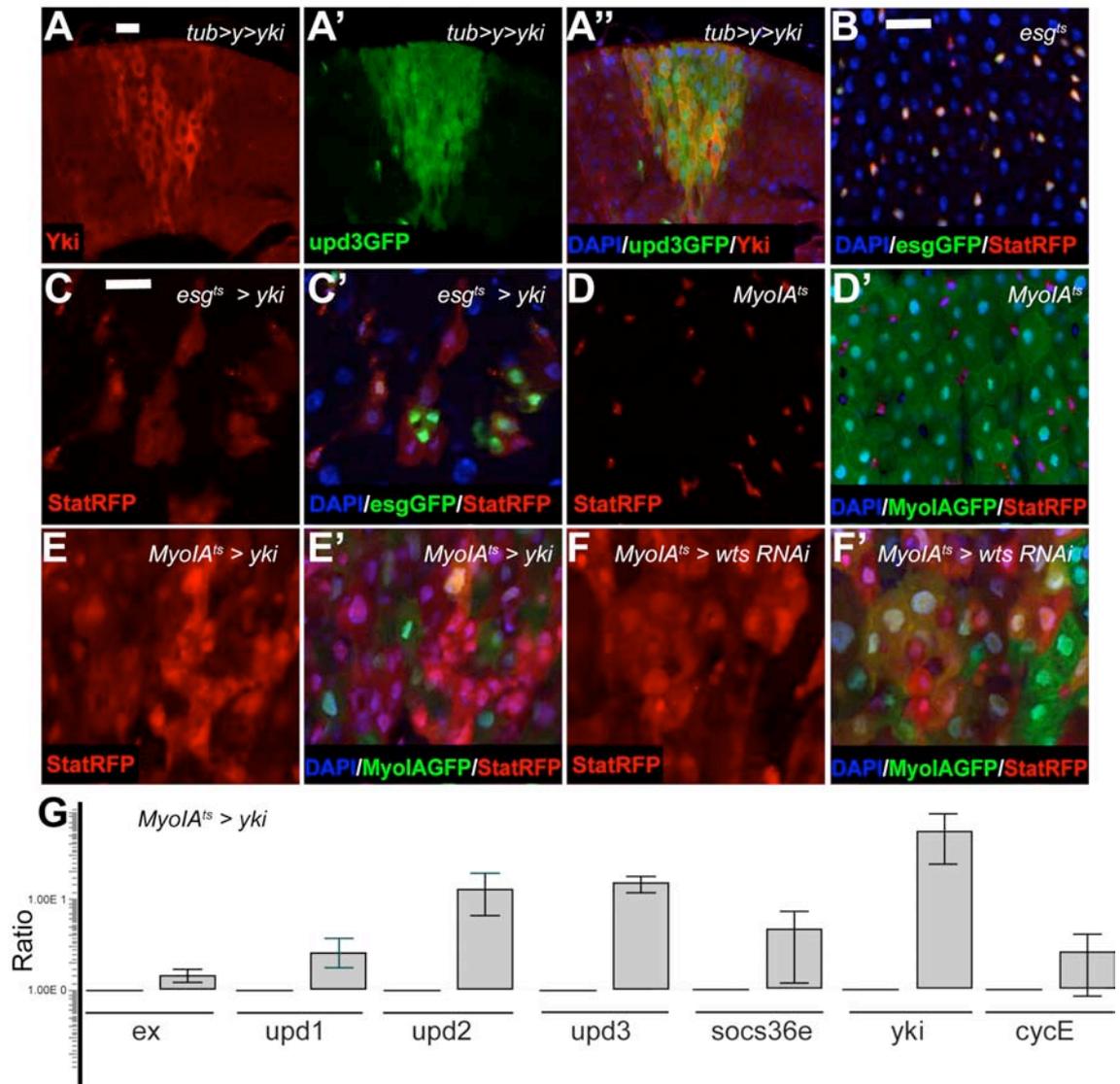
Figure 4.3: Increased Jak/Stat activity following Yki overexpression

(A-A") Clonal overexpression of Yki leads to increased *upd3>GFP* expression. Nuclei are stained with DAPI (blue), GFP is green and Yki is in red. (B-C') *esg^ts*-driven expression of Yki (C-C') increases StatRFP reporter levels compared to control (B). (D-F') *MyoIA^ts*-driven expression of Yki (E-E') or *wts*-RNAi (F-F') increases StatRFP reporter levels compared to control (D-D'). Nuclei are stained with DAPI (blue), RFP is in red and ECs marked by GFP are in green. Scale bars = 20μm. (G) Induction of all three Upd cytokines following *MyoIA^ts*-driven expression of Yki measured by RT-qPCR. Panel G was generated by A.Kohlmaier.
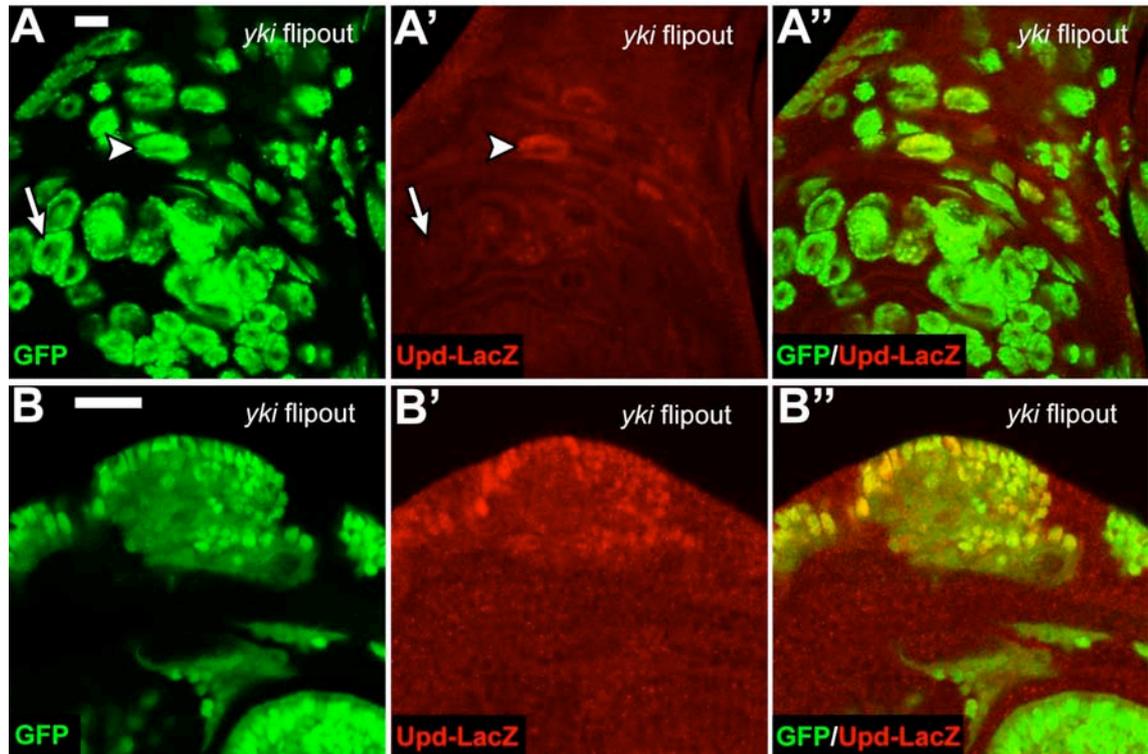
Figure 4.4: Hpo pathway inactivation in wing imaginal discs alters Upd expression

Confocal micrographs of wing imaginal discs. **(A-B'')** Clones overexpressing Yki (B-B'') in wing imaginal discs show an increase in levels of the Jak/Stat cytokine *upd*. Note that increased *upd-LacZ* was not seen in all *yki* overexpressing clones (arrowhead compared to arrow in A-A'). *upd* expression is increased when *yki* flipout clones are in regions where *upd* is developmentally expressed. Cells overexpressing Yki are marked with GFP (green) and β-Galactosidase is in red. Scale bars = 20μm.

4.2.5 Jak/Stat signalling is required in ISCs for Yki-induced proliferation

To test whether the Hpo pathway is dependent on Jak/Stat signalling in order to induce ISC proliferation, I performed genetic epistasis experiments (Figure 4.5 and 4.6). Expression of a *stat*-RNAi construct suppressed the increased proliferation and Dl levels seen upon Yki overexpression in progenitor cells (Figure 4.5). In contrast, *stat*-RNAi expression in the ECs did not suppress the Yki induced non cell-autonomous regenerative response (Figure 4.6), suggesting that Yki-induced ISC proliferation requires Stat activity in the ISCs, but not the ECs.

4.3 Yki is activated by intestinal stress

Jak/Stat signalling plays a key role in midgut regeneration following various forms of stress (Buchon et al., 2009b, Jiang et al., 2009). Having linked Yki activation to Jak/Stat signalling activity, I tested whether Hpo signalling plays a role in responding to intestinal stress.

4.3.1 Verification of Yki antibody staining in the midgut

Yki transcriptional activity is dependent on its nuclear localisation, which is antagonised by Hpo signalling. Characterisation of any possible role of Yki in the intestinal regenerative response therefore involved examining its expression and subcellular expression pattern. I began by testing two different Yki antibodies (Figure 4.7). Endogenous Yki is 418 amino acids long. Yki69 is an antibody generated in the Tapon lab recognizing amino acids 381-395 in the C-terminus of Yki (referred to as Yki69 in all figures). The other Yki antibody used in this study (referred to as Yki in all figures) was generated in the laboratory of Kenneth Irvine using the full-length bacterially expressed protein as an immunogen (Oh and Irvine, 2008).

In WT guts, Yki expression with both antibodies is relatively low in all cell types (Figure 4.7A and E). Yki levels are often slightly higher in small cells – presumably
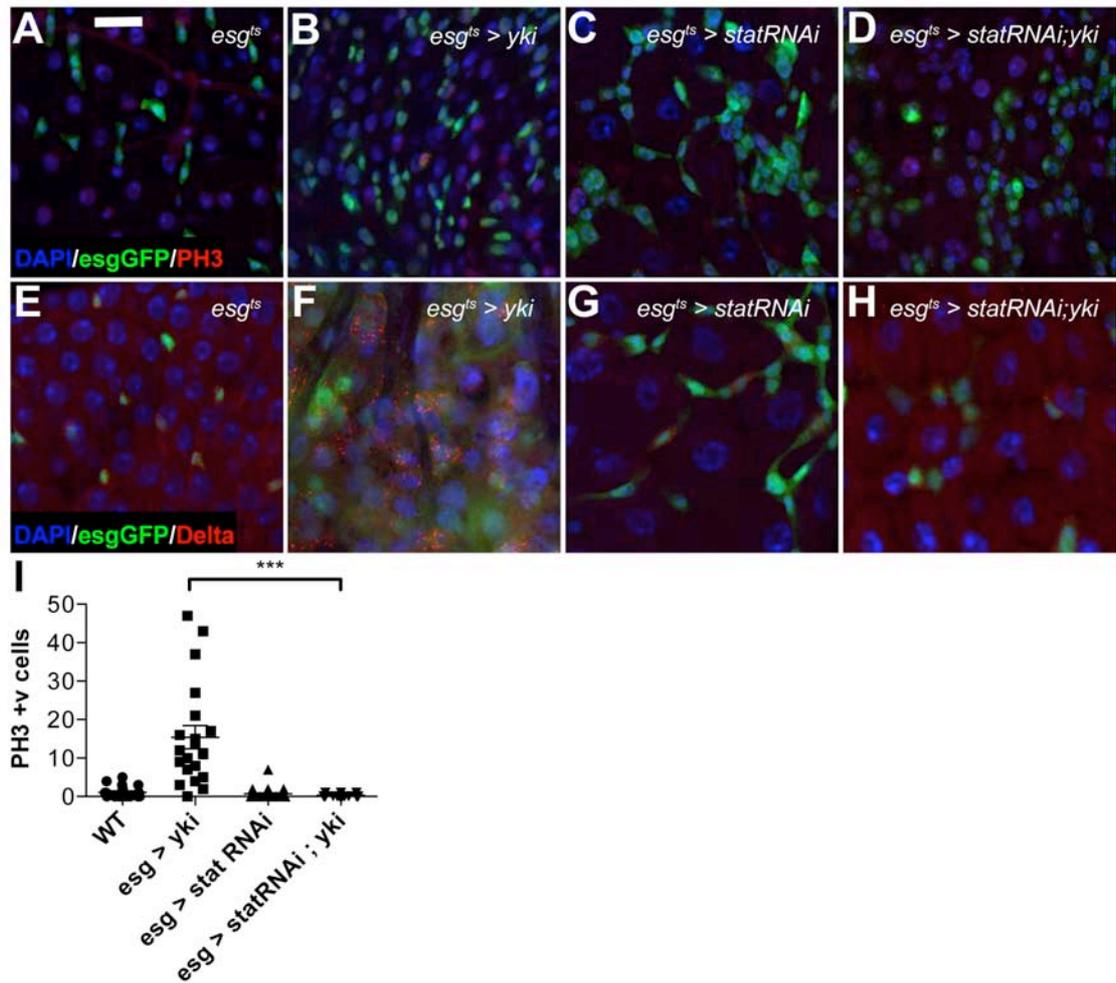
Figure 4.5: Yki-induced proliferation is dependent on Jak/Stat signalling

(A-H) The *esg^{ts}*-Yki-induced overproliferation and increase in Dl+ cells (B and F) is rescued by co-expression of *stat*-RNAi (D and H). Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green), PH3 (A-D) and Dl (E-H) are in red. Scale bar = 20μm. Quantification of PH3+ cells per midgut. **(I)** *esg^{ts}*-driven expression of *stat*-RNAi significantly rescues Yki-induced proliferation ($p < 0.0001$, n>20).
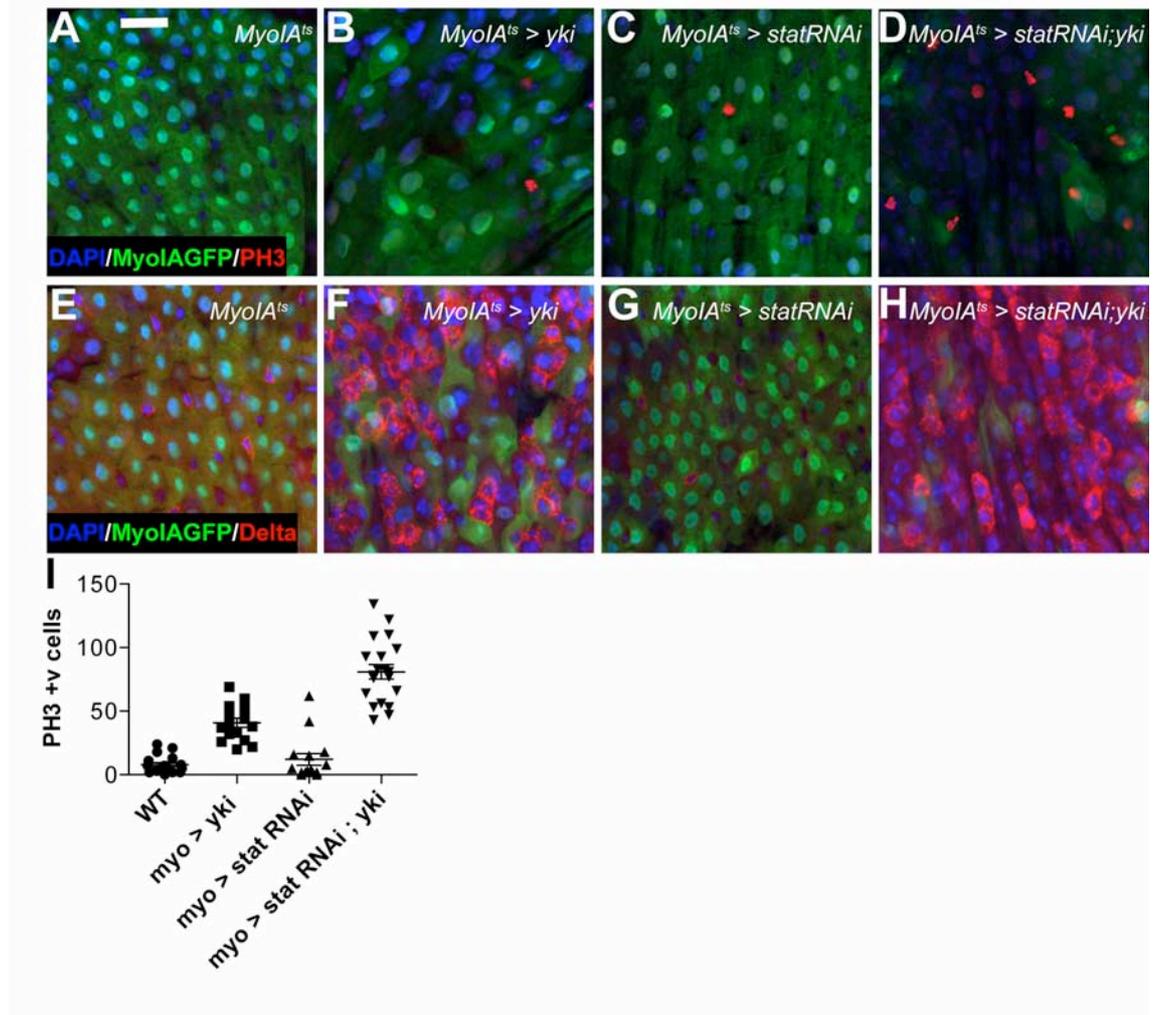
Figure 4.6: Stat-RNAi expression in ECs does not suppress Yki-induced proliferation

