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Investigating Cell Fate Decisions of the Intestinal Stem Cell

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Summary

The development of multicellular organisms, as well as the maintenance of adult tissue homeostasis, requires the production of new cell types that will build up the new organism and maintain it throughout its entire life. Cell proliferation, differentiation and cell death have to be carefully orchestrated during development, requiring cell-cell communication. This is carried out by a small number of cell signaling pathways that are reiteratively employed both during development and during tissue homeostasis in adults. One of these pathways, which plays many critical roles, both during development and in adult stem cells, is the Notch signaling pathway. During my PhD, I become interested in understanding how different cell types can be specified during development, and how do cells ensure that this occurs properly. During the first part of my thesis, I investigated one mechanism of cell type specification – asymmetric cell division. During the second part of my PhD, I studied the maintenance of tissue homeostasis by the adult stem cells of the *Drosophila* intestine.

During development, multipotent cells divide and give rise to cells with more restricted fates, in the process of building differentiated adult tissues. One mechanism employed to specify different cell fates is asymmetric cell division: during mitosis, cell fate determinants are asymmetrically localized to one of the future daughter cells, and will determine the fate of that daughter cell (Knoblich, 2001). A good example of this occurs during the development of the sensory organs in *Drosophila*, where precursor cells, the sensory organ precursors (SOPs), divide asymmetrically to produce two distinct cells, a pIIa cell and a pIIb cell. These will again divide asymmetrically to give rise to the four different cell types of the sensory organs. In this process, the Par6-aPKC complex localizes at the posterior cortex of the SOPs and promotes the actin-dependent localization of the cell fate determinants Numb, Partner of Numb (Pon) and Neuralized to the opposite anterior pole. Both Numb and Neuralized regulate Notch signaling, promoting its activation only in the posterior cell, which will become the pIIa cell (Bardin et al., 2004). The plasma membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) regulates the plasma

membrane localization and activity of various proteins, including several actin regulators, thereby modulating actin-based processes (Di Paolo and De Camilli, 2006). I examined the distribution of PIP2 and of the PIP2-producing kinase Skittles (Sktl) in mitotic SOPs, and found that both Sktl and PIP2 reporters were uniformly distributed in dividing SOPs. However, in the course of this study, I unexpectedly observed that overexpression of full-length Pon or its localization domain (LD) fused to the Red Fluorescent Protein (RFP::Pon^{LD}) resulted in asymmetric distribution of Sktl and PIP2 reporters in dividing SOPs. This finding that Pon overexpression alters polar protein distribution is important because RFP::Pon^{LD} is often used as a polarity marker of dividing progenitors (Perdigoto et al., 2008). However, since Sktl and PIP2 do not play any detectable role in the asymmetric localization of the cell fate determinants, I decided to not further pursue this project.

The second part of my PhD focused on how adult stem cells maintain tissue homeostasis. The maintenance of adult tissue homeostasis during the whole life span of the organism (which in some vertebrates can be more 200 years) requires that stem cells divide to replace the differentiated cell types of the organ, as well as to maintain the stem cell pool. In order to do so, adult stem cells utilize many of the same signaling pathways that are employed during embryonic development to regulate cell growth, proliferation, differentiation, death and morphogenesis. The adult *Drosophila* Intestinal Stem Cells (ISC) divide throughout the lifetime of the adult fly to replenish gut tissue by producing two differentiated cell types. The ISC division is thought to be asymmetric concerning the fate of the two daughter cells: one remains an ISC and the other becomes a progenitor cell, termed the enteroblast (EB). The ISC is the only dividing cell in the adult gut, and the EBs go on to differentiate directly, either into an enterocyte or an enteroendocrine cell. Enterocytes are polyploid absorptive epithelial cells, while the enteroendocrine cells are diploid cells that express peptide hormones. Lineage labeling analysis demonstrated that ISCs are multipotent stem cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) and Notch signaling has been shown to play a role in regulating cell fate decision of the ISC lineage. The Notch ligand Delta is expressed in the ISCs and Notch signaling is activated in the EB to promote EB, enteroendocrine and

enterocyte fates (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). In my work, I have shown that the transcriptional repression of Notch target genes is required to maintain the ISC identity (Bardin et al., 2010). However, how Notch signaling is modulated to control the acquisition of the EB fate and two distinct differentiated cell fates is not understood. It has been proposed that the specification of the differentiated fates could be related to the level of Notch signaling (Ohlstein and Spradling, 2007), a mechanism that has also been proposed to be employed by other stem cell lineages (Mazzone et al., 2010). Given the role of Notch signaling in many stem cell fate decisions, it is important to understand how it can promote alternative fates in a single precursor cell. To answer this question and to identify novel regulators of the cell fate decisions in the ISC lineage, we carried out a chemical mutagenesis screen in *Drosophila*. During this screen, random mutations were induced and mitotic clones, in the F2 generation, were screened for mutants in which intestinal cell type specification was affected. Several complementation groups with defects in cell type specification were identified, which can be divided into three groups: mutants in which the stem cell is lost, mutants with overproduction of enteroendocrine like cells and mutants with excess of stem cell-like cells. I characterized briefly and mapped some of these mutations. I further investigated the role of one of the genes identified in this screen.

One complementation group was found to specifically affect the self-renewing of ISCs without affecting the production of differentiated cells. I mapped this complementation group to the *Gmd* (GDP-mannose 4,6-dehydratase) gene. Clones of *Gmd* mutant cells have an overproduction of Delta positive, proliferative, multipotent stem cell-like cells in the intestine. However, unlike the loss of Notch signaling, loss of *Gmd* does not affect differentiation, as shown by the finding that enterocytes and enteroendocrine cells are normally specified in *Gmd* mutant clones. This suggests that in *Gmd* mutants ISCs, the ISC can divide asymmetrically, in relation to the fate of the daughter cells, to self-renew and produce EBs, or symmetrically to give rise to two ISCs.

Gmd is required for the production of fucose and has been shown to be required for the *O*-fucosyltransferase 1 (Ofut1)-dependent fucosylation of the Notch

receptor (Okajima and Irvine, 2002; Okajima et al., 2003; Sasamura et al., 2003). It is thought that *O*-fucosylated Notch protein serves only as a subsequent substrate for further glycosylation by the glycosyltransferase Fringe (Fng), since the loss of fucosylation results in defects only in developmental contexts that require Fng-dependent modification of Notch (Okajima et al., 2008; Okajima et al., 2005). Interestingly, I found that *Gmd* plays a *fringe*-independent, but *Ofut1*-dependent, role to limit symmetric ISC divisions. Furthermore, the *Gmd* phenotype can be suppressed by the expression of active nuclear Notch. In addition, I found that Notch signaling could still be activated in *Gmd* mutant clones, but that precise asymmetric fate decisions in the daughter cells do not occur. Studies in mammalian cells found that *O*-fucosylated Notch receptor is more strongly activated by its ligands (Chen et al., 2001; Moloney et al., 2000; Stahl et al., 2008). My data are consistent with *Gmd* being required for high levels of Notch activation in the ISC lineage, which is necessary to limit symmetric division of the ISC. To test how the levels of Notch signaling affect the ISC lineage, I analyzed the temperature-dependent effect of *rumi* mutations in the intestine. Rumi is a component of the Notch signaling pathway but the requirement for Rumi is temperature-dependent: at 18ºC *rumi* mutants have a very mild loss of Notch signaling phenotype while at 28ºC they have a stronger phenotype (Acar et al., 2008). I found that *rumi* mutant clones in the intestine are wild-type at 18ºC but have a stronger phenotype at 28ºC. At 21ºC and 22ºC, *rumi* mutant clones have an overabundance of ISCs but differentiation is essentially unaffected, similar to *Gmd* mutants. This data support a model in which different cell fate decisions require different levels of Notch signaling: while the asymmetric decision between ISC and EB requires high levels of Notch signaling, further differentiation of the EB into enterocyte or enteroendocrine cells can occur with lower levels of Notch activity. Such requirement of high levels of Notch signaling for daughter cell commitment, that is, exit from self-renewal, could be a mechanism to prevent loss of the ISC. I propose that this could be a general mechanism utilized by stem cells to ensure their maintenance, in which high threshold signaling prevents noisy, low levels of signaling that could lead to the loss of the stem cell.

Sumário

A produção de diferentes tipos celulares é um processo fulcral para o organismo, quer durante o seu desenvolvimento, quer na regulação da homeostase adulta, para a qual contribuem os processos de renovação celular. A coordenação de diferentes eventos celulares, tais como a proliferação, diferenciação e morte celular, envolve sofisticados processos de comunicação celular. Para este efeito, existem diferentes vias de sinalização inter-celular que são utilizadas, primeiro durante o desenvolvimento, para orquestrar a embriogénese, e posteriormente no adulto, para manter os diferentes tecidos. A via de sinalização 'Notch' é uma das principais vias de sinalização celular, sendo essencial tanto durante o desenvolvimento como para a regulação de vários tipos de células estaminais. O principal objectivo da minha actividade científica tem sido investigar a origem dos diferentes tipos celulares e os mecanismos que asseguram a sua correcta especificação num tecido em homeostase. Numa fase inicial, estudei a divisão celular assimétrica, um dos mecanismos utilizados para gerar diferentes tipos celulares. Na segunda e principal parte dos meus estudos doutorais, investiguei a manutenção do epitélio intestinal em *Drosophila melanogaster* por células estaminais intestinais.

Durante o desenvolvimento dos metazoários, células progenitoras com potencial para gerar diferentes tipos celulares dão origem a células com potencial mais restrito que, eventualmente, estarão na origem dos diferentes tipos de células diferenciadas constituintes dos vários tecidos do organismo adulto. A divisão celular assimétrica é um dos mecanismos utilizados para gerar diferentes tipos celulares. Quando uma célula se divide assimetricamente, ocorre uma distribuição assimétrica de factores citoplasmáticos durante a mitose, que são herdados diferencialmente pelas células filhas e determinam a identidade dessas células (Knoblich, 2001). A divisão da célula precursora dos órgãos sensoriais (SOPs) em *Drosophila* é um exemplo de divisão celular assimétrica. A célula SOP divide-se assimetricamente produzindo uma célula pIIa e uma pIIb, que se dividem também assimetricamente, originando os quatro tipos de células dos órgãos sensoriais. Durante a mitose, o

complexo Par6-aPKC, um complexo de proteínas que participa em processos de polarização celular, localiza-se no pólo posterior da célula SOP e promove a localização, através dos microfilamentos de actina, dos determinantes Numb, Partner of Numb (Pon) e Neuralized no pólo oposto da célula. As proteínas Numb e Neuralized são reguladoras da via de sinalização Notch, promovendo a activação assimétrica do receptor Notch na célula posterior, que adopta a identidade de pIIa (Bardin et al., 2004). O fosfolípido fosfatidilinositol (4,5)-bifosfato (PIP2) é um componente do folheto interno da membrana plasmática que regula a localização e actividade de várias proteínas, incluindo reguladores dos microfilamentos de actina (Di Paolo and De Camilli, 2006). Para testar o papel do PIP2 na divisão assimétrica das células SOP, determinou-se a localização do PIP2 e da proteína Skittles (Sktl), a cinase que produz PIP2. Observei que tanto Sktl como repórteres de PIP2 se localizam simetricamente no córtex de células SOP em mitose. No entanto, observei também que a expressão da proteína Pon ou do seu domínio de localização (LD) ligado com uma proteína fluorescente (RFP::Pon^{LD}) resultam na localização assimétrica dos repórteres de PIP2 e de Sktl no pólo posterior da célula SOP em divisão. Como o RFP::Pon^{LD} é um repórter de divisão assimétrica frequentemente utilizado, o seu efeito na localização da cinase que produz PIP2 e do PIP2 é relevante (Perdigoto et al., 2008). No entanto, como o PIP2 não parece estar implicado na localização assimétrica de determinantes durante a divisão celular, o projecto foi concluído com a publicação destes resultados.

Durante a segunda e principal parte dos meus estudos doutorais, interessei-me pela problemática da manutenção da homeostase por células estaminais adultas. A manutenção dos diferentes orgãos ocorre continuamente durante toda a vida do organismo que, em certos animais, pode ultrapassar os 200 anos. Durante esse tempo, as células estaminais têm que renovar os diferentes tipos celulares e, simultaneamente, manter a sua própria população. Para tal, as células estaminais adultas utilizam as mesmas vias de sinalização celular que são utilizadas durante o desenvolvimento embrionário para comunicar entre si e regular a proliferação, diferenciação e morte celular. As células estaminais intestinais (ISCs) presentes no intestino de *Drosophila* proliferam durante toda a vida adulta, produzindo os dois

tipos celulares diferenciados que constituem este epitélio. As ISCs são as únicas células a proliferar no intestino e têm uma linhagem muito simples: uma das células filhas permanece como ISC enquanto que a outra célula filha, denominada enteroblasto (EB), se diferencia. O EB adopta um de dois tipos celulares, convertendo-se quer numa célula enteroendócrina, produzindo hormonas peptídicas, quer numa célula epitelial absortiva, designada de enterócito. As ISCs exprimem Delta, um ligando da via Notch, e foram recentemente identificadas como células estaminais proliferativas e multipotentes (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). A via Notch tem um papel essencial na regulação dos diferentes tipos celulares no intestino, na medida em que esta via é activada no EB e promove a diferenciação desta célula, regulando também a sua diferenciação numa célula enteroendócrina ou num enterócito (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). A repressão da transcrição de genes alvo da via Notch é essencial para a manutenção da célula estaminal (Bardin et al., 2010). No entanto, ainda não foi determinado como é que a via Notch regula estas três decisões. Foi proposto que o EB poderá receber diferentes níveis de sinalização Notch, determinando assim qual dos tipos celulares será produzido (Ohlstein and Spradling, 2007). Um mecanismo semelhante foi proposto para a regulação da diferenciação na glândula mamária (Mazzone et al., 2010).

