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Intestine Homeostasis and the Role of Tumor Suppressor Gene 101 in Drosophila Melanogaster: A Dissertation

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INTESTINE HOMEOSTASIS AND THE ROLE OF TUMOR SUPPRESSOR GENE 101 IN DROSOPHILA MELANOGASTER

A Dissertation Presented

By

MADHURIMA CHATTERJEE

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Interdisciplinary Graduate Program

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ABSTRACT

Tissue homeostasis in the adult *Drosophila melanogaster* intestine is maintained by controlling the proper balance of stem cell self-renewal and differentiation. In the adult fly midgut, intestinal stem cells (ISCs) are the only dividing cells and their identity maintenance is crucial to the proper functioning of the fly gut. Various pathways such as Notch, JAK-STAT and Wingless are known to regulate ISC division and differentiation.

Here I used a pathogen feeding model to study conditions that accelerate ISC division and guide intestinal cell differentiation favoring enterocyte development. I also examined the role of Tumor Suppressor Gene 101 (TSG101) in ISC maintenance and function. TSG101, a part of the ESCRT1 complex. It is known to stimulate the Notch pathway and to play a role in endocytIc trafficking. TSG101 loss-of-function mutants show developmental defects in various fly and mammalian tissues. The protein also plays a role in virus abscission from host cells. In my experiments I have observed that TSG101 is required for ISC maintenance. TSG101 knockdown and loss of function mutant clones have defects in ISC proliferation that hinder the normal intestinal responses to oral pathogen ingestion.

Based on these results I conclude that TSG101 is needed in the adult fly intestine for proper ISC maintenance and function, thereby being an important player in intestinal homeostasis.

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LIST OF ABBREVIATIONS

Bmi1	Bmi Polycomb Ring Finger Oncogene
BMP	Bone morphogenetic Protein
CD8	Cluster of Differentiation 8
CFU	Colony Forming Unit
DAPI	4',6-DIamIdino-2-Phenylindole
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulphate Sodium
E(spl)	Enhancer of Split
EB	Enteroblast
EC	Enterocyte
EE	Enteroendocrine cell
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ept2	*Erupted2*
ESCRT	Endosomal Sorting Complex Required For Transport
Esg	Escargot
FLP	Flippase
FRT	Flippase Recognition Target

GFP	Green Fluorescent Protein
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
HS	Heat Shock
Ig	Immunoglobulin
ISC	Intestinal Stem Cells
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5
P.e.	*Pseudomonas entomophilla*
PQ	Paraquat
Pros	Prospero
RNA	Ribonucleic Acid
RNAi	RNA Interference
S.m.	*Serratia marcescens*
STAT	Signal Transducers and Activators of Transcription protein
Su(H)	Suppressor of Hairless
TS	Temperature Shift
TSG101	Tumor Suppressor Gene 101
^{ts}	Temperature Sensitive
UAS	Upstream Activating Sequence
Upd	Unpaired

UEV	Ubiquitin E2 Variant
UTR	Untranslated Region
VPS	Vacuolar Sorting Protein
Wg	Wingless

CHAPTER 1

INTRODUCTION

Intestinal mucosa in all organisms faces multiple challenges. It has to sustain the organism by absorbing nutrients from food while being continuously exposed to pathogenic agents. The intestinal epithelium like any other living tissue needs constant renewal with cell turnover to maintain its functionality and structural integrity. This process is known as tissue homeostasis. Tissue homeostasis is a highly regulated process. Wnt, BMP and Notch signaling pathways have been Implicated in mammalian intestinal cell proliferation (Fodde 2007; Nakamura 2007). The human GI tract is a relatively under-explored organ due to its complexity which makes experimental manipulation difficult. *Drosophila* has emerged as a powerful tool for analyzing the function of human disease genes, either as fly homologues or by expressing in transgenic flies the mutated forms of human genes. Here, I provide some background on the fly intestine as a model for tissue development and infectious diseases and summarize some of the regulatory pathways in *Drosophila* homeostasis.

Drosophila Intestine and Intestinal Stem Cells

The gastrointestinal (GI) tract in all animals is a major immune and endocrine organ. In addition to absorbing nutrients, the GI tract also serves as a major site of interaction between the host and environmental pathogens. The intestine houses billions of bacteria most of which are harmless while some are useful and yet others are harmful to the host organism (Backhed 2005, Radtke 2005). Food and water borne pathogens and toxins cause diarrhea and inflammatory bowel disease which kill millions of people world-wide each year (Brito 2005). Around 1% of the US population suffers from inflammatory bowel disease (Macdonald 2005). Understanding how the intestine responds to pathogens is therefore a major scientific question which will be useful in providing new ways to treat and manage intestinal diseases.

The intestine is a living, developing organ with different cell types. The Intestinal Stem cells have the ability to divide and differentiate into the different cell types. Mammalian intestinal stem cells (ISCs) are located in bases of crypts but at least two groups of cells have been cited as stem cells. Additionally, precursor cells in the transit amplifying zone can also proliferate (Fig 1.1C). The involvement of multiple cell types makes it difficult to examine tissue damage responses in mammalian intestines.

Flies on the other hand have a simpler intestinal structure. It is composed of a layer of longitudinal and circular smooth muscles that execute peristalsis, and an inner layer of specialized epithelial cells that constitutes the intestinal epithelium. The outer and inner layers are connected by a basement membrane. The intestine is comprised of three distinct anatomical regions: foregut, midgut, and hindgut. The foregut and hindgut are ectodermally derived, while the midgut is of endodermal origin. The epithelium is 1-2 cells thick and the ISCs are the only cell type that has been shown to proliferate (Fig 1.1). Various markers for different cell types in the fly intestine exist, making it easier to manipulate and analyze (Micchelli 2006, Ohlstein 2006, Dionne 2008).

The ISCs in the fly intestine undergo mitosis giving rise to an ISC and an enteroblast. They were shown to reside in within ”cell nests” which are small groups of small cells in the intestine that includes one type of cell that made extensive contact with the intestinal basement membrane (the ISC which is the mother cell), and another that does not (the daughter cell). The cell nest therefore comprises of precursor daughter cells that are still in close contact with the parent ISC. Enteroblasts are non-dividing precursor cells. Ninety percent of enteroblasts differentiate into mature enterocytes and ten percent into enteroendocrine cells (Ohlstein 2007). Enteroblasts that differentiate into enterocytes undergo endoreplication to become polyploid. Escargot is a marker

of ISCs and enteroblasts (EBs). Delta is a known ISC specific marker while Prospero marks enteroendocrine cells (EE). In many of the following experiments I used an escargot promoter-Gal4/UAS-CD8GFP line that marks ISCs and EB's with GFP (Fig 1.4).

Stem cell-mediated tissue repair is a promising approach for many diseases. Mammalian intestine is an actively regenerating tissue such that epithelial cells are constantly shedding and underlying precursor cells are constantly replenishing the loss of cells. An imbalance of these processes can lead to intestinal diseases including inflammation and cancer. It has been shown that ISC division is accelerated when the gut epithelium is disrupted by DSS feeding, while ISC differentiation is affected by enterocyte damage/loss by bleomycin feeding, oxidative stress and aging. Enteroblasts also nonautonomously regulate ISC proliferation in response to nutrition insulin signaling and several other factors. Thus, both enterocytes and enteroblasts contribute to the maintenance of tissue homeostasis in the Drosophila midgut. (Amcheslavsky 2009, Choi 2008).

ISC division and daughter cell differentiation is a highly regulated process. We use various cellular markers and different feeding conditions to study the different cell types in the intestine and their response to pathogens. Our studies here

show that ISCs division and differentiation into enterocytes is accelerated by feeding bacterial and chemical pathogens. This should provide further insight on the uses of the fly gut as a model for food and water borne diseases. In further sections, I describe the mechanisms and pathways regulating ISC fate and function.

Fig 1.1 A

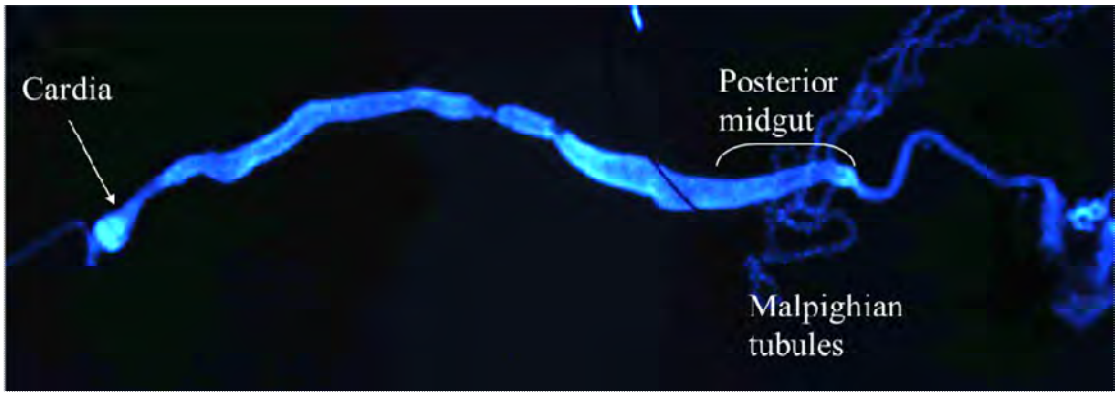


Fig 1.1 B

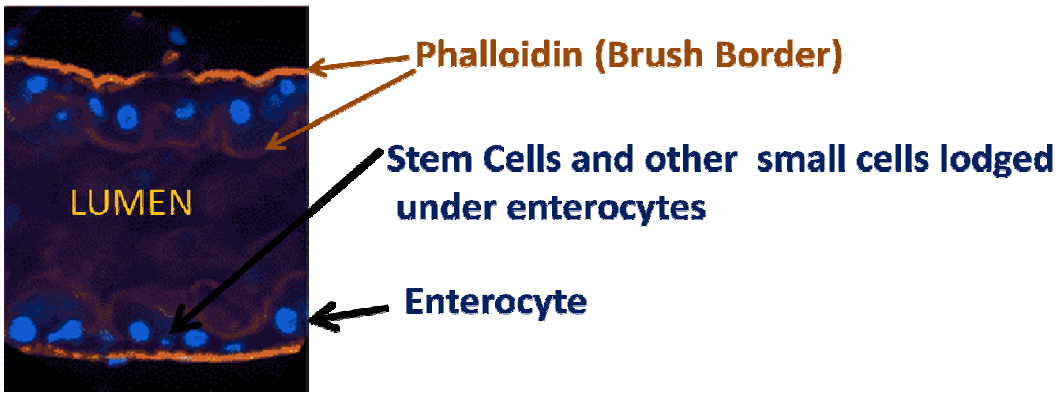
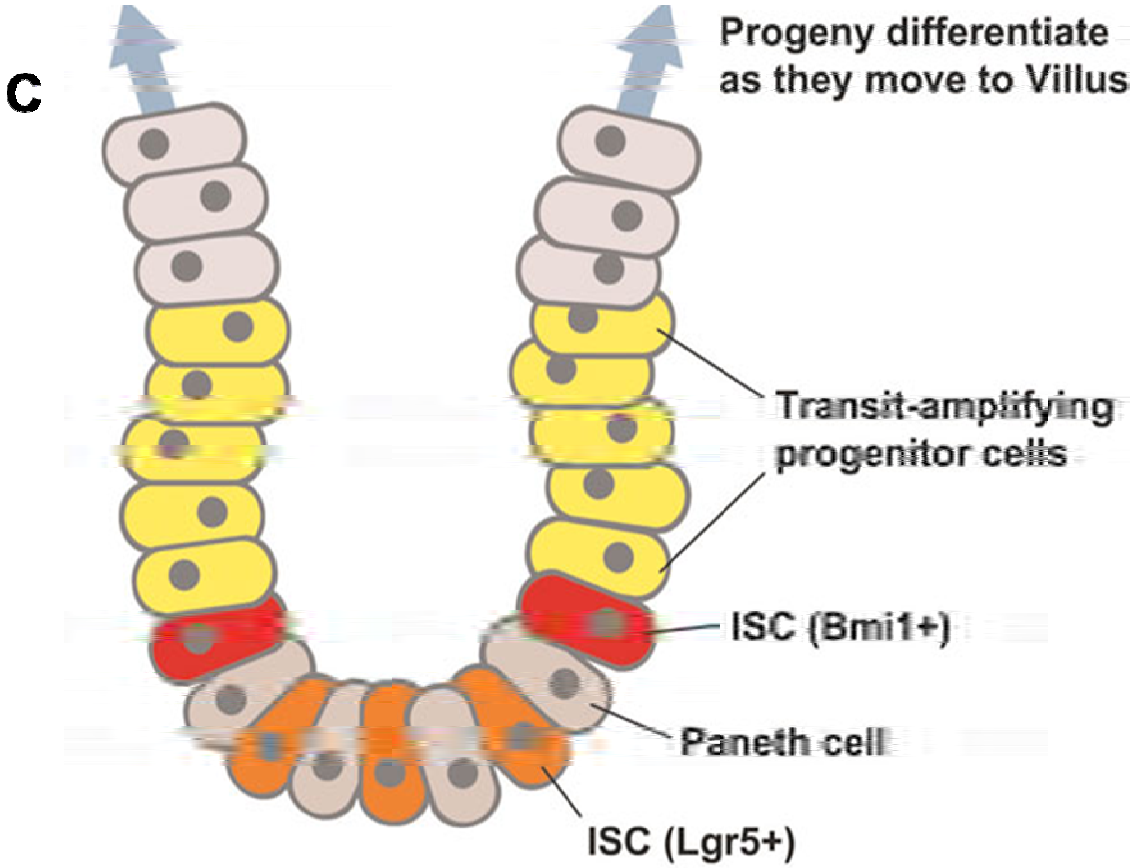


Fig 1.1 A-B The *Drosophila* intestine.

A: Whole mount of adult fly gut with the different regions labeled. The cardia is located anteriorly and is analogous to the mammalian stomach. It is followed by the absorptive midgut, waste removing malphigian tubules and the hindgut. Our experiments deal with the posterior midgut region.

B: Sagittal view of the posterior midgut region with DAPI (blue) marking the nuclei and Phalloidin (orange)staining marking the luminal brush border send the outer layer of the midgut. Large DAPI stained nuclei belong to Enterocytes which also possess the brush border. Small cells (ISC's and early EB's) are located more basally compared to the EC's and are not close to the lumen. These small cells located basally form the cell nest.

Fig 1.1 C-D


C
Progeny differentiate
as they move to Villus
Transit-amplifying
progenitor cells
ISC (Bmi1+)
Paneth cell
ISC (Lgr5+)

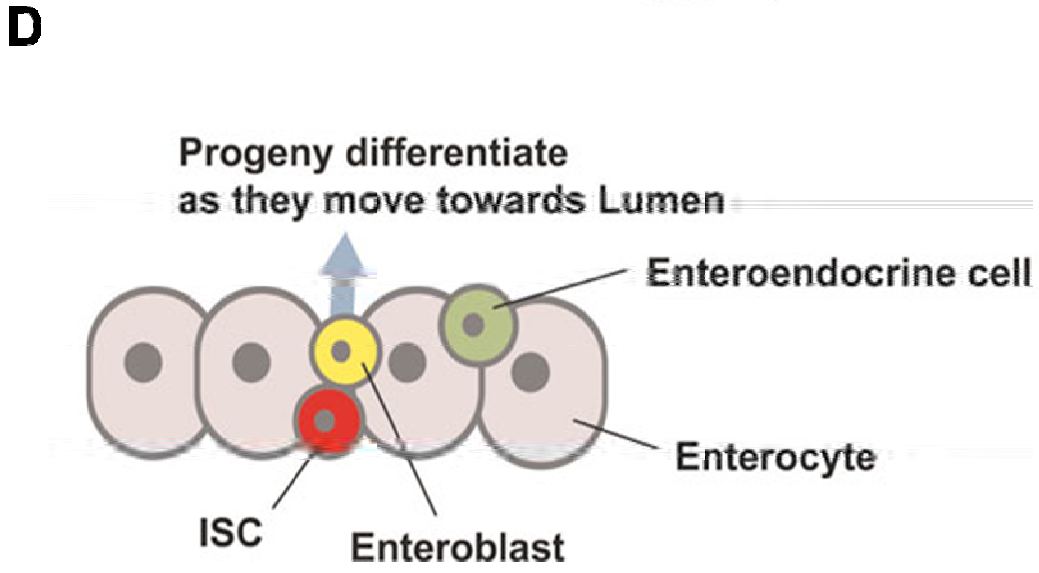

D
Progeny differentiate
as they move towards Lumen
Enteroendocrine cell
Enterocyte
ISC
Enteroblast

Fig 1.1 C-D Schematic representation of different cell types in the mammalian and fly midguts.

In these cross-sectional schematic representations of the intestinal epithelia, the ISCs are located basally, adjacent to the surrounding muscular layer, and the lumen is located at the top. In the mammalian crypt (C), there are two ISC populations at its base; the Bmi1 (red) and Lgr5 (orange) populations. After ISC division, one daughter differentiates into a transit-amplifying progenitor (yellow), which divides and moves upward to the villus. As differentiation progresses the daughter cells leave the crypt to reside in the villi.

D: *Drosophila* ISCs are also located basally (red). ISCs divide and one daughter undergoes differentiation into an enteroblast (yellow). Unlike mammalian ISCs, transit-amplifying progenitors are not produced and the enteroblast differentiates directly into an enteroendocrine cell (green) or an enterocyte (beige) and moves to the lumen.

Figure adapted from Karpowicz 2010

Fig 1.2

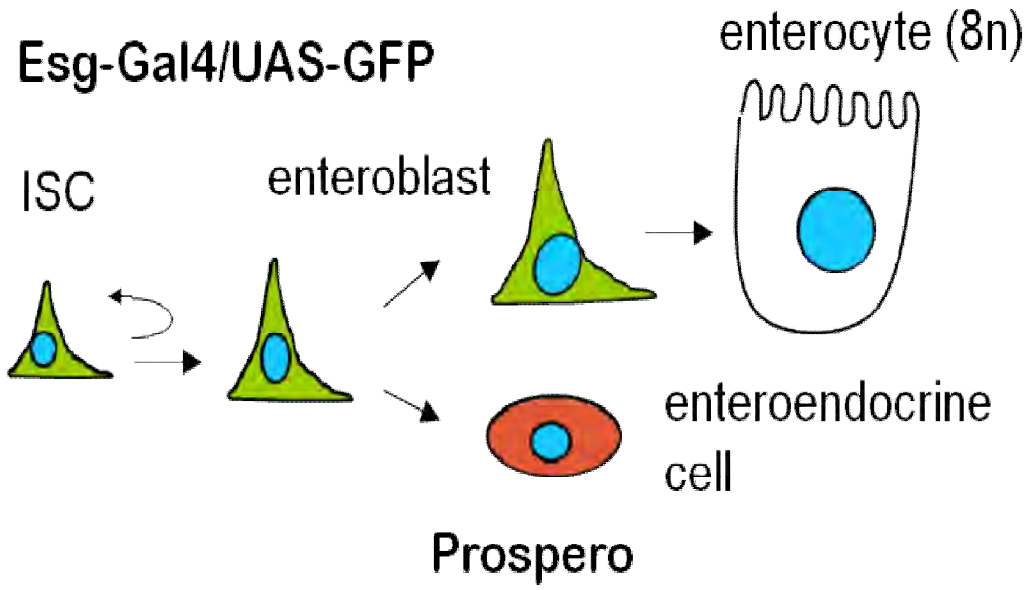


Fig 1.2 Schematic representation of different cell types in the fly midgut.

In the escargot Gal4 UAS CD8 GFP line used for many of the experiments in this thesis, ISCs and EBs which express esg are GFP positive. EE cells are stained by Prospero. ECs can be distinguished by their larger polyploid nuclei.

Drosophila Intestinal Pathogens

The fly intestine has been used as a model for food and water borne diseases. The feeding experiments involved *P. entomophilla* and *S. marcescens* as pathogenic bacteria, as well as paraquat and H_2O_2 as stress-inducing agents. In this thesis I refer to all four agents as “pathogens” since they elicit similar responses in the posterior midgut.

S. marcescens is a Gram negative bacterium which has been isolated from 30 different insect species (Grimont 1978). It is a member of the family Enterobactericeae and is known to cause outbreaks in hospitals which may lead to major clinical infections. Severe illness due to Serratia marcescens is generally seen in immunocompromised patients (Villari 2001, Bollman 1989). The ampicillin and streptomycin-resistant mutant *S. marcescens* Db11 has previously been identified as an oral *Drosophila* pathogen. When introduced into the fly hemocoel via septic injury, *S. marcescens* kills the adult fly in a day. On the other hand, the lethality is gradual in an intestinal infection model, even though the bacterium is present in the hemolymph (Nehme 2007). We therefore used this bacterium to study the fly intestine as a model for response to mammalian pathogens.

Pseudomonas entomophila on the other hand, is an insect pathogen. It is the first known *Pseudomonas* strain to be pathogenic in *Drosophila melanogaster.* It is a Gram-negative bacterium, found in soil, aquatic, or rhizosphere environments. It was first isolated from *Drosophila melanogaster* (Vodovar 2006). Once ingested, it causes lethality in *Drosophila* larvae and adults. *Pseudomonas entomophila'*s genome encodes insecticidal toxins, a diffusible hemolytic activity, lipases, extracellular proteases, and potential adhesions which cluster with type I or II secretion system proteins. *P. entomophila* is harmless to plant life, which makes it useful as an insecticide (Vodovar 2006). *P.e*.'s lethality has been shown in previous studies and in our own results to be less in adult flies than in fly larvae (Buchon 2009). This allows us to use the *P.e.* feeding model to study how the fly intestine responds to an insect specific pathogen.

The production of microbicidal reactive oxygen species (ROS) is a key feature of the host defense response in mucosal epithelia (Foley 2003). However this system has to be carefully regulated to protect the intestine from oxidative stress. Stem cells maintain low levels of reactive oxygen species (ROS) in order to protect themselves from oxidative damage. Persistently high ROS can contribute to ectopic stem cell divisions and misdifferentiation of progenitors, disrupting midgut homeostasis, a phenotype common in aging midguts (Kobayashi 2011). I therefore used 2 chemicals, paraquat and H_2O_2 as stress-inducing agents. Their

ability to induce oxidative stress has been previously described (Liehl 2006; Nehme 2007). The overall aim of pathogen feeding is to further develop the fly gut a model for studying food and water borne diseases and to learn more about the responses and regulatory pathways of the fly intestine as a whole.

Delta Notch Signaling

The ISC niche is known to be a key regulatory factor in their maintenance and functional regulation. The niche is defined as the location (within a tissue) where stem cells reside - because it contains the correct concentrations of cell signaling ligands that stem cells need for their functioning (Bardin 2010). Therefore, investigating the regulatory Interactions between stem cells and their niches is critical for understanding how homeostasis is controlled under normal and challenged conditions. For the ISC, the niche may be maintained by the surrounding cells and basement membrane layer with which the ISC makes extensive contact. The niche plays a key role in maintaining ISC fate and function thereby maintaining intestinal homeostasis.

Insulin signaling has been shown to promote ISC division, at least upon intestinal damage (Amcheslavsky 2009). There are several different Identified pathways and mechanisms required for the development and maintenance of ISCs. The EGF receptor pathway, Wingless pathway, TSC2, Decapentaplegic pathway, and intrinsic chromatin modification by the deubiquitinase Scrawny are required for this process (Amcheslavsky 2011, Lin 2008; Buszczak 2009; Jiang 2009; Lee 2009; Buchon 2010; Mathur 2010; Biteau 2011; Jiang 2011). Many conserved biochemical pathways like JAK, Hippo, and JNK signaling are required for

intestinal cell proliferation during pathogenic stimulation (Maeda 2008, Staley 2010, Shaw 2010). JNK signaling has been shown to upregulate ISC proliferation, causing Delta positive cell numbers to accumulate. This is misdifferentiation is restricted by Delta-Notch signaling that keeps Notch active in the EB's, thus maintaining proper intestinal cellular structure. Old and stressed fly intestines have been shown to lose this balance which results in aberrant ISC morphology and Delta-Notch patterns (Biteau 2008). The ISC niche is therefore maintained by Notch signaling in daughter cells which in turn is activated by Delta expressed by the ISC itself.

Notch signaling is highly conserved through evolution and plays a fundamental role in the determination of cell fate (Artavanis-Tsakonas 1995). It also affects cell cycle progression and apoptosis (Weinmaster 2000). The Delta protein is a Notch ligand and a specific ISC marker. ISCs divide asymmetrically. Delta is expressed In ISCs and activates Notch signaling in EBs (Fig 1.3), thereby making the Notch target gene, the transcription factor Suppressor of Hairless [Su(H)] a marker for enteroblasts. Su(H) turns on the expression of the Enhancer of Split genes in the EB. To maintain ISC fate, Notch activity is inhibited in the ISC by various pathways. For instance, Hairless protein binds to Su(H) and keeps it inactive in the ISCs, thereby rendering it incapable of responding to

Notch signaling (Bardin 2010). This plays a role in inhibiting Notch signaling in the ISCs.

Upon activation, full length Notch protein is cleaved, the activated intracellular domain is endocytosed and processed by presenilin, ubiquitinated, and transported into the nucleus, where it cooperates with a family of transcription factors including Suppressor of Hairless to activate the transcription of downstream target genes, such as the Enhancer of Split complex (Weinmaster 1997).