(A-H) The *MyoIA*^{ts}-Yki-induced overproliferation and increase in Dl+ cells (B and F) is not rescued by co-expression of *stat*-RNAi (D and H). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green), PH3 (A-D) and Dl (E-H) are in red. Scale bar = 20μm. Quantification of PH3+ cells per midgut. **(I)** *MyoIA*^{ts}-driven expression of *stat*-RNAi does not rescue Yki-induced proliferation in ECs (n>15).
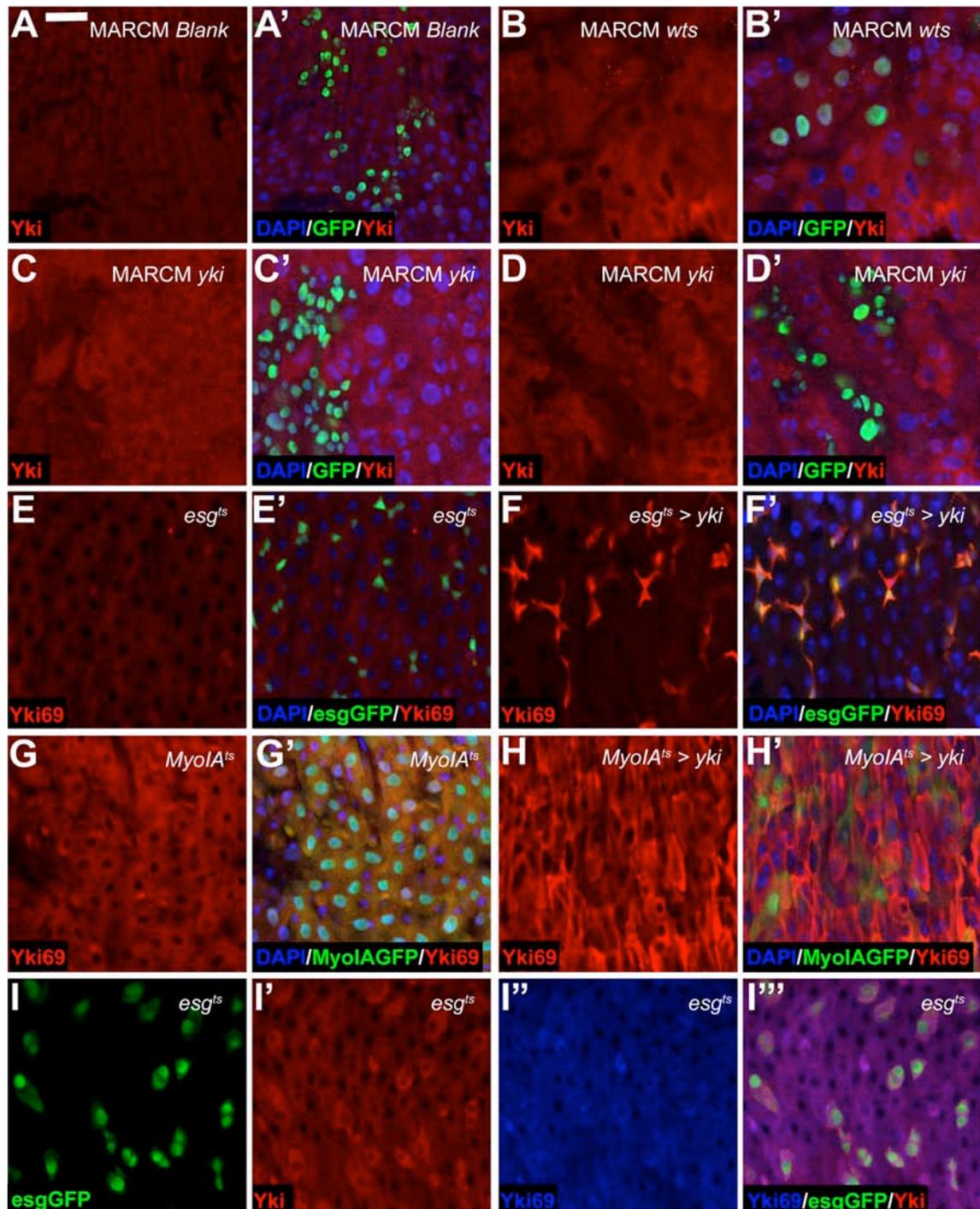
Figure 4.7: Validation of Yki antibody stainings in the midgut

(A-D') Nuclear Yki antibody staining in seven-day-old *wts*-mutant MARCM clones (B-B') compared control clones (A-A'). Yki antibody staining is reduced in *yki*-mutant MARCM clones (C-D'). **(E-H')** *esg*^ts or *MyoIA*^ts-driven expression of Yki increases Yki69 antibody staining. Nuclei are stained with DAPI (blue), GFP is green, Yki staining is in red. **(I-I''')** Overlapping expression of two different Yki antibodies. GFP is green, Yki staining is in red and Yki69 staining is blue. Scale bar = 20μm.

ISCs. The specificity of the Irvine Yki antibody was confirmed in *wts*-mutant MARCM clones. Wts is known to phosphorylate Yki resulting in Yki inactivation and retention in the cytoplasm (Oh and Irvine, 2008). In the GFP positive clones mutant for *wts,* Yki nuclear staining was increased compared to the surrounding WT tissue (Figure 4.7B-B'). Complementary to this first result, examination of clones mutant for *yki^{B5}*, a protein null allele generated by homologous recombination (Huang et al., 2005), showed a decrease in Yki antibody staining (Figure 4.7C-D').

The Yki69 antibody did not give such clear results when tested in *wts* MARCM clones (data not shown) but can be used effectively when studying high levels of Yki expression (Figure 4.7E-H'). Figure 4.7I-I''' illustrates the increased levels of Yki antibody staining occasionally apparent in WT guts and demonstrates the corresponding staining patterns observed with the two different Yki antibodies.

Interestingly, *esg^{ts}*-driven Yki expression in the ISCs did not appear to affect Yki staining levels in the ECs (Figure 4.7E-F'). This would suggest that the proliferative response observed following *esg^{ts}*-driven Yki expression (see Chapter 3) is indeed due to a progenitor-specific effect and not a result of any persistence in protein expression in the differentiated ECs.

4.3.2 Increased Yki antibody staining following *P.e* infection

Ingestion and enteric infection with *Pseudomonas entomophila* (*P.e*) is reported to kill ECs and activate JNK signalling (Vodovar et al., 2005). *P.e* infection leads to induction of Upd/Jak/Stat signalling, which drives ISC mitoses and intestinal regeneration (Buchon et al., 2009b, Jiang et al., 2009).

I examined Yki protein expression using the Irvine anti-Yki antibody, which had performed best in the antibody validation tests (Figure 4.7) (Oh and Irvine, 2008). Yki expression was mainly cytoplasmic under standard conditions (Figure 4.8A-A'). Upon 24hr *P.e* infection, a strong increase in Yki levels was noted in the *esg+* cells

(ISCs/EBs) and some ECs (Figure 4.8B-B'). Although I could not reliably detect increased endogenous Yki in all EC nuclei after oral *P.e* infection, Yki staining in some EC nuclei did appear to be slightly increased. These experiments suggest that Yki activity is triggered in both the EC and ISC/EB population by intestinal stress. This raises the possibility that Yki functions as a stress sensor, relaying information about the health of the epithelium to the ISCs.

Also of note is the Yki expression pattern seen in a subset of the *pros+* EE cells. On several occasions, in WT non-stressed guts, increased Yki antibody staining was observed not only in *esg+* cells but also in the small *esg-* cells (Figure 4.8C-C'). Co-staining Yki with the EE cell marker Prospero revealed that these small *esg-* cells are a subset of the EE population. The role of Yki in EE cells has not been investigated further. I have since obtained a *pros*-GAL4 line, which will allow me to study Yki function in this cell population.

4.3.3   Increased Yki transcriptional activity following *P.e* infection

To monitor Hpo pathway activity during tissue damage, we also measured the expression of two well-characterised Yki targets, *ex* and *diap1* (Huang et al., 2005, Hamaratoglu et al., 2006) by RT-qPCR at various time points after *P.e* infection (Figure 4.9A – A.Kohlmaier). Both *ex* and *diap1* were significantly and rapidly induced upon *P.e* infection, up to 6 fold and 2.6 fold, respectively. Importantly, this activation preceded the induction of ISC proliferation. As expected for a regenerative response, *upds 2* and *3*, *socs36e* and the JNK target *puckered* (*puc*), are also induced with the same kinetics. *Cyclin E* levels are elevated, presumably as part of the proliferative response. The induction of both *ex* and *diap1* was prevented when the *P.e*-infected flies were treated with antibiotics (Figure 4.9A, lower panel). To determine in which cells Yki activity responds to stress signals, I examined *ex* expression using the *ex-LacZ* enhancer trap line (Hamaratoglu et al., 2006). *P.e* infection induced a rapid increase in *ex* expression in the large ECs (Figure 4.9B-C"). Hence, Yki transcriptional activity is induced in response to intestinal stress.
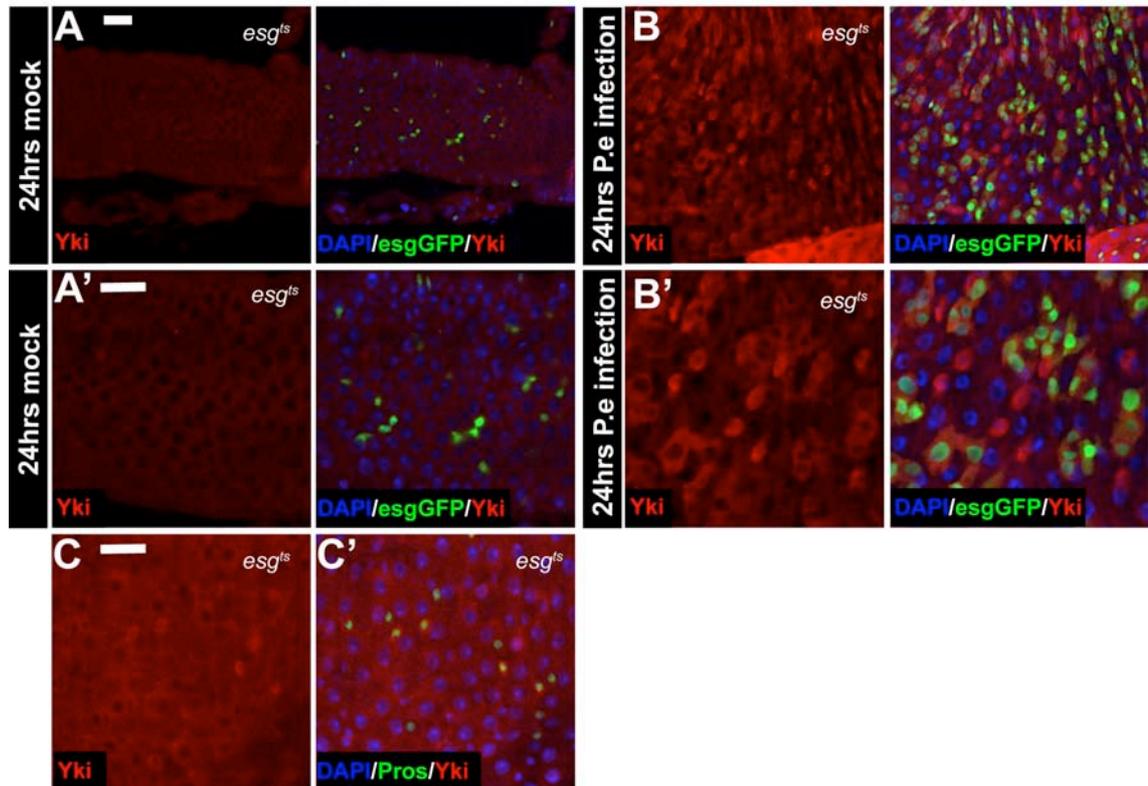
Figure 4.8: Intestinal stress results in increased Yki levels

(A-B') 24 hour *P.e* infection leads to increased Yki levels in *esg+* progenitors and ECs (B-B') compared to mock infected guts (A-A')**.** Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green), Yki is in red. **(C-C')** Yki staining is often increased in a subset of *pros+* cells. Nuclei are stained with DAPI (blue), Pros is green and Yki is in red. Scale bars = 20μm.
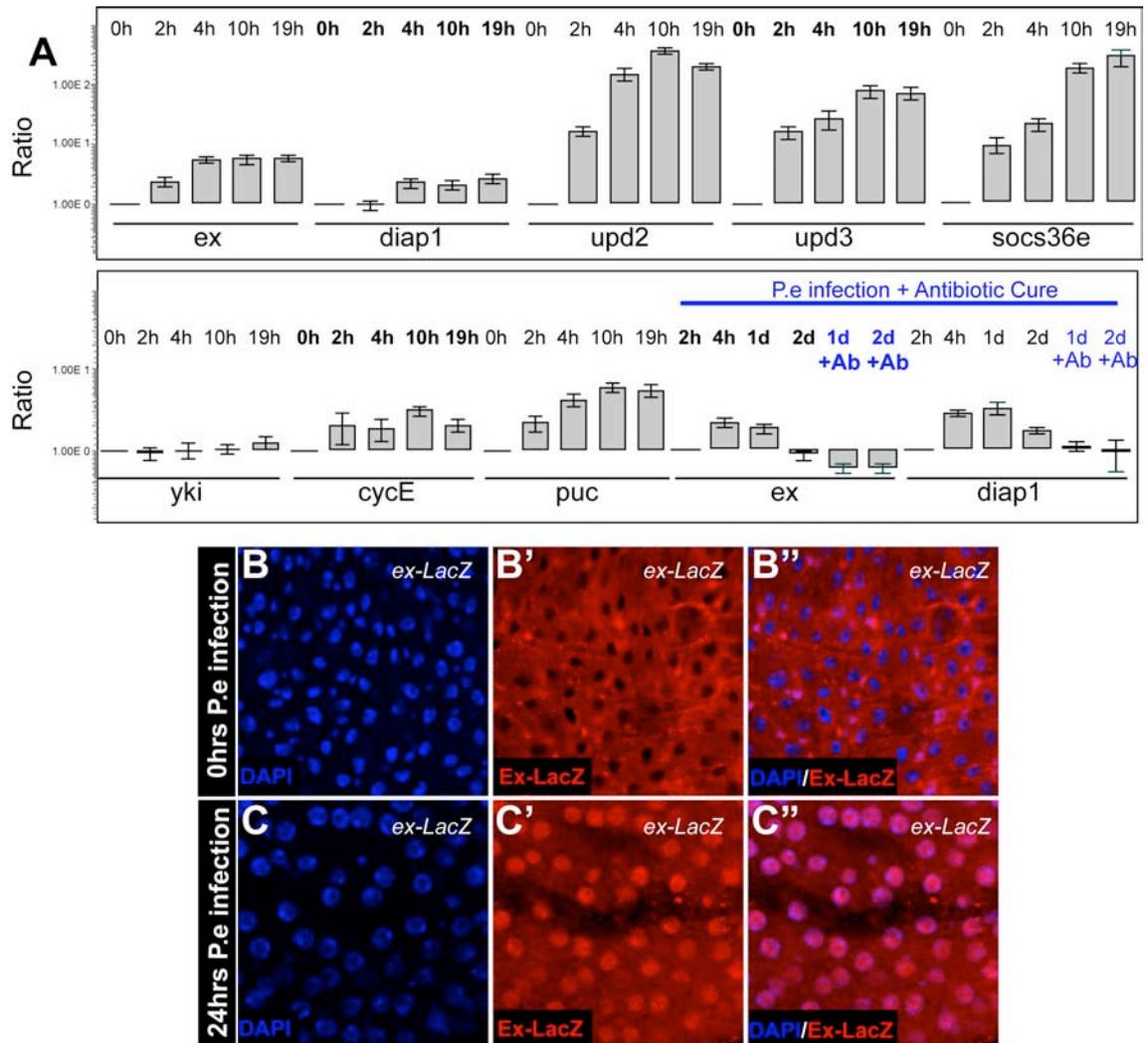
Figure 4.9: Intestinal stress leads to induction of Yki targets

(A) RT-qPCR shows Yki target induction (*expanded, diap1*) immediately upon *P.e* infection (top panel) and reversal of Yki target mRNA abundance following antibiotic treatment (bottom panel). **(B-C'')** Increased *ex-LacZ* activity following 24 hours of *P.e* infection (C-C'') compared to mock infected guts (B-B''). Nuclei are stained with DAPI (blue) and β-Galactosidase is in red. Scale bars = 20μm. Panel A was generated by A.Kohlmaier.