Para identificar novos reguladores do desenvolvimento das células ISC, decidimos fazer um 'crivo' genético através da indução de mutações aleatórias no genoma de *Drosophila,* analisando posteriormente o fenótipo no intestino de moscas adultas. O objectivo deste trabalho foi a identificação de genes que resultam em proliferação ou diferenciação alteradas das células ISC. Neste 'crivo' genético identificámos três tipos diferentes de mutantes: mutantes com hiperproliferação de células estaminais, mutantes com hiperproliferação de células enteroendócrinas e mutantes com número reduzido de células estaminais. Após breve caracterização e mapeamento genético de alguns destes mutantes, concentrei-me no estudo da função dum destes genes na regulação da produção de células ISC.

Num dos grupos de complementação de mutantes identificado, foi observada uma expansão da população de células ISC sem que a sua diferenciação parecesse

afectada. Os mutantes deste grupo de complementação foram mapeados e foram identificadas mutações no gene *Gmd* (codificando a enzima GDP-manose 4,6-desidratase). Os mutantes *Gmd* tem um excesso de células proliferativas multipotentes que expressam o marcador Delta. No entanto, e ao contrário do que foi observado nos mutantes da via Notch (que também revelam excesso de células ISC), as células diferenciadas são normalmente especificadas nos mutantes *Gmd*. Em contraste com o que se verifica em animais controlo, onde as células ISC normais se dividem sempre assimetricamente em relação ao destino das células filhas, as células ISC de animais mutantes *Gmd* podem dividir-se quer assimetricamente, originando uma ISC e um EB, quer simetricamente, produzindo duas células ISC idênticas.

A enzima Gmd é essencial à produção de fucose nas células, sendo necessária para a fucosilação do receptor Notch pela enzima Ofut1 [*O*-fucosyltransferase 1 (Okajima and Irvine, 2002; Okajima et al., 2003; Sasamura et al., 2003)]. A proteína Notch fucosilada pode ser subsequentemente glicosilada por outra glicosiltransferase, conhecida por Fringe [Fng (Okajima et al., 2003)]. Esta modificação de Notch por Fng é necessária em certos contextos celulares e modifica a afinidade de Notch para os seu ligandos; o único contexto descrito em que fucosilação de Notch é indispensável é como substrato para a subsequente glicosilação do recepor pelo Fng (Okajima et al., 2008; Okajima et al., 2005). No entanto, observei que a mutação do gene *fng* no intestino não afecta as células ISC, enquanto que mutações em *Ofut1* resultam no mesmo fenótipo que a perda de função de *Gmd*.

Experiências em células de mamíferos sugerem que o receptor Notch fucosilado pode ser mais eficazmente activado pelos ligandos (Chen et al., 2001; Moloney et al., 2000; Stahl et al., 2008). Isto levou-nos a propor que a intensidade do sinal Notch depende da fucosilação do receptor e que a decisão que as células filhas de uma célula ISC têm que tomar, entre permanecer como ISC ou iniciar a diferenciação, requer uma actividade elevada da via Notch. Pelo contrário, a decisão sobre o destino das células diferenciadas pode ocorrer com uma actividade mais reduzida da via Notch.

A apoiar desta hipótese, verifica-se que a modulação da actividade Notch em mutantes termosensíveis *rumi* [codificando para um componente da via Notch (Acar et al., 2008)], revelam hiperproliferação de células ISC sem afectar a decisão do tipo de diferenciação. Estes resultados levam-me a propor um modelo em que uma actividade elevada da via Notch é necessária para implementar a decisão de diferenciação das células ISC. Esta necessidade de uma actividade elevada da via Notch poderá constituir um potencial mecanismo para proteger a célula estaminal. Assim, será interessante verificar se outros tipos de células estaminais também requerem uma sinalização Notch elevada, antes de se comprometerem com vias de diferenciação.

Concluindo, este estudo põe em evidência um potencial mecanismo de protecção de uma população de células estaminais, em que a diferenciação só pode ocorrer com níveis elevados de comunicação celular mediada pela via Notch ou por outra via de comunicação celular. Este mecanismo confere assim robustez à população de células estaminais, limitando a vulnerabilidade destas células a sinais de diferenciação inespecíficos e que põe em risco a manutenção, a longo prazo, da homeostase dos tecidos e a viabilidade do organismo.

Abbreviation Index

AMG: anterior midgut primordia
AMPs: adult midgut progenitors
ANK: ankyrin repeats
Apc: Adenomatous polyposis coli
APF: after pupae formation
Aph-1: anterior pharynx defective 1
aur-B: aurora-B
bp: base pair
β-gal: β-galactosidase
bHLH: basic helix-loop-helix
BM: basement membrane
BMP: bone morphogenic protein
BrdU: 5-bromodeoxyuridine
Brm: brahma
CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy
CBCs: crypt base columnar cells
CHO: Chinese Hamster Ovary
Da: Daughterless
DAPI: 4,6-diamidino-2-phenylindole
DILPs: *Drosophila* insulin-like peptides
Dl: Delta
DOS: Delta and OSM-11-like proteins
Dpp: Decapentaplegic
DSL: Delta, Serrate and Lag-2
DSS: Dextran Sulfate Sodium
D/V: dorsal/ventral
EB: enteroblast
EC: enterocyte
Ecc15: Erwinia carotovora 15
ee: enteroendocrine

EGF: epidermal growth factor
EGFR: EGF receptor
EMS: ethyl methane sulphonate
ESC: escort stem cells
esg: escargot
E(spl)-C: Enhancer of split complex
Flp: site-specific recombinase
Fng: Fringe
FRT: FLP Recombination Target
Fz: Frizzled
H: Hairless
HD: heterodimerization domain
Hes1: hairy and enhancer of split 1
HS: heat shock
Gbb: glass-bottom-boat
GFP: Green Fluorescent Protein
Gmd: GDP-mannose 4,6-dehydratase
GSC: germline stem cells
Imd: immune deficiency signaling
InR: insulin receptor
ISC: Intestinal Stem Cells
Jak: Janus kinase
JNK: Jun N-terminal kinase
Kis: Kismet
Kuz: Kuzbanian
LD: localization domain
Lgr5: Leucine-rich-repeat-containing G-protein coupled receptor 5
LNR: Lin-12-Notch repeats
LOF: loss of function
LRP: low-density lipoprotein receptor-related protein
MARCM: Mosaic Analysis with a Repressible Cell Marker:
Math1: mouse atonal homologue 1
N: Notch
NECD: Notch Extra Cellular Domain

NEXT Notch Extracellular Truncation

Neur: Neuralized

NICD: Notch Intracellular Domain

NLS: nuclear localization sequences

Ofut1: *O*-fucosyltransferase 1

PMG: posterior midgut primordia

PcG: Polycomb Group

Pdm1: nubbin

PEST: proline/glutamic acid/serine/threonine-rich motifs

PIP2 : phosphatidylinositol (4,5)-bisphosphate

PH3: phospho-histone H3

PoFut1: Protein *O*-fucosyltransferase 1

PoGglut: Protein *O*-glycosyltransferase

Pon: Partner of Numb

Pros: Prospero

Pvf2: PDGF-and VEGF-related factor 2)

RAM: RBPjκ association model

Rel: Relish

RFP: Red Fluorescent Protein

RNSC: renal-nephric stem cells

ROS: reactive oxygen species

SC: stem cells

Sktl: Skittles

Snr1: Snf5-related 1

SOP: Sensory Organ Precursor

Spen: split ends

Su(H): Suppressor of Hairless

TA: transit-amplifying cell

TAD: transactivation domain

TCF/LEF: T cell factor, lymphocyte enhancer factor TCF/LEF

TMD: transmembrane domain

Trx: trithorax

TrxG: Trithorax Group

UAS: upstream activating sequence

Upd: Unpaired

Vg: vestigial

VM: visceral muscle

Wg: Wingless

Chapter I

Introduction

I. Notch Signaling

Cell-cell communication mediated by Notch signaling is essential for the development of metazoans, from the nematode *Caenorhabditis elegans* to humans. Notch signaling plays a critical role in multiple developmental programs, such as the development of neural, vascular, and intestinal tissues, development of the skin, body segmentation, hematopoietic development, amongst others. Not surprisingly, the Notch pathway has also been implicated in the regulation of many adult stem cells, such as intestinal stem cells, skin stem cells, hematopoietic stem cells, mammary epithelial cells as well as many human diseases (Kopan and Ilagan, 2009).

Mutations in the *Notch* genetic locus were originally described, almost 100 years ago, as sex-linked lethal mutations in *Drosophila,* where the females had notches in their wings (Mohr, 1919). Later, *Notch* was characterized as a “neurogenic locus”, as mutations in *Notch* were found to result in cell fate changes during neurogenesis: many more cells gave rise to neural tissue at the expense of the epidermis (Lehmann, 1983; Poulson, 1945). Almost 30 years ago, the *Notch* locus was cloned and sequenced, and the Notch protein was shown to be a transmembrane protein (Artavanis-Tsakonas et al., 1983; Kidd et al., 1986; Wharton et al., 1985). Later, work by Heitzler and Simpson showed that Notch acts as a receptor for the transmembrane ligand Delta (Heitzler and Simpson, 1991).

A. Notch signaling and cell fate specification

In this part I will briefly introduce Notch signaling and overview its role in specifying cell fates during development. The Notch receptor (Notch in *Drosophila,* Notch1-4 in mammals and Lin-12 and GLP-1 in *C. elegans*) is a transmembrane protein that is activated by its ligands of the DSL family (Delta and Serrate in *Drosophila*, Lag-2 in *C. elegans* and Delta-like and Jagged in vertebrates). The Notch ligands are also transmembrane proteins that activate the Notch receptor expressed in neighboring cells. Upon activation of Notch by its ligands, the Notch protein will undergo proteolytic cleavages that will result in the translocation of intracellular Notch (NICD) to the nucleus. In the nucleus, NICD interacts with the DNA binding protein CSL (human CBF1, fly Suppressor of Hairless and nematode Lag-1) and co-activators, promoting the transcription of the Notch target genes [Fig.1, reviewed in (Bray, 2006; Kopan and Ilagan, 2009; Schweisguth, 2004b)]. Besides the activation of the Notch receptor by ligands expressed in neighboring cells, there can also be *cis*-interaction between the receptor and the ligands expressed in the same cell. This *cis*-interaction between the ligands and the receptor functions, in some developmental contexts, to decrease the ability of the signal-receiving to respond to signal from the neighboring cells (Del Alamo, 2010).

Notch signaling has been shown to regulate cell survival, growth, proliferation, cell fate specification and differentiation in a wide range of developmental contexts. However, all these effects can be carried out through, essentially, two modes of Notch signaling: binary cell fate decisions, in which cells make a choice between two possible cell fates or inductive signaling, in which a novel cell fate is specified (Bray, 2006; Schweisguth, 2004b). Notch signaling mediates binary cell fate decisions by ensuring that two interacting equipotent cells do not adopt the same fate. Binary cell fate decisions can be further subdivided into lateral inhibition and asymmetric cell division. Finally, Notch signaling can also play a role in synchronization of a group of cells.

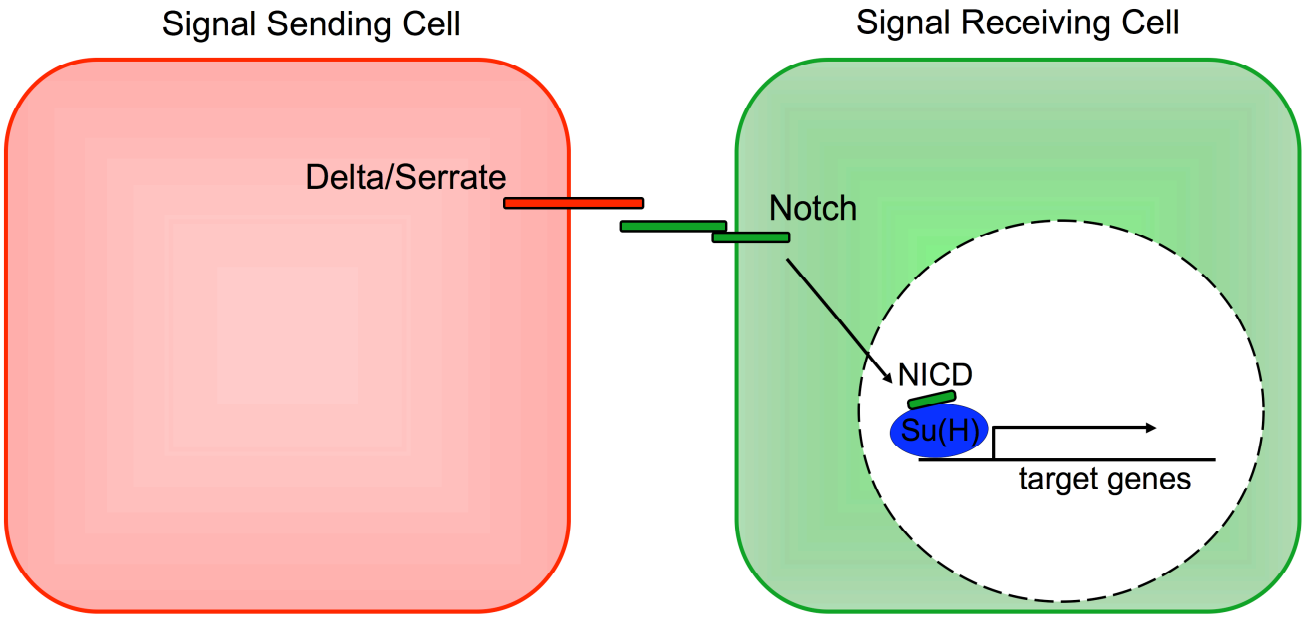


Fig. 1: The Notch ligands, Delta or Serrate, activate the Notch receptor in a neighbor cell. Activation of the Notch receptor will lead to transcriptional activation of the Notch target genes.

A.1. Lateral inhibition

Lateral inhibition is a process during which cells of equivalent developmental potential will signal to each other through inhibitory reciprocal Notch signaling. Both cells start with similar levels of ligand and receptor and the activation of Notch in the signal-receiving cell inhibits, through transcriptional inhibition, the ability of that cell to signal to its neighbor cells. In this way, small differences between initially equipotent cells are amplified by a transcriptional feedback loop. Therefore, signaling that starts as bidirectional, becomes unidirectional (Bray, 2006; Schweisguth, 2004b). In addition to the transcriptional feedback loop, it has also been proposed that *cis*-inhibition of Notch by the ligands can facilitate lateral inhibition by stabilizing small differences between the two cells (Del Alamo, 2010; Sprinzak et al. 2010).