Fig 1.3

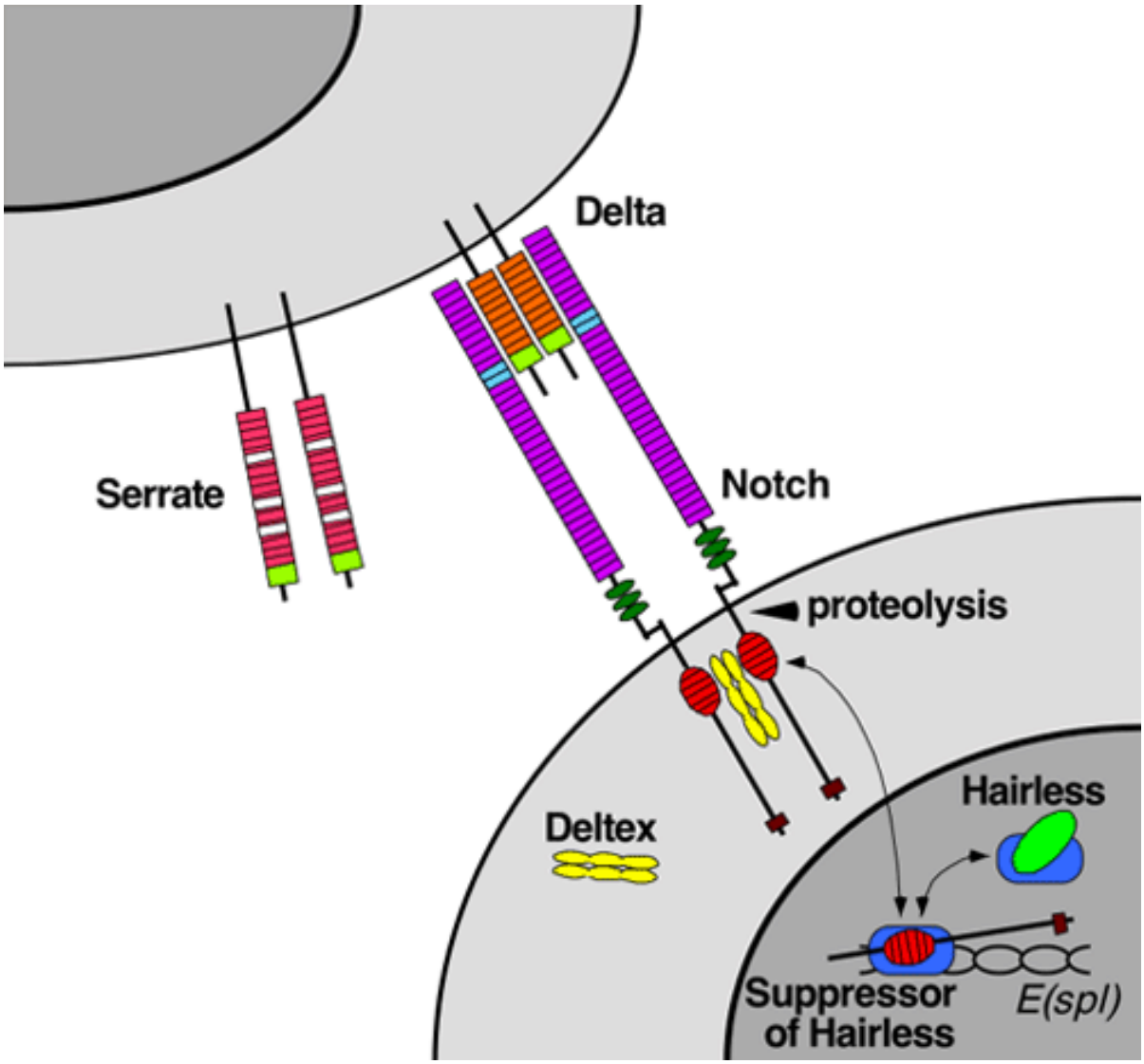

Delta
Serrate
Notch
proteolysis
Deltex
Hairless
Suppressor
of Hairless
E(spl)

Fig 1.3 Schematic representation of the Notch activation pathway and the role of Delta

Delta (orange) is a cell membrane protein and a Notch ligand. Delta binds to the Notch extracellular domain (purple), leading to the cleavage of the Notch receptor. The Notch intracellular domain (red) translocates to the nucleus where it interacts with its target gene Su(H) (in blue), which is then released from its binding with Hairless (green) and can turn on the transcription of the E(spl) genes.

Figure adapted from Artavanis-Tsakonas (1995)

Fig 1.4

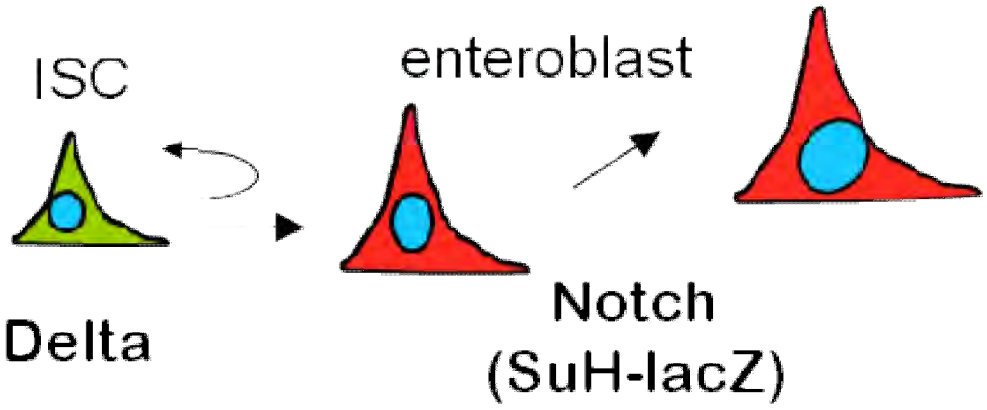


Fig 1.4 Schematic representation of the Delta - Notch segregation during ISC division.

Delta (green) remains in the ISC cytoplasm whereas Notch is detected in the daughter cells.

Fig 1.5

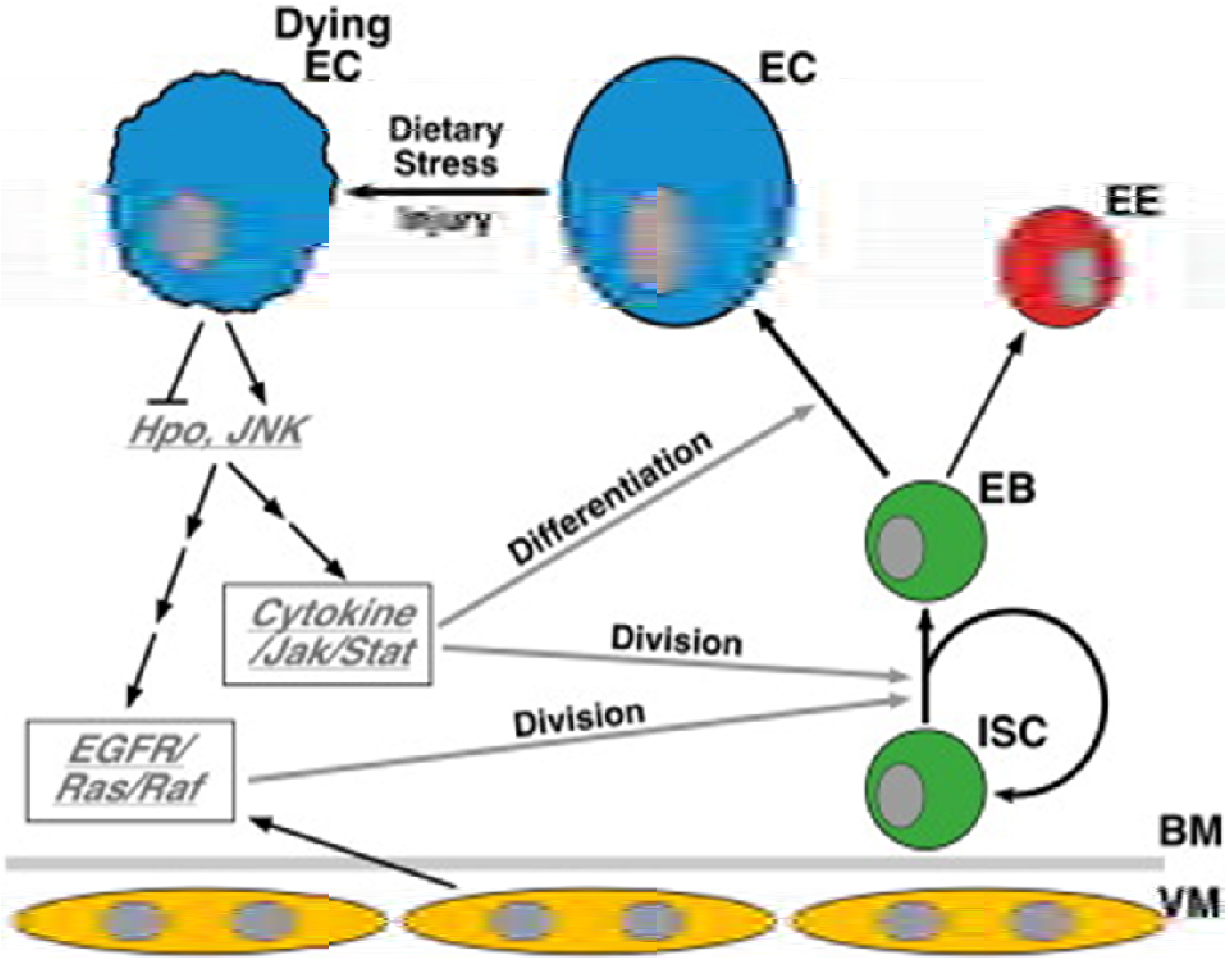

Dying EC
EC
Dietary Stress
EE
Hpo, JNK
Differentiation
EB
Cytokine /Jak/Stat
Division
Division
EGFR/ Ras/Raf
ISC
BM
VM

Fig 1.5 Pathways through which ISC division and differentiation may be accelerated.

EC damage due to oral pathogens can activate the JNK and Hpo pathways. These can activate the expression of secreted mitogen Upd and the EGFR growth factor. This activates the Jak STAT and Ras/Raf pathways in the progenitor cells, thus stimulating ISC division and EB differentiation to replenish the EC population.

Figure from Jiang 2011

TSG101 and ISC maintenance

I studied the role of candidate gene Tumor Suppressor Gene 101 in the fly intestine. An RNAi screen conducted in the lab showed that TSG101 RNAi guts lack cell nests. TSG101 has been previously shown to be required for mouse embryonic development and viability of adult tissues and cells (Ruland 2001, Oh 2007). Its role in the endocytic pathway has been well studied. TSG101 is known in yeast as Vacuolar Sorting Protein (Vps) 23 and is a part of the ESCRT complex, which is responsible for endosomal trafficking (Bishop and Woodman, 2001). Mammalian TSG101 is essential for ESCRT-I function as it binds ubiquitinated receptors and mediates interactions with other ESCRT complexes via its ubiquitin-conjugating enzyme E2 variant domain (Katzmann 2001). Human and *Drosophila* Tsg101 proteins are well conserved and have a shared domain structure so they could be functional analogs and TSG101 knockdown in *Drosophila* shows severe defects in larval development due to the activation of the Notch pathway (Moberg et al 2005). Since the Notch pathway also plays a crucial role in the adult *Drosophila* intestine functioning (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), I was interested in investigating the role of TSG101 in intestinal homeostasis.

The endocytic pathway mediated by the ESCRT complexes is an important regulator of many signal transduction pathways (Gonzalez-Gaitan 2003). Endocytosis involves the turnover of cell-surface proteins (Fig 1.6). This includes ligand-occupied and unoccupied signaling receptors that can continue to signal from within the endosomes (Razi 2008, Vaccari 2008, Seto 2006). Disruption of key ESCRT components can therefore disrupt signaling pathways. These studies can be used to create fly model systems to study cellular trafficking and various tissue developmental defects (Rodahl 2009).

I saw that TSG101 RNAi, when driven in progenitor cells by escargot GAL4, shows defects in adult intestine homeostasis. Flies lack intestinal cell nests, show virtually no cell division in the intestine even after pathogen feeding. I showed that this was due to a severely reduced ISC population in the RNAi fly intestine. TSG101 is known to regulate the Notch pathway as part of its role in endocytosis. The intracellular domain of the Notch receptor in TSG101 mutants was shown to be trapped in endosomes in an active state (Moberg 2005). This interruption of endosomal trafficking maintains the Notch signaling pathway in a constitutively active state. While Notch up regulation can stimulate tissue overgrowth in *Drosophila*, I see that in the adult intestine, it causes ISCs to abandon mitosis, loose Delta staining and the EBs to favor the EC differentiation pathway as opposed to the EE one. ISCs are rendered incapable of responding

to pathogen feeding thus disrupting tissue homeostasis. These findings points to a role of TSG101 in ISC maintenance and intestinal homeostasis.

Fig 1.6

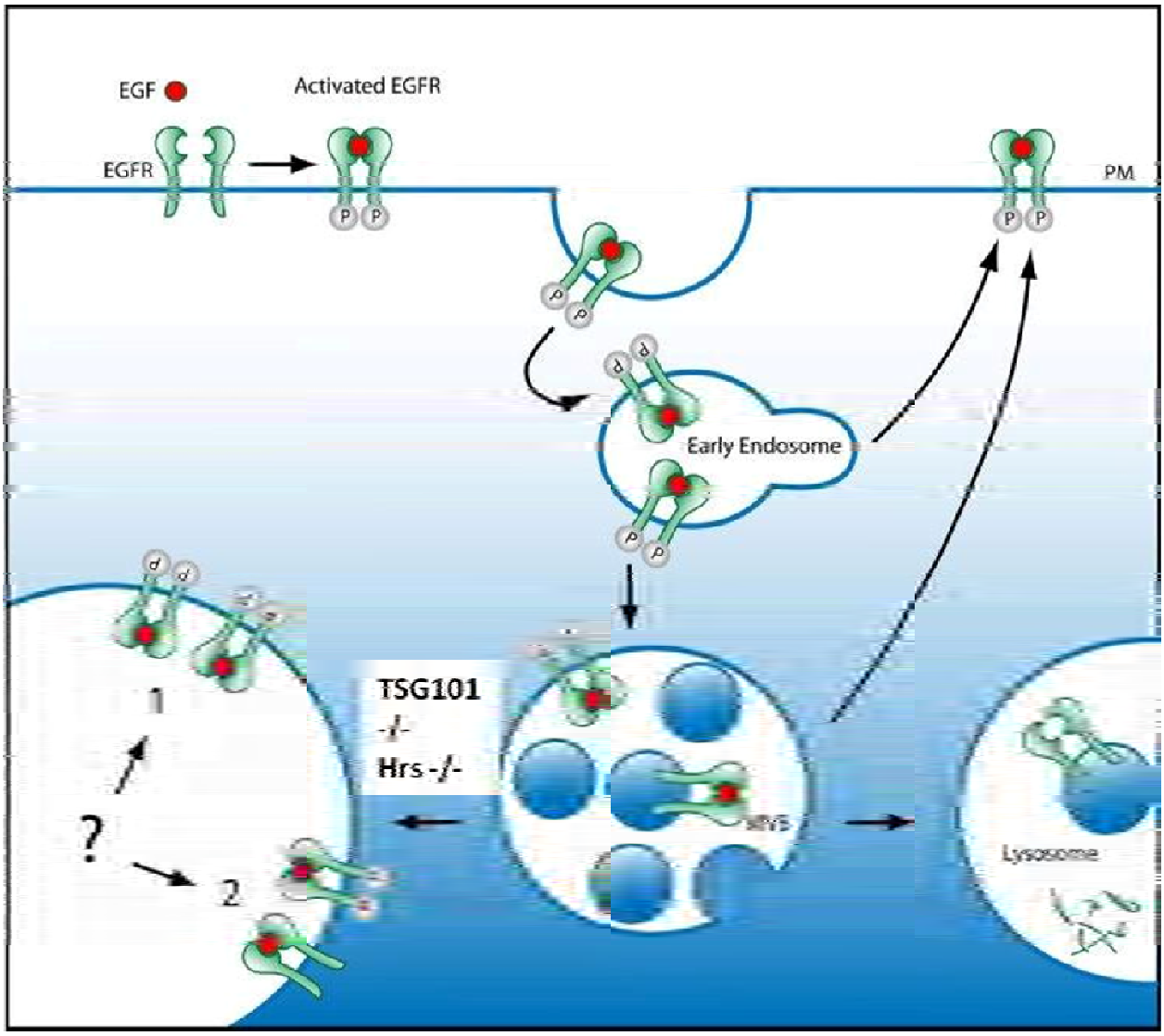

EGF
Activated EGFR
EGFR
PM
Early Endosome
TSG101
-/-
Hrs -/-
1
2
?
Lysosome

Fig 1.6 The endocytic pathway and the role of TSG101

As an example, the endocytic cycling of Epidermal Growth Factor Receptor (EGFR) is shown here. EGFR dimerizes and becomes autophosphorylated starting the signaling cascade. For signal termination, EGFR is ubiquitinated, endocytosed, and targeted to endosomes. From there, it may be recycled back, or, upon interaction with the ESCRT machinery, is further sorted into Multi Vesicular Bodies (MVBs), and finally to lysosomes for degradation. In cells deficient in ESCRT components (Hrs and Tsg101), MVB formation is impaired. This results in inhibition of degradation of EGFR, and other the endocytosed cargo.

Figure adapted from Razi 2008.

The MARCM technique

The ability to create mosaic animals allows the phenotypic analysis of patches of groups of genetically different cells that develop in a wild type environment. It is a valuable tool to study the effects of lethal mutations by controlling the temporal and spatial extent of the expression of mutant genes. The MARCM system is a is a site-directed recombination technology used to manipulate an organism's DNA under controlled conditions in vivo and is widely used to create these mosaics. MARCM stands for “Mosaic Analysis with a Repressible Cell Marker” (Lee 2001). Fig 2.5 shows the chromosomal rearrangements that occur to allow the MARCM labeling to work.

In our variant, the Flippase recombination enzyme (FLP) derived from the 2µm plasmid of the baker's yeast is expressed under the control of the heat shock (hs) promoter on the X chromosome. In one line, the 2nd chromosome contains the Flippase Recognition Target (FRT) sites located upstream of a UASCD8GFP construct driven by Tubulin Gal4 while in another it is located upstream of a tubulin Gal80 construct. When FLP is expressed, the recombination occurs at the FRT sites. Following mitotic recombination, some cells would lose the Tubulin Gal80 and become GFP positive. If that cell happens to be an ISC, all its descendants will also be GFP positive.

I used the MARCM system in two ways. In chapter 2, I used it to study intestinal cell division by marking all the descendants of a few, isolated, GFP expressing parent ISCs (in this case the marked ISC).

Crosses were established at room temperature and cultured at 18 °C, the permissive temperature, until flies reached adulthood. The progeny was kept at 18 °C for 3 day after which they were shifted to 29 °C and dissected at different days (as mentioned in the experiments).

In Chapter 2, I crossed fly stocks to generate offspring with the genotype: hsFLP; FRTG13 UAS-CD8GFP/tubulin Gal4; FRTG13 tubulin-Gal80. These stocks generated small number of GFP positive mitotic clones in the midgut without a heat shock induction of the FLP recombinase. Only flies with all the correct chromosomes exhibited this low level of mitotic recombination and the GFP marked ISC divided and the cell nest gradually grew to include bigger cells as observed in older flies. These are consistent with having successful mitotic recombination, which by chance eliminates the repressor Gal80 in, a mitotic stem cell and allows Gal4 driven GFP expression within that lineage only. I also saw that each cluster only had 1 ISC indicating that that was the original mother cell

of that cluster. After more days however, the clusters grow so large that they are no longer isolated.

In chapter 3, I used MARCM to study the development and morphology of TSG101ept2 expressing ISC's within a wild type adult fly intestine. Since the TSG101ept2 mutant is lethal using the MARCM system I was able to gain some insight in how the TSG101 gene may function in the fly intestine. This system used the mutant located distal to the FRT80B site on the 3rd chromosome. The chromosome containing Tubulin Gal80 carried the wild type copy of the TSG101 gene. Mitotic recombination therefore rendered the marked cell not only GFP positive due to the loss of Tubulin Gal 80 but also a homozygous mutant. So we obtained mutant cells marked with GFP.

The recombination efficiency of different FRT sites varies, so for Chapter 3, flies were grown at 29ºC and heat shocked when they were 3 day old adults. HS was done for 30 minutes 2 times a day at 37ºC for 3 days and flies were left to recover at 29ºC for 7 days before gut dissection. The control carried a non mutant construct downstream of the FRT site and was subjected to the same treatment. This allowed me to compare the GFP marked mutant and control

clones. The formation of each GFP positive cell (even within the same intestine) is an independent event.

COPYRIGHT

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CHAPTER 2

PATHOGENIC STIMULATION OF *DROSOPHILA* ISCs

SUMMARY

By feeding pathogenic bacteria and stress inducing chemicals to adult flies, I demonstrate that *Drosophila* ISCs in the midgut can respond by increasing their division. The resulting enteroblasts differentiate faster to become cells resembling the enterocyte lineage while not significantly changing their rate of differentiation into enteroendocrine cells. These results are consistent with the Idea that *Drosophila* midgut stem cells can respond to tissue damage induced by pathogens and initiate tissue repair. This system should allow molecular and genetic analyses of stem cell-mediated tissue repair.

INTRODUCTION

The gastrointestinal (GI) tract in addition to absorbing nutrients also serves as a major site of interaction between the host and environmental pathogens (Backhed 2005; Macdonald 2005; Radtke 2005). In addition to the numerous microbes and chemicals ingested during daily food intake, the GI tract also houses billions of commensal bacteria, which play important symbiotic roles within the host. The complex interactions between intestinal cells and microbes, both commensal and ingested, are essential for the well being of the host. The epithelial lining of the fly GI tract is essentially one to two-cells thick and the epithelium is constantly shedding cells due to aging or damage. Maintenance of the epithelial integrity requires replenishment of dead cells by proper division and differentiation of precursor cells (Crosnier 2006; Sackville 2008; Casali 2009). This process, known as tissue homeostasis is carried out by adult stem cells that divide to form progenitor cells, which in turn differentiate into various lineages to give rise to the required cell types in the intestine. In the mammalian intestine these stem cells are located at the base of the crypts. Tissue homeostasis is a highly regulated process and Wnt, BMP and Notch signaling pathways have been Implicated in mammalian intestinal cell proliferation (Fodde 2007; Nakamura 2007).

The *Drosophila* midgut has a large number of ISCs located basal to the enterocytes (Micchelli 2006; Ohlstein 2006). The ISC undergoes mitosis giving rise to an ISC and an enteroblast. Enteroblasts are non-dividing precursor cells. Ninety percent of enteroblasts differentiate into mature enterocytes and ten percent into enteroendocrine cells (Ohlstein 2007).

The Delta Notch pathway plays a role in cell fate determination (Micchelli 2006; Ohlstein 2006, 2007). A known ISC specific marker is the punctuate staining of active Delta in the cytoplasm (Bray, 2006). The ISC retains Delta after mitosis while the daughter enteroblast loses Delta, thus stimulating the Notch signaling pathway. Varying Notch levels in daughter cells, regulated by Delta levels in ISC's determine enteroblast fates (Ohlstein 2007). Not much is known about the rate of division of the ISC and to what extent is it regulated. Oxidative stress, tissue damage and aging are considered to have stimulatory effects on ISC numbers and division (Choi 2008, Amcheslavsky 2008). In these studies I show that oral infection by pathogenic bacteria mimics some aspects of the oxidative stress phenotypes. I observe that feeding different oxidizing agents, paraquat and hydrogen peroxide also show similar effects in the fly gut. Paraquat has been used as an herbicide. It is a highly toxic compound that is absorbed rapidly across the mammalian small intestine brush border and is known to trigger Parkinson's disease like symptoms in rats (Ossowska 2006). It has been long

known that the fly gut employs an antioxidant system as an Immune response against ingested microbes and harmful oxidizing agents (Ha 2005a, b).

RESULTS

Feeding of chemical and microbial pathogens causes dose dependent lethality

The uses of P. entomophilla and S. marcescens as pathogenic bacteria, as well as paraquat and H_2O_2 as stress-inducing agents, have been previously described (Liehl 2006; Nehme 2007; Biteau 2008; Choi 2008). However, due to the variability of host response, I performed our lethality study using different doses of these reagents in order to obtain suitable feeding conditions for subsequent cellular assays. The minimum feeding solution contains 5% sucrose alone, which can sustain the viability of flies for more than 7 days albeit under nutritional starvation. The addition of bacteria growth medium 2xYT (2xYT yeast extract and tryptone) in the 5% sucrose provides sufficient nutrients and the flies stay well in this medium for more than 7 days. These two feeding solutions were used as controls. Inclusion of four experimental reagents in our feeding media caused dose dependent lethality when compared to controls (Fig 2.1 A–D). The use of 0.3% H_2O_2 in the feeding sucrose solution killed approximately 50% flies in 4 days. I decided to use this feeding concentration for subsequent experiments because significant pathogenesis could be induced but a substantial number of flies were still alive after 4 days for tissue dissection. Paraquat feeding should induce similar oxidative stress in gut tissue. Indeed, I found that inclusion of 2

mM of paraquat in the sucrose solution caused a killing curve analogous to 0.3% of H_2O_2, thus I chose to use 2 mM paraquat for our subsequent feeding experiments. For bacteria feeding experiments, I included similar volume of 2xYT in the sucrose solution as control. The addition of $3x10^6$ bacteria CFU of *S. marcescens* caused a strong killing effect, such that 60% of flies were killed within 4 days. Serial dilution of this bacteria caused progressively lower lethality. *P. entomophilla* appeared to be less pathogenic, and the use of $9x10^9$ bacteria could only kill approximately 30% of flies in 4 days. This result is consistent with a previous report showing that adult flies have more resistance to *P. entomophilla* than larvae (Liehl 2006). Overall, these results establish that appropriate amount of pathogens can be used for feeding experiments and subsequent intestinal cell analysis.

Pathogen feeding increases the number of precursor cells in midgut

Based on the conditions established in our viability assays, I examined cellular phenotypes of dissected gut from live flies at earlier time of the killing curve, between 2 and 4 days, when most of the flies were still alive. I reason that at earlier time points the intestinal epithelium should be mostly intact and can mount appropriate responses towards pathogenic stimulation, while at later time the intestinal damage may be overwhelming and more complex responses may take

place. The escargot promoter-directed Gal4 expression (esg-Gal4) coupled with UAS dependent mCD8GFP reporter (UAS-CD8GFP) can mark the cell membranes of intestinal precursor cells, including ISCs and enteroblasts (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). In control fly guts, GFP expression can be easily detected in some small cells either as individual cells or as pairs (Fig 2.2A–D). I used 2 to 3 day old flies for experiments but in older flies this esg-Gal4/UAS-GFP expression was detected in more precursor cells, suggesting more rapid division and enteroblast formation in the ISC nest (Biteau 2008; Choi 2008). Meanwhile, bigger nuclei show no such GFP signal as they are mature enterocytes that are polyploid. Some other small nuclei also show no GFP expression but stain positive for another marker Prospero and are thus enteroendocrine cells (Fig 2.2M–P). After feeding with bacterial or chemical pathogens for 3 days, dissected guts show clearly increased GFP signals when compared to control samples (Fig 2.2 E–L). In addition to the apparent increase in the number of GFP positive cells, many GFP positive cells also had bigger sizes. The images shown in Figure 2.2 were all from the posterior midgut region. However, different regions of the midgut showed variable phenotypes. For example, H_2O_2 feeding produced a stronger cell proliferation effect in the anterior midgut, while paraquat shows greater proliferation in the posterior midgut. Nonetheless, feeding of pathogens almost always increased GFP positive cells in some or the other part of the midgut. The increase of GFP-positive cells is a specific response, because the staining of enteroendocrine cells by Prospero did

not show a similar increase (Fig 2.2M–P). Therefore, all pathogen fed samples had detectable phenotypic changes, demonstrating that the pathogens somehow caused cell proliferation in the midgut.