4.4 Is Hpo pathway activity required for the intestinal response to stress?

4.4.1 Requirement for Yki in ISCs for the proliferative response to *P.e* infection

To test Yki function in the regenerative response, I suppressed Yki expression in ISC/EBs or ECs by RNAi, and measured *P.e*-induced proliferation using PH3 staining (Figures 4.10 and 4.11). *P.e* infection is known to lead to an increase in the number of *esg+* progenitors and actively dividing ISCs (compare Figure 4.10 C to A) (Jiang et al., 2009). Yki inactivation by RNAi had no obvious effect in non-stressed guts with regards to D1 or PH3 staining (Figure 4.10 B and F compared to A and E). Given the low levels of proliferation in WT guts, any decrease in proliferation would be difficult to detect. As expected, *P.e* infection led to an increase in the number of PH3+ dividing cells (Figure 4.10I). Interestingly, silencing Yki in the ISCs/EBs using *esg^{ts}* fully suppressed the *P.e*-induced proliferative response (Figure 4.10I). Together with the stress-induced increase in Yki expression in ISCs/EBs shown in Figure 4.8, this demonstrates that Yki is required in ISCs for stress-induced proliferation.

4.4.2 Requirement for Yki in ECs for the proliferative response to *P.e* infection

When Yki expression was suppressed using RNAi in the ECs, only a partial (though statistically significant; p=0.018) suppression of the *P.e*-induced proliferative response occurred (Figure 4.11A-E). Although the Hpo pathway responds to intestinal stress (Figure 4.8 and 4.9), it may not be absolutely required in ECs for triggering Jak/Stat-induced regeneration. A more trivial explanation for this result is that suppressing Yki by RNAi in the dividing ISCs may be more effective than in the post-mitotic ECs, where *yki* transcript and Yki protein are not diluted by cell division. Given that ECs are rapidly shed following infection and replaced by new preECs (which have elevated Yki protein levels due to the infection), it is probably difficult to fully deplete Yki in ECs before they are sloughed off. Yki69 antibody staining is reduced following *MyoIA^{ts}* driven expression of Yki in the ECs, suggesting that Yki protein is indeed at least

Figure 4.10: Yki is required in ISCs for the midgut regenerative response to bacterial infection

(A-H) *P.e* infection induces a proliferative response with increased numbers of *esg+* cells, mitoses, Dl+ ISC-like cells and midgut size (A, C, E, G). *esg*^{ts}-driven expression of *yki*–RNAi (B and F) causes a reduction in the midgut regenerative response to infection (D and H). Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green), PH3 (A-D) and Dl (E-H) are in red. Scale bar = 20μm. Quantification of PH3+ cells upon bacterial infection **(I).** *esg*^{ts}-driven expression of a *yki*-RNAi construct prevents the regenerative response seen in WT midguts upon bacterial infection (I) (p<0.001, n>10).

Figure 4.11: Yki is required in ECs for the midgut regenerative response to bacterial infection

(A-D) *MyoIA^ts*-driven expression of *yki*–RNAi (D) partially prevents the midgut regenerative response to stress (C). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green), PH3 is in red. Quantification of PH3+ cells upon bacterial infection (E). *MyoIA^ts*-driven expression of *yki*-RNAi partially rescues the midgut regenerative response following bacterial infection (E) (p=0.018, n>14). (F-G) *MyoIA^ts*-driven expression of *yki*–RNAi (G) reduces Yki antibody staining compared to control (F). Scale bars = 20μm. Quantification of PH3+ cells upon bacterial infection (H). *MyoIA^ts*-driven expression of Hpo rescues the midgut regenerative response following bacterial infection (H) (p<0.0001, n>11).

partially depleted. Silencing endogenous Yki in the ECs by overexpressing *hpo* significantly reduces the number of proliferating cells upon *P.e* infection (Figure 4.11H); supporting the argument that Yki is required both in the ISCs and ECs for stress-induced proliferation. Yki inactivation by RNAi or via *hpo* overexpression did not induce any effect on proliferation in unchallenged guts, suggesting that the main physiological role of Yki in the midgut is in the regenerative response.

4.4.3   Yki is required in both ISCs and ECs for survival upon *P.e* infection

Having shown that Yki expression is required in the midgut proliferative response, I then addressed whether Yki is needed for survival upon infection. Lifespan was initially monitored in unchallenged flies maintained at 29°C. The doses of *P.e* used in this study are sub-lethal. In keeping with the data from Figures 4.10 and 4.11, Yki inactivation by RNAi in either the ISCs or ECs had no effect on survival in unchallenged flies (Figure 4.12 A and C), again suggesting that Yki is not essential for baseline homeostasis. Interestingly, Yki overexpression in the differentiated ECs almost halved lifespan (Figure 4.12C). Similarly, Yki overexpression in either the progenitor or EC population, when in combination with bacterial infection, significantly reduces lifespan (Figure 4.12 B and D). This reduction in survival is presumably a result of continuously high levels of proliferation and the resultant strain on midgut homeostasis. Silencing Yki by RNAi, with either driver, dramatically reduced survival following *P.e* infection (Figure 4.12 B and D). Yki is therefore required in both ISCs and ECs for survival upon bacterial infection.

4.4.4   Yki is required in ISCs but not ECs for DSS induced regenerative proliferation

Bacterial infection is by no means the only method of inducing a midgut regenerative response. ECs respond to a range of stress stimuli, such as apoptosis, JNK signalling and oxidative stress (Buchon et al., 2009b, Amcheslavsky et al., 2009, Biteau et al., 2008). To build upon the results seen with *P.e* infection, I tested the role of Yki using
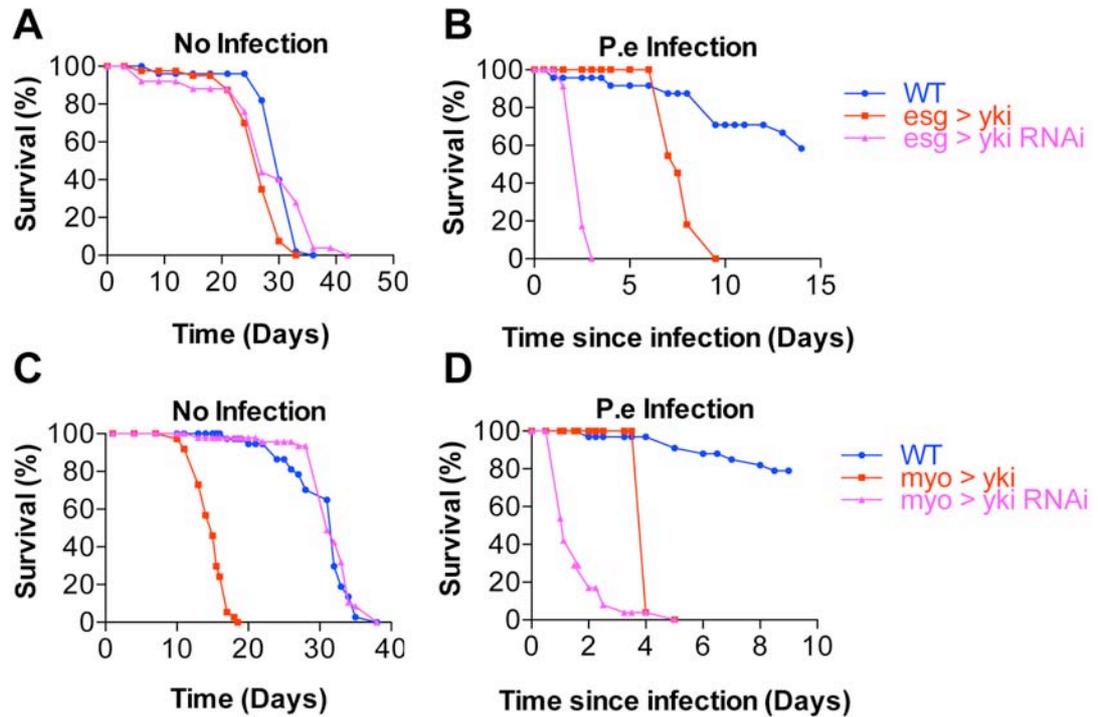
Figure 4.12: Yki is required in both ISCs and ECs for survival upon bacterial infection

(A-D) *yki-RNAi* expression in either ISCs (A) or ECs (C) does not affect lifespan in the absence of infection. *yki-RNAi* expression in either ISCs (B) or ECs (D) reduces survival time following bacterial (*P.e*) infection. *MyoIA^{ts}*-driven expression of Yki reduces lifespan in the absence of infection (C). (n>20).

another means of inducing intestinal stress. Feeding flies Dextran Sulphate Sodium (DSS) stimulates midgut proliferation (Amcheslavsky et al., 2009). When included in the drinking water of experimental mammals, DSS induces intestinal injury resembling ulcerative colitis in humans (Kawada et al., 2007). In flies, DSS feeding disrupts BM organisation, leading to an increase in cell division (Amcheslavsky et al., 2009). In contrast, *P.e* infection is reported to kill ECs and activate JNK signalling (Vodovar et al., 2005).

Following two days of DSS feeding (3% DSS in 5% sucrose solution), a proliferative response is induced (Figure 4.13A and C). Inactivation of Yki by RNAi in the ISCs, but not the ECs, prevents the proliferative response to DSS. Hence, Yki is required in the ISCs for the proliferative response to both *P.e* infection and DSS treatment. Unlike *P.e* infection, which only mildly affects survival, 3% DSS treatment killed flies within only one week. Yki inactivation had no effect on survival, perhaps indicative of the rapid effects of DSS treatment on lifespan (Figure 4.13B and D).

4.5 Conclusions and discussion

In Chapter 3, I characterised the Hpo pathway inactivation phenotype of the adult posterior midgut. Here, I have built upon those findings showing that Yki overexpression triggers increased levels of *upd* transcription and Stat reporter activity. Upon intestinal stress, Yki is activated in ECs and translocates to the nucleus, triggering Upd3 production (Figure 4.14). The secretion of Upd3 from ECs then activates Jak/Stat signalling in the ISCs, inducing their proliferation. Concurrently, Yki also appears to be required in the ISCs in order for regenerative proliferation to occur (Figure 4.14). Whether Yki is a target of Stat signalling in the ISCs is unclear. Hence, Yki appears to function in differentiated ECs as part of the stress response pathway and in ISCs as a driver of the proliferative response to stress.
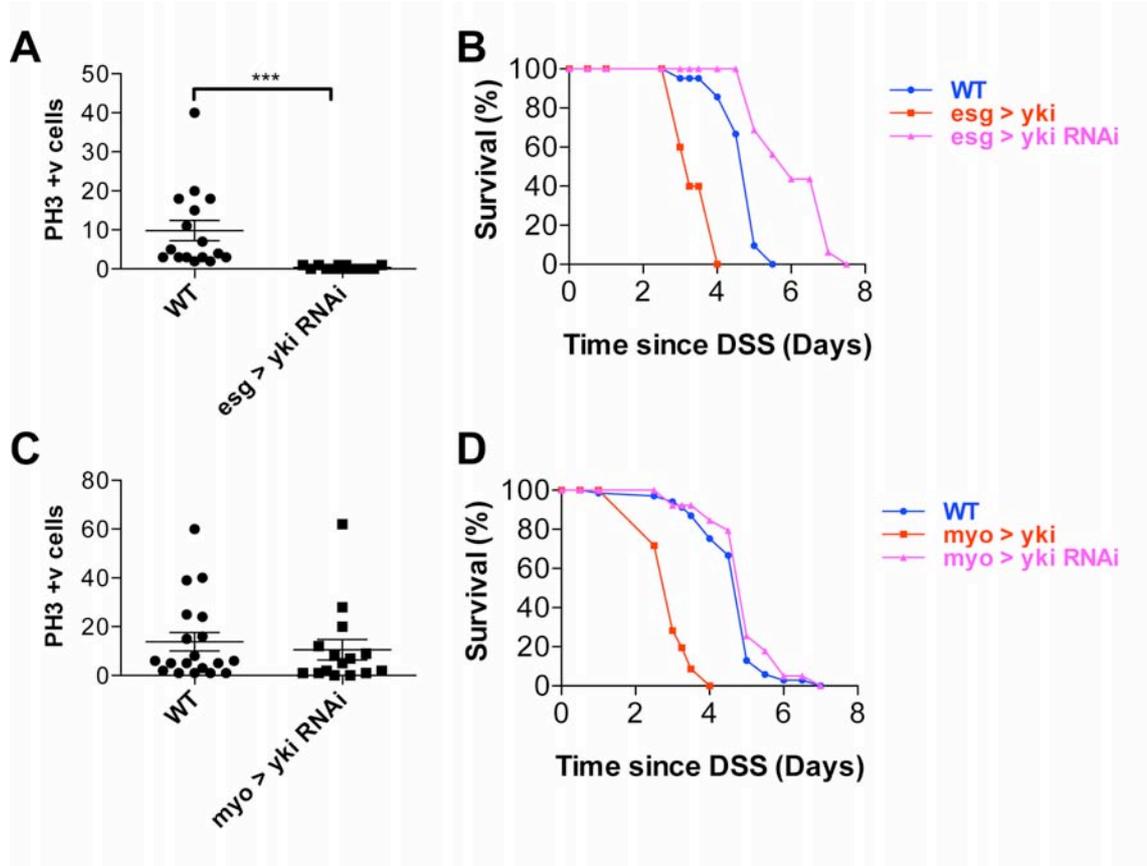
Figure 4.13: Yki is required in ISCs but not ECs for DSS induced regenerative proliferation

(A-D) *esg^{ts}*-driven expression of *yki*–RNAi rescues DSS induced regenerative proliferation (A) (p<0.0001, n>13). *MyoIA^{ts}*-driven expression of *yki*–RNAi (C) did not significantly affect DSS induced regenerative proliferation (B). *yki-RNAi* expression in either ISCs (C) or ECs (D) does not affect survival following DSS feeding. (n>20).
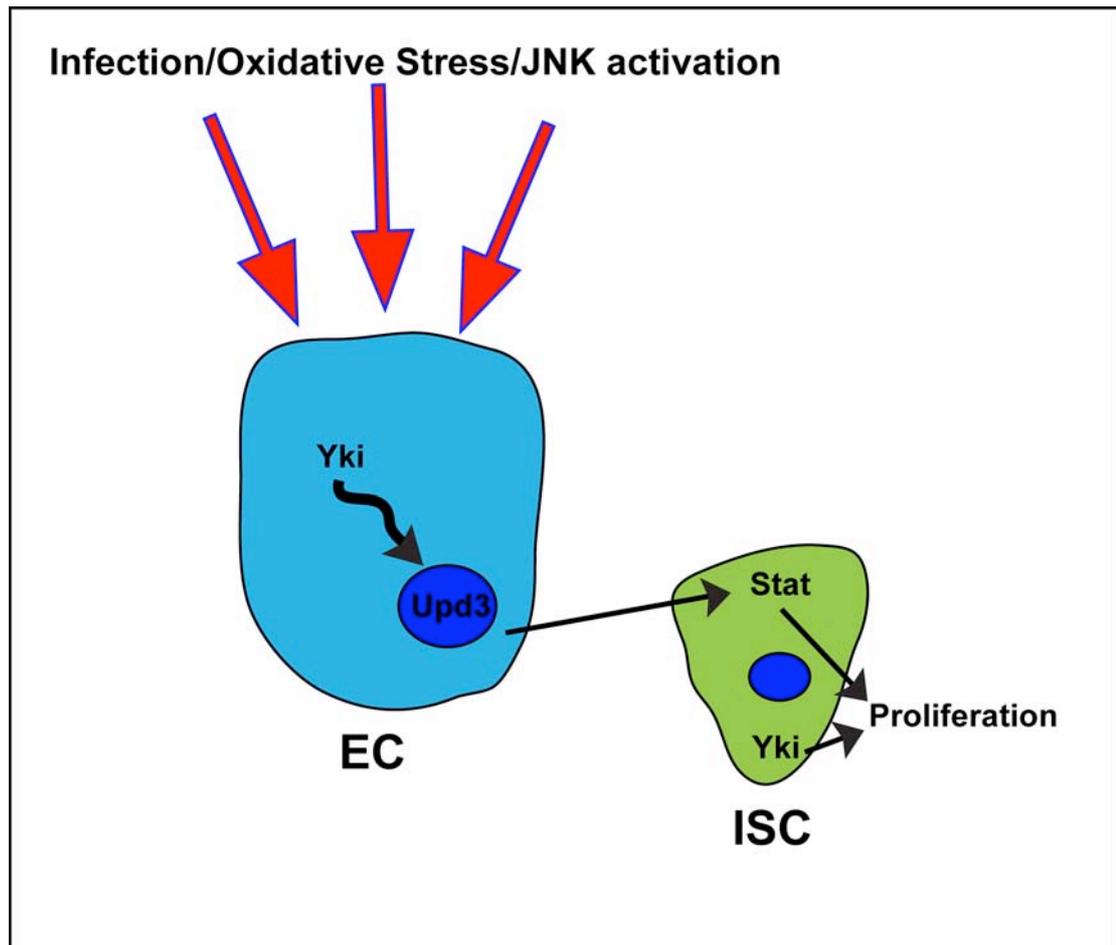
Figure 4.14: Model for the role of Yki in intestinal regeneration

Yki activation in ECs leads to the induction of Upd3, which triggers Jak/Stat signalling activity in the ISCs. Yki is required in the ECs as part of the stress response pathway and in ISCs for proliferative regeneration.

4.5.1 Is the transcriptional upregulation of Upd cytokines by Yki a general phenomenon?

Yki activation in the ECs leads to Upd secretion (Figures 4.1-4.3). There is a precedent for a non-autonomous role of Yki via a secreted factor. Overexpression of YAP1, the mammalian orthologue of Yki, in MCF10A mammary epithelial cells, enhances the proliferation of neighbouring cells through the induction of Amphiregulin, a ligand for the Epidermal Growth Factor Receptor (EGFR) (Zhang et al., 2009a). This mechanism appears to be conserved in flies, where Yki can induce the expression of the EGFR ligand Vein (Zhang et al., 2009a). The Connective Tissue Growth Factor (CTGF) is also a common direct YAP target, which is important for cell growth (Zhao et al., 2008, Urtasun et al., 2011). Thus, non-autonomous functions of the Hpo pathway may prove to be an important feature with respect to growth control.