A.2. Asymmetric Cell Division

Binary fate choices can also be biased, either by extrinsic or intrinsic cues. One example of an extrinsic cue that biases a binary fate decision mediated by Notch signaling is the specification of the vulval precursor cells of *C. elegans*. All vulval precursor cells have the same developmental potential. However, the neighbor anchor cell expresses the EGF/Lin-13 signaling molecule that will activate EGF receptor/Let-23 signaling in the nearest vulval precursor cell, resulting in the up-regulation of the DSL Notch ligands and the down-regulation of the Notch receptor Lin-12 in that cell, which will become the signal-sending cell and will activate Notch in the neighboring vulval precursor cells. Therefore, the directionality of the binary fate decision is biased by extrinsic EGF signaling (Greenwald, 2005; Sternberg, 2005). One example of a binary cell fate choice being biased by an intrinsic cue is an asymmetric cell division where an inhibitor of the Notch pathway is localized asymmetrically to the cortex of the cell in mitosis and is segregated differentially into one of the daughter cells. The daughter cell that inherits the inhibitor of Notch

signaling will be biased to become the signal-sending cell. An example of such a biased cell division is described below (Bardin et al., 2004).

In Fig. 2.A, the development of the *Drosophila* sensory organ precursors (SOPs) illustrates both lateral inhibition and asymmetric cell division. First, the SOP is singled out from the proneural cluster (group of equipotent cells) by lateral inhibition, with the Notch OFF cell becoming the SOP. Then, the SOP divides asymmetrically: during division of the SOP Numb, an inhibitor of activation of Notch signaling, and Neuralized (Neur), which is required for ligand signal sending activity, are localized asymmetrically to the anterior cell. The anterior cell will not receive Notch signal and will signal to the posterior cell, activating Notch in that cell. The Notch OFF cell will adopt the pIIb fate while the Notch ON cell will adopt the pIIa fate. Subsequent divisions in the SOP lineage are also asymmetric (Bardin et al., 2004; Bray, 2006; Schweisguth, 2004b).

A.3. Lateral induction

Lateral Induction mediated by Notch signaling promotes the specification of a new cell type between two different fields of cells that have a pre-existing boundary. Notch signaling is a conserved mechanism to specify cells at boundaries in many contexts and species, such as the induction of the dorsal/ventral (D/V) boundary of the *Drosophila* wing, the segmentation of the *Drosophila* leg, vertebrate somitogenesis, and zebrafish hindbrain segmentation. In Fig. 2.B the D/V compartmentalization of the fly wing disc is shown as an example of boundary formation. Notch is activated at the pre-existing boundary between the dorsal and the ventral compartment, resulting in the induction of a new cell type at this boundary that will function as an organizer, coordinating both growth and patterning of the wing disc. Notch will activate expression of specific targets in these cells, including Wingless (Wg), Vestigial (Vg) and Cut (Bray, 1998; Haines and Irvine, 2003).

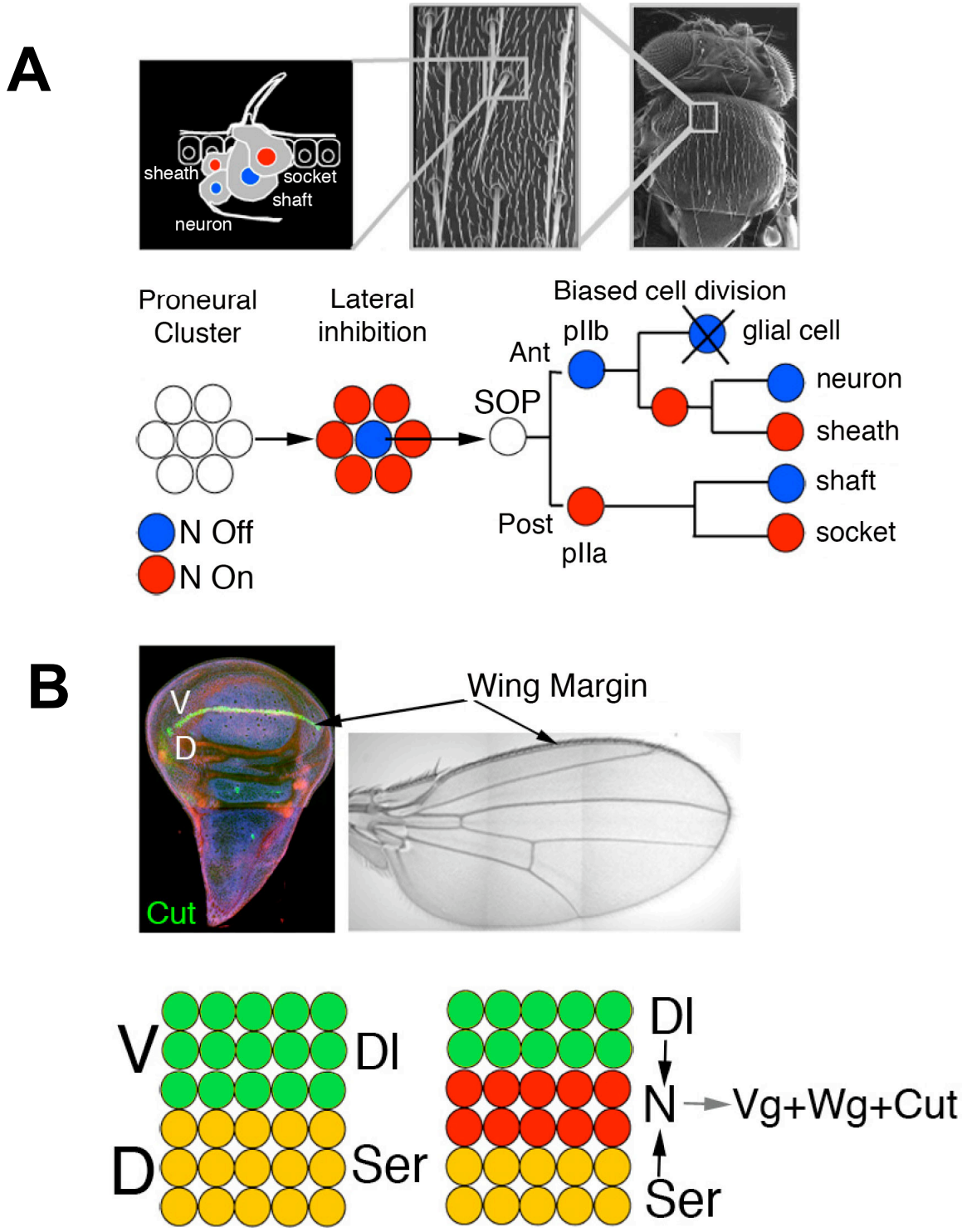


Fig. 2: **A** SOPs are first selected from an equipotent group of cells in the proneural cluster through Notch signaling-mediated lateral inhibition and then the SOP undergoes asymmetric cell divisions to give rise to the four distinct cell types of the sensory organ. **B.** Notch is activated at the boundary between the dorsal and ventral compartment, resulting in the induction of a new cell type, the border cells, that will function as an organizer and will become the future wing margin. Notch will activate expression of specific target genes in these cells, including Wingless (Wg), Vestigial (Vg) and Cut.

A.4. Synchronization of oscillations during Somitogenesis

Finally, Notch signaling also appears to play a role in synchronizing oscillations during somitogenesis. During vertebrate segmentation, the somites, which are epithelial blocks of cells that will give rise to the vertebrae, are generated in a rhythmic manner. During somitogenesis, transcriptional oscillations of genes of the Notch pathway, the Wnt pathway and the FGF pathway were observed. These transcriptional oscillations were proposed do be under the control of the segmentation clock, an oscillator that would generate these pulses of gene expression. The rhythmic signal produced by the clock controls the periodic production of the somites. Since many components of the Notch pathway have been shown to oscillate during somitogenesis, it was thought that Notch signaling could be the generator of the oscillations, acting as a pacemaker of the segmentation clock. However, it has been shown that other oscillating genes are not disturbed in embryos mutant for Notch signaling, which suggests that Notch signaling is not the generator of the oscillations. Instead, Notch signaling seems to play a critical role in the synchronization of the segmentation clock, as loss of Notch signaling results in a progressive loss of synchrony between the oscillating cells. In this context, Notch signaling appears to play a critical role in coupling different cells together, which is essential for synchronous oscillations during segmentation (Jiang et al., 2000; Özbudak and Pourquié, 2008).

B. Molecular Basis of Notch Signaling

Both the Notch receptor and most of its ligands are type I transmembrane proteins. Notch accumulates at the plasma membrane as a heterodimer, composed of a large Notch Extra Cellular Domain (NECD) and a membrane tethered intracellular domain. The NECD and the membrane tethered intracellular domain interact non-covalently in a calcium dependent manner. Figure 3 shows the domain structure of the *Drosophila* Notch receptor. The extracellular domain of Notch contains tandem Epidermal Growth Factor-like (EGF) repeats, which vary in number in the different Notch proteins: 36 in *Drosophila*, from 29 to 36 in the four mammalian Notch receptors and 10 in *C. elegans* GLP-1 (Fleming, 1998). The EGF repeats are followed by the negative regulatory region of Notch, which contains three Lin-12-Notch repeats (LNR) and a heterodimerization domain (HD). The transmembrane domain (TMD) is followed by the RBPjκ association model (RAM), nuclear localization sequences (NLS), a seven ankyrin repeats (ANK) domain and a transactivation domain (TAD) that contains a proline/glutamic acid/serine/threonine-rich motifs (PEST). The TAD also has a glutamine (Q) rich repeat (Kopan and Ilagan, 2009). The structure of *Drosophila* DSL ligands Delta and Serrate is represented in figure 3: DSL ligands contain, at its N-terminus, a DSL motif followed by a EGF repeat called the Delta and OSM-11-like proteins (DOS) motif and EGF repeats. *Drosophila* Serrate as well as mammalian Jagged proteins contain an additional cysteine-rich domain before their transmembrane domain (Kopan and Ilagan, 2009).

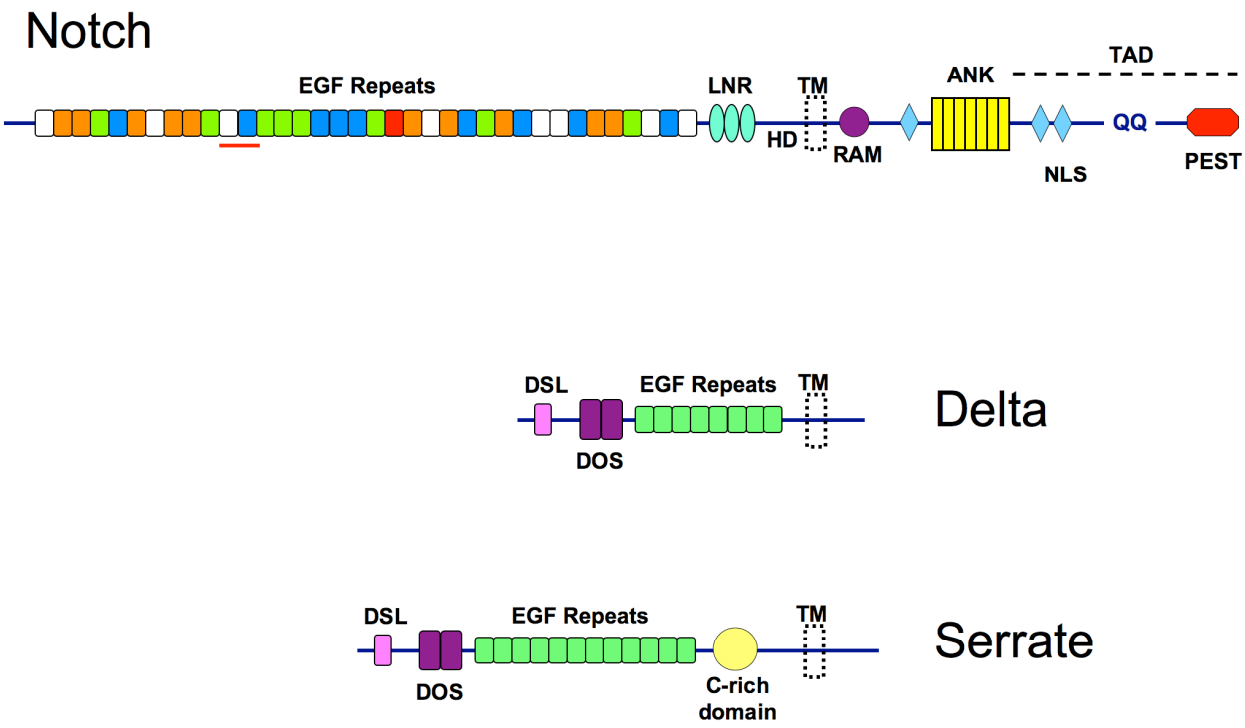


Fig. 3: Structure of the Notch receptor and the Notch ligands, Delta or Serrate (see text for information on the domains). Adapted from (Kopan and Ilagan, 2009).

Upon ligand-receptor binding, the Notch receptor undergoes successive proteolytic cleavages in its transmembrane domain, yielding the Notch Intracellular Domain (NICD) into the cytoplasm. How exactly the interaction of the ligands leads to activation of the receptor is not entirely understood. The negative regulatory region appears to play a critical role in preventing the proteolytic cleavage of the receptor, as the cleavage site is normally protected by a hydrophobic interface between the LNR motifs and the HD domain. It has been proposed that ligand binding induces conformational changes in the negative regulatory region, leading to relaxation of the interaction of the LNR motifs with the HD and exposing the cleavage site (Gordon et al., 2007; Tien et al., 2009). Activation of the Notch receptor by its ligands first leads to cleavage of Notch by the ADAM/TACE/kuzbanian family proteases at site 2 (S2), which releases the ectodomain of Notch and creates an activated, membrane-tethered intermediate called the Notch Extracellular Truncation (NEXT). γ-secretase activity of the Presenilin-Nicastrin-Aph1-Pen2 protein complex cleaves NEXT at two endomembrane sites, S3 and S4, releasing the NICD. NICD is the active form of the receptor and translocates directly to the nucleus, where it acts as a transcriptional regulator, promoting the transcription of the Notch target genes. NICD forms a complex with the DNA binding protein CSL (human CBF1, fly Suppressor of Hairless and nematode Lag-1) and the co-activator Mastermind (Mam). The NICD-CSL-MAM complex binds specific regulatory DNA sequences, activating the expression of the CSL/Notch target genes. Fig.4 represents the here described core Notch signaling pathway (Bray, 2006; Kopan and Ilagan, 2009; Schweisguth, 2004b).