Enteroblast accumulation is the major phenotypic change after pathogen feeding

To further assess the cell proliferation phenotype, I counted the number of GFP positive cells. The counting was performed on microscopic Images taken from the posterior midgut region, as indicated by the bracket in Figure 2.3A. Both GFP-positive and-negative cells were counted. The number of GFP-positive cells per 100 negative cells was plotted as shown in Figure 2.3 B. The result shows that cell numbers are increased 3 fold in case of H_2O_2, 4 fold for paraquat, about 8 fold in case of *P.e*. and 5 fold when fed *S.m*. On the other hand, the number of Prospero-positive enteroendocrine cells did not increase to the same extent as enteroblasts with the greatest increase shown on feeding paraquat which only showed a 2.5-fold increase in cell numbers (Fig 2.3C). The p value for the difference between control and H_2O_2 fed samples show that the difference between the enteroendocrine cells is less significant (p value =0.02) than that between the control and experimental enteroblast numbers ($p<0.01$). This quantification again demonstrates that pathogen feeding caused a cell

proliferation phenotype in the midgut. The expression of esg-Gal4/UAS-CD8GFP marks both ISCs and enteroblasts. To determine which cell type is responsible for the GFP-positive cell increase, I stained for enteroblast-specific marker Su(H)-lacZ and ISC-specific marker Delta. The positively stained cells were then counted and normalized with non-stained cells. The result showed that paraquat and H_2O_2 caused three- to fivefold increase in the number of cells stained positive for Su(H)-lacZ. The two bacteria strains cause two- to fourfold increase of Su(H)-lacZ-positive cells (Fig 2.3D). Cell counts for Delta-positive staining revealed that there was less than twofold increase in the number of ISCs in guts of flies fed with the four reagents (Fig 2.3E). Because the number of enteroblasts increased more than the number of ISCs, it suggests that feeding of pathogens increases ISC division to produce more daughter cells. Therefore, I stained the guts with phospho-histone3 (phospho-H3) antibody to assess cell division. Within the midgut, the only cell type that goes through mitosis is the ISC. Enteroblasts cease mitosis although they still undergo endoreplication. Thus, phospho-H3 staining should mark those ISCs that have condensed chromosomes and are in the process of mitosis. Cell counts showed that paraquat and H_2O_2 treatment increased the number of mitotic cells by approximately threefold. The two bacterial strains used also increased the number by approximately 2.5-fold (Fig 2.3F). Overall, the number of Delta-positive cells did not increase as much while the increase of phospho-H3-posItIve cells correlates better with the increases in

enteroblast accumulation. These data suggest that pathogenic stimulation increases ISC division resulting in the formation of more enteroblasts.

Pathogenic stimulation does not affect cell fate determination

To ascertain that cell fates in midgut are not affected after pathogen feeding, I performed co-Immunoflourescent staining for Delta and Su(H)-lacZ. In midguts of young flies, ISCs and enteroblasts after division are in close contact with each other for a short time. High level of β-catenin is present in the junctions of the two cells and E-cadherin is required to maintain this contact (Maeda 2008). This close contact allows Delta-Notch signaling to occur properly between ISC and enteroblast for correct cell fate establishment. In control guts, Delta is detected only in ISC as punctuate cytoplasm staining (Fig 2.4 A). The neighboring enteroblast has Notch target gene Su(H)-lacZ expression, detected as b-galactosidase staining present in both cytoplasm and nucleus (Fig 2.4B). After H_2O_2 feeding, more cells had nuclear and cytoplasm b-galactosidase staining (Fig 2.4D–F). These staining also became more apparent in cytoplasm likely due to the bigger cell size after pathogenic stimulation. Meanwhile, the cells that had Delta showed no β-galactosidase staining and had clear space surrounding the nuclei (indicated by arrows). This demonstrates that the Delta positive cells have no Su(H)-lacZ expression, and vice versa. The same non-overlap was observed

in paraquat, *P. entomophilla* and *S. marcescens* treated guts. These results suggest that the cell fate decision between ISC and enteroblast remains normal after feeding the various pathogens.

Increased enteroblast differentiation after pathogen feeding

I observed that many of the cells marked by esg-Gal4/UAS-GFP and Su (H)-lacZ were larger in size in pathogen fed samples than in the control samples. This suggests that in addition to the increase of stem cell division, the resulting enteroblasts may have faster differentiation into mature enterocytes, which are substantially bigger in size. To trace the fates of ISC and all subsequent daughter cells, I performed lineage tracing by mosaic analysis with repressible cell marker (MARCM). This technique randomly allows Gal4 driven GFP marking of individual ISC lineage due to FLP-FRT-mediated mitotic recombination that removes the repressor Gal80 (Lee 2001, Micchelli 2006; Ohlstein 2006). Guts of control MARCM flies fed with sucrose showed GFP expression in clusters with small number of cells (Fig 2.6A–C). Under the same feeding condition the H_2O_2 treated flies had more GFP positive cells and were present in bigger clusters (Fig 2.6D–F). Usually I found that one cell exhibited punctuate Delta staining in each cluster (red staining in all parts). Some larger clusters also had 2 or more Delta positive cells (data not shown), but It could be due to fusion of neighboring

clones or some abnormal cell division. Most importantly, the GFP positive cells were mostly of bigger overall cell size and bigger nuclear size, comparing to the control GFP cells. These phenotypic changes were similarly observed in guts of flies fed with paraquat, *P. entomophilla* and *S. marcescens* (Fig 2.6 G–O). I quantified the number of GFP positive cells with bigger cell size. For the quantification, I counted GFP positive cells which had noticeably larger nuclei than the ISC in each cluster (Delta positive) and showed no Delta staining themselves as “large GFP positive cells” namely, they were not ISCs and since the nuclei looked much larger than the ISC they are unlikely to be early EB’s. Since the Notch antibody did not work reliably in the intestine and the MARCM line did not carry Su(H) LacZ, it is difficult to conclusively show that they are not EB’s. However differentiating or newly differentiated cells could still stain positively for Su(H)LacZ due to the stabiltity of the βgalactosidase protein. Therefore other markers like Phalloidin staining would be more useful to conclusively determine the differentiation of the daughter cells.

The result shown in Figure 2.6 Q clearly demonstrates that the number of differentiating or differentiated cells has increased by more than fourfold. Each isolated cluster should represent a single lineage originating from one ISC. Therefore, the result supports the idea that pathogenic feeding increases the number of cells produced by an ISC, which corroborates the results of increased

ISC division. Moreover, the increase in nuclei sizes of most daughter cells suggests that pathogenic feeding also increases differentiation, possibly for tissue repair

DISCUSSION

I have shown that two stress-inducing chemicals and two pathogenic bacteria can induce ISC proliferation and enteroblast differentiation within a few days of feeding. Previous results also demonstrate two other tissue damaging agents in stimulating intestinal stem cells (Amcheslavsky 2009; Buchon, 2009), albeit with different mechanism and responses. These gut phenotypes can be observed in times when less than 50% of fly death occurs. The overall gut morphology of the dissected flies that were still alive appeared rather normal, suggesting that tissue damage is still limited at this time. These results support the Idea that pathogenic feeding causes tissue damage within the midgut and the ISCs respond by increasing their division and the resulting enteroblasts increase their differentiation. While it is also possible that these responses represent non-specific reaction to pathogens, I speculate that the stem cells are actively responding to tissue damage induced by the pathogens and are initiating repair. A report shows that feeding of a non-pathogenic bacterium, *Erwinia carotovora*, can induce the expression of the legend Unpaired 3 for the JAK-STAT pathway, which mediates cell proliferation in the midgut (Buchon, 2009). Moreover, insulin receptor signaling pathway is required for ISC proliferation (Amcheslavsky 2009). Further analysis will show whether similar stimulation and repair mechanism occur after pathogenic bacteria-induced tissue damage.

Food and water borne diseases, as well as intestinal inflammation and cancer, continue to be a major health concern worldwide (Backhed 2005, Macdonald 2005, Radtke 2005). An organism's barrier epithelia are designed to manage continuous contact with microbes and other harmful reagents. Our intention was to use *Drosophila* as a model to study intestinal responses to stress caused by oral ingestion of pathogenic bacteria and compare the phenotype with known stress-inducing agents such as paraquat and hydrogen peroxide. Previous reports show that bacteria and stress-inducing agents cause pathological changes in adult *Drosophila* midgut (Liehl 2006 Nehme 2007 Biteau 2008 Choi 2008). These studies employed different conditions, such as a non-pathogenic bacteria strain *E. carotovora* or a shorter time course for paraquat feeding. Our experimental condition and subsequently induced phenotypes reported here should complement those reports. A detectable phenotype is the increase in cell division, which causes accumulation of enteroblasts in the midgut. The increase in number of ISC based on Delta staining is not as high and cannot account for the increased number of enteroblasts/daughter cells, suggesting that individual ISC division rate has increased. I have also provided evidence that the differentiation of enteroblasts to bigger cells occurs with higher frequency within the same experimental time. On the other hand, the number and morphology of enteroendocrine cells did not show significant difference. Based on these observations I conclude that the oxidative stress caused by bacteria and

chemicals has accelerated cell division as well as differentiation to form more enterocytes, consistent with epithelial repair after pathogenic damage.

Previous reports documented that epithelial damage is associated with feeding of the two pathogenic bacteria, *P. entomophilla* and *S. marcescens* (Liehl 2006; Nehme 2007). These bacteria can elicit complex reactions in the midgut, and thus we remain uncertain of the mechanism by which ISC proliferation is brought about by these pathogens. A logical interpretation of the phenotypes, however, is a damage response where the gut tries to replenish lost enterocytes or those whose functioning is damaged by oxidative stress. It has been shown that the fly gut employs an antioxidant system as an Immune response against ingested microbes (Ha 2005a, b; Lee, 2008). Therefore, bacterial feeding should mimic some aspects of the oxidative stress phenotypes. I observed that feeding the flies with different oxidizing agents, paraquat and hydrogen peroxide, also elicits prominent and similar phenotypes in the fly gut by stimulating ISC division and EB differentiation favoring the EC pathway. This sort of repair process is seen when EC's are damaged. Paraquat has been used as an herbicide. It is a highly toxic compound that is absorbed rapidly across the mammalian small intestine brush border and is known to trigger Parkinson's disease like symptoms in rats (Ossowska 2006).

Various mechanisms can be involved in the effect I have shown here. The Jun N-terminal kinase (JNK) signaling pathway is known to have a cytoprotective role in the fly intestine. It is activated by a variety of environmental challenges, including oxidative stress due to paraquat exposure, and increases stress tolerance and lifespan (Oh 2005). However, it is also seen JNK pathway upregulation due to aging or oxidative stress causes aberrant ISC division and Delta-Notch segregation (Biteau 2008). In our results we see that while mitosis is upregulated, there is no evidence of aberrant cell differentiation. ISC and EB fates remain distinct and EB's appear to differentiate to form EC like cells as seen in the clonal analysis by MARCM. Based on this it is possible that the JNK pathway is less likely to be involved here. It is possible that prolonged periods of oxidative stress will cause the accumulation of misdifferentiated daughter cells implicating the JNK pathway. Further studies can show if other pathways like EGFR, Wingless and JAK/STAT which are known to act synergistically to promote ISC maintenance and proliferation are involved. It is also possible that using more markers will show if there are any defects in cell division and differentiation that are not immediately apparent.

I hope this will lead to a better understanding of the mechanisms that lead to the observed oxidative stress phenotype and further develop *Drosophila* as a model system to study intestinal pathogenesis.

MATERIALS AND METHODS

Drosophila stocks, bacteria strains, and feeding experiments

Information on *Drosophila* genes and stocks is available from Flybase (http://flybase.bio.indiana.edu). y1w*, CantonS and w1118 were used as wild type stocks for gut phenotypic comparison. UAS-mCD8GFP flies were obtained from the Bloomington stock center; esg-Gal4 and Su(H)Gbe-lacZ were as described (Micchelli 2006; Ohlstein 2007). EsgGal4 also known as Esg^{NP5130} is a Gal4 enhancer trap line that reproduces larval and adult esg expression. It was recombined with UASCD8 GFP (II) line to generate the Esg Gal4 UAS CD8 GFP line which expressed CD8 GFP in ISCs and daughter cells. Both lines were obtained from flybase. Flies were maintained on cornmeal-yeast-molasses-agar media. Stocks were maintained at room temperature. For viability tests and feeding experiments, the flies were kept at 29ºC. I used 50–100 flies per vial for viability tests and 10–50 flies per vial for gut phenotype induction. Feeding experiments involved using 3- to 5-day-old flies in an empty vial containing a piece of 2.5 cm x 3.75 cm chromatography paper (Fisher). Five hundred micro liters of 5% sucrose solution alone or with pathogens was used to wet the paper as feeding medium. Sucrose solution alone serves as the control for all experiments. Paraquat (Sigma) and hydrogen peroxide (H_2O_2) (Fisher) were added in different amounts as indicated in the figures to the 5% sucrose solution.

The bacterial growth medium 2xYT broth (MP Biochemicals) was also used as a control for bacteria feeding experiments. A rifampicin resistant *Pseudomonas entomophilla* strain was a generous gift from Bruno Lemaitre; *Serratia marcescens* Db11 was a generous gift from Christine Kocks. The bacteria were cultured overnight in 2xYT, concentrated and resuspended in 2xYT if necessary. The numbers of bacteria as indicated in the figures were mixed with the 5% sucrose solution for feeding. The feeding solution was changed every day.

For lineage analysis, GFP-marked intestinal stem cell clones from MARCM were generated as previously described in Chapter 1. For tissue damage experiments 3-day-old flies were set up for feeding in 29ºC for 3 days before gut dissection.

Immunoflourescent staining and microscopy

Female flies were used for gut dissection, because of the bigger size but male flies were also used occasionally to check the phenotypes. The entire gastrointestinal tract was pulled from the posterior end directly into fixation medium containing 1xPBS and 4% Formaldehyde (Mallinckrodt chemicals). Guts were fixed in this medium for 3 h; except for Delta staining the fixation was for 0.5 h. Subsequent rinses, washes and incubations with primary and secondary antibodies were done in a solution containing 1X PBS, 0.5% BSA, 0.1% Triton X-100 with 1:50 dilution of Horse serum for blocking. The following anti-sera were

used: ante-Delta (monoclonal 1:100 dilution), ante-Prospero (monoclonal 1:50 dilution), all from Developmental Studies Hybridism Bank; anti-phospho-histone H3 (rabbit 1:2,000 dilution) (Upstate Biotechnology); anti-b- galactosidase (monoclonal 1:500 dilution) (Promega); anti-b-galactosidase (rabbit 1:50,000) (Cappel, MP Biomedicals). Secondary antibodies were used in 1:2,000 dilution as follows: goat anti-mouse IgG conjugated to either Alexa 488 or Alexa 568, and goat antI-rabbIt IgG conjugated to either Alexa 488 or Alexa 546 (Molecular probes). DAPI (Vectorshield, Vector Lab) was used at 1:1 dilution in PBS. Most Images were taken by a Nikon Spinning Disk confocal microscope (UMass Medical School Imaging Core Facility).

Fig 2.1

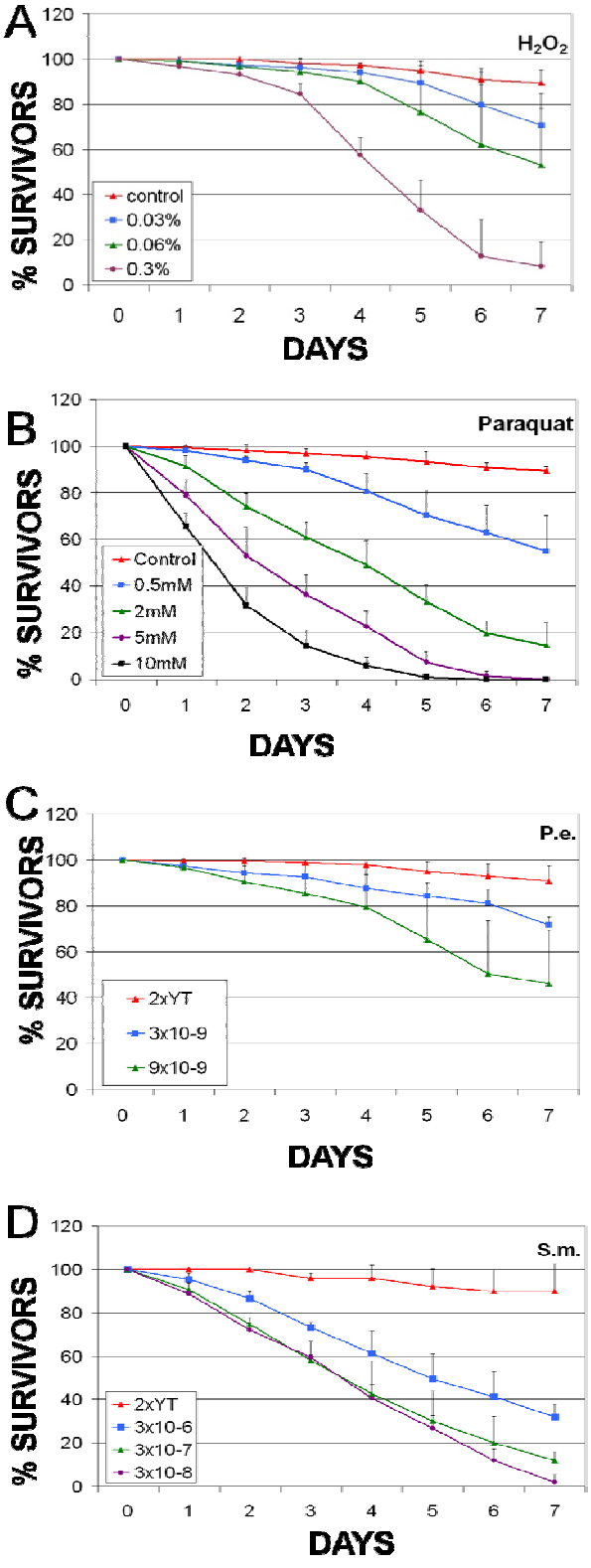

A
H_2O_2
% SURVIVORS
DAYS
control
0.03%
0.06%
0.3%
B
Paraquat
% SURVIVORS
DAYS
Control
0.5mM
2mM
5mM
10mM
C
P.e.
% SURVIVORS
DAYS
2xYT
3x10-9
9x10-9
D
S.m.
% SURVIVORS
DAYS
2xYT
3x10-6
3x10-7
3x10-8

Fig 2.1: Dose dependent lethality on oral ingestion of pathogens.

The four reagents used in the feeding experiments showed a dose dependent mortality when fed to adult flies over seven days. The reagents (A: H_2O_2, B: Paraquat, C: *Pseudomonas entomophilla*, and D: *Serratia marcescens*) were included in various amounts in a 5% sucrose solution. The percentage of flies left alive each day is expressed as survival rate. Fresh feeding solution was prepared every day. The error bars represent standard deviation. The bacteria were cultured in 2xYT medium which is included as a control in A and B. Bacterial amounts are expressed as C.F.U's.

Fig 2.2 A-F

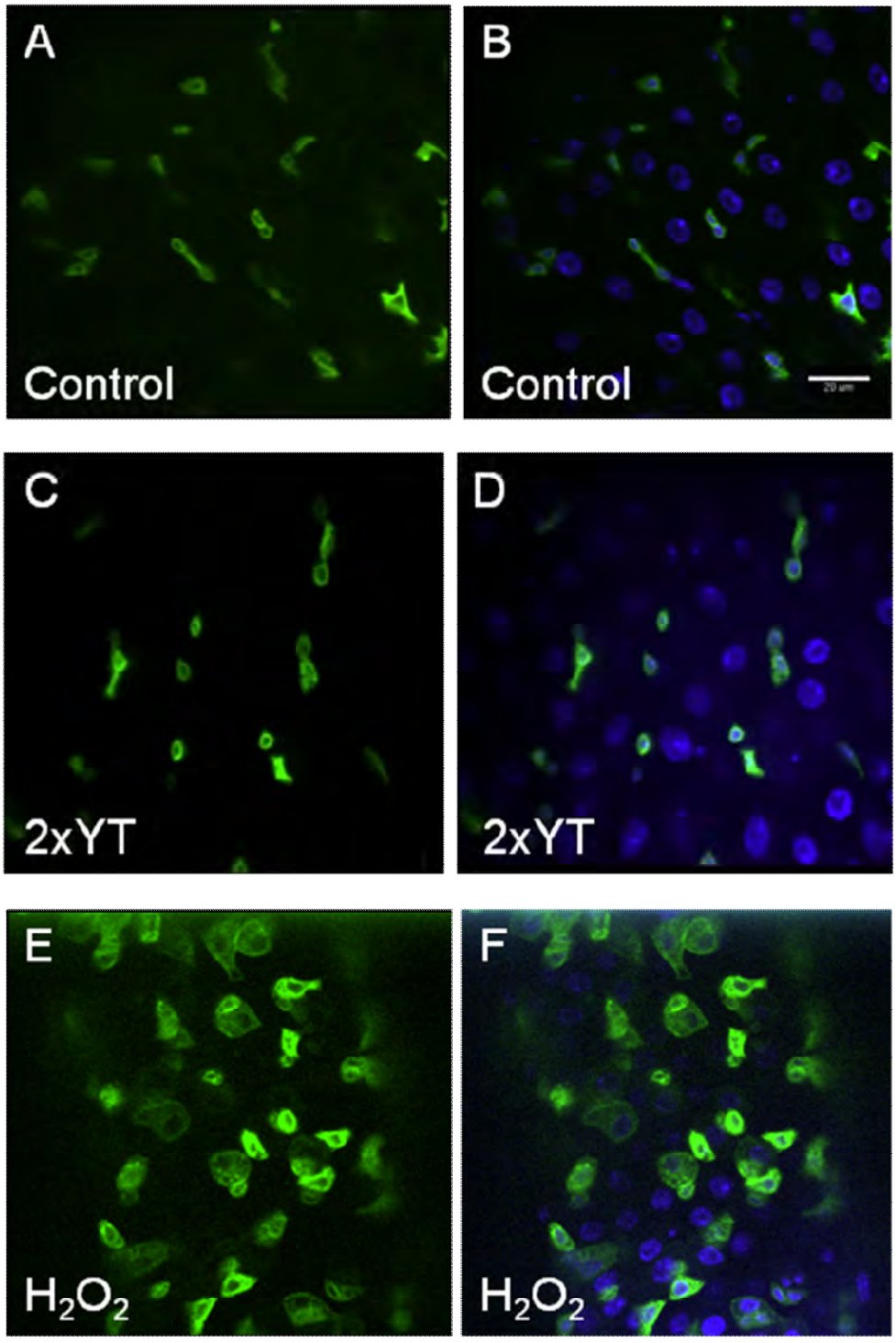


Fig 2.2 G-L

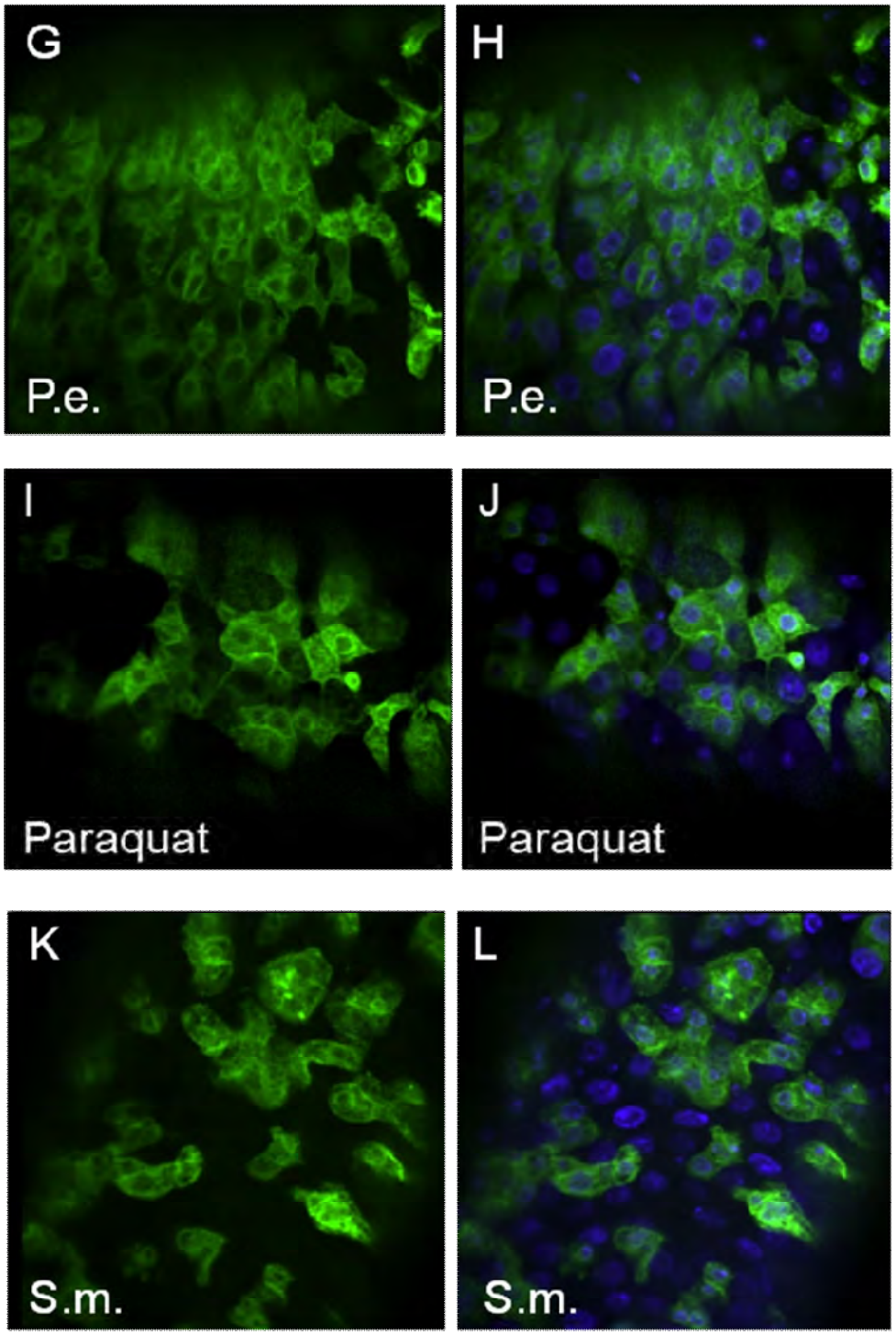


Fig 2.2 A-L: Pathogens increase the number and size of Esg positive cells in the intestine.