It is unclear at this point whether Yki affects Upd secretion directly. Yki is a transcriptional co-activator. The Scalloped (Sd) TF is the best characterised of Yki's TF partners and is required for its growth control function in the wing (Wu et al., 2008, Zhang et al., 2008b, Goulev et al., 2008). However, I did not find any Sd binding sites in the promoter region of any of the three *upd* genes. Although Yki activity clearly responds to stress, it is also possible that inactivation of the Hpo pathway itself causes stress in the ECs (possibly by blocking or delaying physiological cell death or driving inappropriate cell growth), and that this might lead to the upregulation of Upd signalling via a distinct sensor.

Clonal Yki overexpression in wing imaginal discs led to increased *upd-LacZ* levels (Figure 4.4.). Interestingly, *upd-lacZ* upregulation was only seen close to the endogenous Upd expressing areas in the wing hinge (Bach et al., 2007). This could suggest either that Yki can promote Upd expression only in certain cellular contexts (possibly in the presence of other transcription factors) or that Yki promotes sustained expression of Upd but is unable to initiate its transcription.

4.5.2 A dual role for Yki in the intestinal regenerative response

4.5.2.1 Yki function in ISCs

My data point to a clear function for Yki in the ISCs. First, Yki expression is increased in ISCs upon intestinal stress (Figure 4.8). Second, silencing Yki in ISCs abolished the proliferative response to *P.e* infection (Figure 4.10). This 'cell-autonomous' growth-promoting function is similar to the known role of Yki as a growth driver in *Drosophila* imaginal discs and mammalian tissues. Indeed, Yki family proteins have been reported to promote progenitor cell proliferation in the mouse intestine and tissue regeneration in both the Cricket leg and mouse liver (Bando et al., 2009; Camargo et al., 2007; Dong et al., 2007). The possible function of the Hpo pathway as a general regulator of regeneration will be discussed in more depth in Chapter 6.

The results examining the role of Yki in the ISCs (Figures 4.8-4.10) support the argument that the proliferative effect observed following *esg*^{ts}-driven Yki overexpression (see Chapter 3) is in fact due to Yki activity in the ISCs, rather than the *esg*-GAL4 driver leading to unwanted transgene expression in the ECs. This is also supported by the observation that Yki staining does not increase in the ECs when Yki is overexpressed using the *esg*^{ts} driver (Figure 4.7F-F').

Yki overexpression in the ISCs no longer induces proliferation when expressed in combination with a Stat-RNAi construct (Figure 4.5). This result suggests that Yki is acting upstream (or in parallel) to Jak/Stat signalling in the ISCs. It remains unclear whether Yki requires Stat activity in the ISCs in order to drive regenerative proliferation. Numerous signalling pathways play a role in maintaining homeostasis in unchallenged guts, possibly resulting in a higher degree of redundancy between different pathways than is present under stress conditions. I did not observe any effect when Yki was depleted under baseline conditions, suggesting that Yki might only function in regenerative growth or upon loss of a tumour suppressor. However, the possibility of a role for Yki in basal ISC proliferation, or ISC maintenance and survival still warrants further study.

4.5.2.2 Yki function in ECs

The data in Figures 4.11 and 4.12 point to a dual role for Yki in the intestinal regenerative response. Yki's role in the ISCs is in the regulation of regenerative proliferation. The role of Yki in the ECs is less clear and is an area I will further explore in Chapter 5. Several possible explanations could account for the requirement of Yki expression in the ECs for regeneration. Yki expression itself might induce stress in the ECs, leading to *upd* expression and the regenerative response. For example, Yki activation could promote the expression of anti-apoptotic genes (e.g. *diap1*), which lead to the prolonged presence of 'undead' ECs in the epithelium resulting in continued pro-proliferative signalling emanating from the dying cells (Perez-Garijo et al., 2004, Huh et al., 2004). In that case, Yki function might normally be to protect cells from apoptosis, allowing them to survive long enough to send pro-regeneration signals to the ISCs. This would ensure that epithelial integrity is maintained while new cells are being generated. Unfortunately, as discussed in section 3.7.2, my attempts to assess apoptosis in the midgut were unsuccessful.

Alternatively, the Hpo pathway might function as a stress sensor in intestinal cells, triggering Upd cytokine release in response to noxious stimuli. This is a particularly interesting possibility since such sensors have not yet been identified. Importantly, the mammalian Hpo pathway has been implicated in contact inhibition of growth in cultured epithelial cells (Zhao et al., 2007). EC cell loss might therefore inactivate the Hpo pathway due to local changes in cellular density, leading to Yki de-repression and activation of the regenerative response. Further experimentation is required in order to distinguish between these possibilities.

4.5.2.3 Yki function in EE cells

I have not examined the role of Yki in the secretory EE cells. The small, *pros*+ EE cells make up ~10% of the midgut differentiated cell population. Until recently, I had not found a reliable *pros*-GAL4 line for use in the midgut. As shown in Figure 4.7C-C', in several WT guts I have observed increased Yki protein levels in a subset of EE cells.

Within the *pros+* population are subsets of EE cells expressing different peptides including Tachykinin and Allatostatin (Siviter et al., 2000) (Yoon and Stay, 1995, Ohlstein and Spradling, 2006). It is still unclear if regulatory peptides expressed in the midgut EE cells act in a paracrine manner on nearby cells and neurons, or if they act on distant targets such as the brain, in order to influence metabolism or feeding behaviour (Park et al., 2011). Uncovering which members of the *pros+* EE population are expressing the higher levels of Yki protein and whether this expression pattern is altered in situations of intestinal stress could prove informative with regard to the function of different EE subpopulations.

4.5.3 How is Yki activated upon intestinal stress?

How does stress activate Yki in the ISCs and ECs? In the case of the ECs, understanding Yki activation could provide clues as to Yki function in this cell population. My data suggest that at least part of the Yki accumulation occurs due to a post-transcriptional mechanism, since Yki transcript levels were not greatly affected by *P.e* infection (Figure 4.9). Whether this is mediated via canonical Hpo signalling remains to be established.

4.5.3.1 Upstream regulators of the Hpo pathway

The picture surrounding upstream regulation of the Hpo pathway is becoming increasingly complex. Upstream regulators can broadly be divided into three main classes: those acting via the atypical cadherin Fat, the Kibra-Expanded-Merlin (KEM) complex and the apicobasal polarity proteins (aPKC, LGL and Crumbs).

One simple explanation for Yki activation in the ECs would be that disruption to the adherens junctions (AJs) and a resultant loss in epithelial integrity might silence the Hpo pathway. The trans-membrane protein Crumbs, as well as several other apicobasal polarity determinants and apical scaffold proteins, can promote Hpo pathway activity and Yki silencing (Genevet et al., 2010, Yu et al., 2010, Baumgartner et al., 2010, Das Thakur et al., 2010, Chen et al., 2010, Robinson et al., 2010, Ling et al., 2010,

Grzeschik et al., 2010). However, Crumbs is known to be absent from endodermal epithelia, such as the midgut (Tepass et al., 1990, Baumann, 2001).

Like the intestine, the mammalian epidermis is a rapidly regenerating epithelial tissue, whose maintenance is reliant on a population of SCs (Fuchs, 2007). Loss of some AJ components, such as α-catenin, triggers severe epidermal hyper-proliferation (Vasioukhin et al., 2001). Studies in mice have shown that α-catenin can act as a membrane tether for YAP1, the mammalian orthologue of Yki, preventing its nuclear import (Schlegelmilch et al., 2011, Silvis et al., 2011, Zhang et al., 2011a). By inhibiting YAP1, α-catenin regulates epidermal SC proliferation and in doing so prevents skin cancer formation. The relationship between α-catenin and Yki in flies has not been investigated. It is certainly possible that a similar mechanism of Yki activation might be operating in the fly gut.

The mammalian Hpo pathway has been implicated in contact inhibition of growth in cultured cells (Zhao et al., 2007). EC cell loss might therefore inactivate the Hpo pathway due to local changes in cellular density and/or disruption of polarity determinants, leading to Yki de-repression and activation of the regenerative response. In this context, the Hpo pathway might thus be a guardian of "epithelial health", inducing a regenerative proliferative response following cellular damage. Unfortunately, it will always be difficult to directly prove that epithelial damage is causal in Yki stimulation or secondary to Yki activity. The role that upstream regulators of the Hpo pathway might play in the midgut regenerative response will be examined further in Chapter 5.

4.5.3.2 *Interaction with other stress signalling pathways*

A further mechanism by which intestinal stress could induce Yki activity is via interaction with other signalling pathways. The JNK pathway is activated in damaged ECs following intestinal injury and is sufficient to induce ISC proliferation (Amcheslavsky et al., 2009, Biteau et al., 2008, Buchon et al., 2009b, Jiang et al., 2009). Two recent studies have shown a requirement for Yki activity in wing imaginal disc

regeneration (Sun and Irvine, 2011, Grusche et al., 2011). It is suggested that, in the context of wing regeneration, Yki activity is regulated by JNK signalling, although the molecular mechanism remains unclear (Sun and Irvine, 2011).

The JNK pathway could be responsible for Yki activation in the ECs. JNK signalling *is* required for the midgut proliferative response to *Erwinia carotovora* (*Ecc15*) (Buchon et al., 2009b), but *not* to *P.e* infection (Jiang et al., 2009). Given that Yki expression in the ECs *is* required for optimal proliferation upon *P.e* infection, it is unlikely that JNK activity is the sole mechanism for Yki activation. Evidence for a connection between JNK and Hpo signalling in regeneration will be discussed further in Chapters 5 and 6.

The bacteria of the gut also play an important role in intestinal homeostasis. Commensal bacteria must be maintained, microbial pathogens need to be eliminated and the inflammatory response must be tightly controlled. In the *Drosophila* gut, the generation of reactive oxygen species (ROS) by the NADPH oxidase enzyme Duox provides an efficient barrier against microbes (Ha, 2005, Ha et al., 2005). The induction of antimicrobial peptides (AMPs) acts as a second line of defence to gram-negative bacterial infection and is regulated by the Immune Deficiency (IMD) pathway (Nehme et al., 2007). Studies have addressed the role of the midgut immune response in intestinal regeneration and highlighted the fact that epithelial renewal is an essential part of gut host defence (Buchon et al., 2009a, Lee, 2009). Activation of the Duox and IMD pathways occurs at an early stage in the response to infection, raising the possibility that Yki activation may be due to one of these pathways. Alternatively, Yki expression itself might disrupt the epithelium and ROS/AMP production could be by-products of this disruption.

4.5.4 Do different forms of stress activate Yki?

Yki is required in both ISCs and ECs in response to *P.e* infection. A better understanding of Yki activation and function in the regenerative response may come from studying other forms of intestinal stress. DSS is believed to damage BM organisation (Amcheslavsky et al., 2009). Following DSS treatment, Yki is required in

the ISCs and not the ECs for regenerative proliferation (Figure 4.13). Given the basal location of the ISCs, Yki-dependent regenerative proliferation might, in this instance, be activated by damage to the BM, independent of any signal coming from the ECs. Examining to what extent Yki is required in response to other means of inducing intestinal stress, such as bleomycin (a DNA-damaging agent), paraquat (free-radical inducing drug) and JNK pathway activation (Amcheslavsky et al., 2009, Choi et al., 2008, Biteau et al., 2008), could provide further insight into Yki intestinal function.

Chapter 5.   Results

Further investigation into the function and regulation of Yki in the *Drosophila* midgut regenerative response

5.1 Aims of this project

In Chapter 4, I presented results demonstrating a dual role for Yki in intestinal regeneration. In the ISCs, Yki controls proliferation, while Yki's function in the ECs remains less clear. In this final part of my project, I aim to uncover how Yki activation is regulated in the midgut, with a view to gaining a better understanding of Yki function in the ECs. As outlined in section 4.5.3, a number of mechanisms could be responsible for Yki activation. These include regulation by upstream members of the pathway, activation due to a loss of epithelial organisation and interplay with other signalling pathways.

5.2 Role of upstream regulators of the Hpo pathway in midgut Yki activation

Upstream branches of Hpo pathway regulation include the Kibra-Expanded-Merlin (KEM) complex, proteins that signal via the atypical cadherin Ft and the apicobasal polarity proteins (see section 1.2.3). How the different upstream branches of the pathway interact with one another and their relative contribution to Hpo signalling still remains to be fully elucidated. The significance of these different inputs will most likely vary in different organs and contexts. Often the loss of one branch of upstream regulation only has a mild effect on growth, presumably due to redundancy and feedback loops.

5.2.1 Input from the Kibra-Expanded-Merlin (KEM) complex

The KEM complex is predominantly located in the subapical region of the cell. The WW domain-containing protein Kibra binds to the FERM domain-containing proteins

Merlin (Mer) and Expanded (Ex). Together, these three proteins are thought to help recruit the core Hpo kinase cassette to the apical membrane for activation (Genevet et al., 2010, Yu et al., 2010, Baumgartner et al., 2010). The precise mechanism by which the KEM complex is controlled is unclear. Regulation could involve a specific receptor or even a physical property of the cell, such as a change in tension or adhesion. Silencing of *kibra* in the ECs, alone or in combination with *mer*-RNAi, resulted in an increase in the number of Dl+ cells per unit area (Figure 5.1A-D') and higher levels of proliferation (Figure 5.1E), suggesting that the KEM complex is involved in Yki regulation in the adult midgut.

5.2.2 Midgut expression pattern of Hpo pathway targets

The Ft signalling pathway is another major upstream regulator of Hpo signalling (Silva et al., 2006, Willecke et al., 2006, Cho et al., 2006, Bennett and Harvey, 2006). I have not investigated Ft signalling function in the gut but I did examine the expression pattern of the Golgi kinase Four-jointed (Fj), one of its regulators (Villano and Katz, 1995, Ishikawa et al., 2008, Brittle et al., 2010, Simon et al., 2010). Fj, like two other upstream regulators of the Hpo pathway, Ex and Mer, is also a transcriptional target of the pathway (Cho et al., 2006, Hamaratoglu et al., 2006). The Hpo pathway therefore regulates Ft signalling via a negative feedback loop. *fj* expression, revealed by a *fj-LacZ* enhancer trap, appears to be restricted to the small midgut progenitor cells (Figure 5.2A-A'). Increased *fj-LacZ* expression is observed following *P.e* infection, presumably coinciding with the increase in the number of ISCs (Figure 5.2B-B'). A similar expression pattern was apparent for another Yki target, *diap1*. *diap1*-GFP expression appeared to be restricted to a population of small cells (Figure 5.2C-D').