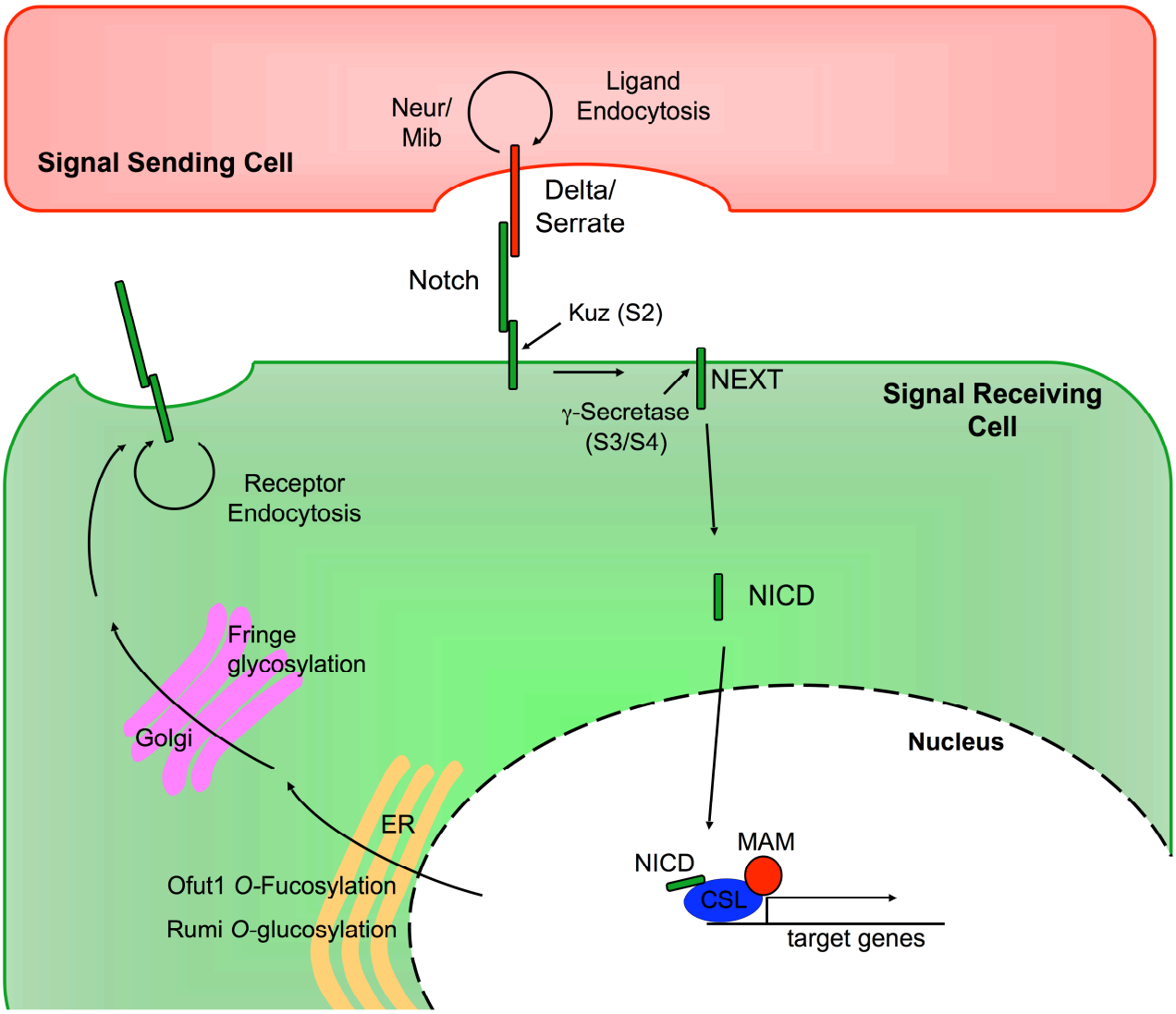


Fig. 4: The main components of Notch signaling. Adapted from (Kopan and Ilagan, 2009).

C. Regulation of Notch Signaling

The activation of the Notch receptor by proteolysis has two immediate consequences: the activation of the receptor by its ligand leads directly to transcriptional activity, dispensing secondary molecules and the activation of the receptor is irreversible, with each receptor protein yielding only one NICD molecule, that will translocate to the nucleus. Notwithstanding, activation of the Notch pathway is tightly regulated by a myriad of mechanisms. It is not the objective of this introduction to describe them exhaustively. I will briefly mention some of the better-characterized mechanisms for regulating Notch activity and will discuss more in depth the modulation of Notch signaling by glycosylation of the receptor, which is of direct interest for the work described in Chapter IV of this thesis.

C.1. Regulation of Ligand activity by endocytosis

Endocytosis of the Notch ligands in the signal-sending cell is essential for Notch signaling activity. E3 ubiquitin-ligases regulate the endocytosis of the DSL ligands in *Drosophila* and vertebrates: Neur (Neuralized), Mib-1 (Mindbomb-1, mindbomb in *Drosophila* and vertebrates) and Mib-2 (mindbomb-2 in *Drosophila* and skelotrophin or mindbomb-like in vertebrates). It has been suggested that these E3 ubiquitin ligases are able to mono-ubiquitinate Delta and Serrate, targeting them to endocytosis and epsin-mediated sorting (Deblandre et al., 2001; Lai et al., 2001; Le Borgne et al., 2005b; Le Borgne and Schweisguth, 2003; Overstreet et al., 2003; Pavlopoulos et al., 2001; Wang and Struhl, 2004; Wang and Struhl, 2005; Yeh et al., 2001). The requirement of endocytosis for ligands to signal is essential for Notch activation, although the mechanism by which endocytosis promotes ligand signaling activity is not known. One model has been proposed in which endocytosis generates a pulling force on the NECD, promoting receptor dissociation and activation. Another,

model has been proposed in which the ligands are produced in an inactive or poorly active state and endocytosis allows ligands to undergo post-translational modifications within endocytic vesicles, which are subsequently recycled to the membrane, with the ligands now in an active form (Kopan and Ilagan, 2009; Le Borgne et al., 2005a).

C.2. Regulation of Receptor activity by endocytosis

The Notch receptor has been shown to be ubiquitinated and targeted to endocytic vesicles by E3-ubiquitin ligases of the Nedd4 family (*Drosophila* Nedd4, Suppressor of deltex and mammalian Itch and Cbl). As a consequence of this ubiquitination, the Notch receptor is targeted to late endosomes and is subsequently degraded in lysosomes (Cornell et al., 1999; Fostier et al., 1998; Le Borgne et al., 2005a; Sakata et al., 2004; Wilkin et al., 2004).

Numb has a role in negatively regulating Notch signaling in a context-dependent manner. Numb is a membrane-associated protein that antagonizes Notch signaling during asymmetric cell division in *Drosophila* by down regulating the activity of Sanpodo, a positive regulator of Notch. Numb removes Sanpodo from the plasma membrane, where promotes Notch signaling, targeting Sanpodo to late endosomes (Berdnik et al., 2002; Dye et al., 1998; Guo et al., 1996; Le Borgne et al., 2005a; O'Connor-Giles and Skeath, 2003; Santolini et al., 2000; Skeath and Doe, 1998). The role of Numb in regulating Notch activity in *Drosophila* appears to be restricted to contexts where cells divide asymmetrically.

D. Regulation of Notch signaling by Glycosylation

The role of protein glycosylation as a way to regulate the Notch pathway first became evident when Fringe, a modulator of Notch activity, was found to be a

glycosyltransferase that modifies the *O*-fucose residues on Notch EGF repeats (Bruckner et al., 2000; Moloney et al., 2000a). The extracellular domain of Notch contains tandem Epidermal Growth Factor-like (EGF) repeats that can be modified by three types of *O*-linked glycans: *O*-linked fucose, *O*-linked glucose and *O*-linked *N*-Acetylglucosamine [Fig. 5, (Matsuura et al., 2008; Moloney et al., 2000b; Shao et al., 2002; Takeuchi and Haltiwanger, 2010)].

O-Fucosylation of serine (S) or threonine (T) residues occurs within a consensus sequence of some EGF repeats of the Notch protein (Moloney et al., 2000b). In some developmental contexts, the *O*-fucose residues have been shown to be elongated by the addition of an *N*-acetylglucosamine (GlcNAc) by Fringe (Fng) in a β1,3 linkage onto *O*-linked fucose (Bruckner et al., 2000; Chen et al., 2001; Moloney et al., 2000a). The Notch ligands, Delta and Serrate, also have been shown to have their EGF repeats modified by *O*-fucose, but the it is not clear whether fucosylation is playing any role since no biological requirement for fucosylation of the ligands has been identified (Panin et al., 2002). The *O*-linked disaccharide GlcNAc-β1,3-Fuc-*O*-S/T can be further elongated by sequential addition of galactose and sialic acid residues but this further elongation does not appear to play any role in Notch signaling activity in *Drosophila* (Xu et al., 2007).

Another modification identified on Notch receptor and that has an important role is *O*-glycosylation (Acar et al., 2008). *O*-Glucose can be added on to a serine in an EGF repeat with the *O*-glucose consensus sequence (Moloney et al., 2000b; Shao et al., 2002). Recently, mouse Notch1 was shown to have the *O*-linked glucose residues extended by two xylose residues but neither the role for these modifications or the enzymes that catalyze them are known (Jafar-Nejad et al., 2010; Takeuchi and Haltiwanger, 2010).

Notch has also been shown to also have *O*-β-acetylglucosamine (*O*-β-GlcNAc) modification on several EGF repeats (Matsuura et al., 2008).

Finally, the Notch receptor has also been found to be modified by *N*-linked glycans but these do not appear do play any role in Notch signaling (Takeuchi and Haltiwanger, 2010).

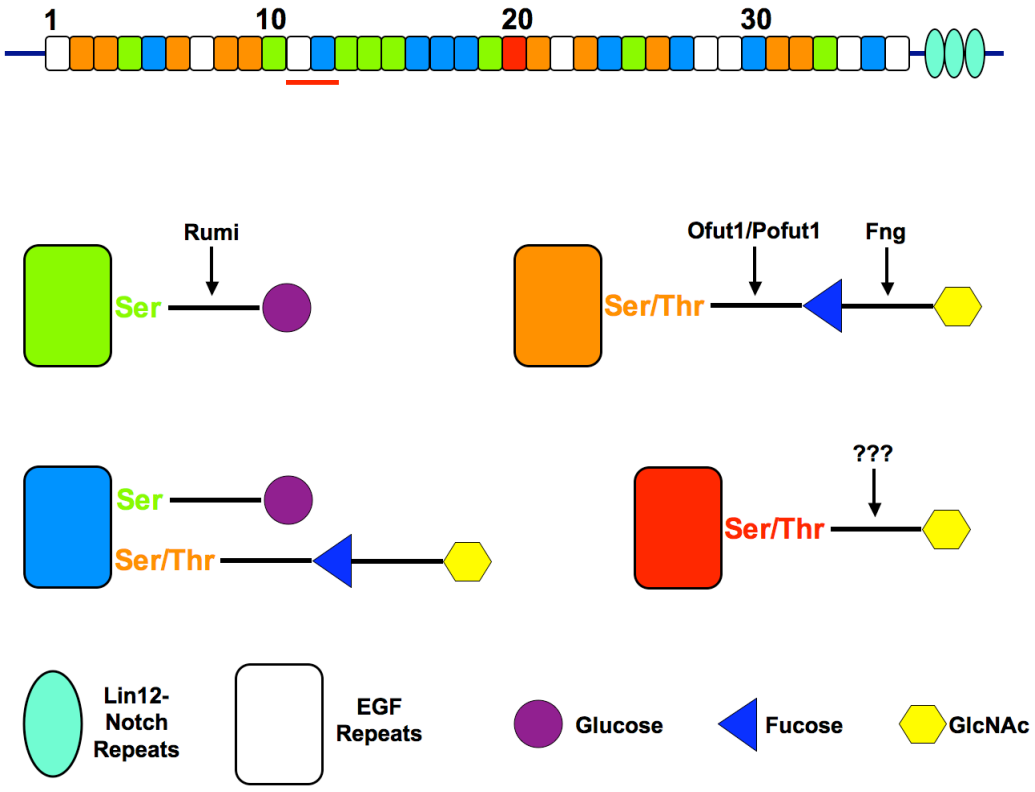


Fig. 5: Glycosylation of the EGF repeats of Notch receptor. The extracellular domain of Notch receptor is represented, with the different EGF repeats and the respective modifications they can undergo. Underlined in red are EGF repeats 11-12, critical for interaction with the ligands. Note that EGF repeat 20, represented in red can also be modified by Rumi and Ofut1/Pofut1 but only *O*-GlcNAcylation is represented, for simplicity. Adapted from (Takeuchi and Haltiwanger, 2010).

Mutations in glycosyltransferase genes that modify Notch and defects in the synthesis and transport of the nucleotide sugars required for synthesis of the glycans lead to defects in Notch signaling (Takeuchi and Haltiwanger, 2010). Therefore, glycosylation of the Notch receptor is an important mechanism to modulate Notch signaling activity.

D.1. *O*-GlcNAcylation of the Notch receptor

Recently, the Notch receptor has been shown to have *O*-β-GlcNAc modification on a serine or threonine located between the 5th and 6th cysteines of several EGF repeats. *O*-GlcNAc seems to be present as monosaccharide on the EGF repeats of Notch and has also been detected on the Notch ligand Delta. This was an unexpected result as Notch is the first protein to be identified as having an *O*-β-GlcNAc modification on an extracellular domain, with all previous known *O*-β-GlcNAc modifications being on intracellular cytoplasmic proteins or nuclear proteins. The role of *O*-GlcNAcylation of Notch and/or its ligands is not known, nor is the *O*-GlcNAc transferase that catalyses this reaction, which has been proposed to be a novel glycosyltransferase [Fig.5, (Matsuura et al., 2008)].