Cell proliferation effect revealed by the esg-Gal4/UAS-CD8GFP marker. The blue color in panel A and all other figures is DAPI staining of DNA. The green staining indicates Esg positive cells. I used 2mM Paraquat and 0.3% H_2O_2 in 5% sucrose and $9x10^9$ C.F.U. of *Pseudomonas entomophilla* and $3x10^6$ C.F.U. of *Serratia marcescens* in 2xYT culture in 5% sucrose. Scale bar is 20 µm.

Fig 2.2 M-P

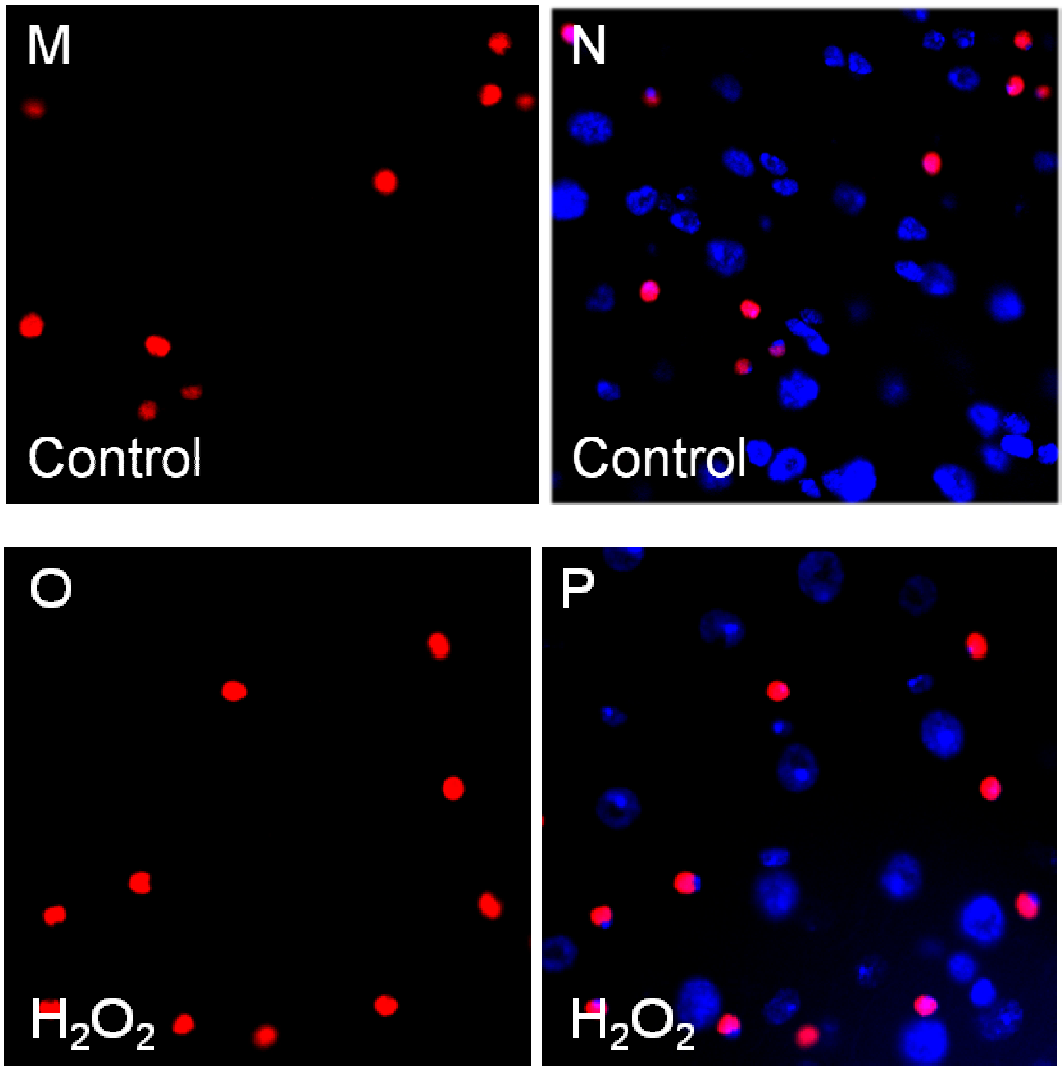
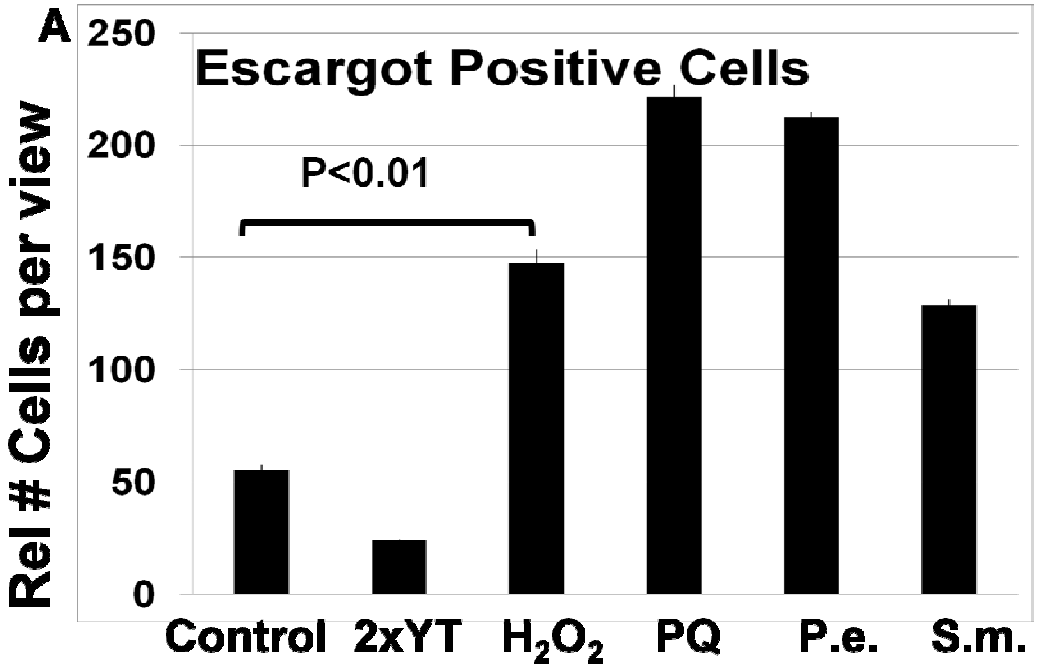
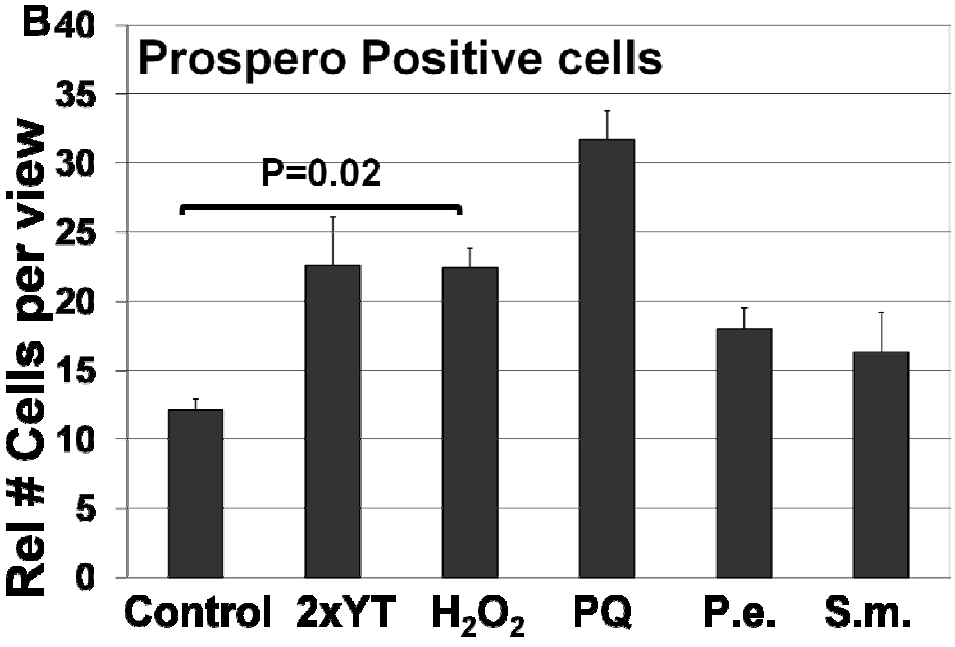


Fig 2.2 M-P: No significant EE proliferation on pathogen feeding.

Enteroendocrine cells (stained red for Prospero) don't show perceptible increase in numbers on pathogen feeding.

Fig2.3A-B


A
Escargot Positive Cells
Rel # Cells per view
250
200
150
100
50
0
P<0.01
Control
2xYT
H_2O_2
PQ
P.e.
S.m.


B
Prospero Positive cells
Rel # Cells per view
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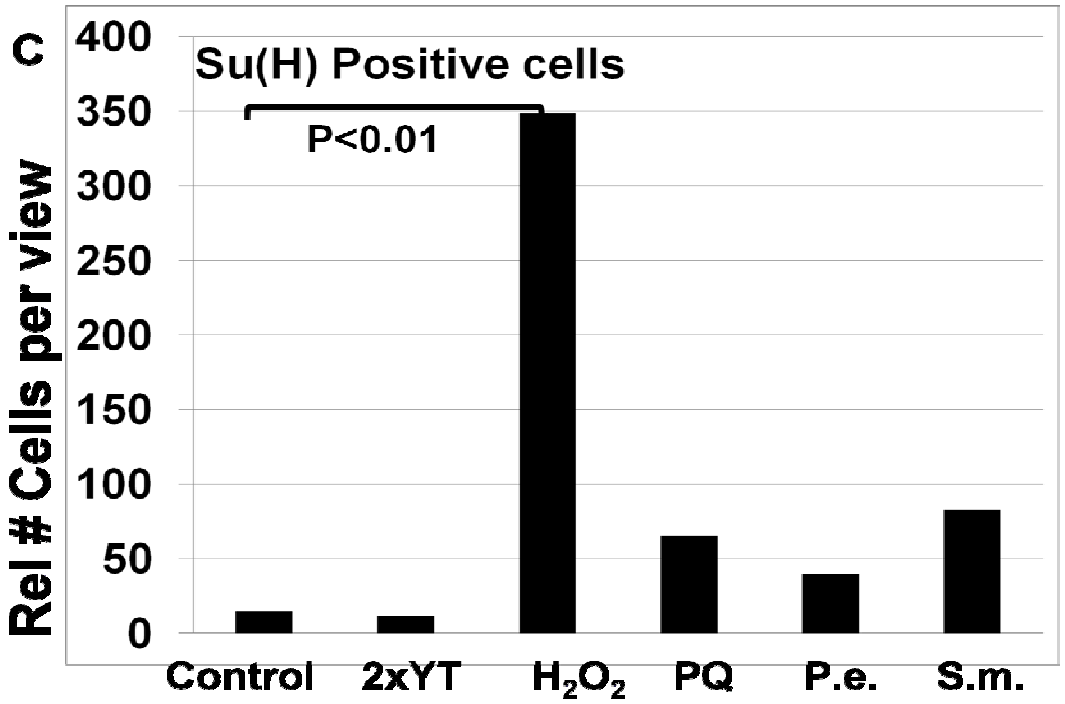
35
30
25
20
15
10
5
0
P=0.02
Control
2xYT
H_2O_2
PQ
P.e.
S.m.

Fig 2.3 C-D


C
Su(H) Positive cells
P<0.01
Rel # Cells per view
400
350
300
250
200
150
100
50
0
Control
2xYT
H_2O_2
PQ
P.e.
S.m.

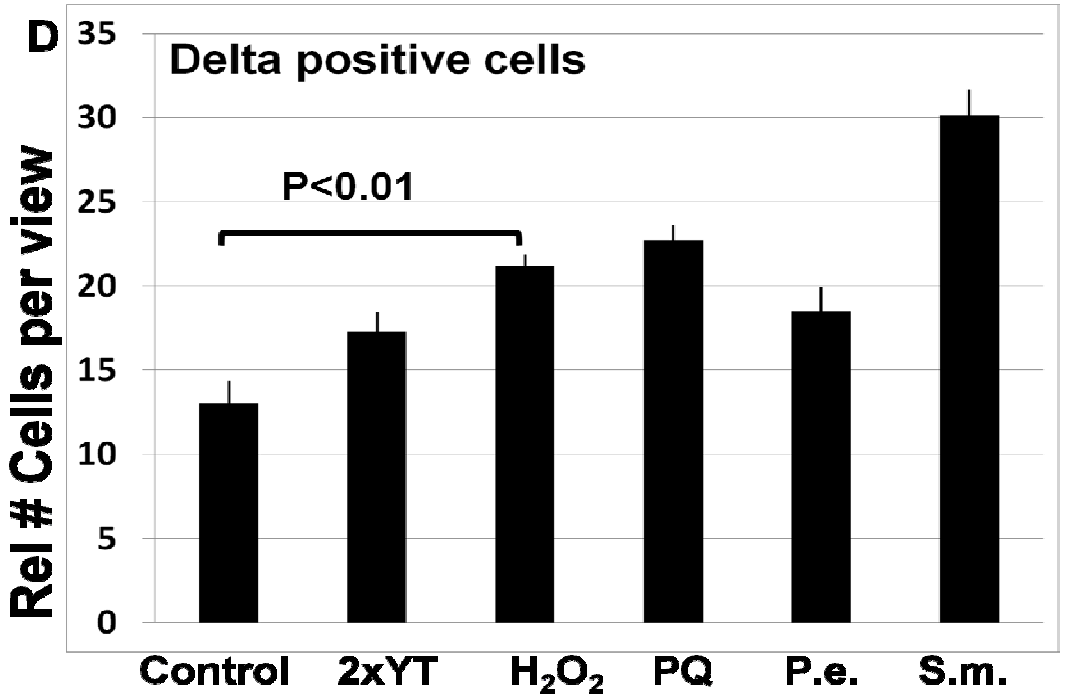

D
Delta positive cells
P<0.01
Rel # Cells per view
35
30
25
20
15
10
5
0
Control
2xYT
H_2O_2
PQ
P.e.
S.m.

Fig 2.3 E

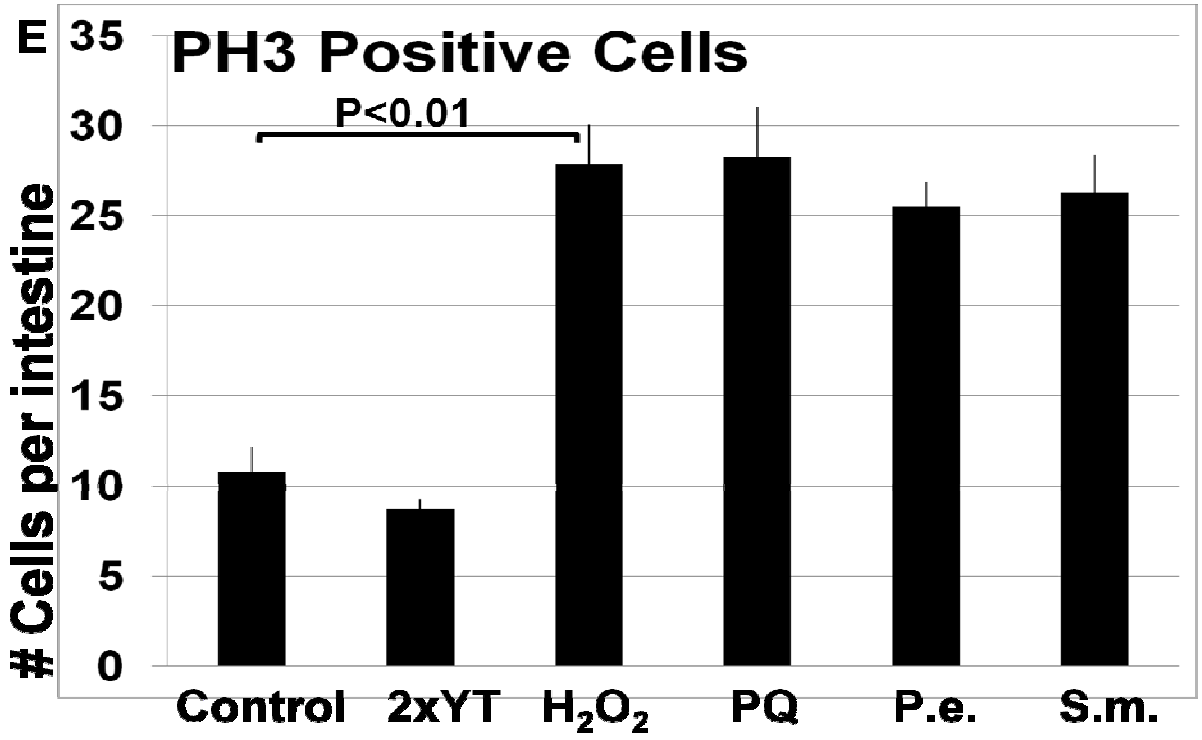

E
PH3 Positive Cells
P<0.01
Cells per intestine
35
30
25
20
15
10
5
0
Control
2xYT
H2O2
PQ
P.e.
S.m.

Fig 2.3: Pathogens increase intestinal cell proliferation.

Quantification of various cell types; A: esg-Gal4/UAS-CD8GFP, B: Prospero, C: Su (H) LacZ, D: Delta and E: PH3, positive cells after feeding with various reagents as indicated. The number of Delta, Su(H) LacZ, esg-Gal4/UAS-CD8GFP and Prospero positive cells were counted in multiple Images for each experiment and normalized by 100 unstained cells revealed by DAPI staining. The resulting number is averaged from several images and plotted as the "Rel # cells per view". In Fig 2.3E, PH3 positive cells were counted in the whole gut and expressed as the average number of mitotic cells per gut. In all experiments I have used 2mM Paraquat and 0.3% H_2O_2 in 5% sucrose and $9x10^9$ C.F.U. of *Pseudomonas entomophilla* and $3x10^6$ C.F.U. of *Serratia marcescens* in 2xYT culture in 5% sucrose. The error bars represent standard error. In Fig 2.3C the error bars are not visible as they are all very small (under 1). The p value is the result of the T test performed between the Control and H_2O_2 feeding samples. The p value for prospero positive cells is 0.02 showing that the increase in EE cells between sucrose (control) and H_2O_2 fed guts is not very significant.

Fig 2.4 A-I

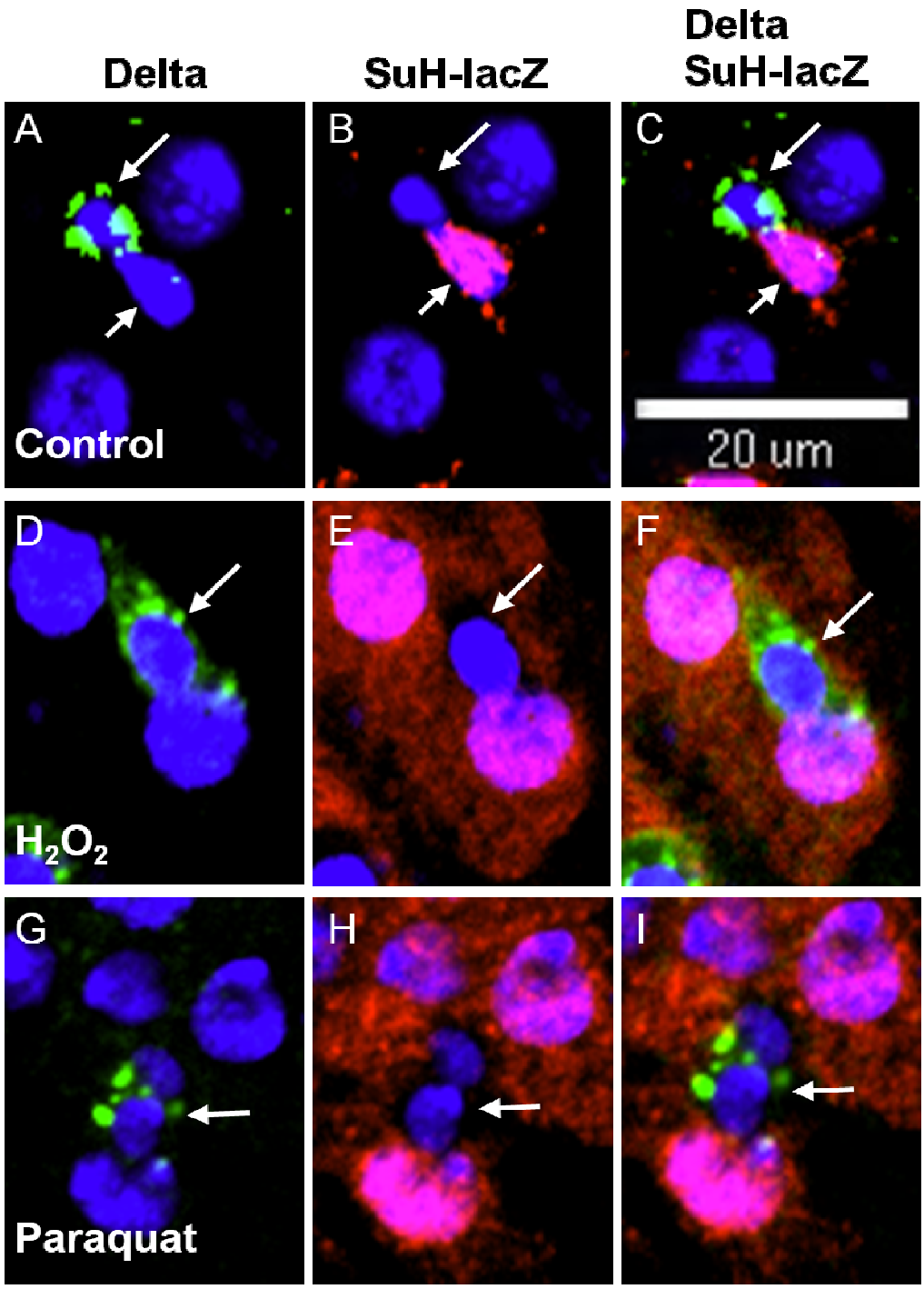

Delta
SuH-lacZ
Delta SuH-lacZ
A
B
C
Control
20 um
D
E
F
H2O2
G
H
I
Paraquat

Fig 2.4 J-O

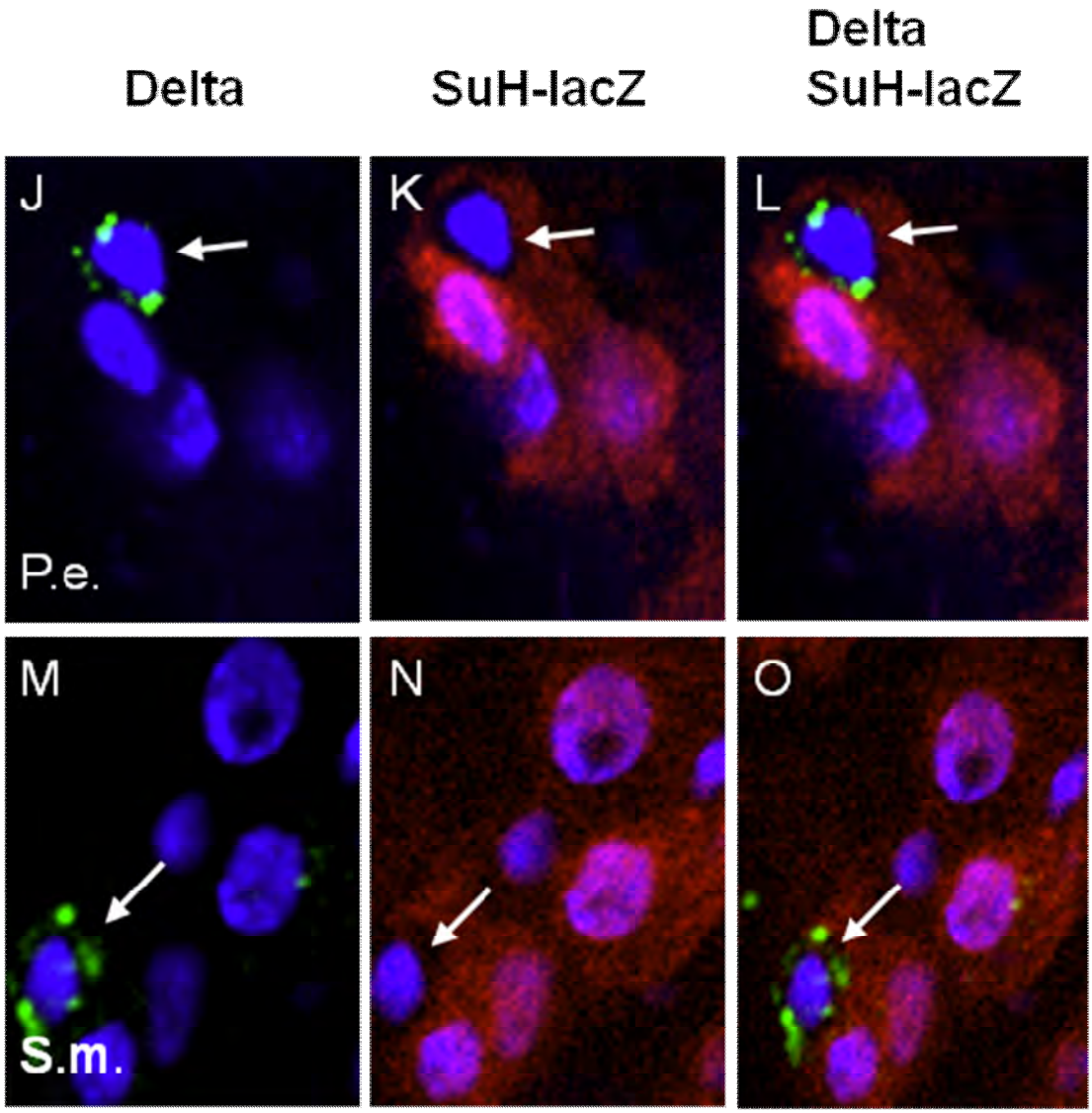


Fig 2.4: Pathogen feeding does not alter the cell fate decision.