5.2.3 Role of epithelial organisation

The apicobasal cell polarity proteins Lgl, aPKC and Crumbs affect cell proliferation and survival by modulating Hpo pathway activity (Robinson et al., 2010, Grzeschik et al., 2010). In addition, the mammalian Hpo pathway has been implicated in contact inhibition of growth in cultured cells (Zhao et al., 2007). A loss of epithelial polarity or
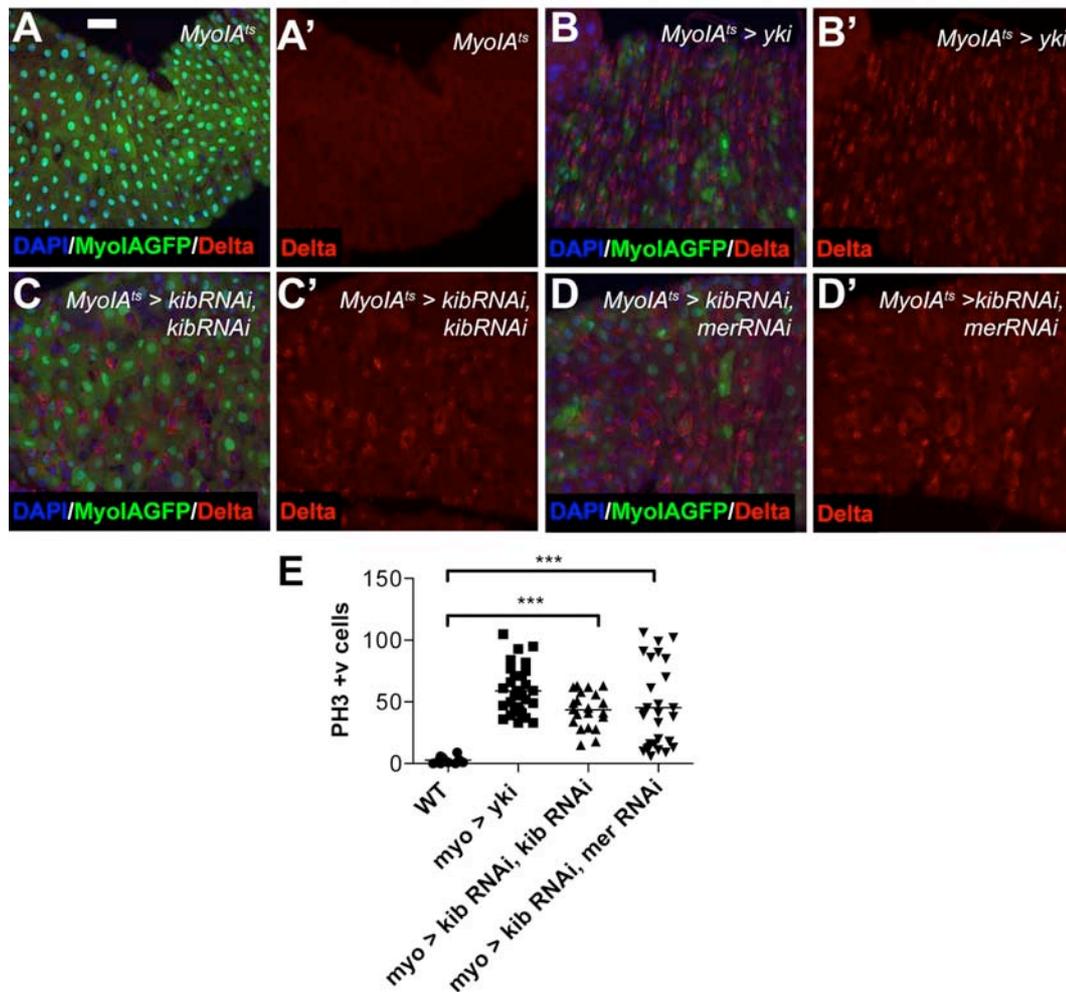
Figure 5.1: Inactivation of upstream components of the Hpo pathway induces Dl expression

(A-D') *MyoIA^{ts}*-driven expression of *kib-RNAi,kib-RNAi* (C-C') or *kib-RNAi;mer-RNAi* (D-D') in ECs induces an increase in the number of Dl+ cells per unit area compared to control (A-A'). Note Dl levels are comparable to *MyoIA^{ts}*-driven expression of Yki (B-B'). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green) and Dl is in red. Scale bar = 20μm. Quantification of PH3+ cells per midgut. (E) *MyoIA^{ts}*-driven expression of *kib*-RNAi,*kib*-RNAi or *kib*-RNAi,*mer*-RNAi in ECs induces ISC proliferation. (p<0.001, n>14).
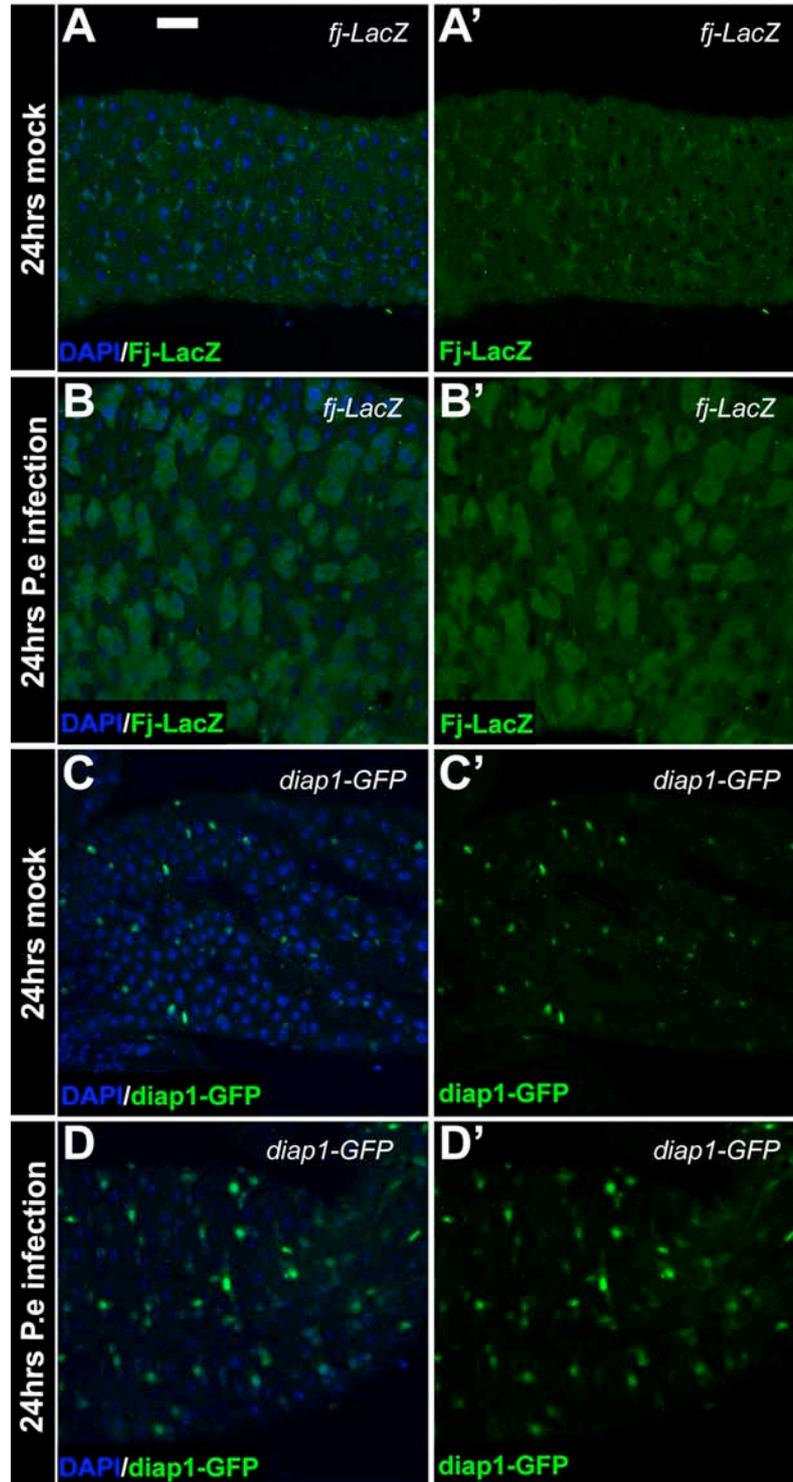
Figure 5.2: Gut expression patterns of Yki targets: Diap1 and Fj

(A-B') *fj-lacZ* expression appears to be restricted to the progenitor cell population (A-A'). *fj-lacZ* expression is increased upon *P.e* infection (B-B' compared to A-A'). **(C-D')** *diap1-GFP* expression is restricted to small cells (C-C'). *diap1-GFP* expression is increased upon *P.e* infection (D-D' compared to C-C'). Nuclei are stained with DAPI (blue), β-Galactosidase (A-B') and GFP (C-D') are in green. Scale bar = 20µm.

integrity is therefore another possible mechanism of Yki activation in the midgut. AJs are composed mainly of homophilic *Drosophila* E-cadherin (DE-cadherin) and the adaptor proteins Armadillo (β-catenin) and α-catenin (Tepass et al., 2001). I therefore examined the localization of AJ components upon bacterial infection. Armadillo and DE-cadherin outline the membranes of all cell-types in the midgut and are most highly expressed at junctions between ISCs (Figure 5.3A-B, E-E', G-H') (Micchelli and Perrimon, 2006, Baumann, 2001). Armadillo staining highlights the increase in ISC number apparent following *P.e* infection and general disruption to cellular organisation (Figure 5.3C-D). A similar effect is observed with DE-cadherin when Yki is overexpressed in the ECs (Figure 5.3F-F'). The extent of midgut overproliferation following Yki overexpression makes it difficult to assess whether the EC AJs are significantly disrupted. Since I did not observe much of a change, it is difficult to argue that disruption to EC junctions or polarity is a key event in Yki activation in this context. I observed no obvious effect when α-catenin was silenced in the gut (data not shown).

Apicobasal polarity is crucial to the formation of cell-cell contacts (Tepass et al., 2001, St Johnston and Ahringer, 2010). The aPKC complex, composed of aPKC, Par6, and Bazooka, localises subapically and is important for the formation and maintenance of the apical domain (Tepass et al., 2001). aPKC has been shown to influence Hpo apical localisation (Grzeschik et al., 2010). In the midgut, aPKC is expressed in *esg+* cells and a subpopulation of EE cells (Figure 5.3I-J"). To assess the role of aPKC and other polarity proteins in the midgut, I expressed various RNAi lines and looked for any resultant effect on proliferation or *esg+* cell number (data not shown). The results were, however, highly variable and as a result this line of investigation was not pursued. It will be difficult to assess whether the dramatic changes in epithelial organisation and polarity are causal in, occur in parallel to, or are a direct consequence of, Yki activation in the midgut. The development of midgut live imaging techniques would presumably help to address this issue.
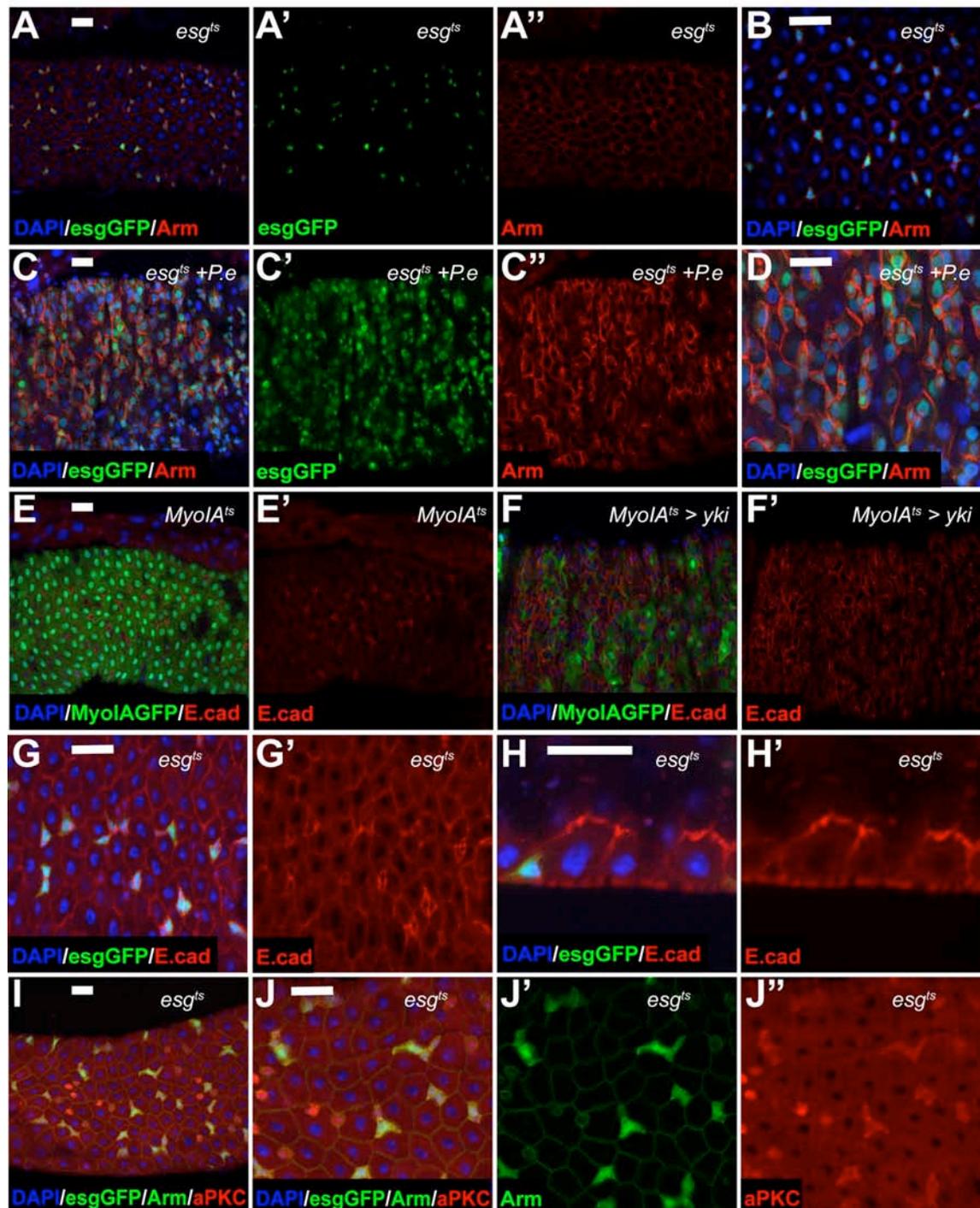
Figure 5.3: Gut expression patterns of epithelial junctional proteins: Armadillo and DE.cadherin

(A-D) Armadillo staining is increased following P.e infection (C-D compared to A-B). **(E-F')** *MyoIA^{ts}*-driven expression of Yki increases DE.cadherin levels (F-F' compared to E-E'). **(G-H')** Apical localisation of Armadillo. **(I-J")** aPKC is expressed in *esg+* and EE cells. Nuclei are stained with DAPI (blue). *esg+* cells (A-D, G-J"), ECs (E-F') and Arm (I-J") are in green. Arm (A-D), E.cad (E-H') and aPKC (I-J") are in red. Scale bar = 20μm.

5.3 The Immune Deficiency (IMD) pathway and Yki activation

ROS generation by the Duox enzyme and the production of AMPs following IMD pathway activation are the first lines of defence against midgut pathogens (Ha et al., 2005). The signalling pathways produced by different types of gut-microbe interaction are involved in determining ISC activity (Buchon et al., 2009a, Chatterjee and Ip, 2009, Cronin et al., 2009, Jiang et al., 2009). As two of the earliest steps in the midgut response to infection, the IMD and Duox pathways are both candidates for having a role in Yki activation.

5.3.1 Influence of the midgut commensal bacteria

The gut commensal bacteria activate basal levels of Upd expression and subsequent Jak/Stat-dependent midgut cell turnover (Buchon et al., 2009b). This role was uncovered by comparing flies reared in germ-free (axenic) conditions to conventionally reared animals. In addition, an alternative scenario to explain the effect of Yki expression in ECs or ISCs would be that excess Yki activity might compromise epithelial integrity, allowing commensal bacteria to penetrate the intestinal barrier, inducing ISC proliferation via an immune response. To test whether the gut commensal bacteria are required for Yki induced proliferation, I overexpressed Yki in the ISCs of flies raised on axenic medium (Figure 5.4). After two generations on axenic medium, guts were dissected, homogenized in PBS and spread on LB plates, showing a dramatic reduction in the number of bacteria present in both the WT and *UAS-yki* fly stocks (Figure 5.4A). *esg*^{ts}-driven *yki* expression induced ISC proliferation even in the absence of bacteria, demonstrating that gut commensals are not required for Yki induced proliferation (Figure 5.4B).

5.3.2 Silencing IMD pathway components during *P.e* infection

IMD pathway activation is a possible candidate for Yki activation, since IMD activity corresponds to *P.e* infection. Interestingly, TAK1, a member of the IMD pathway, is a MAPKKK for JNK, presenting the possibility that the IMD pathway could affect Yki
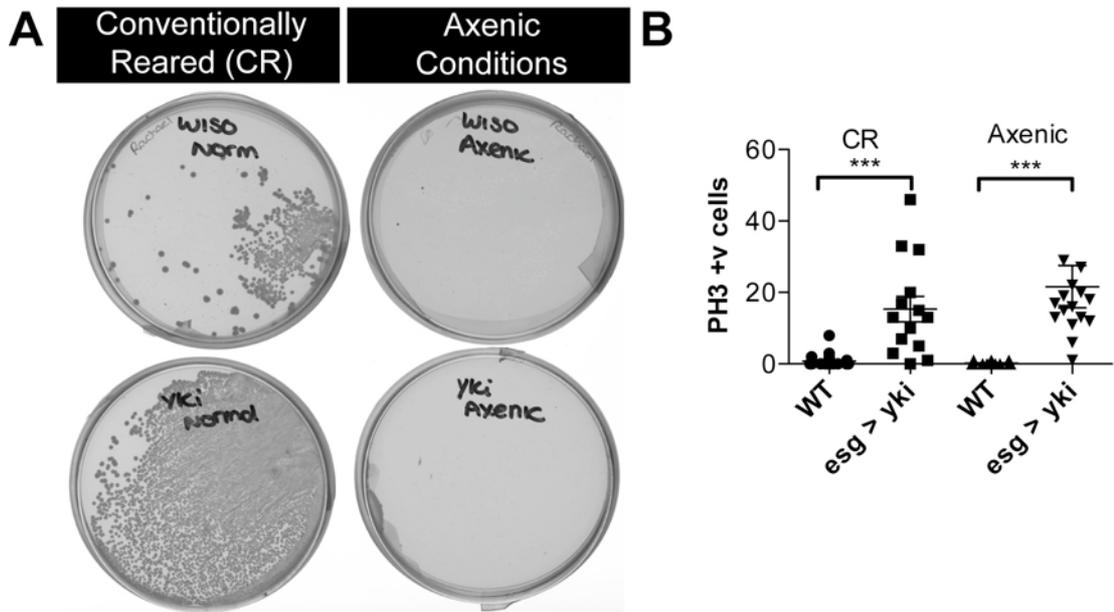
Figure 5.4: Intestinal commensal bacteria are not required for the proliferative response to Hpo pathway inactivation

LB plates showing gut bacteria levels **(A)**. Flies raised in axenic conditions have reduced gut bacteria levels (A). **(B)** *esg*^{*ts*}-driven expression of Yki is still able to induce proliferation in the absence of the gut commensal flora. (P<0.0001, n>14).

via JNK (see section 5.5). With this in mind, I examined the proliferative response to *P.e* infection whilst silencing different members of the IMD pathway by RNAi. In the absence of Yki, proliferative regeneration does not occur (Figure 4.10). Inactivation of the IMD pathway did not significantly affect the proliferative response, suggesting that IMD pathway activity is not required for Yki expression (Figure 5.5). A reduction in the number of PH3 positive cells was observed 12 hours after infection when the IMD caspase Dredd was silenced; however after 24 hours this was no longer the case and this was not investigated further (Figure 5.5A compared to B). Thus, Yki activation upon infection seemingly occurs independently of the IMD pathway.