D.2. *O*-Glucosylation of the Notch receptor

The role of *O*-glucosylation in the Notch pathway had been unknown until quite recently. Biochemical studies of the Notch protein showed that the some of the EGF repeats are modified by *O*-Glucose glycans and that *O*-glucose can be added only on to a serine within the *O*-glucose consensus sequence C1-X-S-X-P-C2 (C, cysteine; X, any amino acid; S, serine; P, proline) between the first and the second cysteines conserved in EGF repeats (Moloney et al., 2000b; Shao et al., 2002). However, it was the isolation of mutations in the gene *rumi* that demonstrated that *O*-glucose

modifications are essential for Notch function (Acar et al., 2008). *rumi* was identified in a screen for genes that affect SOP development in *Drosophila*. *rumi* mutant flies had a loss of Notch signaling phenotype when reared at 25 ºC, but not at 18 ºC. *In vivo* experiments carried out in *Drosophila* showed that Rumi activity was required in the signal receiving cell but not in the signal sending cell for proper Notch signaling activity, suggesting that Rumi is acting on the Notch receptor but not on its ligands (Acar et al., 2008). Rumi was predicted to have a signal peptide, a CAP10 domain, and a C-terminal KDEL ER-retention signal and was shown, *in vitro,* to be a protein *O*-glucosyltransferase (Poglut), catalyzing the addition of *O*-glucose to EGF repeats (Fig. 5). Protein extracts from cells where *rumi* was knocked-down had reduced *O*-glucose on the Notch protein (Acar et al., 2008).

The molecular mechanism by which Rumi affects Notch activity is not entirely understood. Analysis of the strong *rumi* mutant allele $rumi^{79}$, which has a G189E point mutation that results in Rumi protein still being expressed but having no enzymatic activity, suggests that it is indeed the Poglut activity of Rumi that is required and not an independent, potential function as an ER chaperone, as has been described for Ofut1 [see below, (Okajima et al., 2008; Okajima et al., 2005)]. In *rumi* mutant cells, Notch protein accumulates both inside the cells but also at the cell surface, which indicates that Rumi affects the trafficking and/or stability of Notch. Although the Notch receptor is present at the cell surface in *rumi* mutant cells reared at restrictive temperatures, expressing Delta ligand in neighboring cells cannot activate it. However, cell-based assays that measure the interaction between cells expressing Delta and secreted Notch-alkaline phosphatase fusion protein (Bruckner et al., 2000) suggested that reduction in *O*-glucose on Notch does not affect binding to Delta (Acar et al., 2008). Finally, experiments in *Drosophila* wing discs, showed that the phenotype arising from the overexpression of the NEXT, the membrane tethered form of Notch that still requires S3 cleavage to be activated, was not suppressed in *rumi* mutants. These data place the requirement for Rumi between ligand-receptor interaction and S2 cleavage (Acar et al., 2008).

The temperature-dependent requirement for *rumi* function indicates that addition of *O*-glucose is essential for Notch activity at high temperatures, and that

preventing the addition of *O*-glucose to Notch results in temperature-sensitive Notch phenotypes. Temperature sensitivity is frequently related to changes in the protein stability (Sadler and Novick, 1965). One possibility is that the *O*-glucose glycans may be required for the proper folding of Notch at higher temperatures or may hold the extracellular domain of Notch in a stable conformation, essential for proper function at higher temperatures. In this way, the *O*-glucose would function to facilitate the proper folding of Notch or to maintain the receptor in a conformation that would allow for ligand binding to lead to S2 cleavage (Takeuchi and Haltiwanger, 2010).

D.3. *O*-Fucosylation of Notch

In recent years a considerable amount of evidence has emerged on the critical role *O*-fucosylation and *O*-fucosyltransferases play in Notch signaling (Okajima and Irvine, 2002; Okajima et al., 2005; Sasamura et al., 2007; Sasamura et al., 2003; Shi and Stanley, 2003). However, exactly how *O*-linked fucose (abbreviated *O*-fucose) and its extended forms regulate the Notch pathway is not entirely understood.

Protein *O*-fucosyltransferase-1 (Pofut1 in mammals and Ofut1 in *Drosophila*) catalyzes the addition of GDP-fucose to a serine or a threonine in the *O*-fucose consensus sequence C2-X-XX-X-(S/T)-C3 (C, cysteine; X, any amino acid; S, serine; T, threonine) between the second and the third cysteines conserved in the EGF repeats of the Notch receptor [Fig. 5, (Wang et al., 2001)]. Human Pofut1 was initially identified through classical molecular cloning: an enzyme with protein *O*-fucosyltransferase enzymatic activity was purified from Chinese Hamster Ovary (CHO) cells. The N-terminus of Pofut1 protein was sequenced and used to produce a DNA tag that led to the cloning of the human Pofut1 from a cDNA library. Also, the mouse, fly and worm homologues of OFut1 were identified by sequence analysis and these proteins were also found to have *O*-fucosyltransferase activity (Wang et al., 2001). Ofut1/Pofut1 is retained in the ER by its C-terminal KDEL-like ER-retention sequence (Luo and Haltiwanger, 2005; Okajima et al., 2005).

To study the role of Ofut1 in *Drosophila,* Okajima and Irvine first knocked-down the *Ofut1* gene using RNAi and observed that Ofut1 activity is required for Notch signaling in all contexts studied (Okajima and Irvine, 2002). In parallel, a mutation in Drosophila Ofut1, *neurotic*, was identified and further demonstrated that Ofut1 function is essential for Notch signaling activity in flies (Sasamura et al., 2003). Both groups showed that Ofut1 was required cell-autonomously for Notch signaling in the signal-receiving cells, suggesting that the Notch receptor is the likely substrate of Ofut1 (Okajima and Irvine, 2002; Sasamura et al., 2003). Simultaneously, Shi and Stanley generated the Pofut1 knockout mouse. Mouse embryos lacking Pofut1 died during embryogenesis with severe defects in vasculogenesis, cardiogenesis, somitogenesis and neurogenesis. This phenotype is equivalent to the phenotype of mice lacking core components of the Notch pathway, such as the presenilins or the CSL protein, which suggested that all Notch signaling events require Pofut1 activity (Shi and Stanley, 2003). Together, these studies as well as subsequent work on Ofut1 demonstrated that Ofut1/Pofut1 is a core component of the Notch pathway (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003)

Although Ofut1 has been shown to be required for Notch signaling *in vivo*, studies on the activation of Notch1 receptor in CHO cells that lack GDP-fucose, the Lec13 cell line (explained in more detail below), suggested that, while activation of the Notch receptor by its ligands is diminished in the absence of fucose, it is not completely abolished (Chen et al., 2001; Moloney et al., 2000a). This apparent contradiction was resolved by the identification of an ER chaperone activity for Ofut1 (Okajima et al., 2008; Okajima et al., 2005). Okajima and Irvine observed that, in flies, cells mutant for *Ofut1* had reduced levels of Notch protein at the cell-surface and that the cell-surface expression of Notch could be rescued by the overexpression of a catalytically dead form of Ofut1 ($Ofut1^{R245A}$), which suggested that the *O*-fucosylation of the Notch receptor *per se* is not required for cell surface expression. Ofut1/Pofut1 is an ER protein and it has been demonstrated that *O*-fucosylation of the EGF repeats occurs in the ER (Luo and Haltiwanger, 2005; Okajima et al., 2005). Furthermore, it has been suggested that Ofut1/Pofut1 can distinguish properly folded from unfolded EGF repeats, as it only *O*-fucoslyates properly folded ones, which

suggests that Ofut1/Pofut1 might play a role in the ER in quality control (Wang and Spellman, 1998). These data, together with the observation that Ofut1 binds the Notch receptor, probably in the ER, and that it can promote secretion of Notch independently of its *O*-fucosylatransferase activity, led Okajima and Irvine to propose that Oftut1 has an essential role as a chaperone of Notch (Okajima et al., 2008; Okajima et al., 2005).

Experiments to test the role of the catalytic domain of Ofut1 also demonstrated that the catalytically dead form of Ofut1 rescued the neurogenic phenotype of the *Ofut1* null (Okajima et al., 2008; Okajima et al., 2005). Embryos mutant for *Ofut1* but expressing Ofut1^{R245A} through a genomic rescue construct had no detectable neurogenic phenotype, similarly to what has been observed in *Fringe* (*fng*) mutants (Okajima et al., 2008), further suggesting that Fringe protein is not required for Notch signaling during embryonic development (Irvine and Wieschaus, 1994; Panin et al., 1997). To test the requirement for *O*-fucosylation of Notch during wing development, *Ofut1* mutant clones, in which the Ofut1^{R245A} construct was expressed, were generated in the wing discs and found to have the same phenotype as *fng* mutant clones: loss of *Ofut1* results in a complete loss of Notch activation while *fng* mutant clones, as well as *Ofut1* + Ofut1^{R245A} mutant clones, result in ectopic activation of Notch signaling at the clone margin and ectopic expression of Notch target genes such as Wng and Cut (Okajima et al., 2008; Okajima et al., 2005). These data suggest that the main function of the *O*-fucose on Notch is to serve as a substrate for Fng.

The hypothesis that the *O*-fucosylated Notch is only required as a substrate for Fng was further tested by investigating mutants lacking GDP-fucose, the donor substrate for Ofut1 and all fucosyltransferases (Okajima et al., 2008; Okajima et al., 2005; Sasamura et al., 2007). GDP-fucose is synthesized in the cytoplasm from GDP-mannose in a two-step reaction catalyzed by two enzymes: GDP-mannose 4,6-dehydratase (Gmd) and GDP 4-keto 6-deoxymannose epimerase-reductase, known as FX (Becker and Lowe, 2003; Haltiwanger and Lowe, 2004; Ma et al., 2006a). Since enzymes for a salvage pathway for GDP-fucose, which synthesize GDP-fucose from imported fucose in the cytosol, are lacking in *Drosophila,* cells lacking

Gmd have no GDP-fucose available and fucosylation events are not expected to occur (Roos et al., 2002; Sasamura et al., 2007). *Gmd* mutants die as larvae and do not have a neurogenic phenotype. Instead, *Gmd* mutants have a phenotype consistent with loss of Fng phenotype: *Gmd* mutant wing discs fail to grow and do not express any Notch target genes at the dorsal-ventral boundary, such as Wingless (Okajima et al., 2008; Okajima et al., 2005; Sasamura et al., 2007). This is consistent with the absence of the Fng-dependent glycosylation of Notch. Furthermore, the Notch receptor is normally expressed at the surface of *Gmd* mutant cells, consistent with proper folding and trafficking occurring, which would be dependent on the chaperone activity of Ofut1 but not the *O*-fucosyltransferase activity (Okajima et al., 2008; Okajima et al., 2005). *Gmd* mutant clones in the wing disc, however, do not have the same phenotype as *fng* mutant clones: *Gmd* clones only resulted in a mild disruption of the wing margin (Okajima et al., 2008). This weak phenotype is possibly explained by the hypothesis that nucleotide sugars cans diffuse from cell to cell through gap junctions (Okajima et al., 2008). Finally, *Pofut1* knock-out mice also have a decrease in Notch protein localized at the surface of cells in the somites (Okamura and Saga, 2008). Together, these data suggest that *O*-linked fucose modifications on the Notch receptor may only be necessary in contexts where Notch signaling is modulated by Fringe (see next section). Therefore, Ofut1/Pofut1 would play two roles in Notch signaling: *O*-fucosylation of Notch, which is dispensable in some contexts, and a separate but essential role as a chaperone of Notch.

Studies in mammalian cells suggest that *O*-fucose might play a direct role in ligand-receptor binding. Stanley and colleagues used Chinese hamster ovary cells that are mutant for the *Gmd* and therefore lack GDP-fucose (Lec13 cells) to address the role of *O*-fucosylation of Notch1 receptor. It was shown that both ligand binding and activation of the Notch receptor were diminished, while cell-surface expression of Notch1 was normal in these cells (Stahl et al., 2008). These results suggest that *O*-fucose is required for Notch function in a Fng-independent manner (Chen et al., 2001; Moloney et al., 2000a; Stahl et al., 2008). Consistent with these observations, embryonic stem cells that lack Pofut1 had cell-surface expression of Notch proteins equivalent to wild type cells, but again, both ligand binding and activation of the

Notch receptor were reduced in these cells (Stahl et al., 2008). Similarly to what has been observed in *Drosophila*, Notch activity could be partially restored by expressing the catalytically dead form of Pofut1 (Pofut1^{R245A}). However, the expression of an unrelated, inactive ER glucosidase protein also partially rescued the loss of Pofut1 in embryonic stem cells, probably due to an increase in chaperone activities in the ER or in the ER-*cis*- Golgi that can occur when cells overexpress ER glycoporteins (Stahl et al., 2008). Together, these data indicate that interaction of the Notch receptor with its ligands leads to receptor activation in an optimal way if Notch is *O*-fucosylated. These results also suggest that chaperone activity is not specific for Pofut1, since overexpression of other ER proteins could also partially rescue the loss of Pofut1 function and that the requirement of Ofut1/Pofut1 as a chaperone of Notch might vary between *Drosophila* and vertebrates.