Dissected guts from Su(H)-lacZ flies fed with the various agents as indicated were used for Immunoflourescent staining. Delta staining (green) and β-galactosidase staining (red) were performed together on the guts. Representative confocal Images are shown here. In control samples, the Delta-positive cells (A, arrow) and the Su(H)-lacZ positive cells (B, arrowhead) are found next to each other and almost never overlap. The Delta protein appears as punctuate cytoplasm staining. The β-galactosidase staining is both cytoplasm and nuclear, thus overlaps extensively with DAPI staining (blue). In pathogen fed flies, the β-galactosidase staining increased substantially, consistent with the accumulation of more enteroblasts surrounding Delta-positive ISCs. There was also more obvious β-galactosidase staining (red) in cytoplasm, suggesting the cell size of enteroblasts has also increased. However, all Delta-positive cells clearly had no cytoplasm β-galactosidase staining (indicated by arrows in panels D-O), and had non-fluorescent space surrounding the nuclei. Over 100 Delta positive cells were counted in each experiment and no overlap of the staining was observed. Scale bar is 20 μm.

Fig 2.5

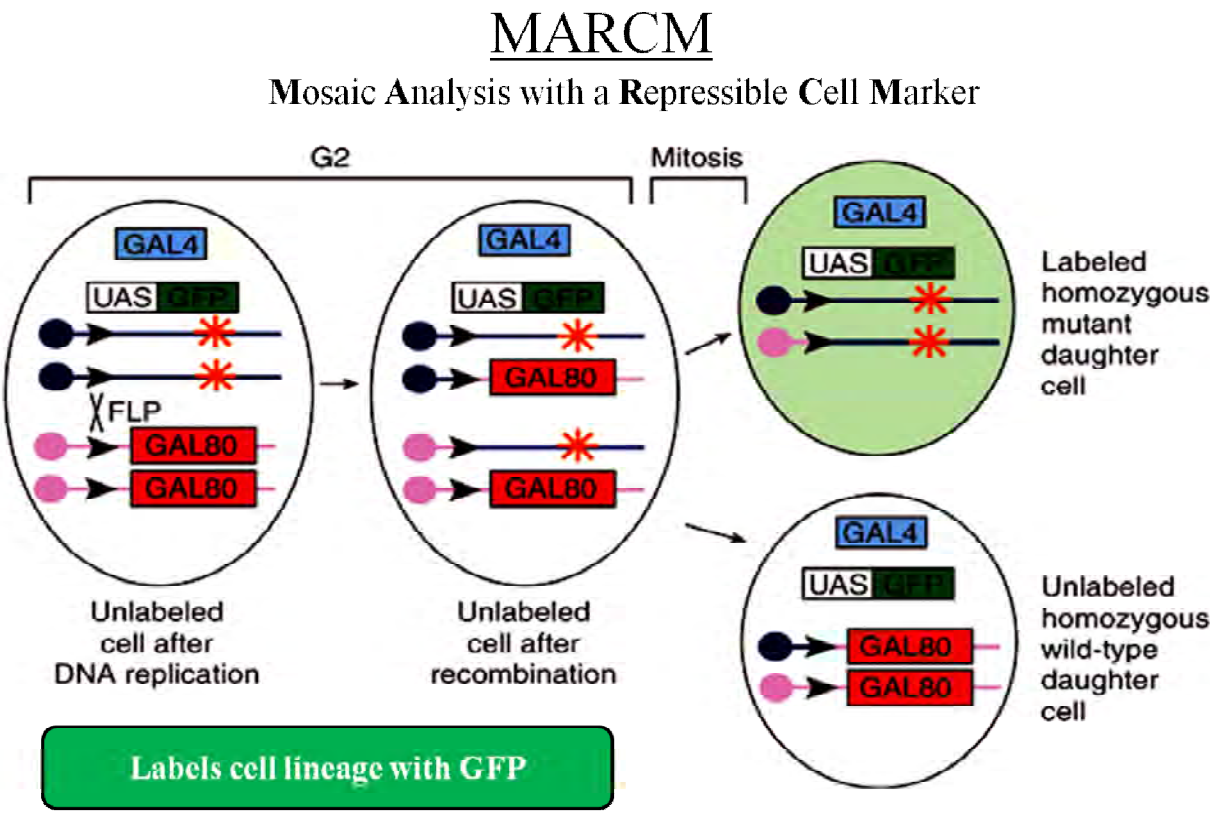


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Fig 2.5 Schematic overview of the MARCM system

FLP-FRT-mediated mitotic recombination coupled with the Gal4/Gal80 chromosomes allows one of the two cells of a recent division to expressed GFP. If the newly formed ISC is genetically marked with this GFP expression, all subsequently derived cells will all be GFP positive, thus marking the whole lineage. If the newly formed enteroblast is genetically marked, it will not divide again and the GFP-marked cell will differentiate as an isolated cell.

Fig 2.6 A-I

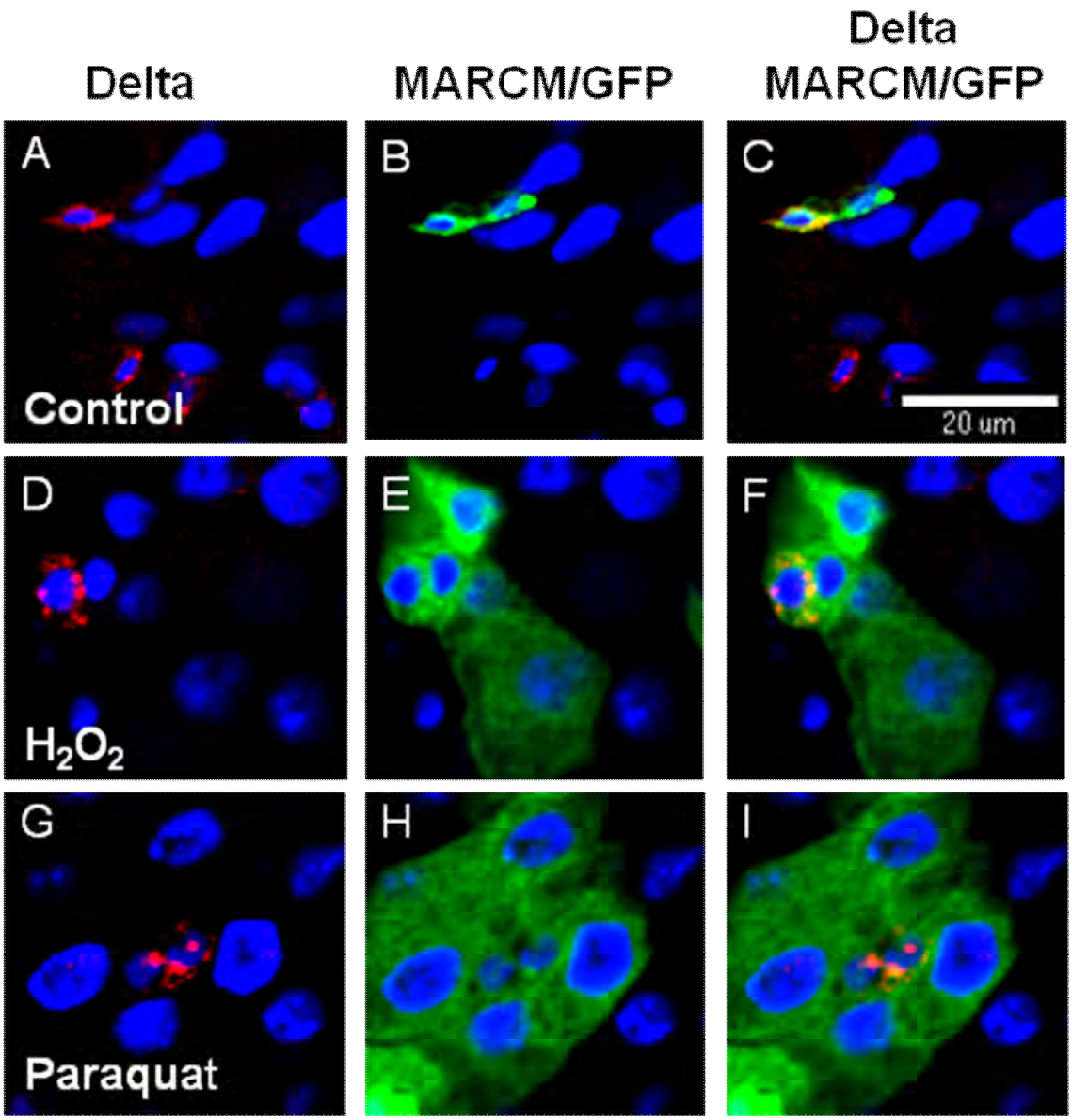

Delta
MARCM/GFP
Delta
MARCM/GFP
A
B
C
Control
20 um
D
E
F
H_2O_2
G
H
I
Paraquat

Fig 2.6 J-O

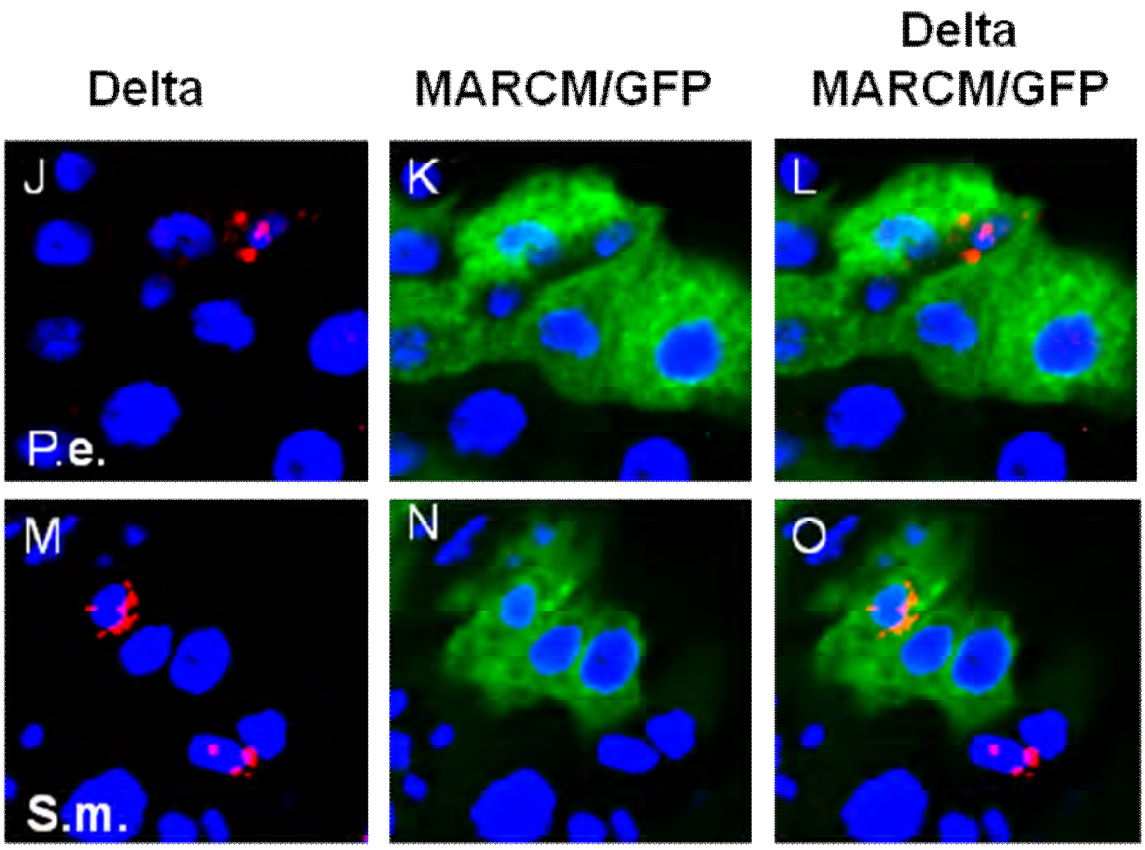
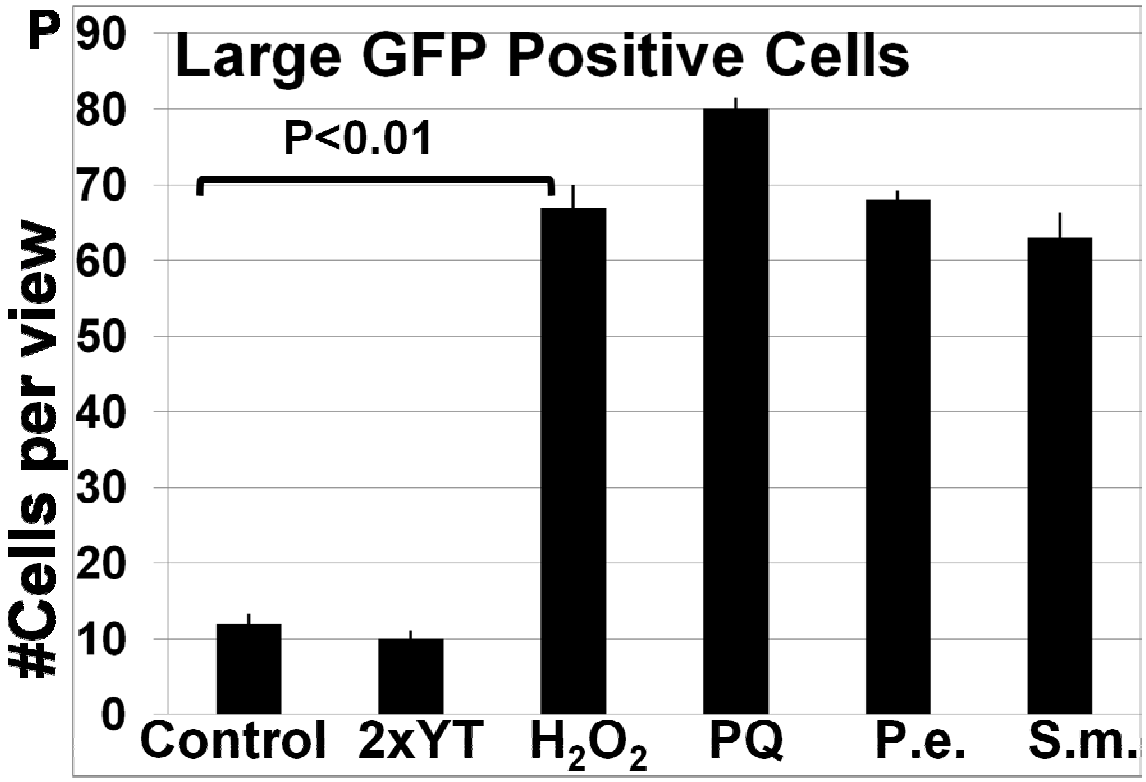


Fig 2.6 P-Q

P


Large GFP Positive Cells
P<0.01
#Cells per view
90
80
70
60
50
40
30
20
10
0
Control
2xYT
H_2O_2
PQ
P.e.
S.m.

Q

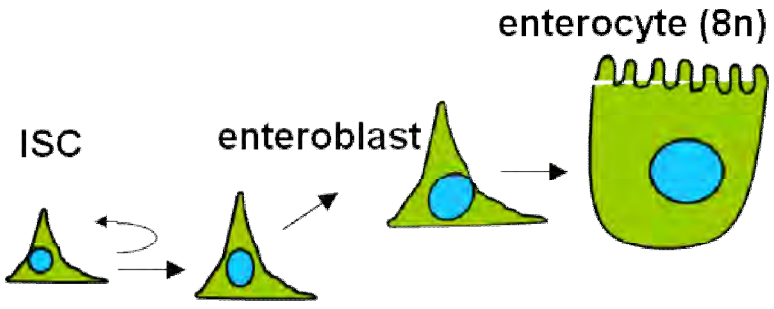

enterocyte (8n)
ISC
enteroblast

Fig 2.6 MARCM clonal analysis shows an increase in enteroblast differentiation.

FLP-FRT-mediated mitotic recombination coupled with the Gal4/Gal80 chromosomes allows one of the two cells of a recent division to expressed GFP. If the newly formed ISC is genetically marked with this GFP expression, all subsequently derived cells will all be GFP positive, thus marking the whole lineage. If the newly formed enteroblast is genetically marked, it will not divide again and the GFP-marked cell will differentiate as an isolated cell. I counted only GFP-positive clusters, thus only events that mark ISCs initially. The guts were also stained for Delta (red). In control guts, the MARCM GFP-positive clusters had one Delta positive cell (A, red staining) and very few GFP positive cells that were also small and should represent enteroblasts. Feeding with any of the 4 reagents increased the number of GFP-positive cells in each cluster (panel D-O), consistent with increased cell division. In isolated clusters, usually one Delta-positive cell was present, suggesting one parental ISC gave rise to the other GFP-positive cells in the cluster. Moreover, the sizes of many of these GFP-positive daughter cells were bigger. Because the control and pathogen feeding were preformed for the same time interval (3 days) and at the same temperature (29ºC), the results suggest that pathogen fed samples have increased enteroblast differentiation into bigger cells. Panel P shows a schematic representation of differentiation from an ISC to a mature enterocyte. Panel Q

shows the counting of large GFP-positive cells per gut in the indicated feeding experiments. Large GFP positive cells are defined as those that do not stain for Delta or Su(H)LacZ. “p” values show the results of the T test results performed between the control and H_2O_2 sets. Error bars represent standard error.

CHAPTER 3

Role of TSG101 in *Drosophila* Intestinal Homeostasis

SUMMARY

Correct tissue development and homeostasis require that cells maintain their fates. Given the crucial role of ISCs in *Drosophila* intestine homeostasis, it is essential that the ISCs maintain their own fate and division potential. Here I show the role of TSG101 in ISC fate maintenance and tissue homeostasis. Using an RNAi approach I have observed that the loss of TSG101 greatly reduces the numbers of ISCs and EE's, severely inhibits mitosis in the intestine, and prevents cell proliferation even after pathogen feeding. A TSG101 mutant line called *erupted2* was used for clonal analysis of TSG101 mutants and showed smaller cell nests. Taken together, I propose that TSG101 is needed for cell division and intestinal homeostasis.

INTRODUCTION

Tissue homeostasis requires regulated receptor signaling and the maintenance of cell polarization. Receptor signaling often involves the endocytIc pathway. Proteins can either be recycled back to the cell surface, or they are internalized in the early endosome which matures into Multi Vesicular Bodies which fuses with lysosomes for proteolytic degradation (Gruenberg 2007). Tumor Suppressor Gene 101 (TSG101) has been known to have a role in tumurogenesis. Dampening TSG101 expression transformed marine 3T3 fibroblasts, causing metastatic tumors in nude mice. (Oh 2007, Ruland 2001).

TSG101 is located on the 3rd chromosome of the fly genome (Fig 3.1). Mammalian and *Drosophila* TSG101 contains an amino terminal ubiquitin (Ub)-conjugating domain. However, due to the active site cysteine in the (Ub)-conjugating domain being replaced by a tyrosine, the fly TSG101 lacks Ub-conjugating activity (Sancho 1998) but can bind monoubiquitinated substrates (Sundquist 2004).

TSG101 plays a role in the endocytIc pathway as a component of the ESCRT1 complex. The ESCRT machinery is known to be involved in various cellular

processes. It has been shown that disrupting ESCRT subunits leads to loss of apico-basal cell polarity, cytoskeletal disruption and increased Notch signaling (Sevrioukov 2005, Vaccari 2005). Clonal analysis of TSG101 mutants has shown an increase in Notch accumulation leading to hyperplasic overgrowth in the surrounding tissues caused by Notch targets JAK STAT pathway (Moberg 2005). On the other hand, the mutant cells themselves are apoptotic (Pfleger 2007). The ubiquitination and endosomal processing of the Notch receptor is a highly regulated process (Mukherjee 2005).

In our studies I actually noted a slowing down of cell proliferation when I used an RNAi line for TSG101. The guts don't even respond to agents previously known to stimulate epithelial cell proliferation. Clonal analysis did not show any overgrowth of surrounding tissues but the clones themselves were smaller than control clones.

Fig 3.1

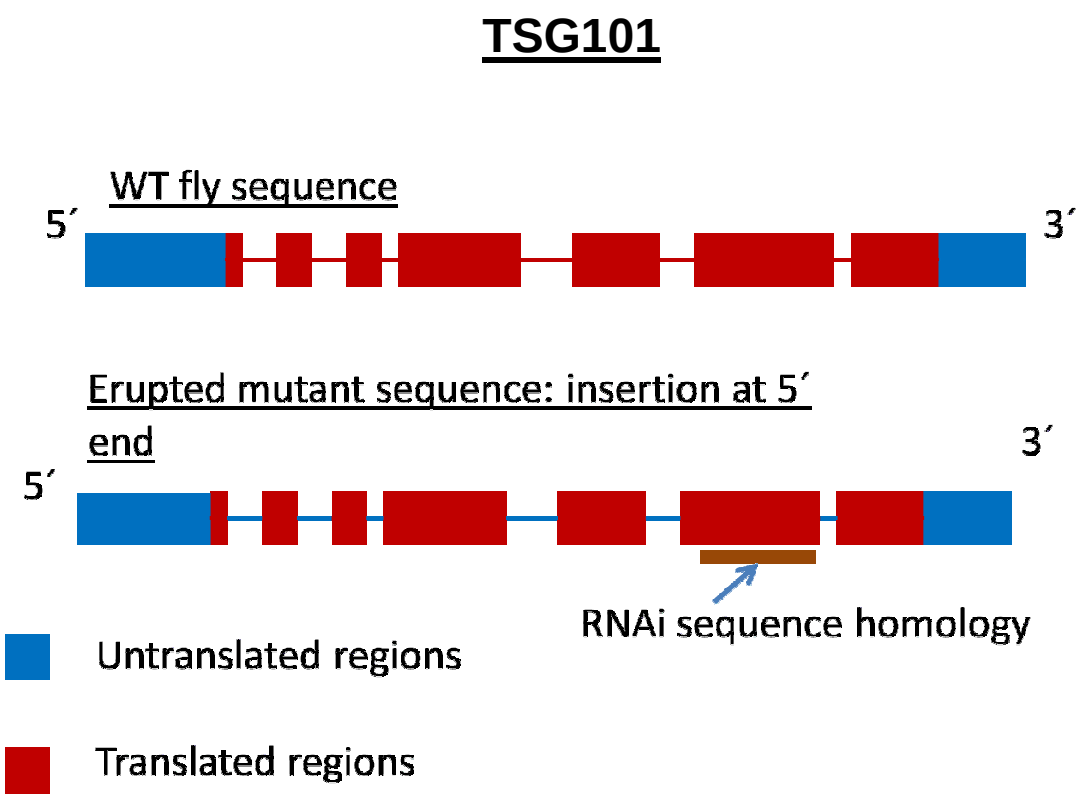


Fig. 3.1 TSG101 Gene Structure

Schematic representation of the TSG101 gene showing exons (rectangles) flanking the introns (lines). The gene is located on the third chromosome and is approximately 2.4 KB in length. The *erupted* mutant sequence has an insertion at the 5' UTR. The RNAi sequence in the RNAi line has sequence homology with a part of the sixth exon.

Fig 3.2

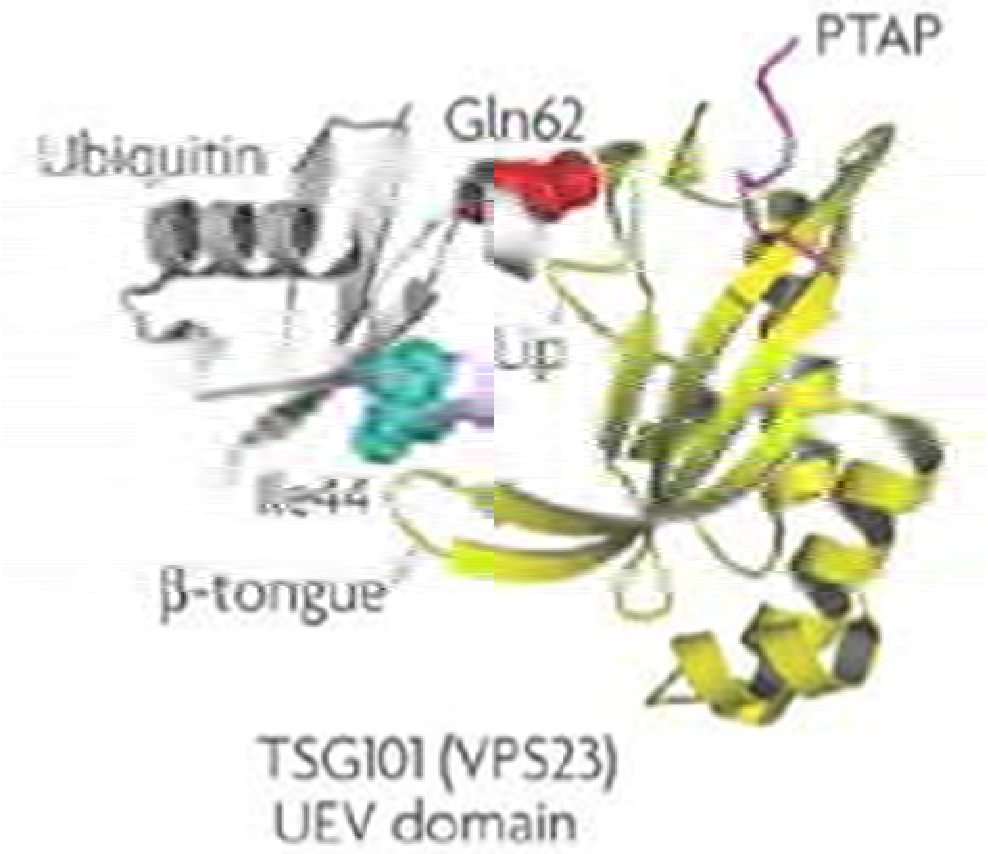


Fig 3.2 TSG101 Protein structure and Ubiquitin binding.