5.4 Generation of ROS by the Dual Oxidase enzyme and Yki activation

ROS, generated by the Duox enzyme, are highly reactive and diffusible molecules with an ability to act as intracellular messengers. In an infectious microbial situation, the number of infectious bacteria becomes dominant over harmless commensals and the DUOX-expression pathway is activated to enhance DUOX activity (Ha et al., 2009). The ROS levels required to eliminate bacteria must be balanced with ROS-induced damage to the epithelium. Silencing Duox activity by RNAi significantly reduced the midgut proliferative response to infection (Figure 5.6A). Duox inactivation in the presence of Yki overexpression does not reduce regenerative proliferation (Figure 5.6A), suggesting that Yki functions either downstream or independently of Duox activity. To address this issue further, I examined Yki expression following *P.e* infection in the absence of Duox (Figure 5.6B-E). Duox inactivation did not appear to prevent Yki induction upon infection entirely, with Yki staining perhaps less nuclear when compared to WT stressed guts (Figure 5.6E compared to 5.6C). Further study is needed to confirm any change definitively, given the variability of Yki staining and the absence of a null mutant for Duox.
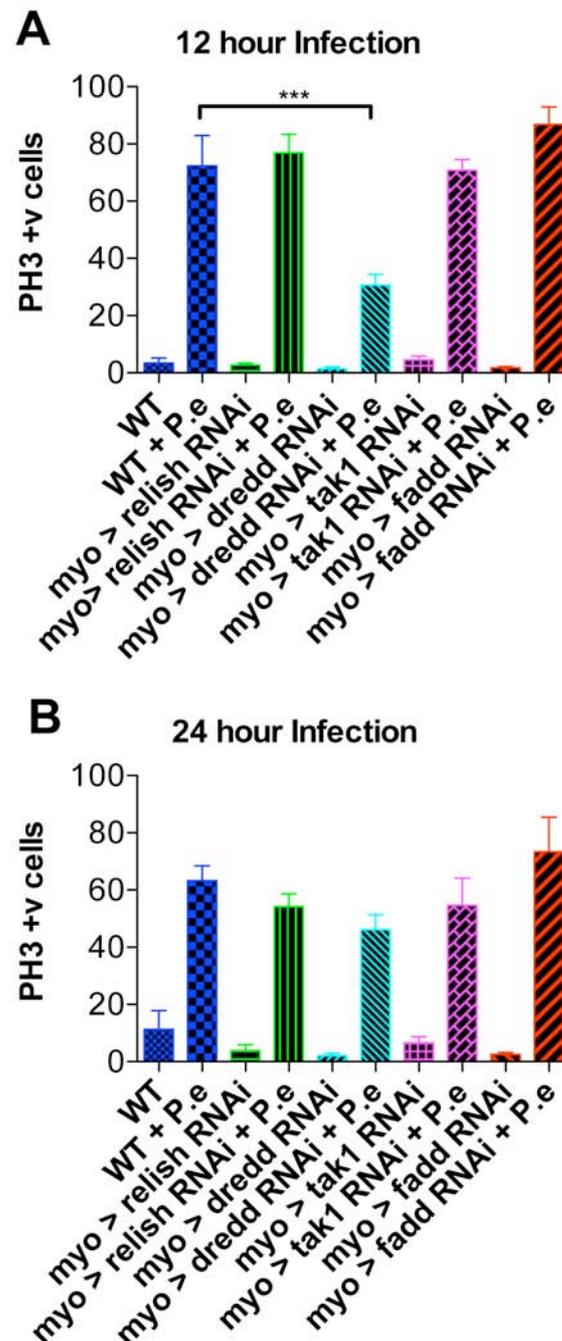
Figure 5.5: Silencing members of the IMD pathway does not severely affect the proliferative response to infection

Quantification of PH3+ cell numbers following *P.e* infection when members of the IMD pathway have been silenced **(A-B)**. *MyoIA^{ts}*-driven expression of *dredd*-RNAi reduces the proliferative response following 12-hour *P.e* infection (p<0.004, n>12). Silencing members of the IMD pathway in ECs using the *MyoIA^{ts}* driver does not significantly affect levels of proliferation 24 hours after *P.e* infection (B).
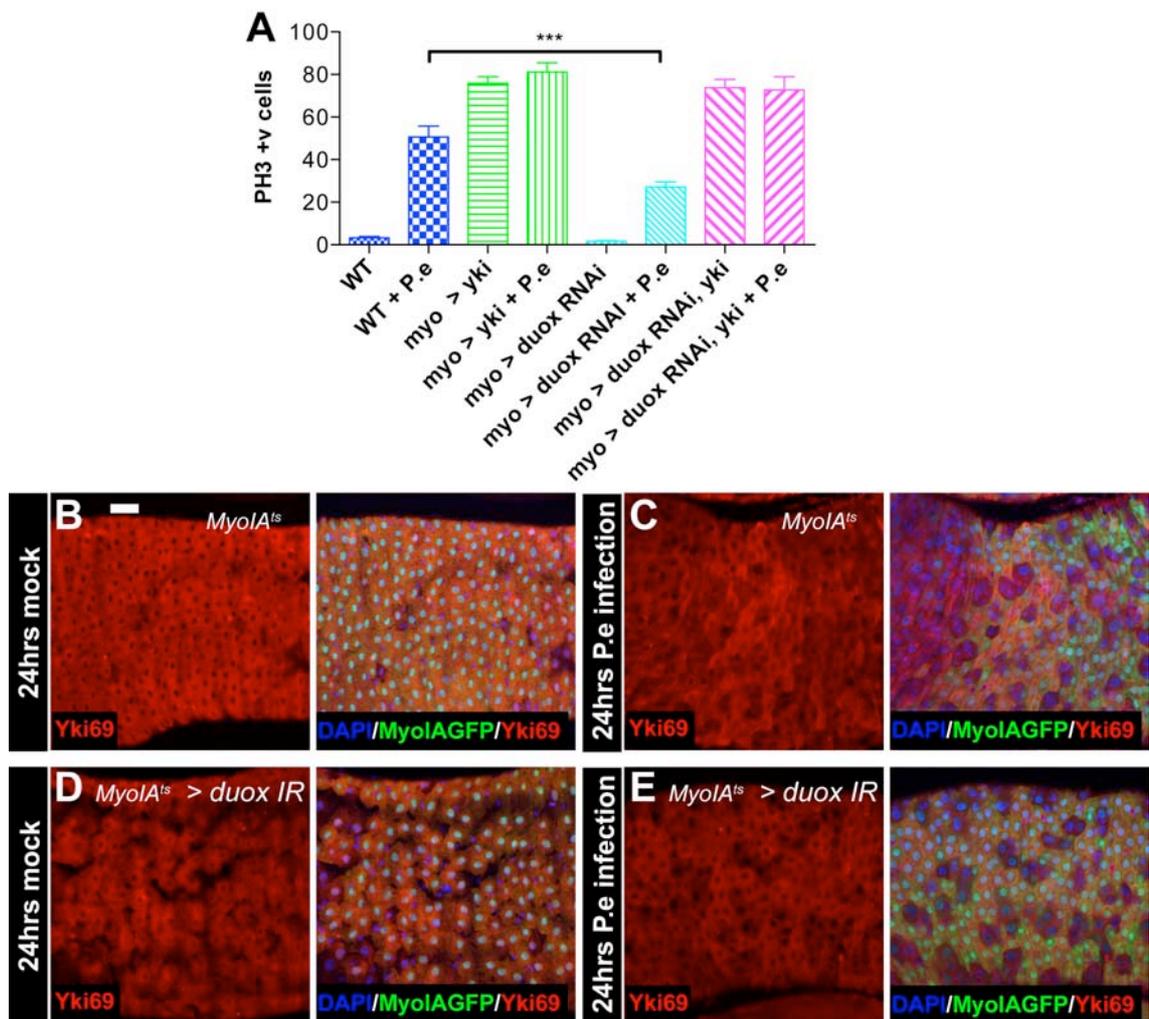
Figure 5.6: Loss of Duox enzyme activity reduces the proliferative response to infection

Quantification of PH3+ cell numbers following *P.e* infection **(A)**. *MyoIA^{ts}*-driven expression of *duox*-RNAi reduces the proliferative response to infection (P<0.0006, n>18). *MyoIA^{ts}*-Yki-induced overproliferation is not rescued by co-expression of *duox*-RNAi. **(B-E)** *MyoIA^{ts}*-driven expression of *duox*-RNAi might reduce Yki levels following *P.e* infection (E compared to C). Nuclei are stained with DAPI (blue), ECs are marked by GFP (green) and Yki69 is in red. Scale bar = 20μm.

5.4.1 Midgut response to Hydrogen Peroxide treatment

Duox has both a trans-membrane NADPH oxidase domain, which produces Hydrogen peroxide (H_2O_2) and an extracellular peroxidase homology domain (PHD), which breaks down H_2O_2 (Ritsick et al., 2004, Ha, 2005). 0.1% H_2O_2 treatment increases the number of dividing PH3 positive cells (Figure 5.7A) (Buchon et al., 2009b). Interestingly, silencing Duox by RNAi prevented H_2O_2 induced proliferation (Figure 5.7A), suggesting that H_2O_2 treatment cannot be used to mimic Duox enzyme activity in the midgut. The requirement of Duox for H_2O_2 induced proliferation is presumably because H_2O_2 is a substrate for Duox's extracellular PHD (Ha, 2005). This finding suggests that either the generation of microbicidal secondary oxidants, such as HOCl, or Tyrosine crosslinking of extracellular matrix/membrane proteins, both of which can be catalyzed by the Duox PHD using H_2O_2 as a substrate (Ha, 2005, Donko et al., 2005), are responsible for ISC proliferation in response to H_2O_2 treatment or Duox activation. It would be interesting to probe this mechanism further and identify the signalling pathways in question. H_2O_2 treatment did not affect Yki-target expression, as assayed by *ex-LacZ* expression, again suggesting that H_2O_2 treatment alone is not sufficient to mimic the effects of Duox activation (Figure 5.7B) (Hamaratoglu et al., 2006).

5.5 Does the Hpo pathway interact with JNK signalling in the midgut?

The JNK pathway is also part of the regenerative response and its ectopic activation is sufficient to induce ISC proliferation (Amcheslavsky et al., 2009, Biteau et al., 2008, Buchon et al., 2009a, Jiang et al., 2009, Cronin et al., 2009). Two recent studies in wing imaginal discs have shown a requirement for Yki activity in wing disc regeneration (Sun and Irvine, 2011, Grusche et al., 2011). In the context of wing regeneration, Yki activity is thought to be regulated by JNK signalling (Sun and Irvine, 2011).
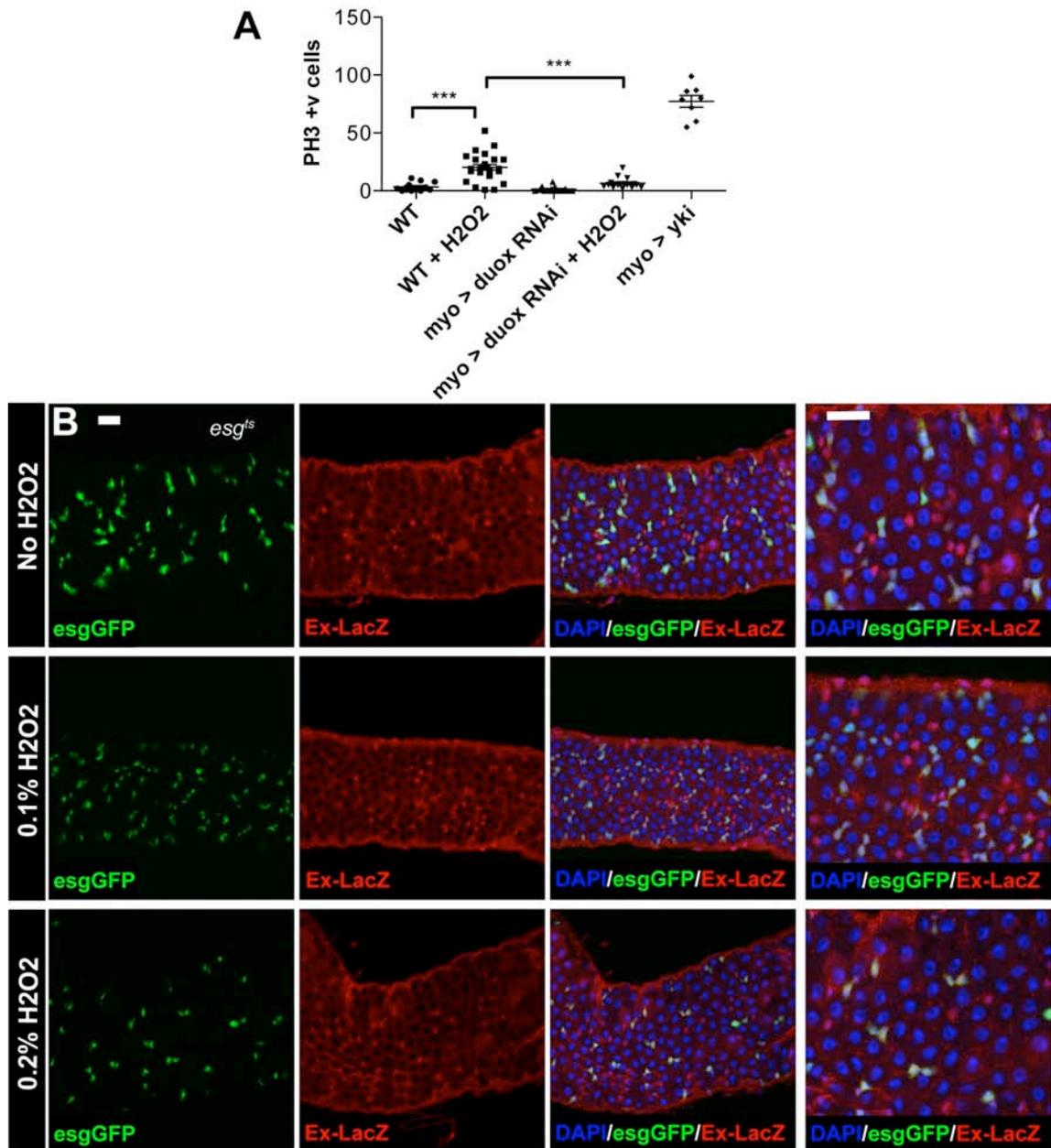
Figure 5.7: Hydrogen Peroxide treatment induces proliferation but does not affect Hpo pathway target expression

Quantification of PH3+ cells following 0.1% H_2O_2 treatment **(A).** H_2O_2 treatment induces proliferation but not when Duox is silenced in the ECs. (P<0.004, n>12). **(B)** H_2O_2 treatment does not result in an increase in *ex-LacZ* reporter levels. Nuclei are stained with DAPI (blue), *esg+* cells are marked by GFP (green) and β-Galactosidase is in red. Scale bar = 20μm.

5.5.1 JNK pathway activation increases Yki target expression

In an experiment carried out by our collaborator Alexander Kohlmaier, JNK activation induced by silencing of the JNK phosphatase Puc, led to a three-fold increase in Yki target gene expression (Figure 5.8). These results provide a further example of Yki transcriptional activity being induced in response to intestinal stress and suggest that JNK signalling could regulate midgut Yki activity directly.

5.5.2 Yki activity is downstream or in parallel to the JNK pathway

The relationship between the JNK pathway and Yki activation was examined further using immunofluorescence. Expression of a constitutively activated version of the JNK kinase Hemipterous (Hep) in the ECs induces gut regeneration within 24 hours of transgene expression. HepCA expression also induced Yki expression (Figure 5.9 A-B'). Yki expression was visibly increased in the ISCs and became more nuclear in the ECs (Figure 5.9 B-B'). Silencing JNK activity either by overexpression of Puc or inactivation of Basket (*bsk*, the *Drosophila* JNK) had no obvious effect on Dl expression at baseline (Figure 5.9C-D). In genetic epistasis experiments, a reduction in JNK activity did not affect the ability of Yki overexpression to induce a proliferative response (see increased number of Dl positive ISCs in Figure 5.9 E-F), consistent with Yki activity functioning downstream of JNK signalling.