A function for Ofut1 in transport and localization of Notch in *Drosophila* has also been proposed. Two studies from Matsuno and colleagues have proposed that Ofut1 is a secreted protein that interacts with the extracellular domain of Notch when the receptor is localized at the plasma membrane. Furthermore, these authors argue that Ofut1 is not required for initial delivery of Notch to the apical plasma membrane but is instead required for the transcytosis from the apical plasma membrane to the adherens junctions and for constitutive trafficking of Notch into early endosomes (Sasaki et al., 2007; Sasamura et al., 2007). The authors carried out surface staining experiments and found that Notch is still present on the surface of *Ofut1* mutant cells, although the level of Notch at the surface is reduced (Sasaki et al., 2007). Uptake experiments with antibodies that bind the extracellular domain of Notch showed that Notch receptor is still internalized in *Ofut1* mutant cells (although at lower levels) but did not localize to early endosomes (Sasamura et al., 2007). These data, together with the observation that Notch protein fails to localize at the adherens junctions in *Ofut1* mutant cells, led to a model in which Ofut1 is not required for the delivery of Notch to cell surface but instead binds to Notch extracellularly and promotes its transcytosis to the adherens junctions (Sasaki et al., 2007; Sasamura et al., 2007). However, it is unclear how Ofut1 could arrive at the cell surface, with the endogenous protein having been shown to localize to the ER (Okajima et al., 2005). Experiments

with *Drosophila* S2 cells in which knock down of Ofut1 could be rescued by supplementing the medium with Ofut1 suggests an extracellular role for Ofut1, although it cannot be excluded that the supplemented Ofut1 was taken up by the cells and transported back to the ER (Sasamura et al., 2007; Vodovar and Schweisguth, 2008). Finally, the observation that Ofut1 is required in a cell-autonomous manner argues against a role for Ofut1 as a secreted protein that acts on Notch outside the cells (Okajima and Irvine, 2002; Sasamura et al., 2003). Analysis of *Gmd* mutant wing discs led Matsuno and colleagues to further propose that this role for Ofut1 is independent of its enzymatic activity as *Gmd* wing discs did not have the same defects on Notch localization as *Ofut1* and *Ofut1* + *Gmd* double mutants (Sasamura et al., 2007). Part of the discrepancy between different proposed roles for Ofut1 can be explained by technical differences: Matsuno and colleagues did not observe that Notch protein co-localized with ER markers in *Ofut1* mutant cells while Okajima and Irvine observed that Notch appeared to be retained in the ER in the same mutants; however, the two labs used different ER markers and different immunohistochemistry protocols (Okajima et al., 2005; Sasamura et al., 2007; Vodovar and Schweisguth, 2008). Also, the surface stainings of Notch in *Ofut1* differ between the two labs, which can also be explained by the difference in protocols (Okajima et al., 2008; Sasamura et al., 2007; Vodovar and Schweisguth, 2008).

Additional experiments are required to explain the inconsistencies between these different observations. Although Ofut1/Pofut1 does appear to play a role as a chaperone for Notch in the ER, all of the experiments to address this role were done in overexpression contexts. It would therefore be interesting to test the function of a catalytically dead Ofut1/Pofut1 expressed at endogenous levels. Furthermore, it should be clarified whether this chaperone function is specific to Ofut1/Pofut1 or if the rescue of chaperone function of Ofut1, in *Drosophila,* could also be done with the overexpression of ER proteins. Finally, the role that *O*-fucose modification alone plays on the structure of extracellular domain of Notch, the interaction with its ligands and the activation of the receptor have not yet been fully addressed.

D.4. Modulation of Notch receptor signaling by Fringe

Fringe (Fng) was first described as a protein required for interaction between the dorsal and the ventral cells in the wing disc and boundary formation during wing development in *Drosophila* (Haines and Irvine, 2003; Irvine and Wieschaus, 1994). Irvine and colleagues then showed that Fng modulates the interactions between Notch and its ligands, which is required for the formation of the wing-boundary (Panin et al., 1997). Fng is co-expressed with Serrate in the dorsal compartment of the wing disc while Delta is expressed in the ventral compartment. Fng enhances, in a cell autonomous manner, the ability of Delta to activate Notch and inhibits the ability of Serrate to activate Notch. In this way, Fng ensures that Serrate and Delta only activate Notch at the boundary between the dorsal and the ventral compartment, with the expression of Notch target genes, such as Wingless and Cut, therefore establishing the boundary between the two compartments (Haines and Irvine, 2003; Panin et al., 1997). Later, Fng was shown to be a glycosyltransferase that modulates Notch signaling by catalyzing the addition of a *N*-acetylglucosamine (GlcNAc), through β1,3 linkage, onto *O*-linked fucose (Bruckner et al., 2000; Chen et al., 2001; Moloney et al., 2000a). This sugar modification of Notch was demonstrated to enhance Delta binding to Notch and decrease Serrate binding to Notch by modifying the affinities of individual EGF repeats of Notch to the ligands (Bruckner et al., 2000; Chen et al., 2001; Moloney et al., 2000a; Xu et al., 2005).

Mammals have three Fringe proteins: Lunatic, Manic, and Radical Fringe. Knock out of Lunatic Fringe in mice results in defects in somitogenesis, a process that is Notch1-dependent (Evrard et al., 1998; Zhang and Gridley, 1998). Lunatic Fringe plays an essential role during somitogenesis, and was shown that periodic inhibition of Notch signaling by Lunatic Fringe was required for the segmentation clock in chick embryos (Dale et al., 2003). Loss of function studies for Radical and Manic Fringe in mice could not identify any obvious developmental function for these genes (Tan et al., 2009; Zhang and Gridley, 1998).

D.5. Ligand-Receptor interaction and the role of glycosylation

In vitro studies in *Drosophila,* using purified Notch receptor and ligands demonstrated that the addition of GlcNAc alone on *O*-fucose is sufficient to enhance Notch binding to Delta and to inhibit Notch binding to Serrate (Xu et al., 2007; Xu et al., 2005). Many EGF repeats were shown to be modified by Fng but it is unclear whether all of these sites participate in the Notch-ligand interactions and subsequent activation of the receptor (Shao et al., 2003; Xu et al., 2007; Xu et al., 2005). Through analysis of the interaction between S2 cells expressing the fly Notch receptor in which regions of the extracellular domain had been deleted with S2 cells expressing Delta, the EGF repeats 11–12 were shown to be necessary and sufficient to mediate interactions with Delta, in this particular essay (Rebay et al., 1991). In accordance with this observation, fragments of human Notch1 containing only EGF repeats 11-13 were shown to interact with the DSL domains of the ligands (Hambleton et al., 2004).

Later work by Xu and Irvine showed that, while EGF repeats 11-12 of Notch are essential for ligand binding, multiple EGF repeats effectively contribute to ligand binding. The authors also showed that the influence of Fng on ligand-Notch interaction is mediated by multiple EGF repeats, both for the enhancement of the Delta-Notch interaction and for the inhibition of Ser-Notch interaction (Xu et al., 2005). Xu and Irvine carried out extensive deletion mapping of the extracellular domain of Notch, as well as site-specific mutagenesis of specific serine or threonine residues, predicted to be sites of *O*-fucosylation, and measured the interaction of these extracellular domains of Notch with the ligands by carrying out cell-based binding essays in which the soluble extracellular domains of Notch are fused to alkaline phosphatase and their interaction with cells that express the ligands Delta or Serrate was measured by the activity of the alkaline phosphatase. This essay, the authors argue, is much more sensitive than the cell-cell aggregation essay previously used by (Rebay et al., 1991). Importantly, testing the interaction between a mini-Notch receptor composed only of four EGF repeats (EGF10-13) and the ligands showed that glycosylation of a single EGF repeat can influence the strength of the

interaction, even if the same EGF repeat is not critical for interaction of the full-length Notch receptor. Therefore, the glycosylation of a single EGF domain can directly modify binding of that EGF to the ligands (Xu et al., 2005).

Two possible models can be proposed for the role of glycosylation in mediating the interaction between Notch and its ligands. Either glycosylation affects ligand-receptor interaction by only determining the global structural conformation of the Notch extracellular domain or the glycosylation of individual EGF repeats can modify the affinity of each EGF repeat for the ligand, with multiple EGF repeats working cooperatively during ligand-receptor interaction. These two models are not mutually exclusive but the data argues against the model first model alone, suggesting that, while glycosylation may influence the structural conformation of Notch, multiple EGF domains of Notch do indeed participate in interaction with the ligands (Xu et al., 2005). Although these studies clearly showed the ability of GlcNAc added on to fucosylated Notch to modulate the interaction of Notch with its ligands, the specific role of *O*-fucose on Notch and whether it can also modulate the strength of the interaction of individual EGF repeats with the ligands has not yet been addressed. Experiments in mammalian cells mentioned above indicate that fucosylated Notch can bind Delta more strongly (Stahl et al., 2008) but it would be interesting to understand if this is mediated by multiple EGF repeats. It would be important to carry out similar cell-based interaction studies to elucidate this.

E. Other roles of fucosylation in mammalian development and disease

Studies in *Drosophila* for the role of fucosylation during development have shown that the main requirement for fucosylation is for the *O*-linked fucosylation of the Notch EGF repeats. *O*-linked fucosylation of Notch receptors is also essential in mammals. However, other roles for fucosylation during development have also been identified in mammals. While the fucose residues added on to Notch are *O*-linked, that is, the fucose is attached to the hydroxy oxygen of a serine or threonine, other

fucosylated proteins have *N*-linked fucose attached to a nitrogen of asparagine or arginine side chains. I will briefly summarize some of the Notch-independent roles fucose plays during mammalian development, adult life and disease.

The Lewis^x epitope, also known as the stage-specific embryonic antigen-1 or CD15, is an α(1,3)-fucosylated glycan expressed during early embryonic development and thought to play a role in cell-cell adhesion as blocking this epitope leads to loss of embryo compaction (Ashwell and Mai, 1997a; Ashwell and Mai, 1997b; Ashwell and Mai, 1997c; Ashwell and Mai, 1997d; Becker and Lowe, 2003). The Lewis^x epitope is also present in the developing brain and thought to play and is thought to play important roles during neurogenesis (Becker and Lowe, 2003).

In adult mammals, the most well-known fucosylated epitopes are the ABO blood group antigens, expressed on the surface of the erythrocytes. The H antigen is α(1,2)-fucosylated in all individuals and is then further elongated by the *ABO* locus-encoded glycosyltransferase to form the A and B antigens in individuals of the A, B or AB blood group. In individuals type O, the H antigen is not further modified (Lowe, 1993). Although the role of the of the ABO antigens in the erythrocytes is not fully understood, they have been proposed to play a role in self-recognition, which facilitates the detection of pathogens (Seymour et al., 2004). It is also possible that these antigens play a role in other cells.

The *ABO* and the *Se* loci have also been implied in the regulation of the levels of the von Willebrand factor, an essential coagulation protein. The levels of this coagulation factor have been associated with many human blood clotting-related diseases, with the ABO antigens playing a potentially important role in this process (Becker and Lowe, 2003).

Fucose is also an essential component of the oligosaccharide ligands for selectins, which are cell adhesion receptors expressed in platelets, endothelial cells and leukocytes. Selectins bind the oligosaccharides that decorate the cell surface of specific cells or proteins secreted by leukocytes. The interaction of selectins with these oligosaccharides promotes the rolling of the leukocytes on the endothelium, the first step of the exit of the leukocytes from the blood vessels (Springer, 1994).

Fucosylated glycans also play critical roles in host-microbe interaction. The Lewis^b antigen, a fucosylated antigen, is required for the attachment of the pathogenic bacteria *Helicobacter pylori* to the gastric epithelium and this interaction is thought to be important for the development of *H. pylori*-mediated peptic ulcer disease (Hooper and Gordon, 2001b).

In the adult mammalian intestine, epithelial cells express glycans that contain α(1,2)-linked fucose. These are thought to contribute to the colonization of the developing gut by the indigenous microbial flora by providing a favorable ecological environment for the non-harmful commensal bacteria that use fucose as a source of carbon (Hooper and Gordon, 2001a).

Fucosylated glycans have also been implicated in many human diseases. Loss of A and B blood group antigens expression, accompanied with the concomitant increase in the expression of the H and Lewis^y antigens has been observed in many tumors and is usually associated with a bad clinical prognosis. Also, increased expression of the Lewis^x and Lewis^a antigens has also been observed in many tumors, correlating as well with poor clinical prognosis, probably by playing a role in aiding metastasis by facilitating the adhesion of the cancerous cells to the endothelium (Becker and Lowe, 2003). The increase in fucosylation of the α-fetoprotein observed during hepatocellular carcinoma is used as a marker to distinguish this carcinoma from chronic liver disease (Miyoshi et al., 1999).

Increased fucosylation has also been observed in adult rheumatoid arthritis, an autoimmune disorder. Finally, as a consequence of its role in the selectin dependent recruitment of leukocytes, fucosylation has also been associated with pathologies that involve this process, including atherosclerosis, reperfusion injury following ischemic damage, inflammatory skin diseases and asthma (Becker and Lowe, 2003).

F. Role of Notch in human disease

Because of the role Notch signaling plays in so many fundamental processes during development and in adult cells, it is not surprising that the misregulation of

Notch signaling activity has been associated with multiple human disorders (Gridley, 2003); (Rampal et al., 2007).

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy) syndrome is an adult-onset hereditary disease that is characterized clinically by recurrent transient ischemic attacks, strokes, subcortical dementia, migraine with aura and psychiatric disturbances. Most CADASIL patients have either a gain or a loss of cysteine residues in the EGF repeats of *Notch3* (Gridley, 2003) (Rampal et al., 2007; Tien et al., 2009).

Spondylocostal Dysostosis (SCD) is a congenital disorder that is characterized clinically by short trunk, short stature (due to axial skeletal defects), vertebral malformations, rib fusions and deletions, hemivertebrae, and nonprogressive kyphoscoliosis. SCD can be associated with neural tube defects, genitourinary defects, torticollis, limb abnormalities, hernia, and cardiac abnormalities. Mutations in the ligand *Delta-like3* were identified in autosomal recessive SCD families (Rampal et al., 2007).

Alagille syndrome is an autosomal dominant, developmental disease with multi-organ involvement that is characterized by a lack of intrahepatic bile ducts resulting in neonatal presentation of chronic cholestasis and jaundice. Mutations in the ligand *Jagged1* have been identified in patients with this syndrome (Gridley, 2003); (Rampal et al., 2007).

The Notch pathway has been implicated in many cancers. It has been reported that Notch signaling is often deregulated in human malignancies, functioning as an oncogene in cervical, lung, colon, head, neck, and renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer. Also, in a small group of tumors, including human hepatocellular carcinoma, skin and small lung cancer, Notch has a tumor suppressor role (Gridley, 2003); (Rampal et al., 2007).