Schematic representation of the interaction between TSG101 and Ubiquitin. TSG101 is shown in yellow, with its UEV, β-tongue and Lip domains pointed out below. Ubiquitin is shown in grey.

Figure adapted from Williams 2007

RESULTS

TSG101 RNAi guts show reduced numbers of progenitor cells

I used an esg GAL4 line to drive TSG101 RNAi expression in ISCs and EBs. The adult RNAi intestine showed a markedly reduced numbers of escargot positive cells (marked with GFP) (Fig 3.3 A-B') when compared to the control fly gut. Areas of the TSG101 RNAi midgut lack esg>GFP cells altogether whereas they are very evenly dispersed in the control line. The 3rd instar larval guts did not show any perceptible difference (Fig 3.3 C-D'). Quantitatively I see that Esg>GFP positive cells show an approximately 2 fold decrease in the RNAi intestine (Fig 3.3 F).

Loss of GFP positive cells correlates with lower overall cell numbers in the RNAi gut compared to the control. This is seen in Fig 3.3 A'-B' and quantified in Fig 3.3 E. As mentioned before esg marks ISCs and EBs

ISCs and EE cell numbers greatly decrease in TSG101 RNAi intestines

Since esg is expressed in both ISCs and EBs, I carried out specific staining to identify the cell type that is reduced in the RNAi gut. As the RNAi guts have fewer

cells overall, I expressed the cell numbers in absolute (cells per view) and relative (compared to total cells per view) terms.

I see that esg positive cells don't show a significant relative decrease in the RNAi line (Fig 3.4 A). Relative EB numbers also don't decline in the TSG101 RNAi midgut. This is shown by Su(H)-LacZ staining in Fig 3.4 E', F' and expressed quantitatively in Fig 3.4 B.

Delta staining shows that ISC numbers decline markedly in the RNAi line in absolute and relative terms (Fig 3.4 C). The relative numbers of ISC positive cells drop 5 fold in the RNAi line. The remaining ISCs in the RNAi line also show reduced Delta staining individually (Fig 3.4 F'' arrows). Therefore, it is the ISCs that mainly contribute to the decline in esg positive cells in the RNAi intestine. EE cells are practically absent in the RNAi line (Fig 3.4 D,G,H).

Due to the lower number of ISCs the RNAi intestine generally lacks cell nests. Esg>GFP cells in the RNAi line mostly appear singly and often have altered cell shapes compared to the control progenitor cells (Fig 3.4 E- F'''). In the control I mostly see esg>GFP positive cells in pairs or clusters with a Delta positive ISC

and a Su(H)-lacZ positive EB next to each other (arrows in Fig 3.4 E, E' E''' E''''). Taken together I observe a cell proliferation defect in the TSG101 RNAi intestine.

TSG101RNAi intestine lacks cell division and does not respond to pathogen feeding:

To verify the cell proliferation defect I suspected in the previous section, I carried out PH3 staining in the intestine. The RNAi intestine had no PH3 staining. Since mitosis is a transient event, PH3 staining is often low in control guts. Therefore, I fed the flies with the pathogenic agents that are known to stimulate mitosis in the intestine. I used *Serratia marcescens (S.m.)*, which has been described in Chapter 2, and DSS, which causes ulcerative colitis in mammals and has been shown to greatly increase PH3 counts in fly guts (Amcheslavsky 2009). Feeding these pathogens also failed to induce proliferation in the intestine compared to the control. I used sucrose as the control for DSS feeding whereas bacterial medium 2xYT served as the control for S.m. feeding in both sets of flies (Fig 3.5).

TSG101 knockdown in adult flies showed increasing EB numbers

I used a temperature shift assay to knock down TSG101 in adult flies. Esg is expressed all through the fly's development and I used esgGAL4 to drive the TSG101 RNAi. So the TS experiment can show us some intermediate phenotypes. I crossed in Tubulin Gal80^{ts} and shifted adult flies to 29ºC dissecting guts at various intervals. After 9 days of TS the flies showed the TSG101 phenotype like the previous line with no Tubulin Gal80^{ts}. Control flies show increased cell division when they are kept at 29ºC for longer durations (Fig 3.6 C). The RNAi line shows no such cell division increase (3.6 B, D). The numbers of esg>GFP positive cells in the RNAi line don't increase with TS (Fig 3.6 E). After staining I saw that ISC numbers begin to decline in the RNAi line as the TS progresses, becoming lower and lower than the control line (Fig 3.6 G). EE numbers also begin to show a decline as the TS progresses (Fig 3.6 B, D, and F).

EBs remained the only cell type in the RNAi gut to show an increase over the TS duration. EB numbers, while still lower than in the control guts showed a regular increase over the 9 days of TS (Fig 3.6 H). These results show that EBs is the only cell type that is maintained and increases in number in the TSG101 RNAi midgut.

Delta staining is lost from esg positive cells in TSG101 RNAi guts

In the control fly gut, all esg positive cells are either ISCs staining for Delta or EBs staining for Notch target gene Su(H)-lacZ as shown by arrows and an enlarged area in Fig 3.7 A-A''. However the RNAi line has some small, faint GFP positive cells that don't stain for Delta and are also not Su (H)-lacZ positive. I am unsure if these were former ISCs that have lost their fate and Delta expression thus becoming quiescent or if they were headed towards apoptosis.

TSG101 mutant mimics the RNAi cell proliferation defect

To corroborate our RNAi results I used a TSG101 mutant line for clonal analysis. The mutant is called *erupted2*. The *ept2* allele contains an approximately 8 kb insertion that disrupts the continuity of sequences within the 5' UTR region of TSG101 (Moberg 2005). Since the *ept* mutant is lethal, I used a MARCM approach described in chapter 2 to analyze mutant clones in the adult midgut. The mutant clones were also rendered GFP positive by losing the Tubulin $Gal80^{ts}$ repressor due to FLP recombination. A control construct FRT80B PIM75C was used for comparison.

I see that after HS and recovery the mutant clones are smaller than the control clones (Fig 3.8). Fig 3.8 A shows the number of cells in each mutant and control clone and B shows the average number of cells per clone. Representative pictures in Fig 3.8 C-D'' show the smaller mutant clone of GFP positive cells versus the larger control gut clone of GFP positive cells.

DISCUSSION

Endocytosis is an important regulator of cellular processes. Notch signaling has been shown to be affected by ubiquitination and endosomal sorting of the activated, internalized Notch receptor (Gupta-Rossi 2004). TSG101 controls Notch signaling in developing fly tissues so its knockdown causes non-cell-autonomous overgrowth. However the role of Notch in the organism can be highly context dependent and Notch and Delta signaling have to be maintained at different levels in the fly intestine to preserve the asymmetry of ISC division.

I see that TSG101 RNAi lines lack ISCs and EEs. Both these cell types are increased in number when Notch is knocked down in the fly gut (Liu 2010). Loss of ISC fate is the main result of Notch up regulation. Notch signaling also makes the newly formed EB to choose the EC differentiation pathway and not become EE (Liu 2010). It is therefore possible that Notch is also up regulated in our RNAi system. The RNAi line showed no phenotype in the larval intestine.

The TSG101 RNAi is driven by esgGAL4 to see its effects on intestinal progenitor cells. However esg is expressed from the embryonic stage of the flies so I felt that the observed RNAi phenotype could be the end stage of a

complicated mechanism spanning the embryonic, larval and pupal stages. To rectify this, I performed a temperature shift (TS) assay where the RNAi was only activated in 5 day old adult flies. I saw the phenotype develop slowly as TS progressed with decreasing ISC and EE cells while the esg cell numbers remained stable. The EBs actually show an increase as TS progresses which explains the stable numbers of esg positive cells as ISCs are gradually reduced (Fig 3.5). Activation of Notch signaling requires Delta which is a Notch ligand. The presence of Su (H) LacZ cells in the RNAi line can be explained in different ways. Firstly, there are instances of Notch signaling mediated by ADAM metalloproteases occurring in a Delta independent manner in *Drosophila* cell cultures (Delwig 2008). Secondly, we may only be seeing the Lac Z staining as an artifact of previously present Delta-activated Notch signaling and the Notch pathway was not active while the cells were still staining for Lac Z.

The TSG101 mutant did not show the hyperplasic phenotype in clonal analysis. The wild type cells surrounding the *ept2* mutant clones look similar to those surrounding the control clones (Fig 3/7 C-D'') but the mutant clones themselves showed slower cell proliferation compared to the control. This corroborates the cell proliferation defect seen in the RNAi line and is in line with published observations about TSG101 mutant cells (Moberg 2005).

Loss of ISCs in the TSG101 RNAi line can occur in 2 ways: the ISCs could become terminally differentiated into ECs due to Notch signaling or they could simply undergo apoptosis. The RNAi line showed some small esg>GFP positive cells that don't stain for Delta or Su (H)-lacZ. So based on our current definitions they are neither ISCs nor EBs. They cannot be EE since they don't stain for Prospero and EE cells would lack esg>GFP anyway. I hypothesize that these are former ISCs who have lost Delta and therefore their ISC fate rendering them incapable of cell division. Since I rarely see these cells, I would conclude that these cells could slowly undergo apoptosis.

Although the RNAi line does not respond to pathogen feeding by increasing ISC division as the control flies do, there nothing to suggest that TSG101 is directly involved in the gut response to pathogens. The RNAi gut is so severely depleted of ISCs in the first place that the loss of ISC maintenance seems a more plausible explanation.

Taken together, our results show that TSG101 plays an important role in the adult *Drosophila* intestinal homeostasis. The exact mechanism is not clear but it seems to help maintain ISC fate, which is crucial for preserving tissue responses to injury and repair.

MATERIALS AND METHODS

Drosophila stocks and feeding experiments

UAS-mCD8GFP and UAS TSG101 RNAi flies were obtained from the Vienna *Drosophila* RNAi Center; FRT80B, pIM75c, esg-Gal4 and Su (H) Gbe-lacZ were as described (Xu 1993, Micchelli 2006; Ohlstein 2007). RNAi sequence homology is shown in Fig 3.1. *FRT80B, erupted2* flies were a kind gift from Kenneth Moberg (Emory University). Flies were crossed to generate the following lines:

- Esg-Gal4 UAS-mCD8GFP/ UAS TSG101 RNAi; Tubulin Gal80^{ts}/ Tubulin Gal80^{ts} for Temperature shift experiments with esg-Gal4 UAS-mCD8GFP/ Cyo; Tubulin Gal80^{ts}/ Tubulin Gal80^{ts} as Its control.
- esg-Gal4 UAS-mCD8GFP/ UAS TSG101 RNAi and Su(H)Gbe-lacZ/X; esg-Gal4 UAS-mCD8GFP/ UAS TSG101 RNAi for the RNAi line experiments with Su(H)Gbe-lacZ/X; esg-Gal4 UAS-mCD8GFP/Cyo and esg-Gal4 UAS-mCD8GFP/Cyo as Its control lines respectively.
- hsFLP,UASCD8GFP/X; esg-Gal4/Cyo; FRT80B,erupted2/FRT80B Tubulin Gal80^{ts} for the mutant clone analysis with hsFLP,UASCD8GFP/X; esg-Gal4/Cyo; FRT80B,pIM75c/FRT80B Tubulin Gal80^{ts} as Its control.

For the mutant clone analysis flies were grown at 29ºC and heat shocked when they were 3 day old adults. HS was done for 30 minutes 2 times a day at 37ºC for 3 days and flies were left to recover at 29ºC for 7 days before gut dissection.

For the TS experiments, flies were allowed to mate and lay embryos at 25ºC for 5 days. The larvae were allowed to develop further and adults hatched at 18ºC. 5 day old adults were shifted to 29ºC and dissected on days 0 (before shift), 3, 6 and 9.

Feeding experiments were as described in chapter 1. 3% Dextran Sulfate Sodium was used in a 5% sucrose solution and *Serratia marcescens* was used at $3x10^6$ CFU. Control for DSS feeding is 5% sucrose and 2xYT in 5% sucrose served as the bacterial feeding control. Guts were dissected after 3 days of feeding at 29ºC.

Immunoflourescent staining and microscopy

Female flies were used for gut dissection, because of the bigger size but male flies were also used occasionally to check the phenotypes. Dissection and staining protocols and the antibodies used are described in Chapter 1.

Fig 3.3 A-D'

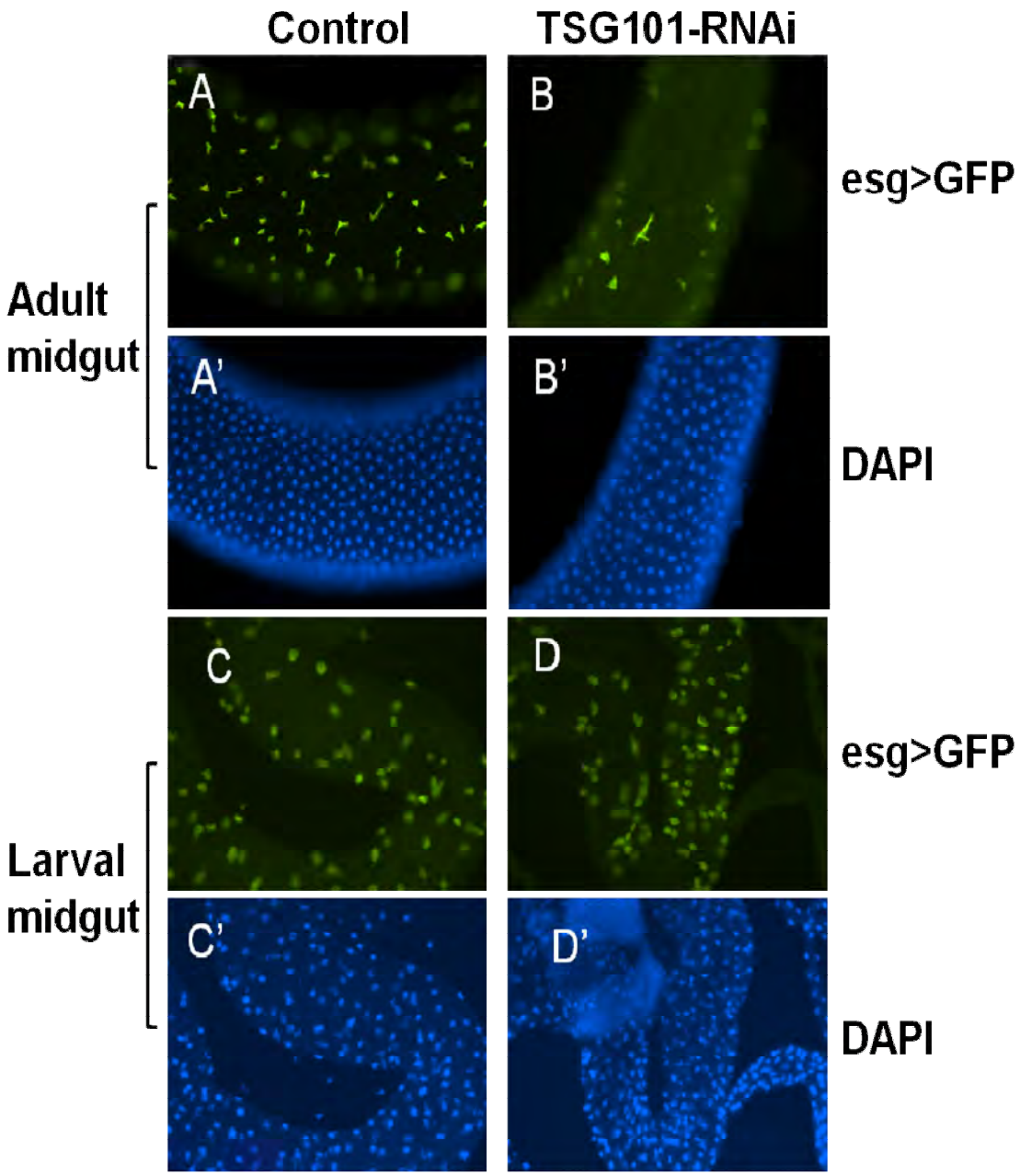

Control
TSG101-RNAi
A
B
esg>GFP
Adult midgut
A'
B'
DAPI
C
D
esg>GFP
Larval midgut
C'
D'
DAPI

Fig 3.3 E-F

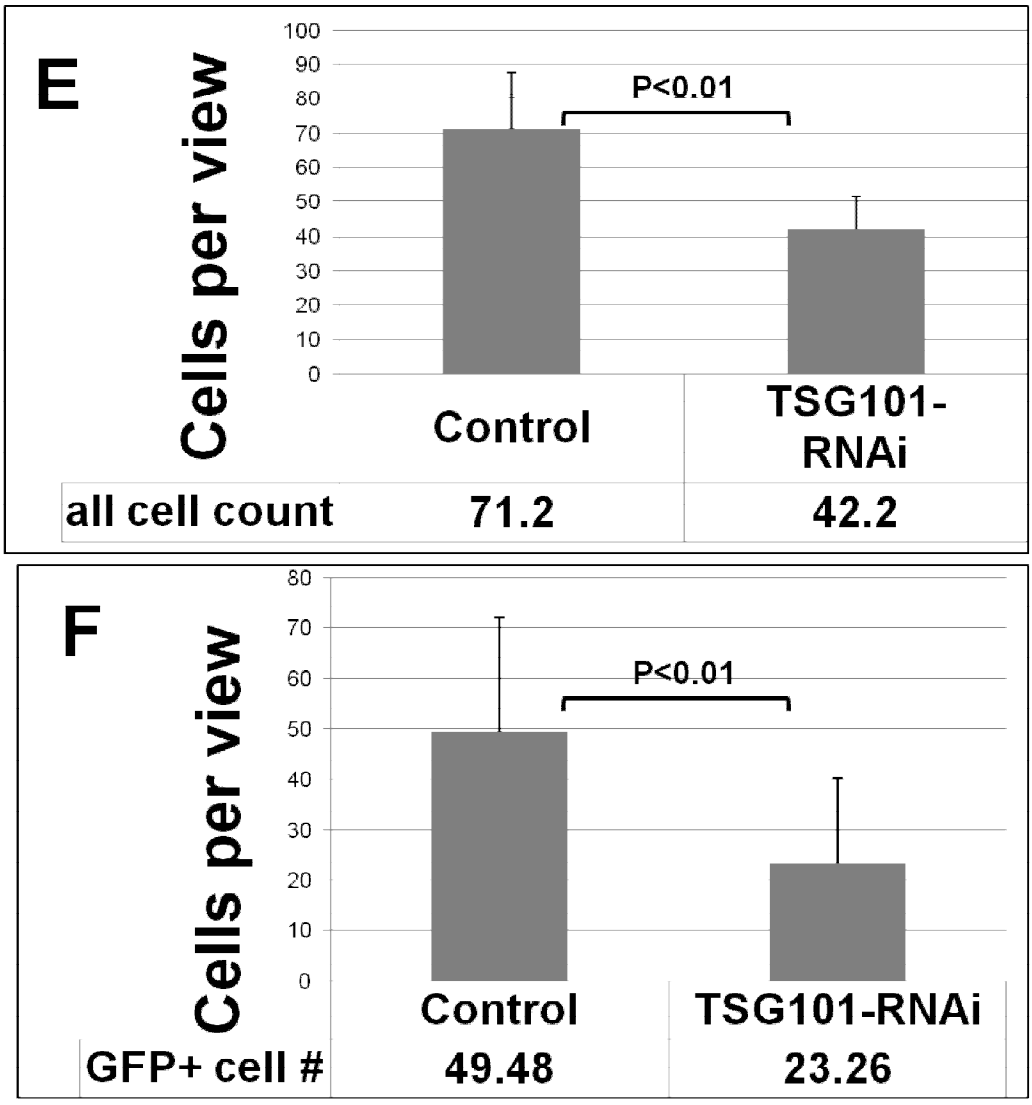
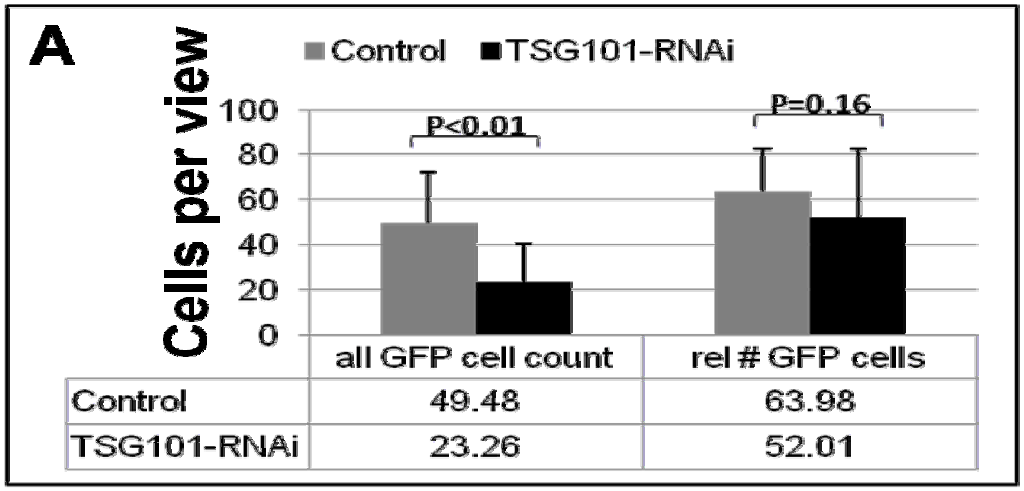

E
Cells per view
100
90
80
70
60
50
40
30
20
10
0
P<0.01
Control
TSG101-RNAi
all cell count
71.2
42.2
F
Cells per view
80
70
60
50
40
30
20
10
0
P<0.01
Control
TSG101-RNAi
GFP+ cell #
49.48
23.26

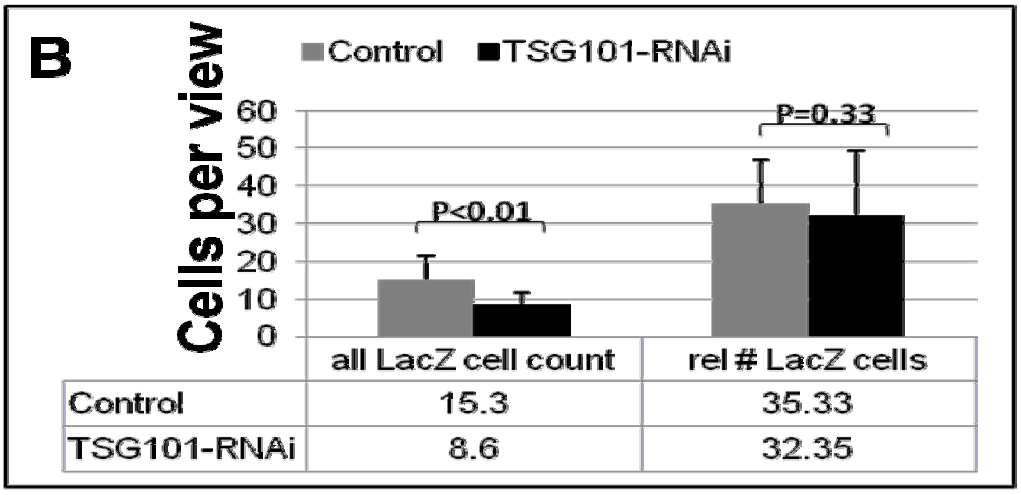
Fig 3.3: Fewer epithelial cells in the adult Escargot-driven TSG101 RNAi fly intestine.

Whole mount of adult fly intestines (A-B') and larval intestines (C-D'). The control adult intestine (A) esg-Gal4/UAS-CD8GFP shows the normal number and distribution of escargot driven GFP positive cells. The number is severely reduced in B. The blue staining in all pictures is DAPI DNA staining. The 4^{th} larval intestines don't show any such phenotype. Quantification is shown for all cells (E) and GFP positive cells (F) which marks ISCs and EBs. Cells are counted per view. Overall there is a 50% reduction in ISC and EB numbers in the RNAi line. Error bars represent standard error. P values are derived from the Student's T test perfomed between control and RNAi samples.