Puc is not only a potent suppressor of JNK activity but also a downstream target of the pathway (Martin-Blanco et al., 1998). A *puc-lacZ* reporter line can be used to assess JNK activity levels in the midgut epithelium (Martin-Blanco et al., 1998, Wang et al., 2005). *Puc-lacZ* expression in the midgut increases with age and when flies are given the ROS-inducing drug paraquat (Biteau et al., 2008). Yki expression in ECs led to an increase in *puc-lacZ* expression (Figure 5.9 G-H'), adding another degree of complexity to the interaction between the Hpo and JNK pathways. When considered alongside the fact that Yki expression in the ECs reduces lifespan (Figure 4.12), it is interesting to speculate that Yki expression speeds up the midgut ageing process. Most likely, the
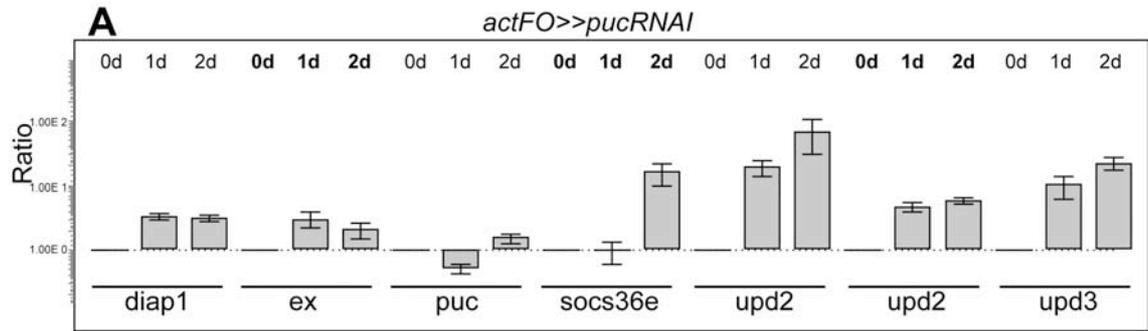
Figure 5.8: JNK signalling promotes Yki activity in the midgut

(A) RT-qPCR analysis in adult midguts. Increased abundance of *expanded* and *diap1* (Yki target genes) mRNAs are observed upon de-repression of JNK pathway activity by RNAi mediated inhibition of Puckered expression. The regenerative response is documented by increased Jak/Stat signalling (*upd* cytokines, *socs36E*). Data in this figure were generated by A.Kohlmaier.
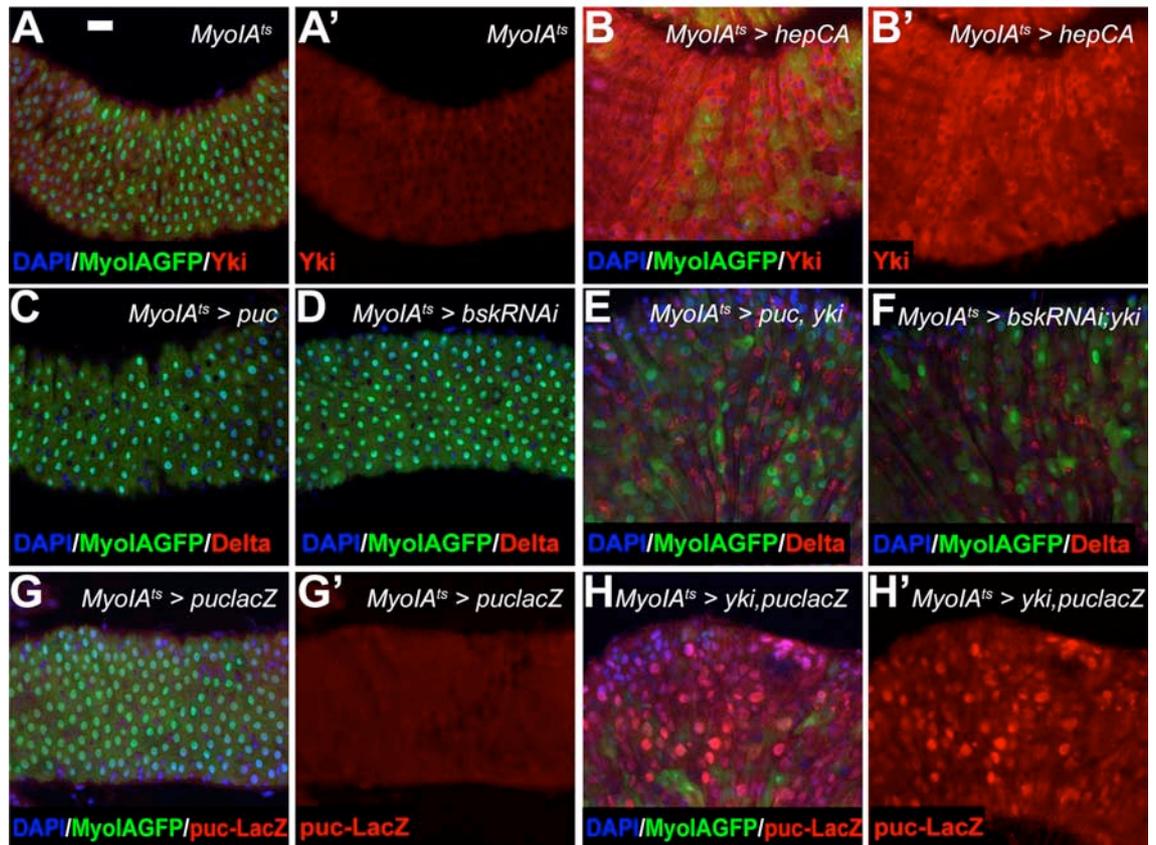
Figure 5.9: Yki functions downstream of JNK pathway activity

(A-B') *MyoIA^{ts}*-driven expression of constitutively activated Hep (B-B') increases Yki staining levels compared to control (A-A'). **(C-F)** Silencing JNK signalling activity (C and D) does not prevent the increase in Dl staining seen following *MyoIA^{ts}*-driven expression of Yki (E and F). **(G-H')** *MyoIA^{ts}*-driven expression of Yki increases *puc-lacZ* levels (H-H') compared to control (G-G'). Nuclei are stained with DAPI (blue). ECs are marked by GFP (green). Yki (A–B'), Dl (C-F) and β-Galactosidase (G-H') are in red. Scale bar = 20μm.

resultant overproliferation following Yki expression in the ECs triggers JNK pathway activity indirectly.

5.6 Conclusions and discussion

I investigated potential mechanisms of Yki activation in the midgut regenerative response. However, the function and mechanism of Yki activation in the ECs remains unclear. While inactivation of members of the upstream KEM complex induced midgut proliferation, I was not able to fully address the contribution of other Hpo pathway upstream inputs on midgut Yki activity, such as Ft signalling. Signalling by the IMD pathway does not appear to affect Yki activation. ROS generation, by the Duox enzyme, remains a possible mechanism for triggering Yki expression, as does JNK signalling, which was shown to induce Yki activity.

5.6.1 Yki activation upon intestinal stress

Although Yki activity clearly responds to stress, it is also possible that inactivation of the Hpo pathway itself causes stress in the ECs (possibly by blocking or delaying physiological cell death or driving inappropriate cell growth), and that this might lead to the upregulation of Upd signalling via a distinct sensor. As a result, it will be difficult to delineate whether factors such as loss of cell polarity and epithelial integrity are responsible for, or occur as a result of, Yki activation.

5.6.2 The JNK pathway as a regulator of Hpo pathway activity in the midgut

JNK activation can induce Yki activity and expression (Figures 5.9 and 5.10). JNK pathway inactivation did not affect the ability of Yki to induce proliferation, suggesting that Yki acts downstream of JNK in the midgut. A more informative experiment would be to test whether JNK activation can induce regenerative proliferation in the absence of Yki, however this is technically challenging. JNK activation in the ECs for only a short

period of time is enough to kill the flies. This would not allow sufficient time for downregulation of Yki protein by RNAi. A better approach would be to temporally separate Yki RNAi from JNK activation by using both the Gal80^{ts} and the Q systems (Potter et al., 2010). This would enable Yki inactivation prior to JNK activation. A second approach would be to generate MARCM clones mutant for *yki*, whilst activating JNK activity. As described in section 3.3.1, I have had difficulty with the generation of MARCM clones in the midgut.

JNK pathway activity is dispensable for ISC division during infection with sub-lethal doses of *P.e* (Buchon et al., 2009b, Jiang et al., 2009). Since Yki *is* required for regenerative proliferation, JNK signalling alone cannot be solely responsible for Yki activity. JNK activation in the midgut is also shown to induce Jak/Stat signalling (Jiang et al., 2009), while my results also suggest that Yki promotes JNK activity (Figure 5.9H-H'), making it difficult to delineate a straightforward hierarchical relationship between JNK and Hpo pathway activity.

5.6.3 Duox enzyme activity as a regulator of Yki activation in the gut

Jak/Stat signalling and ISC division are not dependent on the IMD pathway in order to respond to gut-microbe interactions (Jiang et al., 2009). Silencing the IMD pathway did not appear to affect Yki activation in the midgut (Figure 5.5-5.6), suggesting that the IMD pathway does not regulate Hpo pathway activity.

Duox enzyme activity and ROS generation remain possible mechanisms of Yki activation. ROS have been shown to regulate a variety of signalling pathways through oxidative inactivation of critical redox-sensitive signalling proteins (Lee, 2009, Rhee et al., 2000). Duox enzyme activity is required for the proliferative response to both *P.e* (Figure 5.6) and *Ecc15* infection (Buchon et al., 2009b). *Duox*-RNAi expression no longer prevented regenerative proliferation when expressed alongside UAS-*yki* (Figure 5.6).

ROS generation can lead to cellular and tissue damage, while tissue damage is in turn known to provoke ROS generation (Niethammer et al., 2009). Given the pleiotropic effects of ROS on cell physiology, it will be difficult to clarify whether ROS directly induce SC signalling or simply cause tissue damage, which in turn signals to SCs via other means. Since it is currently unknown how tissue damage can be transformed into a physiological signal for SC activation, it is tempting to speculate that ROS induced tissue damage is sensed by Yki.

Wnt, Jak/Stat and JNK signalling have all been shown to be under redox control in various contexts (Funato et al., 2006, Junn et al., 2000, Liu et al., 2004). ROS production occurs at injury sites in the epithelia of humans, plants and zebrafish and is important in wound healing (Kanta, 2011). Recent studies demonstrating the importance of JNK-dependent Yki activation in *Drosophila* wing imaginal disc regeneration provide further support for a possible link between ROS generation, JNK signalling and Yki activation in the midgut (Grusche et al., 2011, Sun and Irvine, 2011).

5.6.4 Future experimental directions

The range of upstream branches known to regulate the Hpo pathway in developmental contexts would suggest that regulation of Yki activation in the midgut is most likely dependent upon numerous inputs. Yki is required for midgut regeneration in response to several different forms of stress, again suggesting that regulation is not via a single mechanism. Two of the best candidates for Yki activation in the context of the midgut are Duox-dependent ROS generation and JNK signalling activity. Since H_2O_2 treatment did not appear to mimic Duox activity, it will be important to test whether Duox expression is itself sufficient to trigger Yki activity and to also address whether Duox expression can induce proliferation in the absence of Yki. Given the role of Yki in wing imaginal disc regeneration, the influence of Duox expression on Yki activity should also be examined in this context.

Chapter 6. Discussion

During my PhD, I have uncovered a role for the Hpo pathway in the regulation of *Drosophila* intestinal regeneration. In summary, I have shown that Yki, the downstream transcriptional co-activator for the Hpo signalling pathway, is required both in ISCs and ECs in order for regenerative proliferation to occur following the induction of intestinal stress. My study not only suggests an important role for Hpo signalling in regeneration, but also provides a novel link between Hpo signalling and Jak/Stat pathway activity, whereby Yki expression in the ECs leads to secretion of Jak/Stat pathway ligands (see Figure 4.14), thereby enhancing Jak/Stat signalling activity. Numerous mammalian studies have begun to address the role of the Hpo pathway in various SC populations, including the intestine (see section 1.2.7). However, unlike my work, the majority of these studies have not examined the different possible roles the pathway might play in SCs versus their differentiated progeny.

6.1 The Hpo pathway as a component of the intestinal regenerative response

6.1.1 Other studies examining Yki activity in the *Drosophila* posterior midgut

Alongside my own work, which was published in collaboration with Alexander Kohlmaier (Edgar lab, Heidelberg) (Shaw et al., 2010), three other groups have also published similar findings regarding the Hpo pathway and intestinal SC regeneration (Staley and Irvine, 2010, Ren et al., 2010, Karpowicz et al., 2010). I will use these publications as a basis for my discussion of our current understanding of Hpo pathway function in the midgut.

6.1.1.1 The role of the Hpo pathway in the intestinal regenerative response

In general, all four publications regarding Hpo pathway activity in the midgut are in agreement that, while endogenous levels of Yki are not required for maintaining baseline intestinal homeostasis (Shaw et al., 2010, Staley and Irvine, 2010, Karpowicz et al., 2010), Yki activity is required for proliferative regeneration upon intestinal stress. My work, and that of Ren and colleagues, demonstrates that the Hpo pathway exhibits both autonomous and non-autonomous effects on ISC proliferation. Karpowicz et al focussed their study on the cell-autonomous requirement for Yki in the ISCs. In contrast, Staley and Irvine did not observe any cell-autonomous effect when Yki was overexpressed in the progenitor population. This discrepancy probably reflects the relative strength of the Yki transgenes used in our various studies. I used a WT *UAS-yki* transgene (Huang et al., 2005) whereas Staley and Irvine drove expression of *UAS-yki-S168A:V5*, a targeted insertion (Oh and Irvine, 2009). Although in principle the S168A mutant should be more potent, we have observed that the untagged transgene has a stronger effect in imaginal discs, either because of the insertion site or because the tag disrupts a C-terminal motif required for full Yki function.

Staley and Irvine report a non-autonomous function for the Hpo pathway in midgut regeneration, consistent with my work. Interestingly, they observed that the regenerative response elicited by the DNA damaging agent bleomycin was also partially reduced by Yki depletion. Similar to our study, this suggests a general role for Yki in the intestinal damage response. In Figure 4.13, I demonstrated that Yki activation is required specifically in the ISCs/EBs for ISC proliferation stimulated by DSS, a notion supported by work from Ren and colleagues.

I have demonstrated a requirement for Yki in both the ISCs and ECs upon bacterial infection (Figures 4.10 and 4.11). Karpowicz et al also observed reduced levels of infection-induced proliferation in the absence of Yki in the ISCs. However, they did not test the requirement of Yki expression in the differentiated ECs. Interestingly, Ren et al observed no effect on regenerative proliferation in response to either bleomycin or bacterial infection when Yki was silenced in either the ISCs/EBs or ECs. They instead

suggest that bleomycin and *P.e*, which are both known to induce damage in the ECs, stimulate ISC proliferation via a Yki-independent process. The increased nuclear Yki expression observed in the ECs upon either infection or bleomycin treatment (Figure 4.8 and (Staley and Irvine, 2010)), coupled with the non cell-autonomous effects of Yki activation in the ECs, would argue against this suggestion. I have since obtained the Yki RNAi fly line used in the Ren study and intriguingly also find it to have no effect on regenerative proliferation in response to bacterial infection.

6.1.1.2 *Regulation of Yki activity in the midgut*

In Chapter 5, I began to address several of the possible upstream mechanisms of Yki regulation. Hpo pathway activity in the midgut is most likely regulated by a range of inputs. A branch of upstream Hpo pathway regulation, which I have not investigated, is Ft/Ds signalling. Karpowicz and colleagues have shown that knockdown of Hpo, Wts, Mer, Ex or Ft can increase the frequency of midgut ISC division (Karpowicz et al., 2010). Ds is believed to act as a ligand to Ft (Willecke et al., 2008, Matakatsu and Blair, 2006, Rogulja et al., 2008). In the Karpowicz study, ISCs/EBs were shown to express Ft, whilst their differentiated progeny exhibited higher levels of Ds, suggesting that ISCs/EBs might signal via the Ft/Ds cadherins in order to regulate tissue expansion. Damage caused by intestinal stress most likely affects cell-cell junctions and polarity. Given Ft is an adhesion molecule one can speculate that a loss of polarity or cell-cell contact might lead to disruption of the Ft-Ds interaction, which would result in reduced Hpo pathway activation and thus proliferation.

Interestingly, both my work and that of the Irvine laboratory shows that increased JNK signalling can drive Yki activation, suggesting a link between the two pathways in mediating the proliferative stress response (Figures 5.8-5.9 and (Staley and Irvine, 2010)). JNK activation induces nuclear Yki expression. However, unlike JNK activation, Yki expression does not induce a visible increase in apoptosis or caspase cleavage (Staley and Irvine, 2010). JNK mediates the response to tissue damage in a diverse range of contexts, such as ageing, toxins and infection. The fact that Yki is activated in response to JNK in the midgut raises the possibility that Hpo signalling

might play a general role in the regenerative response to tissue damage. Thus, it is important that the link between JNK and Hpo signalling in regeneration is studied further (see section 6.2.2).

Another outstanding question is whether Yki activity is regulated by phosphorylation or whether increased activity upon midgut stress merely reflects increased Yki protein levels (Figure 4.8). Upon bacterial infection, Yki transcript levels are not greatly affected, suggesting that at least part of the Yki accumulation occurs due to a post-transcriptional mechanism (Figure 4.9). Typically, Hpo signalling regulates Yki activation via phosphorylation, resulting in cytoplasmic retention. In mammals, YAP stability is regulated by coordinated phosphorylation of a phospho-degron by Lats and CK1 through SCF$^{\beta\text{-TRCP}}$. I found that silencing the *Drosophila* homologue of SCF$^{\beta\text{-TRCP}}$, *slimb1* had no obvious effect on Yki levels or proliferation (data not shown). The YAP phospho-degron does not appear to be conserved in *Drosophila*, suggesting that other E3 ligases could be acting on Yki in the gut (T.Maile, personal communication) (Liu et al., 2010a). An *in vivo* or cell-based screen for ubiquitin ligases or deubiquitinating enzymes involved in Yki degradation may provide interesting insights into Yki regulation in the midgut.