Targeting genes that modulate of the Notch pathway has been proposed as a significant therapeutic target for pharmacological treatment of Notch associated pathologies. In recent years, components of the Notch pathway have started to be tested as specific therapeutic targets for T cell acute lymphoblastic leukemia and colon cancer, as well as potential targets for inhibiting tumor angiogenesis. One

possible group of modulators are the enzymes that modulate Notch activity through glycosylation the Notch receptor (Rampal et al., 2007). Therefore, investigating the mechanisms of how the Notch pathway is regulated is critical both for the understanding and treatment of pathologies associated with misregulation of the Notch pathway.

II. The Adult Intestinal Stem Cells of *Drosophila*

Somatic stem cells are responsible for the maintenance of adult tissues and they proliferate to renew the stem cells as well as to produce cells with more restricted potential that may continue to divide but will eventually differentiate into the cells that make up the tissue. Stem cells exist in virtually all mammalian adult tissues and are the basis for the development and maintenance of these tissues. Through out the adult lifetime, stem cells must maintain themselves, produce differentiated cells to renew the adult tissue, as well as respond to damage. Loss of the stem cells' ability to maintain tissue homeostasis and regulate their own proliferation can lead to aging as well as other pathological alterations, particularly cancer.

In recent years, many studies have suggested that multiple cancers have, at their origin, cancer stem cells. Cancer stem cells are proposed to originate from transformation of the somatic stem cells and to proliferate to produce more cancer stem cells as well as differentiated-like cells that make up the majority of the tumor mass, with the tumoral cells being produced in a hierarchy similar to the production of normal differentiated cells (Bonnet and Dick, 1997; Lapidot et al., 1994; Lobo et al., 2007). Increasing evidence has been recently provided in support of this hypothesis (Barker et al., 2009; Bonnet and Dick, 1997; Gil-Perotin et al., 2006; Holland et al., 2000; Lapidot et al., 1994; Singh et al., 2003). If cancer stem cells are responsible for the origin and maintenance of most cancers, this has important clinical implications since most treatments for cancer target all the cells in the tumor as if they have equal malignant potential. These treatments are often successful in reducing the bulk of the

tumor size but not in preventing recurrence of the tumor, which could be explained by the inability of these treatments to eliminate the cancer stem cells (Lobo et al., 2007). Therefore, tumors that have at its origin cancer stem cells might be more aggressive and difficult to treat. Understanding the biology of cancer stem cells could lead to new treatments that target the bulk of the cancerous mass as well as the cancer stem cells. Moreover, understanding stem cell biology, how tissue homeostasis is maintained and also how it can be perturbed is critical to understanding diseases that result from misregulation of stem cell physiology.

A. General principles of adult Stem Cells

Before going into the introduction of the intestinal stem cells of *Drosophila*, I would like to overview some of the basic principles of adult stem cells. Many of the terms are used as proposed by Austin Smith in a glossary for stem cell biology (Smith, 2006). Adult stem cells (SC) are cells that can produce, throughout the lifetime of the organism, daughter cells that will renew the stem cell, as well as daughter cells with more restricted potential. These daughter cells will renew the tissue or organ, maintaining tissue homeostasis. The daughter cells with more restricted potential are already committed to differentiation. They may differentiate directly but may also continue to proliferate, being termed transit-amplifying cells (TA). Eventually, the TA cells will stop dividing and will engage in a differentiation programme. In order for a stem cell to maintain tissue homeostasis, it must maintain stem cell homeostasis, that is, maintain the stem cell pool throughout the whole life of the organism. For this purpose, the stem cell must balance proliferation to renew the stem cell with proliferation to produce cells that will differentiate (Fig. 6). The stem cell may divide asymmetrically in respect to the fate of its daughter cells, where one will self-renew the stem cell while the other will go on to differentiate, or it might divide symmetrically, producing two daughter cells that become stem cells or, the converse, producing two daughter cells that will go on to differentiate. Maintenance of

stem cell homeostasis also requires balancing these different types of divisions (Smith, 2006).

Stem cells can be qualified depending on their potency, that is the array of cell-fate options available to the stem cell. A pluripotent stem cell can generate all the cell lineages that make-up the organism, with the best example being the embryonic stem cells. A multipotent stem cell can generate multiple lineages that build-up an entire tissue or multiple tissues, with a good example of these being the haematopoietic stem cells. An oligopotent stem cell can generate two or more lineages within a given tissue while an unipotent stem cell generates a single lineage within a tissue (Smith, 2006).

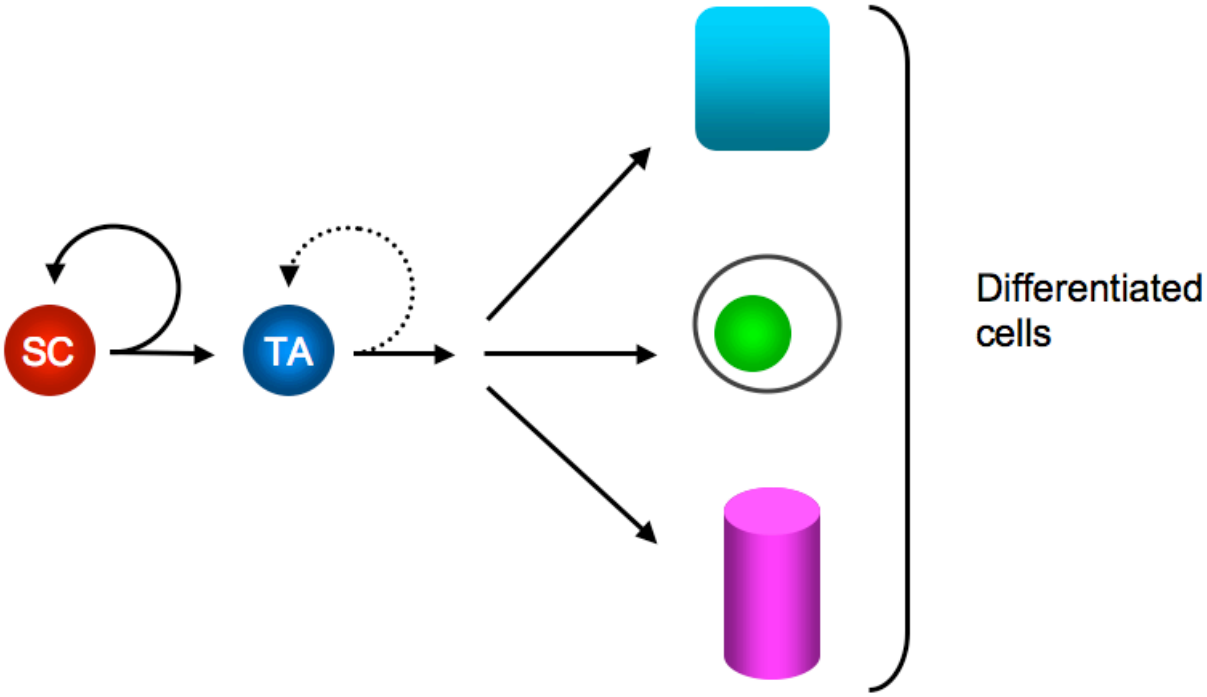


Fig. 6: Outline of a somatic stem cell lineage: the stem cell (SC) divides to renew the stem cell and to produce cells with more restricted potential, the transit amplifying cells (TA). The TAs continue to divide but will eventually differentiate into the different differentiated cell types.

Frequently, cells have been identified, within a tissue, which can proliferate and produce differentiated cells. These are termed progenitor cells and may be potential stem cells but in order to be considered *bona fide* stem cells, their ability to maintain the stem cell pool must be verified. The best way to test this is through clonal analysis, a powerful technique through which the properties of single cells are investigated. By tracking a single cell and its progeny throughout the lifetime of the organism, both the ability to self-renew the stem cell pool and the potency of the cell can be addressed and the lineage of the stem cell can be established (Smith, 2006).

Stem cells have been found to be located in a niche, which is a microenvironment that provides the stem cell with anatomical support and stimuli necessary for stem cell self-renewal, survival and, sometimes, differentiation (Jones and Wagers, 2008; Smith, 2006).

B. Adult Stem Cells in *Drosophila*

Stem cell research in invertebrates has had a central role in the identification of basic concepts in stem cell biology. *Drosophila,* not surprisingly, has been found, in more recent years, to harbor a considerable number of adult stem cells (Kohlmaier and Edgar, 2008). The study of adult stem cells in *Drosophila* has allowed the understanding, at a cellular and molecular level, of how stem cells renew tissues and are, at the same time, regulated. Often, adult stem cells have been shown to be regulated by a niche. For simplicity, I will briefly summarize the different adult stem cell models in *Drosophila*, and then concentrate on the intestinal stem cells of the fly midgut.

One stem cell-like cells in Drosophila are the larval neuroblasts, which have shown to be post-embryonic neural stem-like cells that divide mostly during larval development of *Drosophila*. In other insect such as crickets, neuroblast proliferation continues during the lifetime of the individual. Neuroblasts have been shown to divide asymmetrically, with the glial cells functioning as a niche. The neuroblasts divide to

produce a cell that maintains the neuroblast fate and an intermediate progenitor called ganglion mother cell, which will divide only once more into two differentiating neurons or glia (Chia et al., 2008). Recent work has identified a second type of neuroblasts in the larval brain, the posterior-asense-negative (PAN) neuroblasts, which display an intermediate, transit-amplifying lineage, that generates asymmetrically dividing secondary neuroblasts (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008).

Female germline stem cells (GSC) are one of the best-characterized stem cell models in *Drosophila*. Female GSCs are localized in a cell niche at the tip of the ovariole, the fly ovary. The niche, formed by cap cells, is in direct contact with the GSCs. The GSCs divide in a coordinated manner to the somatic escort stem cells (ESC) that will produce squamous non-dividing daughter escort cells. These escort cells will encyst the differentiating GSC daughters, called cystoblasts. After four transit-amplifying divisions with incomplete cytokinesis, one cystoblast is determined as the oocyte. Inhibitory bone morphogenic protein (BMP) signaling, via the ligands decapentaplegic (Dpp) and glass-bottom-boat (Gbb) secreted by the niche cap cells, triggers transcriptional inhibition of differentiation in the GSCs. After cell division of the GSCs, the daughter cell further away from the niche does not receive sufficient inhibitory BMP signal and starts to differentiate to become a cystoblast. The cap cells of the ovariole are a good example of a niche that is required for stem cell maintenance and proliferation and that regulates differentiation (Xie, 2008).

Male GSCs have also been thoroughly studied in *Drosophila*. The male GSCs are localized adjacent to the somatic hub cells, which function as the niche and are localized at the apical tip of the testis. The hub cells secrete Unpaired (upd), the Jak/Stat ligand. Activation of Jak/Stat signaling in the GSCs promotes self-renewal of the GSC by blocking differentiation. As in the ovary, the daughter cells further away from the niche undergo transit-amplifying divisions and start to differentiate, being termed gonialblasts (Xie, 2008).

The adult posterior midgut has recently been shown to be renewed by intestinal stem cells (ISCs) which divide, approximately once a day, to renew the stem cell and to produce a cell committed to differentiate, the enteroblast [EB; (Micchelli and

Perrimon, 2006; Ohlstein and Spradling, 2006)]. The enteroblast differentiates directly into large absorptive cells or into enteroendocrine cells. The biology of these ISCs is described in detail below.

The intestinal stem cells (ISC) of the adult hindgut were only recently identified. The ISCs of the adult hindgut were identified as slow cycling cells, being localized in an anterior ring in the hindgut. They were reported to self-renew in response to short-range Wingless ligand released from niche cells localized more anteriorly. Then, the hindgut ISCs were shown to produce fast cycling hindgut progenitors that would finally exit cell cycle and differentiate to enterocytes (Takashima et al., 2008). However, later work has proposed that these stem cells are normally quiescent and that only severe damage to the hindgut promotes proliferation of these progenitor cells (Fox and Spradling, 2009).

Adult renal-nephric stem cells (RNSCs) have also been recently identified in the Malpighian tubules, the fly excretory organ. These stem cells were observed to proliferate in the lower tubules and urethras of the Malpighian tubules and produce the renalblast daughters, which differentiate into renalcytes in the lower tubules or into type I and type II cells in the upper tubules. Autocrine Unpaired secretion and activation of Jak/Stat signaling in the stem cells was observed to promote proliferation (Singh et al., 2007).

C. Development of the *Drosophila* digestive track

The adult *Drosophila* digestive system consists of the alimentary canal and the organs associated to it, which are the mouthparts, the labellar glands, the salivary glands and the Malpighian tubules [Fig.7, (Miller, 1994)]. The alimentary canal is essentially a tube that extends from the mouth to the anus and is divided in three main domains: the foregut, the midgut and the hindgut. The foregut comprises the esophagus, the crop and the stomodaeal valve part of the cardia. The midgut consists solely of one structure, the ventriculus, which is the longest part of the alimentary canal. The ventriculus forms at its anterior most end the cardia and

terminates at its posterior end in the pyloric region. The hindgut consists of the anterior intestine and the posterior intestine or rectum, which terminates in the anus. The two pairs of Malpighian tubules arise each from a single stalk the stems out at the pyloric region of the midgut (Fig.7). Both the foregut and the hindgut have a chitinous intima layer lining the epithelium while it is absent in the midgut (Miller, 1994).