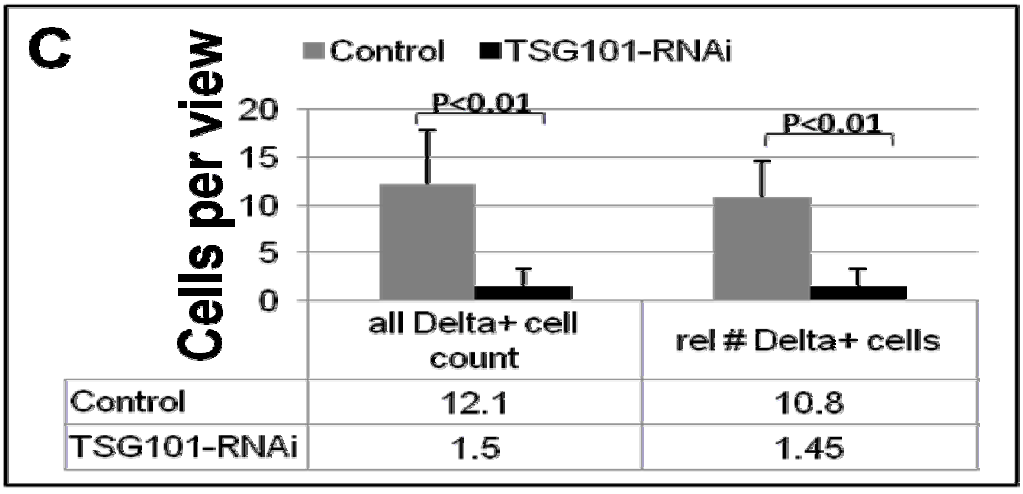
Fig 3.4 A-C



	all GFP cell count	rel # GFP cells
Control	49.48	63.98
TSG101-RNAi	23.26	52.01



	all LacZ cell count	rel # LacZ cells
Control	15.3	35.33
TSG101-RNAi	8.6	32.35



	all Delta+ cell count	rel # Delta+ cells
Control	12.1	10.8
TSG101-RNAi	1.5	1.45

Fig 3.4 D

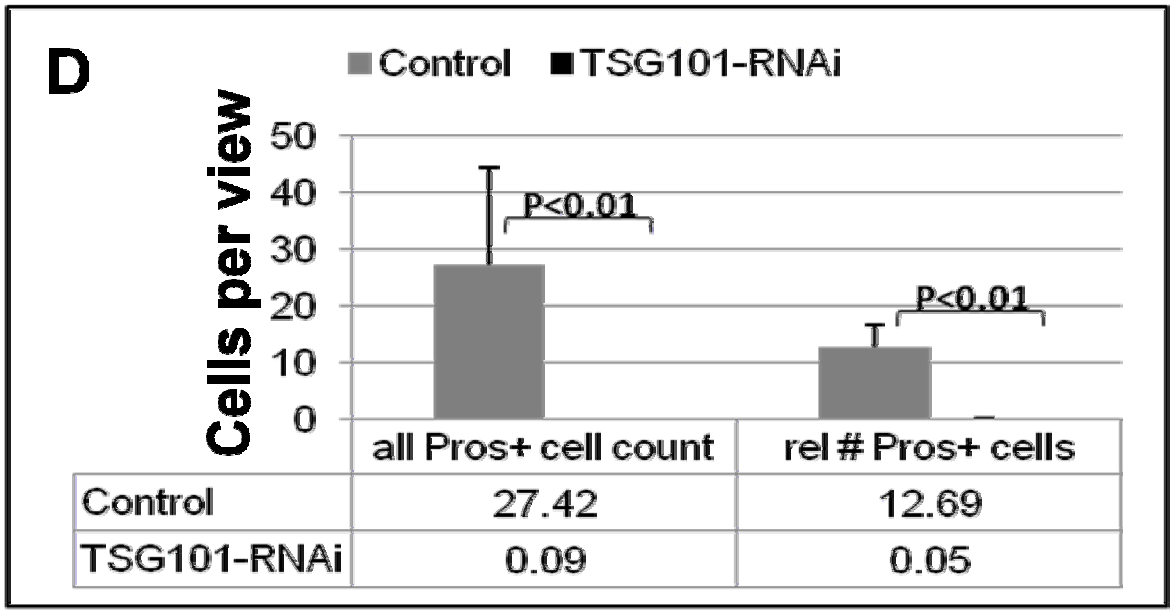


Fig 3.4 (A-D): ISCs and Enteroendocrine cell numbers greatly decrease in TSG101 RNAi intestines.

Quantification of total (per view) and relative numbers of various cell types in control and TSG101RNAi intestines. A: GFP positive cells represent ISCs and EBs. B: LacZ positive cells are Su (H) positive and represent EBs. C: Delta positive cells are ISCs and (D). Prospero positive cells are enteroendocrine cells or EE. Error bars represent standard error. P values show the result of the Student's T test performed between control and RNAi data sets.

Fig 3.4 E-F''

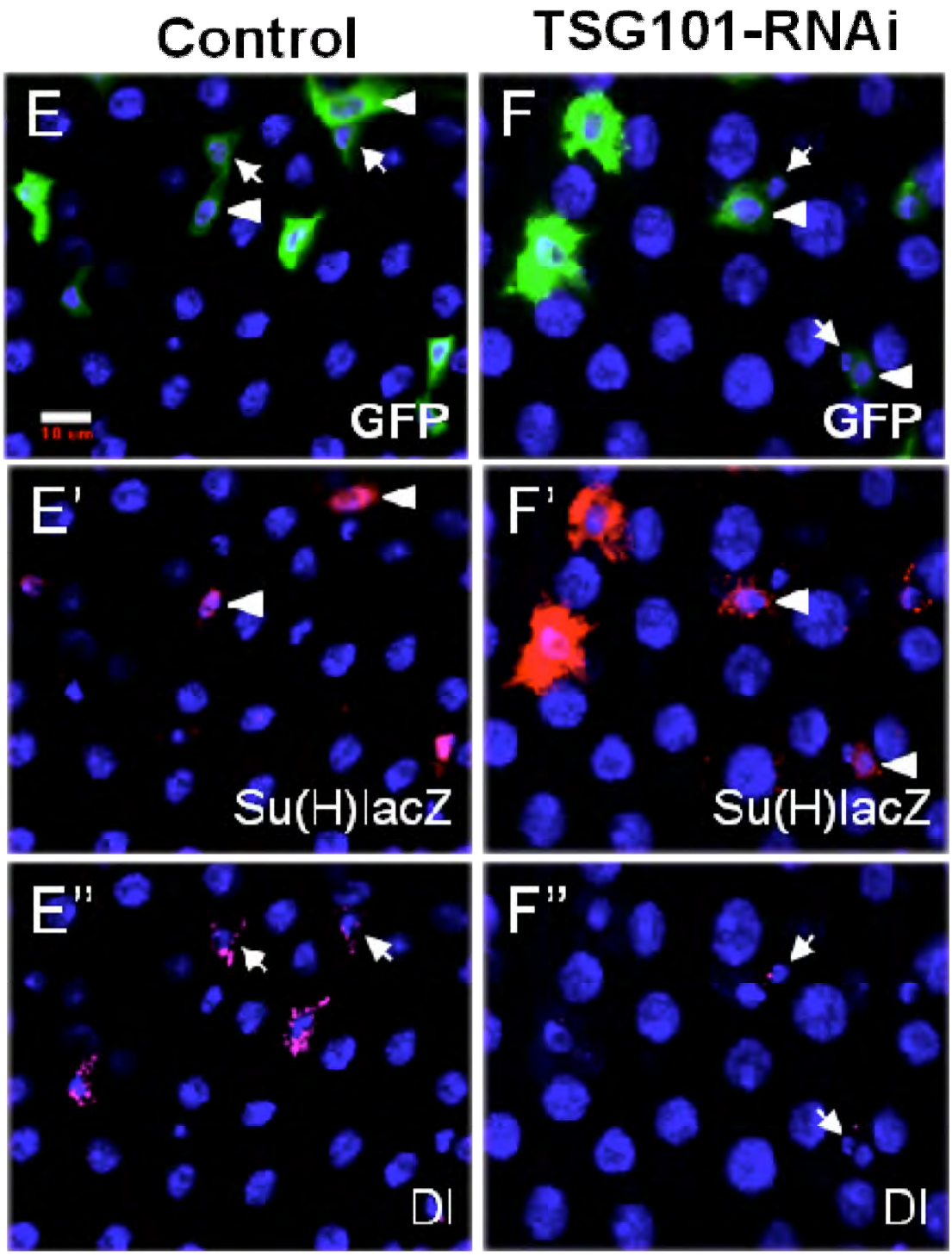


Fig 3.4 E'''-F'''

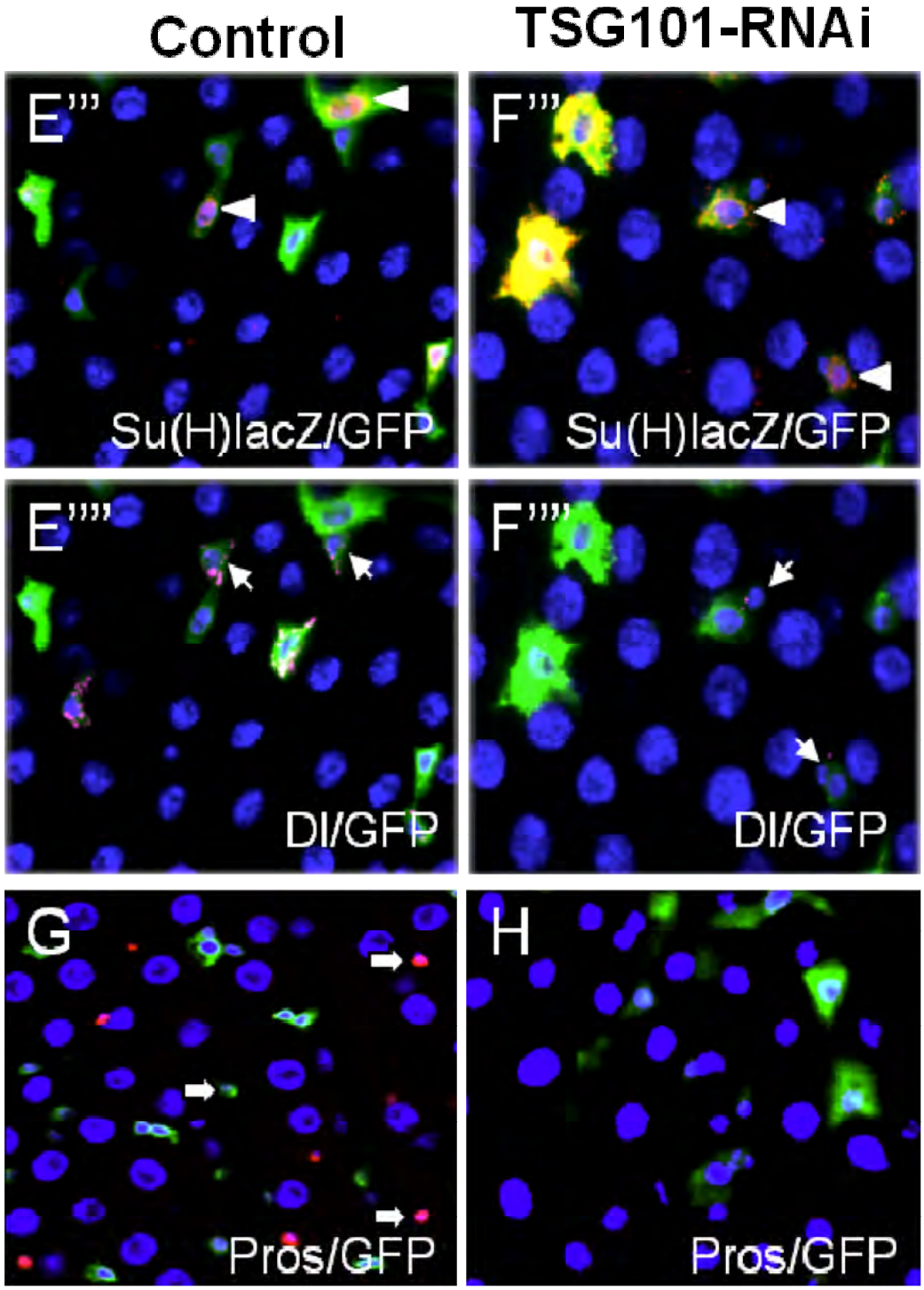

Control
TSG101-RNAi
E'''
Su(H)lacZ/GFP
F'''
Su(H)lacZ/GFP
E''''
Dl/GFP
F''''
Dl/GFP
G
Pros/GFP
H
Pros/GFP

Fig 3.4 E-H: Loss of cell nests In TSG101RNAi intestine.

Panels E-H depict morphological differences between control and RNAi intestines. GFP marked Esg positive cells mostly occur singly in the RNAi gut (F) compared to control (E). The RNAi line shows intestinal EBs which are stained red and marked by arrows as in control (E'-F'). ISCs are greatly reduced in the TSG101RNAi gut as marked by arrows and stained red in E''-F''. The level of Delta also appears lower in the few TSG101 ISCs. Panels E'''-F'''' show overlays of red stained EB's (E'''-F''') or red stained ISCs (E''''-F'''') with green stained esg positive cells that mark both ISC and EB and blue stained DNA. Note that in the control ISC and EB mostly appear in pairs (arrows in E''' and E'''') while in the RNAi line they are located singly (F'''-F''''). Panels G and H show that prospero positive EE's are absent in the RNAi line. They are shown in the control intestine by red staining and arrows (G). Scale bar shown in E is 10µm.

Fig 3.5

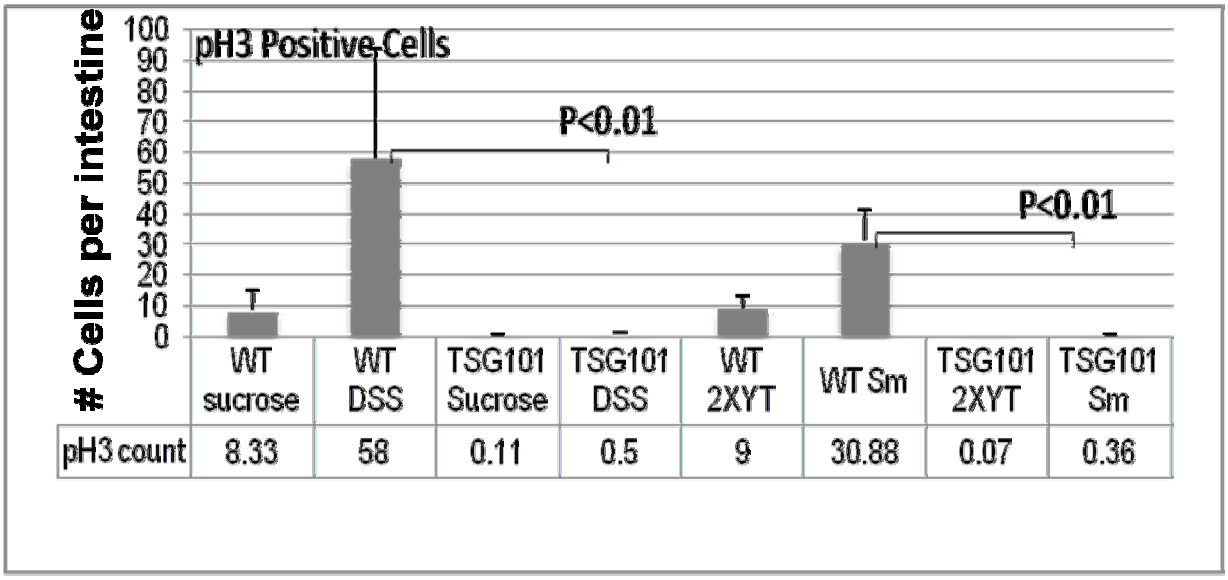


Fig 3.5: TSG101RNAi intestine lacks cell division and does not respond to pathogen feeding.

The RNAi intestine shows no PH3 staining. Feeding pathogens known to cause intestinal cell division (DSS and *Serratia marcescens*) also fail to induce proliferation in the intestine compared to the control. Sucrose is the control for DSS feeding whereas bacterial medium 2xYT is the control for S.m. feeding in both sets of flies. Error bars show standard error. P values show the result of the Student's T test performed between control and RNAi data sets.

Fig 3.6 A-D

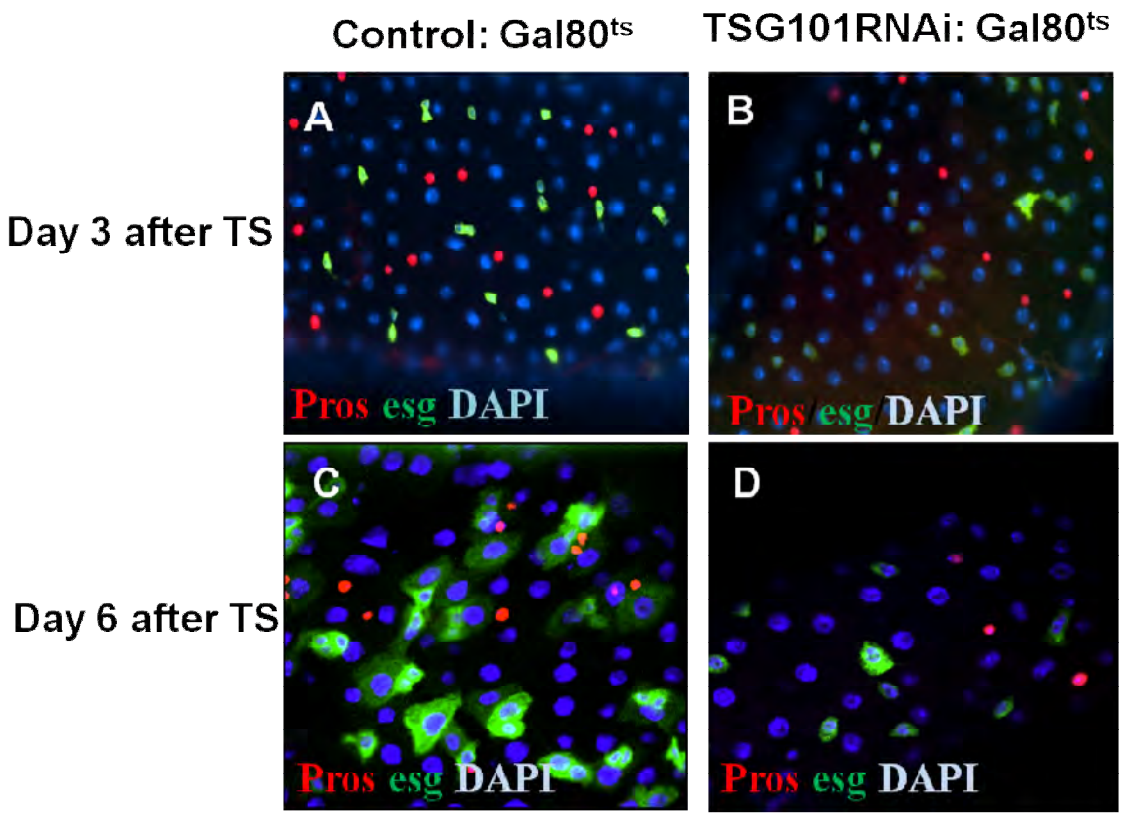

Control: Gal80ts
TSG101RNAi: Gal80ts
Day 3 after TS
Day 6 after TS
A
B
C
D
Pros esg DAPI
Pros esg DAPI
Pros esg DAPI
Pros esg DAPI

Fig 3.6 A-D: TSG101RNAi phenotype emerges after temperature shift.

Control flies of genotype esgGal4UASCD8GFP/Cyo; TubulinGal80^{ts} and flies carrying the RNAi line (UAS TSG101RNAi/ esgGal4UASCD8GFP, TubulinGal80^{ts}) were grown at 18ºC and shifted to 29 ºC as 5 day old adults. Guts dissected on days 3 and 6 show that the RNAi line has reduced prospero positive cells (stained red) compared to the control and in D the gut shows no cell proliferation and greatly reduced esg positive cells (stained green) compared to control (C).

Fig 3.6 E-F

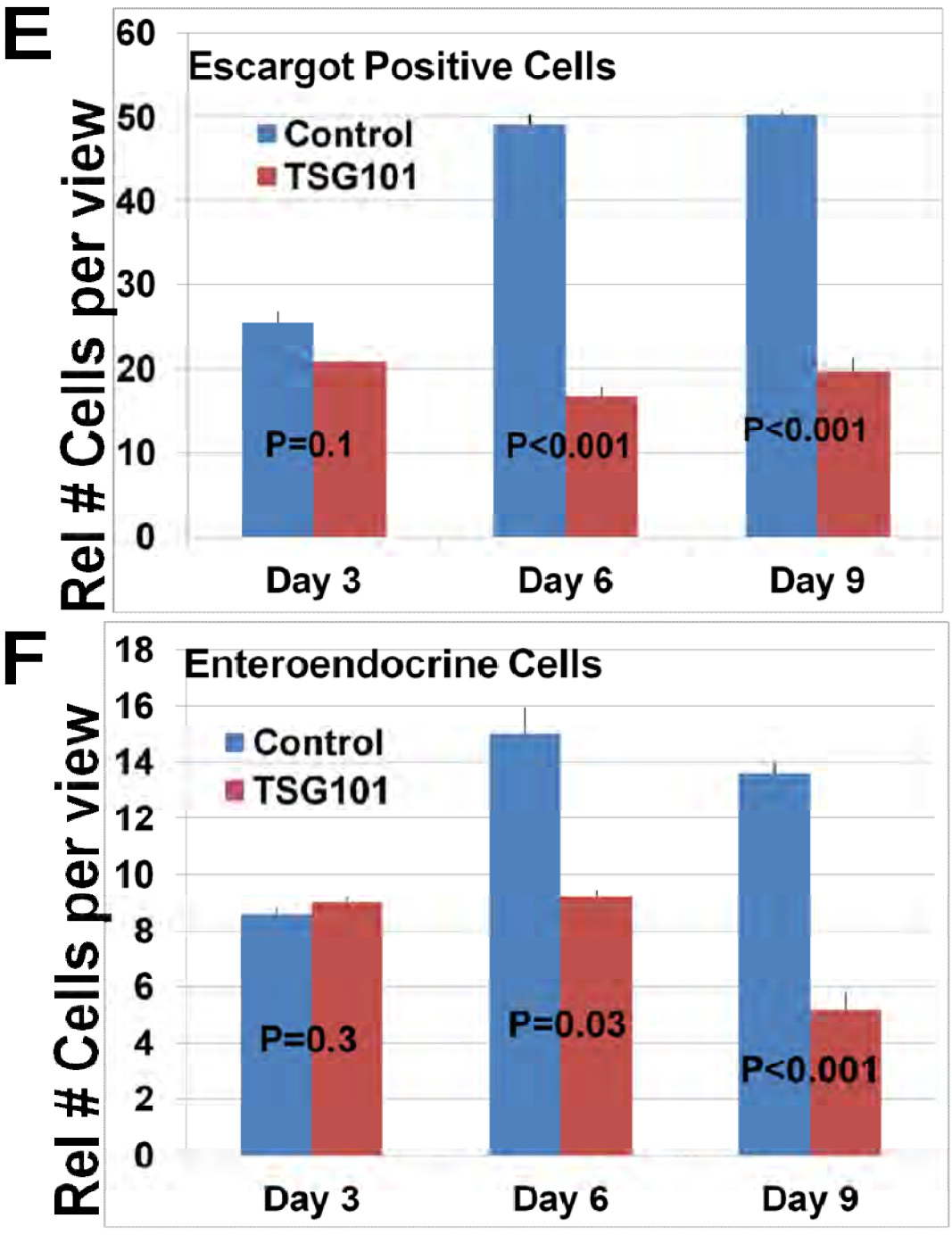

E
Escargot Positive Cells
Rel # Cells per view
60
50
40
30
20
10
0
Control
TSG101
P=0.1
P<0.001
P<0.001
Day 3
Day 6
Day 9
F
Enteroendocrine Cells
Rel # Cells per view
18
16
14
12
10
8
6
4
2
0
Control
TSG101
P=0.3
P=0.03
P<0.001
Day 3
Day 6
Day 9

Fig3.6G-H

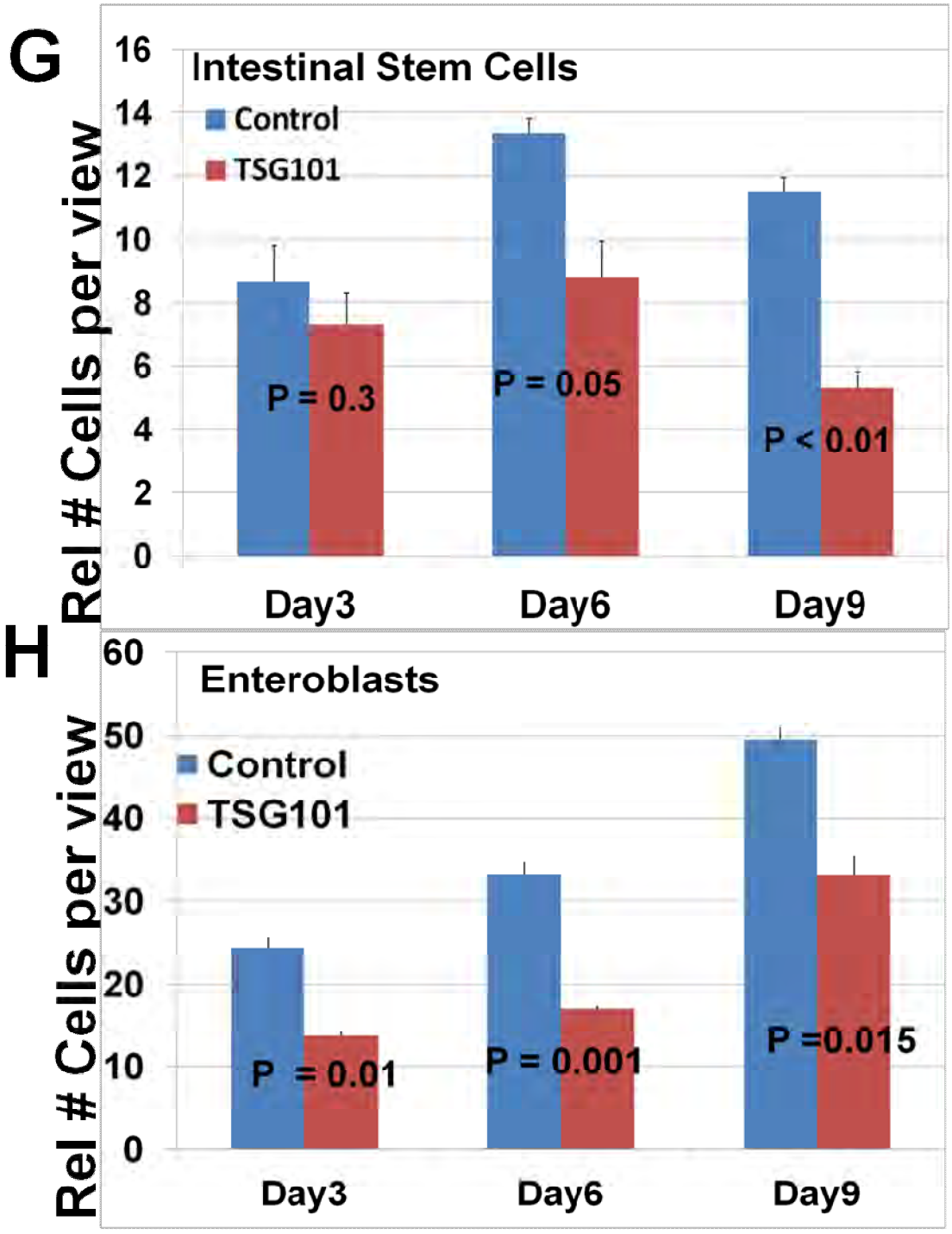

G
Intestinal Stem Cells
Control
TSG101
Rel # Cells per view
16
14
12
10
8
6
4
2
0
P = 0.3
P = 0.05
P < 0.01
Day3
Day6
Day9
H
Enteroblasts
Control
TSG101
Rel # Cells per view
60
50
40
30
20
10
0
P = 0.01
P = 0.001
P =0.015
Day3
Day6
Day9

Fig 3.6 E-H: Enteroblasts are the only cell type maintained in TSG101 RNAi guts.