6.1.1.3 Signalling downstream of Yki

Loss of Hpo activity results in elevated Jak/Stat signalling levels, suggesting a link between these two pathways in mediating the proliferative response to stress (Staley and Irvine, 2010, Shaw et al., 2010, Karpowicz et al., 2010, Ren et al., 2010). Yki induced proliferation is blocked in the absence of Jak/Stat signalling activity in the ISCs (Figure 4.5 and (Karpowicz et al., 2010)). Hpo signalling is reported to have both autonomous and non-autonomous effects on growth (Zhang et al., 2009a) and in Figures 4.1 to 4.3, I demonstrated that Yki activation in the ECs induces proliferation via the secretion of Upd cytokines. This is particularly relevant to tumour formation, where the release of secreted molecules by tumour cells has been shown to induce autocrine tumour growth and to recruit stromal cells, which promote metastasis (Hanahan and Weinberg, 2000, Orimo and Weinberg, 2006, Wu et al., 2010). A microarray study in cultured

mammalian cells has shown that YAP can regulate members of the interleukin family of cytokines, suggesting that a regulatory connection between Hpo and Jak/Stat signalling might be conserved (Hao et al., 2008). Stat regulation in Hpo-driven cancers is yet to be evaluated.

As discussed in Chapter 1, several points of potential crosstalk have been identified between the Hpo pathway and various other signalling networks. Ren et al found that alongside elevation of all three Upd cytokines, Yki overexpression in the ISCs/EBs also caused an increase in levels of all three EGFR ligands. While Yki expression exerted no significant effect on *wg*, *upd3* and *vn* mRNA levels were increased 40 and eight-fold respectively, while inhibition of EGFR signalling activity was found to prevent proliferation upon Yki expression in MARCM clones (Ren et al., 2010). YAP activation in cultured breast epithelial cells drives proliferation non cell-autonomously via EGFR ligand expression, demonstrating a conserved link between Hpo and EGFR (Zhang et al., 2009a).

Yki activation in the ISCs induces expression of common Hpo pathway targets including *ex, cycE, bantam*, and *diap1*. It is unclear whether Yki affects *upd* and *vn* secretion by directly binding to their promoter region. Knockdown of Sd, the best characterised of Yki's TF partners, suppresses Yki induced proliferation, suggesting that Sd plays a role in regulating Yki target gene expression in the midgut (Ren et al., 2010). However, Yki overexpression in the wing pouch, where Sd levels are high, does not lead to *upd* expression (Figure 4.4), arguing against a general role for the Yki/Sd complex in *upd* transcription. Since Yki is a co-activator and has a range of TF binding partners, its target genes are dictated by which of its partners are expressed in a particular tissue. It is therefore possible that Yki has a direct role on Upd/EGFR ligand transcription, but only when the right combination of factors is present. In this respect, Teashirt and Homothorax, which are expressed in the wing hinge where I observed upregulation of *upd-lacZ* in Yki clones, would be interesting candidates to test in the midgut. Alternatively, Yki could regulate *upd* indirectly by promoting the expression of one or more TF(s).

6.1.1.4 Pez function is essential to Hpo pathway activity in the midgut

Recently, a new Hpo signalling component was identified in the *Drosophila* midgut (Poernbacher et al., 2012). Pez is an evolutionarily conserved FERM domain containing protein, which also contains a protein tyrosine phosphatase (PTP) domain (Edwards et al., 2001). Pez function, but not its PTP domain, is essential to Hpo signalling, specifically in the midgut. The identification of a seemingly gut-specific member of the Hpo pathway raises the possibility that other, as yet unidentified, gut-specific Yki regulators, binding partners and targets may exist. Pez binds to Kibra, but not to Ex or Mer, and in doing so restricts Yki activity. Hence, Pez and Kibra appear to act in a complex to regulate Hpo signalling in the midgut. Pez inactivation results in increased proliferation and expression of both Upd and Vn. Kibra inactivation, as shown in Figure 5.1, also results in a proliferative phenotype, as does mutation of Ex or Mer (Karpowicz et al., 2010). Thus, the KEM complex clearly has an effect on Yki activity in the midgut. Whether this is solely through Pez or involves other mechanisms, for example, links with apicobasal polarity proteins, as has been demonstrated in other contexts (see section 1.2.3.4), is still to be clarified.

6.1.2 Role of the Hpo pathway in the mammalian intestine

Recent studies have begun to assess the role of the Hpo pathway in the mammalian intestine. Endogenous YAP expression appears to be restricted to the crypt compartment (Camargo et al., 2007). Intestinal expression of inducible YAP-S127A protein results in the expansion of undifferentiated progenitor cells, a phenotype similar to that observed following YAP activation in the skin (Zhang et al., 2011a, Schlegelmilch et al., 2011). Aberrant Notch signalling was shown to be potentially responsible for YAP-mediated proliferation in the small intestine (Camargo et al., 2007).

In the colon, YAP is detected in the nuclei of cells but does not appear to be driving proliferation, since YAP elimination does not affect the abundance of dividing cells (Zhou et al., 2011). Similarly, biallelic deletion of YAP causes no obvious intestinal defects under normal homeostasis (Cai et al., 2010). Thus, in contrast to ES and iPS

cells, where YAP is required for maintaining pluripotency, under normal conditions YAP appears to be largely dispensable in the intestine.

When the Hpo pathway is inhibited, intestinal homeostasis is disrupted. Conditional deletion of Mst1/2 results in a phenotype similar to that observed upon YAP overexpression, with the expansion of progenitor cells and onset of colonic polyps (Zhou et al., 2011). Mst1/2 LOF also coincides with a loss of secretory cell lineages, a phenotype observed upon Notch activation. This differs from the Hpo pathway inactivation phenotype in the fly midgut where cell fate and terminal differentiation do not appear to be altered. Deletion of Sav1 in the intestine results in a similar but milder phenotype when compared to Mst1/2 LOF (Cai et al., 2010). In both cases, Hpo pathway inactivation leads to a reduction in YAP phosphorylation and increase in YAP nuclear localisation (Zhou et al., 2011, Cai et al., 2010). Genetic ablation of one YAP allele is sufficient to suppress the excess proliferation observed in both Mst1/2 and Sav1 knockout mice. Given the large amounts of constitutively active 36-KDa Mst1 polypeptide observed in WT crypts, it would appear that under baseline conditions canonical Hpo pathway components actively restrict Yap nuclear localisation and transcriptional activity to a level, which is insufficient to promote proliferation. This regulation appears to be similar to the situation in the fly midgut where Wts activation is required to restrict Yki activity and proliferation during baseline homeostasis (Figure 3.5C).

The aberrant proliferation induced by YAP in ISCs is, to some extent, dependent on activation of the Wnt and Notch signalling pathways. In Mst1/2 null mice, β-catenin levels are largely unchanged but its transcriptional activity is enhanced, leading to the induction of Wnt target genes (Zhou et al., 2011). Likewise, depletion of YAP in SW480 colon adenocarcinoma cells causes an 80% reduction in β-catenin transcriptional activity (Zhou et al., 2011) and endogenous YAP co-precipitates with β-catenin in CRC cells (Imajo et al., 2012). It is unclear whether this relationship between β-catenin and Hpo signalling is conserved in flies. A greater understanding of Hpo pathway interaction with other signalling pathways, such as Notch, Wnt and Jak/Stat, in

the intestine should hopefully shed light on possible therapeutic strategies for the treatment of intestinal diseases.

Although seemingly dispensable for normal homeostasis, YAP protein levels are dramatically increased in the crypts of mice treated with DSS (Cai et al., 2010). Similar to the requirement of Yki in flies treated with DSS, YAP deletion leads to a significant increase in mortality and reduced body weight in mice subjected to DSS treatment. The possibility that Yki/YAP might only be required to drive growth under regenerative conditions or upon loss of a tumour suppressor suggests that YAP may prove to be an interesting therapeutic target. This is similar to the case of Focal Adhesion Kinase, which is reported to be required for intestinal regeneration and tumour growth, but not homeostasis (Ashton et al., 2010). The fact that Yki/YAP is dispensable for homeostatic ISC proliferation does not mean it has no role in this process. It is plausible that maintenance of baseline homeostasis is so crucial to survival that it is placed under the control of several redundant signalling pathways (e.g. Yki, EGFR, Wg, IIS, Jak/Stat). In agreement with this notion, combined inactivation of Wg, Jak/Stat and EGFR leads to rapid stem cell attrition, while separate inactivation does not (Xu et al., 2011).

6.1.3 Clinical implications of Hpo pathway activity in the intestine

Colorectal cancer is commonly associated with persistent inflammation and a continual regenerative response. In the mammalian intestine, YAP appears to be present in a poised state. Loss of cell-cell contact/intestinal damage results in a lack of Hpo pathway input, enabling increased YAP abundance and nuclear activity. YAP, in turn, cooperates with β-catenin in order to activate genes involved in SC expansion and intestinal repair. The activation of YAP in regulating ISC regeneration indicates that Hpo deficiency may contribute to tumourigenesis in the intestine.

Deletion of Mst1/2 results in the formation of colonic adenomas within 3 months (Zhou et al., 2011). Similarly, when exposed to DSS induced injury, Sav1 mutant mice exhibit a YAP-dependent enhancement of tumourigenesis (Cai et al., 2010). High levels of YAP expression are also present in human colonic cancers and cancer derived cell lines

(Steinhardt et al., 2008, Zhou et al., 2011, Konsavage et al., 2012). A two-fold increase in YAP mRNA levels has been observed in human CRC, although YAP phosphorylation levels are yet to be evaluated (Zhou et al., 2011). Diminished YAP phosphorylation in hepatocellular carcinoma suggests a loss of inhibitory input from the core Hpo kinase cascade (Camargo et al., 2007). Interestingly, whereas in previous studies the Hpo pathway has been shown to restrict YAP nuclear localisation when cells are contact inhibited (Zhao et al., 2007), studies in plated colon cancer cells show YAP nuclear localisation, even at high cell density (Konsavage et al., 2012). Resistance to contact inhibition of growth is a known feature of cancer cells. It is therefore possible that nuclear accumulation of YAP might be a general mechanism enabling this cancer cell characteristic.

Identifying the cell of origin in carcinomas resulting from mutations in upstream members of the Hpo pathway is another important area of future research. For example, in some cases, NF2 mutation has been shown to result in hepatocellular carcinoma, originating from hepatocytes (Zhang et al., 2010). In other cases, NF2 mutation is reported to result in mice, which simultaneously exhibit a mixture of both hepatocellular carcinoma and cholangiocarcinoma, originating from the oval (precursor) cells (Benhamouche et al., 2010). Thus, a better understanding of the physiological role of Hpo pathway activity in progenitors versus their progeny will have important conceptual implications for our understanding of Hpo-induced tumourigenesis. YAP overexpression across a number of human cancer types is observed at a higher frequency than can be explained by amplification of the *YAP* locus or LOF mutations in Sav1 and NF2, which appear relatively uncommon in comparison. The mechanisms leading to YAP activation/overexpression across the tumour spectrum is another area for future inquiry.

The most direct therapeutic strategy for YAP depletion would be the use of anti-sense RNA or RNAi. Given the apparent dispensability of YAP function in the healthy adult colon, YAP inactivation might not cause major toxicity in healthy cells. The complexity and range of upstream inputs acting on the Hpo pathway core kinase cascade, along with the range of determinants affecting YAP nuclear localisation, makes the

manipulation of upstream members a less attractive therapeutic approach. Indeed, Pan and colleagues have recently reported a screen for small molecules inhibitors of the YAP/TEAD interaction. Using this approach, they identify verteporfin as a YAP/TEAD interaction inhibitor, and show that this compound can revert the hepatomegaly induced by loss of *NF2/Merlin* in mice (Liu-Chittenden et al., 2012). Given the range of YAP-interacting TFs, this approach may need to be tailored to block different YAP/TF interactions, since the identity of YAP binding partners could differ from one cancer to the next.

6.2 The Hpo pathway as a regulator of regeneration

My study is amongst the first suggesting a non-autonomous role for the Hpo pathway in regeneration, rather than a direct cell-autonomous effect on proliferation. It will be interesting to see how the Hpo pathway is regulated in the midgut and if this non-autonomous, regenerative function is conserved in other contexts and organisms.

6.2.1 Yki and regeneration

Alongside the *Drosophila* midgut, a role for the Hpo pathway has also been demonstrated in both Cricket leg and wing imaginal disc regeneration (Bando et al., 2009, Grusche et al., 2011, Sun and Irvine, 2011).

Compensatory cell proliferation, the ability of dying cells to stimulate the proliferation of their neighbours, was first characterised in wing imaginal discs. Two studies have demonstrated that Yki is activated in wing discs in response to tissue damage (Sun and Irvine, 2011, Grusche et al., 2011). The regenerative response to tissue damage (whether it be induced surgically, genetically or by irradiation) is substantially impaired in the absence of just one copy of Yki. Mechanisms shown to contribute to Yki regulation during normal development, such as Ft-Ds signalling, were found to affect Yki activity during regenerative growth but are seemingly not sufficient to account for Yki hyperactivation, since regeneration still occurs in the absence of Dachs (Grusche et al., 2011). The most likely scenario is that multiple upstream inputs are responsible for

sensing tissue damage and, in turn, activating Yki.

6.2.2 JNK signalling and the regulation of Yki activity

Wing imaginal disc compensatory proliferation and regeneration is known to involve JNK signalling activity (Bergantinos et al., 2010, Ryoo et al., 2004). While JNK signalling is not necessary for Yki activation during normal development (Igaki, 2009), Sun and colleagues demonstrated a requirement in wing disc regeneration (Sun and Irvine, 2011), suggesting that JNK activity provides a context-specific input on the Hpo pathway. Similar to my observations in the gut (see section 5.6.2), it is unclear whether JNK influences Yki activity directly in this context. JNK might regulate Yki via a single mechanism or might alternatively affect several upstream Hpo pathway regulators. In mammals, JNK has been shown to directly phosphorylate YAP in order to regulate apoptosis (Tomlinson et al., 2010). A more in depth biochemical understanding of how JNK regulates Yki in other contexts is clearly required.

The role of JNK signalling is clearly complex, since JNK is known to be pro-apoptotic in some contexts (Kanda and Miura, 2004). This complexity is demonstrated by the JNK-Yki interplay in different classes of tumour. Yki is activated in tumours associated with *lgl* mutation (Grzeschik et al., 2010, Ling et al., 2010, Menendez et al., 2010). In wing imaginal discs, this activation requires JNK (Sun and Irvine, 2011). However, in tumours associated with *scrib* mutation, JNK is pro-apoptotic and exhibits anti-proliferative effects. In this context, it is in fact downregulation of JNK activity, which results in Yki activation, suggesting that the JNK pathway can repress Yki activity in certain contexts (Chen et al., 2012).

6.3 Progress in the *Drosophila* midgut field

Since the initial discovery of a population of adult SCs in the fly midgut in 2006, work in the field has focussed mainly on identifying the signalling pathways regulating ISC activity. Understanding the processes governing ISC lineages is vital to the study of SC dynamics and there still remain fundamental questions pertaining to how the different

signalling pathways regulate these processes. Studying the emergence of ISCs during development, which would be easier in flies than mammals, might offer clues as to how they are programmed and thus regulated. In particular, finding the origins of the adult ISCs and their larval precursors (the AMPs), when these cells are specified (embryo or larvae) and what elements in the environment (ECM, other cells, secreted factors) they interact with could provide insight into the mechanisms regulating SC behaviour in the adult. Given the role of the Hpo pathway in gut regeneration, one might imagine that Hpo pathway function could also contribute to adult gut development during the pupal stages, but this remains to be investigated.

Numerous markers are now available for studying intestinal homeostasis and these can be used in combination with ever improving lineage-tracing techniques. Most recently, Twin-spot MARCM, a new lineage tracing method based on mitotic segregation, was used to highlight the presence of both symmetric and asymmetric divisions in the midgut (Yu et al., 2009, de Navascues et al., 2012, O'Brien et al., 2011). A similar approach has been utilised in the mouse intestine and yielded complementary results (Snippert et al., 2010). It is unclear at this point how the ratio of symmetric to asymmetric divisions is regulated in different contexts and whether, as would appear to be the case in the mammalian intestine, some ISCs are mainly homeostatic, whilst others are predominantly active during adaptive growth.

Alongside developing our understanding of SC homeostasis, studies in the midgut have provided insight into processes such as ageing and regeneration. Often, as is the case with the Hpo pathway, whilst researchers have been able to identify the signalling pathways involved in these processes, it has remained difficult to elucidate how they are activated and in what order. The development of midgut *ex vivo* culture and live imaging will hopefully enable some of these questions to be answered.

6.4 Concluding remarks - The Hpo pathway as a general sensor of epithelial health

The growing evidence for Hpo pathway activity in regeneration, coupled with numerous studies demonstrating interplay between the Hpo pathway and polarity/cell-cell contact, has led to the concept that the Hpo pathway might function as a general sensor of epithelial integrity and health. The emerging picture of a pathway regulated by numerous inputs including polarity, mechanical forces and stress signals, raises the possibility that, by integrating these signals, the Hpo pathway regulates not only growth during development but also tissue homeostasis through its function in regeneration. In the future, it will be interesting to assess whether Hpo pathway activity can regulate regeneration in organisms exhibiting more extensive regenerative capacities, such as urodeles, hydra or planaria.

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