Temperature shift experiments were performed on control flies (genotype esgGal4 UASCD8 GFP; Tubulin Gal80^{ts}) and the RNAi line (esgGal4 UAS CD8 GFP/UAS TSG101 RNAi; Tubulin Gal80^{ts}) with different cell types counted on days 0, 3, 6 and 9 after the shift from 18ºC to 29ºC. Relative numbers of Esg positive cells (ISC an EBs) don't increase in the RNAi line as TS progresses compared to the control (E). F: EE cell numbers show a slight decline in the RNAi gut compared to the control as TS progresses. G: TSG101 RNAi gut shows progressively lower ISC numbers compared to control. H: EB numbers in TSG101 RNAi guts while still lower than control, increase as TS progresses. EB's are the only cell type to show an overall increase with TS. Error bars show standard error.

Fig 3.7

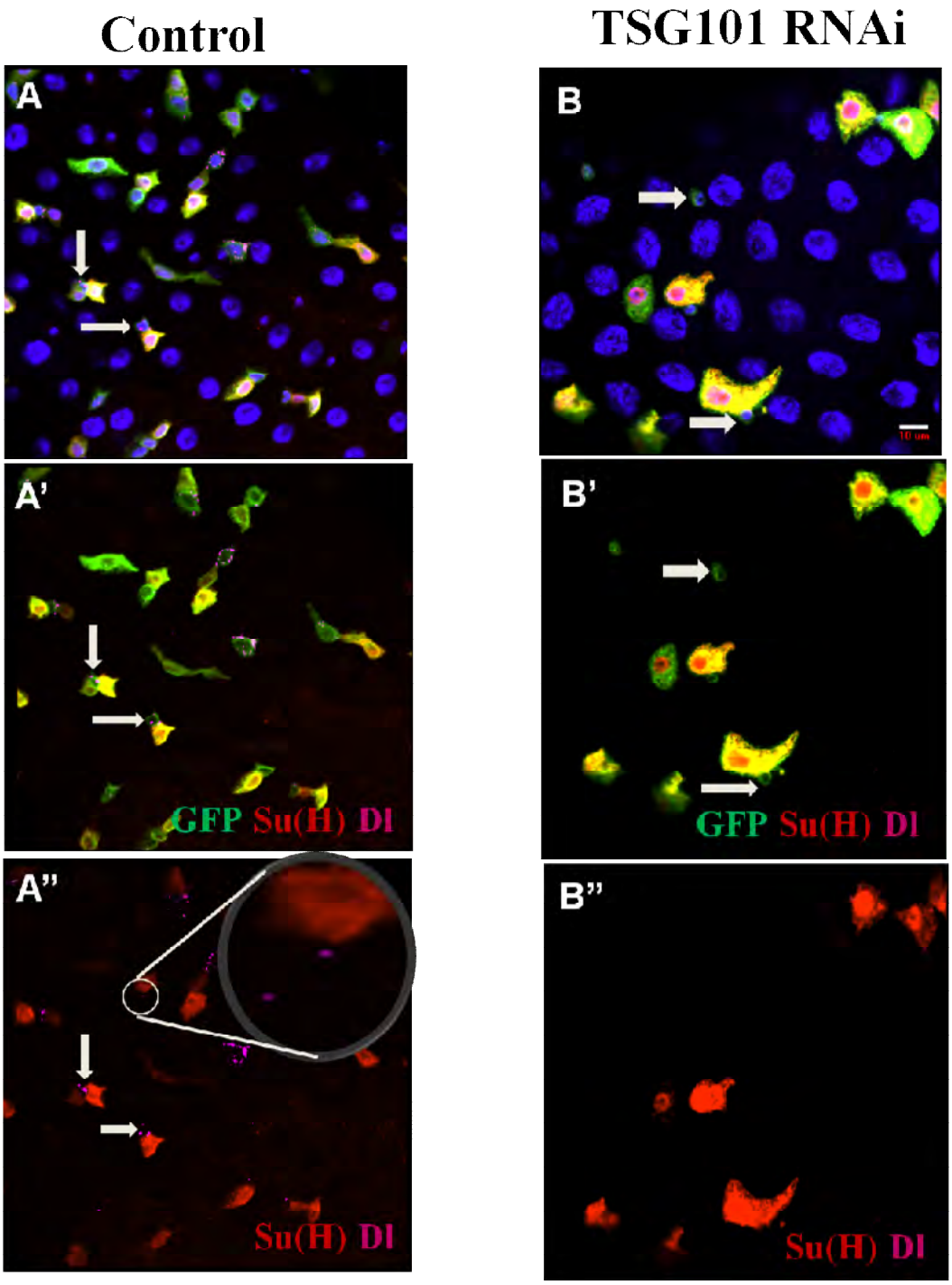
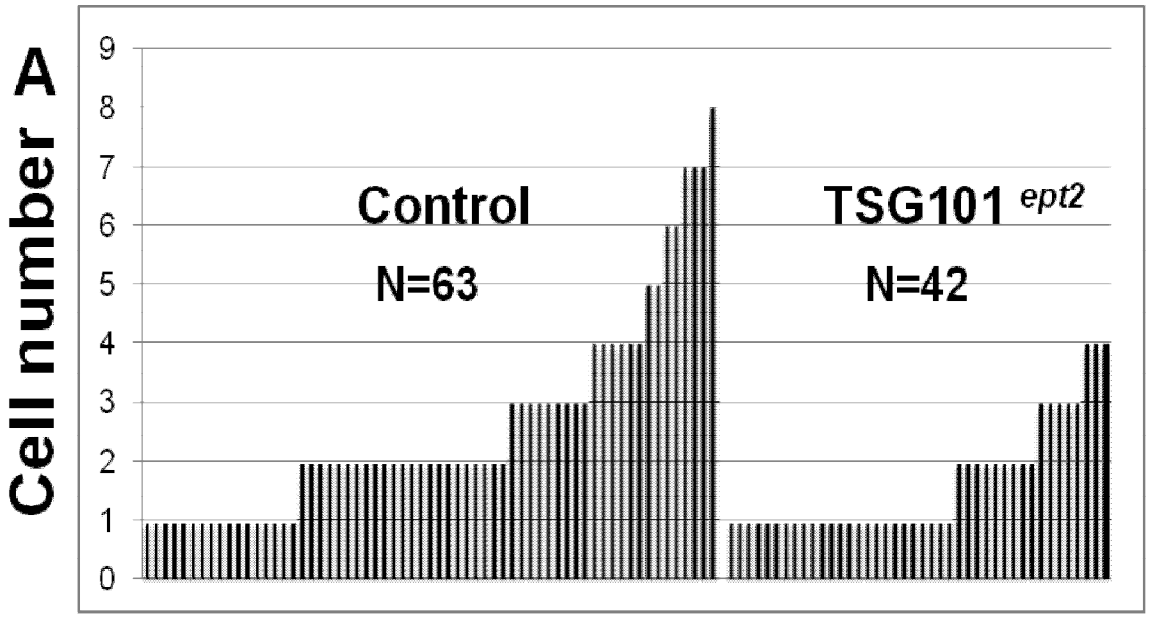


Fig 3.7: Delta staining is lost from esg positive cells in TSG101 RNAi guts.

Delta (pink) and Su(H) LacZ (red) co-staining in control (A-A'') and RNAi (B-B'') guts. in the control gut, all esg driven- GFP positive cells have Delta (pink) or Su(H) lacZ (red) staining as shown by arrows and enlarged area in panel A''. In the TSG101 RNAi gut, some small and faint GFP positive cells (arrows in B and B') lack Delta and Su(H) lacZ (red) staining.

Fig 3.8 A-B


A
Cell number
9
8
7
6
5
4
3
2
1
0
Control
N=63
TSG101 ept2
N=42

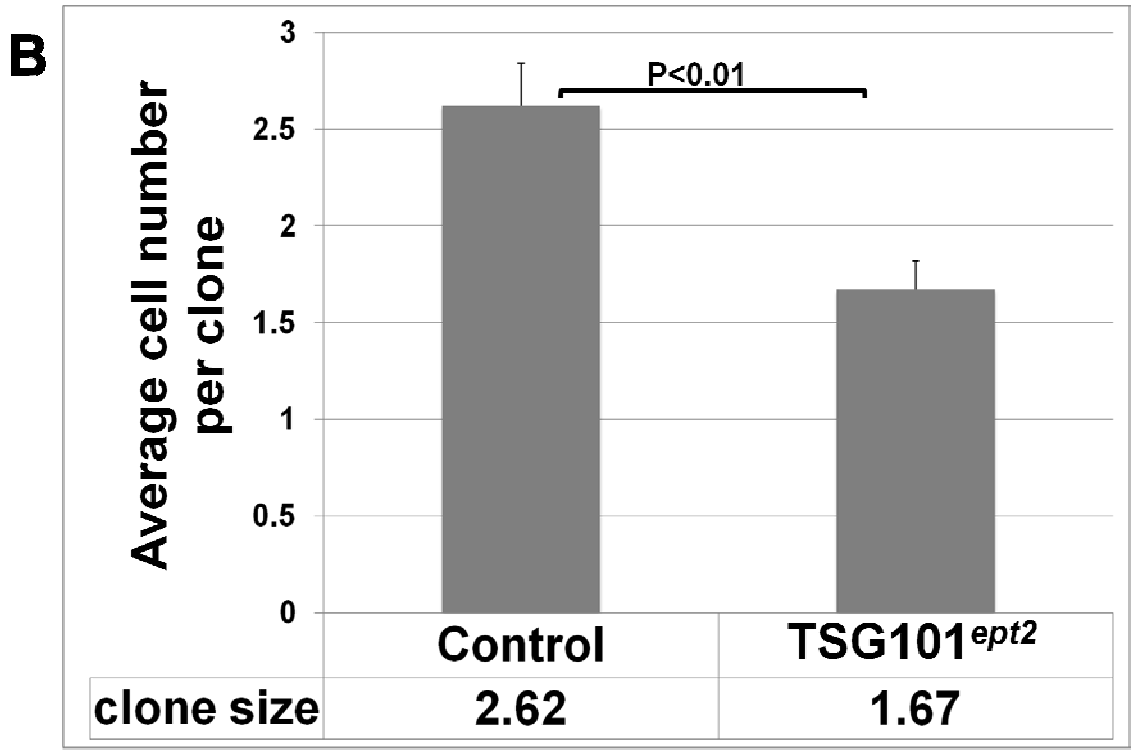

B
Average cell number per clone
3
2.5
2
1.5
1
0.5
0
P<0.01
Control
TSG101 ept2
clone size
2.62
1.67

Fig 3.8 C-D''

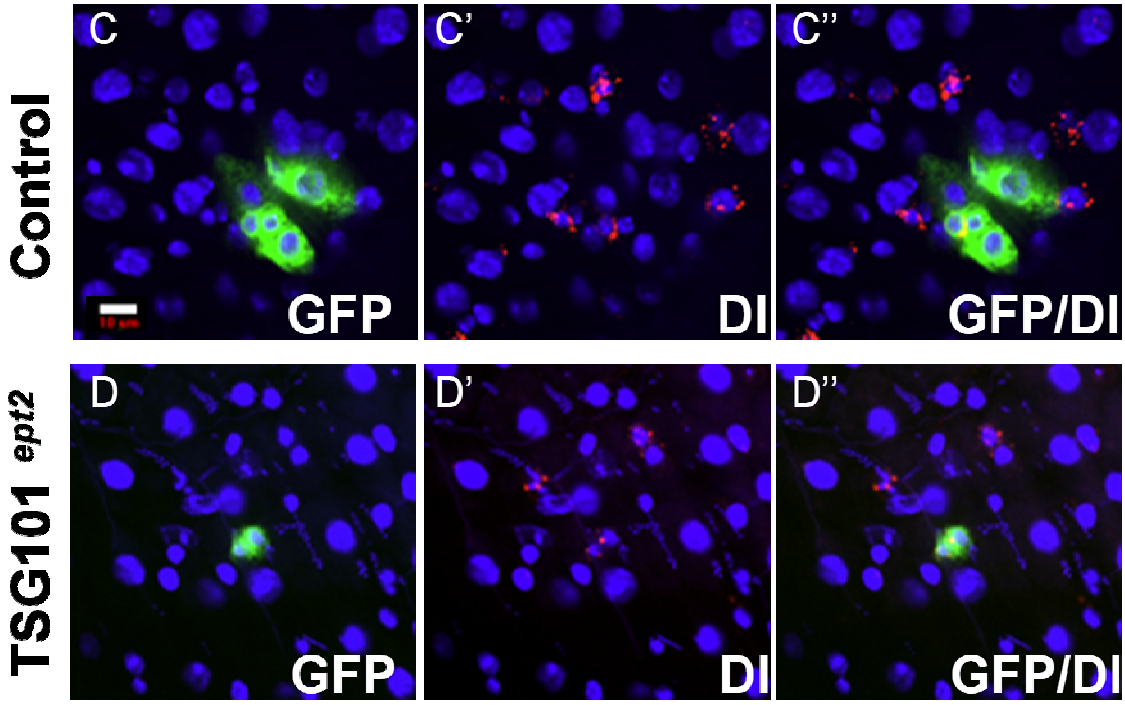


Fig 3.8: TSG101 mutants have reduced cell division.

A: Number of cells per GFP positive clone in *erupted2* mutant and control guts. In the *erupted2* line, the GFP positive cells are TSG101 mutants. Total 63 control clones and 42 mutant clones were counted, as a result of three sets of experiments. B: Average GFP positive cell per clone in control vs. mutant guts. Each clone is counted independently. Error bas show standard error. C-D''' show a representative view of a mutant and control midgut after HS showing a GFP positive cell cluster in each midgut with Delta positive (red stained) ISCs. Scale bar is 10μm.

CHAPTER 4

CONCLUSIONS

Food and water borne diseases cause millions of deaths worldwide (Brito 2005). The human GI tract is a very complex endocrine organ and also a primary site for host pathogen interaction. However the mammalian GI tract is very complex, leading to difficulty in experimental manipulation. The goal of this project was to use the fly intestine as a model system to gain insight into the complicated mechanisms and pathways of intestinal tissue homeostasis especially in response to pathogenic challenge. The easy availability of genetic constructs, conserved biochemical pathways like JAK-STAT make *Drosophila* an attractive model system.

In the fly intestine, the ISCs are the only dividing cells (Ohlstein 2005). Their characteristics were described by Micchelli and Perrimon in 2006 (Micchelli 2006). Therefore, their functional regulation must play a vital role in tissue homeostasis. The adult *Drosophila* midgut has approximately 1,000 ISCs that are distributed evenly along the gut and located basally to mature enterocytes. ISC is the only cell type that undergoes mitosis, while the differentiating enteroblasts

undergo endoreplication. ISC functioning has been shown to be greatly affected by surrounding cells and its microenvironment or niche (Amcheslavsky 2009). I therefore attempted to study how the ISC responds to pathogenic challenges.

I fed the flies with pathogenic bacteria*, P. entomophilla* and *S. marcescens* known to cause epithelial damage (Liehl 2006; Nehme 2007). Feeding oxidizing agents, paraquat and hydrogen peroxide, also show strong cell proliferation effects in the fly gut which mimic the effect seen after feeding bacteria to flies. Paraquat is a highly toxic compound which is quickly absorbed across the mammalian small intestine brush border and is known to trigger Parkinson's disease like symptoms in rats (Ossowska 2006).

I showed that ISCs respond to bacterial and chemical pathogens by increasing their division rate. There is also some evidence of cell differentiation with the EB nuclei increasing in size showing that the daughter cells prefer the EC differentiation pathway. EE and ISC numbers did not increase significantly. It is known that while ISC division can be upregulated by perturbation of the basement membrane, the resulting EBs in the fly midgut remain at a small to intermediate size. EB's don't differentiate further unless there is EC loss/damage (Amcheslavsky 2009). It is therefore possible that the ISC division I observed is

part of the intestinal repair process, which is part of gut homeostasis as a whole. It will be interesting to see if the JNK pathway which is known to play a cytoprotective role in the fly intestine is upregulated in this case, together with JAK/STAT, EGFR and Wingless pathways which could maintain the normal cell differentiation pattern under oxidative stress.

The second part of the project deals with the role of candidate gene TSG101 in intestinal functioning. Loss of TSG101 expression produced tumors in nude mice which led to it being labeled as a tumor suppressor (Li 2006). However, a conditional Tsg101-knockout in mouse primary glands did not cause the formation of tumor cells so the role of Tsg101 as a tumor suppressor became controversial but it was still considered essential for tissue development and survival (Wagner 2003).The protein was later identified as a subunit of the ESCRT1 complex and together with several other components, is crucial for its trafficking function (Luyet 2008). TSG101 is analogous to the yeast Vps 23 protein and it is its role as a trafficking protein that has implicated TSG101 in viral abscission. TSG101 is known to be required for the budding of HIV. Studies showing that TSG101 depletion inhibits HIV budding has made it an attractive drug target (Garrus 2001). This also highlights the crucial role of the ESCRT machinery itself. The role of ESCRT in regulating cell proliferation by the

differential accumulation of cell signaling ligands and receptors suggests a possible role in cancer.

The ESCRT pathway has been shown to affect various cellular processes including the Notch signaling pathway in *Drosophila*. TSG101 is known to play an important role in regulating ESCRT function (Moberg 2005, Gilbert 2009). These studies demonstrated heightened Notch signaling due to increased localization of activated Notch receptor in early endosomes. The role of Notch and the outcomes of Notch signaling remain highly context dependent. It is known that the ubiquitination and endosomal processing of the Notch receptor is tightly regulated and affects its signaling capacity (Mukherjee 2005). Notch can remain active after its internalization within endosomes, thus effects of Notch signaling can continue much after Notch has been activated at the cell membrane. The upregulation of Notch signaling caused by the *ept2* mutant allele of TSG101 was seen to cause non autonomous tissue overgrowth in the larval and adult fly eye discs. However, the overgrown tissue was composed of wild type cells as the mutant was expressed in a mosaic system. I used the RNAi approach to study the effects of TSG101 knockdown in the fly intestine.

I observed a different result using a TSG101 RNAi line in the adult fly intestine. The RNAi was driven in esg positive ISCs and EBs only since they are the key

players in tissue repair and homeostasis. I saw a loss of cell proliferation, loss of EE cells, severe reduction in ISCs and aberrant cell morphology. The RNAi intestine failed to respond to pathogen feeding which has been previously shown to greatly accelerate cell proliferation. This phenotype is consistent with the known effects of increased Notch signaling in the adult fly intestine. The knockdown of TSG101 has been already shown to allow the activated Notch receptor to accumulate in the EB endosomes from where it can potentially continue to signal even if the ISC (and therefore the Delta ligand) is no longer present to drive Notch signaling.

Notch is known to induce terminal differentiation in ISCs and inhibit the differentiation of EBs into EEs (Liu 2010, Perdigoto 2011). I am not certain if there is a fate switch between ISC and EB in the RNAi line or if the ISCs just loose Delta staining and slowly undergo apoptosis. There is some evidence for the latter as I see small faint GFP positive cells which are neither Delta nor Su(H)-lacZ positive in the fly midgut. To analyze this phenotype further I used a Tubulin Gal80^{ts} system to specifically knockdown TSG101 RNA in esg positive cells only after the flies had developed into mature adults. I was able to use TS to induce a similar phenotype as the esg driven TSG101 RNAi line. The ISC numbers dropped and EB numbers increased while EE numbers dropped slightly. I don't expect the EE cells to completely disappear as in the esg>GFP

line which has a much longer knockdown of TSG101, but the slight decrease in their numbers points to their non-replacement which is a known effect of increased Notch signaling (Park 2009). The increase in EB numbers in the TS experiments can be explained by the role of TSG101 as an ESCRT1 component. As the RNAi effect kicks in during TS, Notch can be increasingly trapped within the endosomes and continue to signal and recruit Su(H) even as ISC numbers begin to decrease.

Another interesting phenomenon was the absence of the RNAi phenotype in the larval guts which looked no different from the control. I verified that the EsgGal4 driver was still active in the larval guts since they showed GFP expression in the progenitor cells (Fig 3.3). One explanation could be the different ISC niche in the larval guts where the progenitor cells are located in larger, tighter clusters than in the adult. It would be interesting to see the actual time during development when the TSG101RNAi effect begins to kick in and study why it becomes active then.

The RNAi results were corroborated by TSG101 mutant clonal analysis. Contrary to previous reports in other fly tissues, I did not observe a non cell autonomous hyperplasia in the surrounding cells. This is expected in the adult fly intestine since only the ISC's have the ability to divide, so even if the *ept2* mutant cells

express Upd as they do in the fly eye, the intestinal ISCs (which were expressing the *ept2* allele are scattered and perhaps unlikely to affect each other's proliferation rate via secreted mitogens.

However, consistent with the results shown by Moberg et al (2005), the TSG101 mutant cell clones are smaller, indicating slower proliferation rates. I cannot say if some mutant cells are killed and removed by apoptosis before I can even see them but that remains a possibility. I was unable to detect change of cell fate within the mutant clones with each clone still containing one Delta positive ISC. It can be useful to test these cells for apoptotic markers. There could be additional defects within the *ept2* mutant ISC itself.

I therefore propose our model for TSG101 function in fly gut homeostasis. I propose that TSG101 is needed to maintain ISC fate by controlling Notch signaling. Loss of TSG101 therefore leads to phenotypes that correlate with arrested Notch receptor processing and up regulation of Notch signaling (Fig 4.1).

Fig 4.1

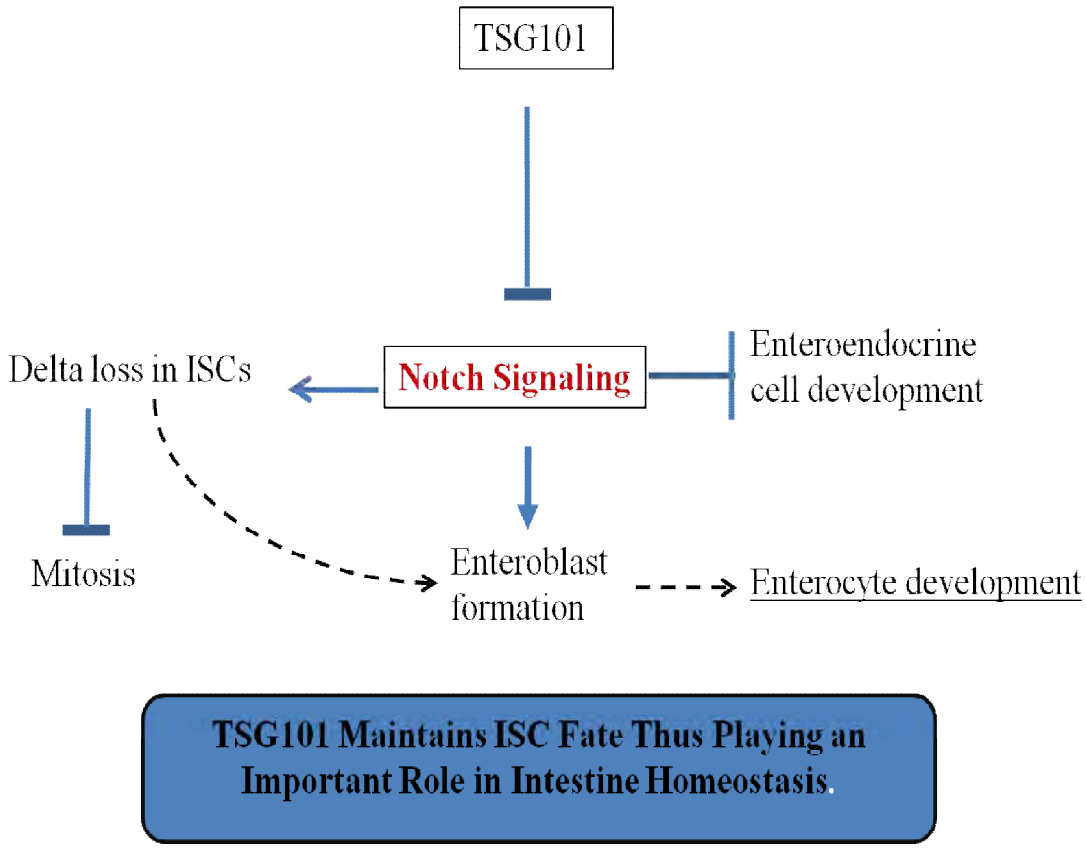


Fig 4.1 Proposed model.

TSG101 is needed for ISC maintenance and intestine homeostasis. It acts via limiting Notch signaling. Upregulation of Notch signaling in a TSG101 deficient system leads to loss of ISC fate and lack of EE cells. Loss of ISC fate disrupts tissue homeostasis.

Further investigation of TSG101 mutants can be done together with other mutants known to disrupt ISC fate and Notch pathway. How does the RNAi line fare in a Notch gain or loss of function background? Cellular markers for other components of the ESCRT pathway like Hrs will shed more light on the actual mechanistic details of TSG101 in the context of the fly intestine, both in adults and in larvae. Markers for regulators of the Notch and ESCRT pathway like Unpaired, STAT, and Wg etc will also reveal more details about the possible targets of TSG101 and how it ties into pathways that are concerned with ISC fate maintenance. Does TSG101 also affect the trafficking of Delta? Is it possible that like Notch, Delta in the lack of TSG101 may also get trapped in endosomes but remain in an inactive state since all ligands can certainly not signal from within endosome. Moreover, Delta needs to be expressed on the cell membrane to activate the Notch pathway in the adjacent daughter cell.

The lack of observed effects of the RNAi in the larval gut is also worth investigating. It would be interesting to see if TSG101 has different roles in the larval intestine. More cellular markers can be used to test mutant and RNAi expressing cells for apoptosis.

TSG101 affects the very survival of the only dividing cells in the gut, namely the ISCs, which play a crucial part in tissue homeostasis. I feel this provides more insight into the highly context dependent function and effects of TSG101 and Notch signaling.

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