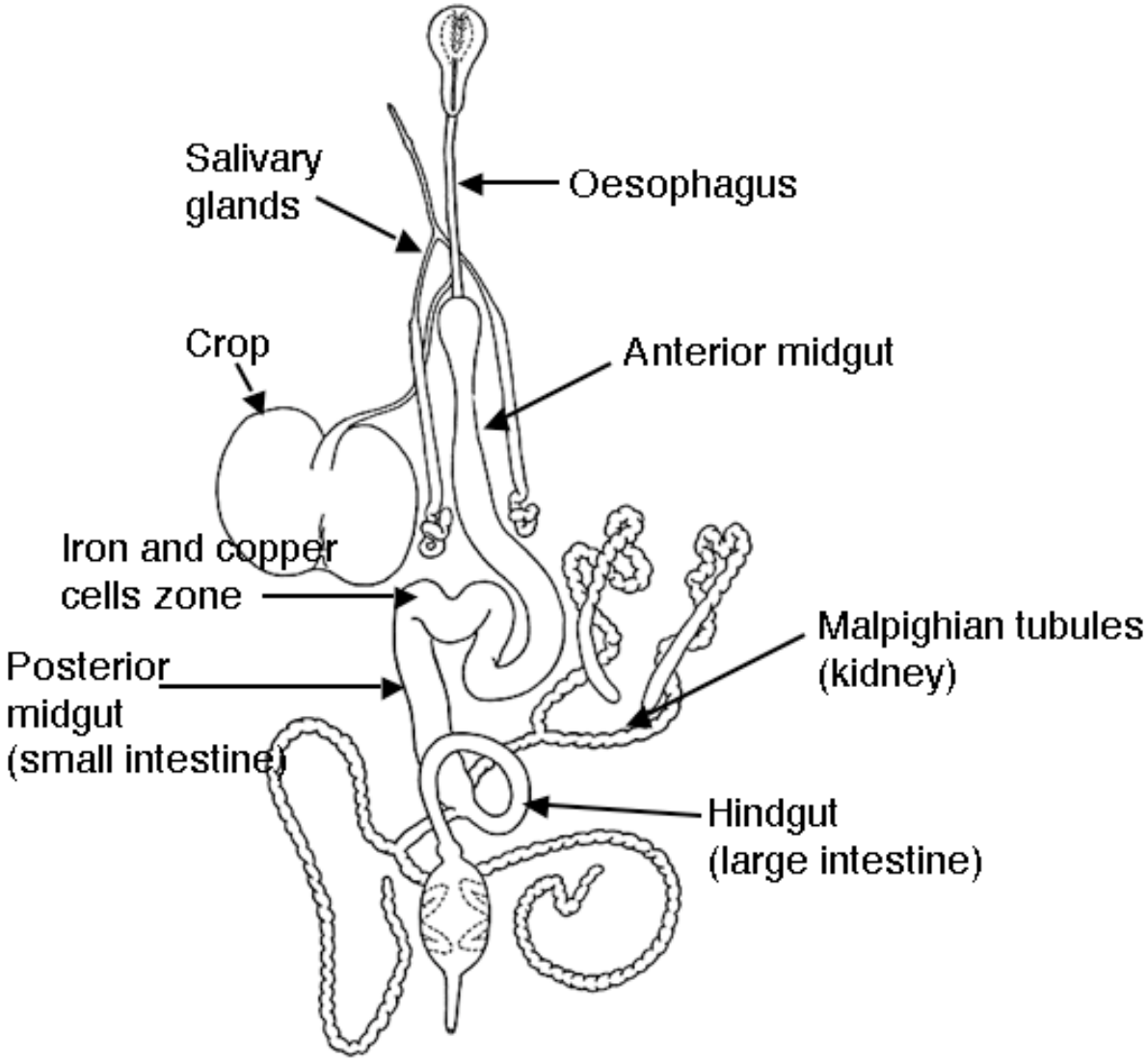


Fig. 7: A drawing of the *Drosophila* alimentary canal and the organs associated to it. Adapted from (Miller, 1994).

During embryonic development, the epithelial layer of the alimentary canal of the larvae arises from both the endoderm and the ectoderm (Hartenstein, 1993; Poulson, 1994). For simplicity purposes, I will only elaborate on the development of the midgut. The posterior midgut is orthologous to vertebrate small intestine, with the epithelial lining of both organs being of endodermal origin. The transcription factors Forkhead and GATA play conserved roles in the specification and formation of the digestive systems of both vertebrates and *Drosophila* (Stainier, 2005). During gastrulation, the anterior and posterior midgut primordia (AMG and PMG respectively), which are localized at opposite poles of the embryo, invaginate and extend toward each other to give rise to most of the midgut (Hartenstein, 1993; Poulson, 1994; Reuter et al., 1993; Tepass and Hartenstein, 1994). The ectoderm that surrounds the AMG and PMG primordia gives rise to the foregut and the hindgut respectively. After gastrulation, the endodermal cells of the AMG and PMG primordia undergo an epithelial to mesenchymal transition, losing their epithelial characteristics and forming the midgut rudiments. Later during development, the midgut rudiments stretch along the longitudinal axis of the organism and then approach each other to start to fuse to form again an epithelial tissue that will constitute the larval midgut (Hartenstein et al., 1992; Reuter et al., 1993; Tepass and Hartenstein, 1994). This mesenchymal to epithelial transition to form the epithelial midgut requires cross-talk of the endodermal midgut rudiments with the adjacent visceral mesoderm, that provides a basal substratum for the epithelium formation, as well as interactions between the endodermal cells amongst themselves (Reuter et al., 1993; Tepass and Hartenstein, 1994). This interaction of the endodermal gut precursors and the mesodermal cells is also observed during the development of the vertebrate gut (Stainier, 2005). The larval midgut has now a typical columnar epithelial structure and is surrounded by mesodermal tissue (Tepass and Hartenstein, 1994). The epithelial cells that will build up most of the larval midgut are called larval enterocytes and are absorptive cells. Intercalated between them are the undifferentiated adult midgut progenitors (AMPs) also known as midgut histoblast islets or midgut imaginal islets (Hartenstein et al., 1992). The AMPs are initially spindle-shaped cells localized to the

apical surface of the epithelium; later they migrate to the basal surface, where they stay during larval development (Jiang and Edgar, 2009). Notch signaling has been shown to mediate the development of the AMPs since in embryos mutant for *Notch*, the number of AMPs increases at the expense of the larval enterocytes (Hartenstein et al., 1992).

During larval development, the enterocytes grow by increasing its cell size and undergoing endoreplication, becoming polyploid cells (Bodenstein, 1994; Jiang and Edgar, 2009). Two recent studies have focused on the AMPs during larval development and pupation. Jiang and Edgar have shown that, during larval development, the AMPs appear as islets of cells that are distributed among the enterocytes and that divide extensively, remaining as diploid cells (Jiang and Edgar, 2009). During an initial phase of larval development, the AMPs divide and the daughter cells migrate away from each other, resulting in an increase in the number of islets of AMPs. In later larval stages, the AMPs continue to proliferate while remaining close together and increasing the number of cells *per* islet, with each islet containing from eight to thirty cells at the onset of metamorphosis [0h after pupae formation or APF, (Jiang and Edgar, 2009)]. These authors showed that the enhancer of the *escargot (esg)* gene driving the expression of the *lacZ* gene or *GFP* via the *Gal4/UAS-GFP* reporter system (which will be referred to as *esg-lacZ* or *esg-GFP* for here on) promotes specific expression of LacZ or GFP protein in the AMP cells (Jiang and Edgar, 2009). Interestingly, *esg-lacZ* and *esg-GFP* are also specifically expressed in the intestinal stem cells of the adult midgut and its daughter cell (Micchelli and Perrimon, 2006). EGF signaling was found necessary and sufficient for the proliferation of the AMPs. The EGF ligand *vein* is expressed in the visceral muscle surrounding the larval midgut and is required for the early proliferation of the AMPs. Autocrine production of other EGF ligands, *spitz* and *Keren*, by the AMPs themselves is also thought to contribute to the proliferation of the AMPs (Jiang and Edgar, 2009). In another study, Ohlstein and colleagues identified a transient niche that is generated in the larval midgut during development. In early larval stages, when only one AMP is present, that single AMP expresses the Notch ligand Delta (Mathur et al., 2010). Interestingly, Delta was also found to be

specifically expressed in the stem cells of the adult midgut (Ohlstein and Spradling, 2007). Later, when the AMP islands contain two cells, one expresses Delta and one expresses a *lacZ* reporter of Notch transcriptional activity, which indicates that after mitosis, one of the AMP daughter cells has received a Notch signal (Mathur et al., 2010). Morphologically, this Notch ON cell corresponds to a previously described cell type, the peripheral cell (El Shatoury and Waddington, 1957). The peripheral cells have extended processes that involved the AMPs and as the AMP islets increase in size, the peripheral cells encapsulate all the AMPs (Mathur et al., 2010). Loss of Notch signaling in the larval midgut during early larval development resulted in loss of the peripheral cells while activation of Notch in the AMPs suggests that Notch signaling is necessary and sufficient to promote the peripheral cell fate (Mathur et al., 2010).

During metamorphosis, most AMPs stop proliferating and start forming the adult midgut epithelium. First, the larval midgut shortens itself, becoming compacted while the visceral muscles around it contracted. In parallel, the AMP clusters fuse to form the new midgut epithelium and engulf the larval epithelium, which is histolyzed and shed into the lumen (Jiang and Edgar, 2009). During metamorphosis, the visceral muscle de-differentiates: the fibers histolyze but the muscle cells do not die and, instead, will redifferentiate in to the adult midgut visceral muscle at the later stages of metamorphosis (Klapper, 2000). As the AMPs start to form the new epithelium of the adult midgut, most of these start to differentiate as enterocytes and lose *esg-GFP* expression. Some AMPs retain *esg-GFP* expression and are thought to become the future stem cells of the adult midgut (Jiang and Edgar, 2009). As AMPs differentiate into enterocytes, some AMPs retain Delta expression and are thought to become the future stem cells (Mathur et al., 2010). It is likely that the remaining *esg-GFP*+ AMPs correspond to the AMP population that retains Delta expression. These *esg-GFP*+ pre-stem cells are thought to continue to divide and start producing Prospero positive enteroendocrine cells as well [more on the adult midgut cells below, (Jiang and Edgar, 2009)]. Interestingly, Ohlstein and colleagues observed that the peripheral cells play a critical role at the onset of metamorphosis: during early metamorphosis, the processes of the peripheral cells retract, releasing the AMPs that start to

differentiate, while the peripheral cells undergoes programmed cell death (Mathur et al., 2010). Furthermore, these authors found that the peripheral cells acts as a temporary niche that maintains the AMPs in an undifferentiated state during larval development (Mathur et al., 2010). In other stem cell systems, the stem cell niche has been shown to maintain the stem cells in an undifferentiated state through BMP signaling. Ohlstein and colleagues demonstrated that indeed inhibitory Dpp signal from the peripheral cells to the AMPs is the inhibitory signal that the peripheral cell niche utilizes to keep the AMPs in an undifferentiated state until morphogenesis (Mathur et al., 2010).

D. The adult posterior midgut

The adult *Drosophila* midgut is a pseudo-stratified, brush-border epithelium resting on a continuous basal lamina or basement membrane. Adjacent to the basal lamina are two layers of visceral muscle, with an inner circular layer and an outer longitudinal layer (Lehane, 1996; Miller, 1994). The lumen of the midgut is lined by the peritrophic membrane, which is a thin cylindrical sheath composed of chitin and proteins and that is secreted by cells in the anterior-most region of the cardia. The peritrophic membrane surrounds the food and protects the epithelium from abrasion and microorganisms (Miller, 1994). For simplicity, I will concentrate on the biology of the posterior midgut, as this is the region of the midgut that harbors the stem cells that I have studied during by PhD. The epithelium of the posterior midgut is mostly constituted by large enterocytes [ECs, (Baumann, 2001; Lehane, 1996; Miller, 1994)]. ECs have a cuboidal or low columnar shape and have a large, polyploid nucleus (Baumann, 2001; Lehane, 1996). The apical, luminal surface of the ECs is covered by microvilli and the basal plasma membrane extends infoldings into the cell, which form a labyrinth of membranes in the basal part of the cells (Baumann, 2001). The apical microvilli are rich in F-actin, α-spectrin, β_H-spectrin and myosin-II, which can be used as markers for the ECs (Baumann, 2001). The ECs have also been shown to express the nuclear transcription factor Pdm-1, another EC marker; since

they are the only polyploid cells in the midgut epithelium, their large nucleus can also be used as a marker (Beebe et al., 2010; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The ECs are absorptive cells and their particular epithelial architecture is essential for vectorial transport of ions and other molecules (Baumann, 2001). Interspaced between the large ECs are small diploid cells. Some of these cells are enteroendocrine (ee) cells, as they are positive for the peptidic hormones such as Allatostatin and Tachykinin (Lehane, 1996; Ohlstein and Spradling, 2006). The ee cells have been shown to express the transcription factor Prospero (Pros), which is the generally used ee marker (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The ee cells have been reported to have different cell morphologies, having pyramidal, oval, fusiform or bowl shape, a basolateral membrane without many infoldings and lying tightly adjacent to the basal lamina. Some ee cells appear to contact directly the midgut lumen via a narrow extension while others do not appear to extend a process to the lumen (Lehane, 1996).

The adult posterior midgut is maintained by recently identified adult intestinal stem cells (ISCs) that maintain tissue homeostasis throughout the whole life of the fly (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). However, it has been known for many years that the fly midgut was maintained by regenerative cells found scattered throughout the midgut as single cells or in pairs or groups called nidi. Histologically, these cells were known to be small cells with a small, basal nucleus and denser cytoplasm than the surrounding epithelial cells (Baumann, 2001; Lehane, 1996; Miller, 1994). Also, they are wedged-shaped, with a basal membrane containing no infolds, they are localized adjacent to the basal lamina and have a thin process that extends toward the lumen and is covered with thick microvilli; these cells strongly express armadillo (the fly β-catetin homologue), which marks the nidi (Baumann, 2001; Lehane, 1996; Miller, 1994; Ohlstein and Spradling, 2006). In many insects, such as the beetle, the cockroach and the moth, these regenerative cells were know to renew the adult midgut continuously throughout the whole lifetime of the organism as well as regenerate the midgut in response to damage (Lehane, 1996). However, since no mitotic figures had been observed in the adult *Drosophila*

midgut, it was thought that the *Drosophila* midgut was not renewed by somatic cell divisions, having been suggested that the midgut was maintained only by cell growth of the ECs (Bozcuk, 1972). In Fig. 8.A. there is a schematic drawing of the posterior midgut histology.

E. Identification of the adult Intestinal stem cells in the midgut

A field was born with the recent break-through discovery, made by both the Spradling and the Perrimon labs, of the proliferating stem cells in the adult intestine (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Both groups unambiguously identified proliferating stem cells in the adult intestine by lineage labeling analysis (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Lineage labeling analysis consists of marking a single cell and its progeny clonally, either with GFP or with βGal expression (Harrison and Perrimon, 1993; Lee and Luo, 2001). When analyzing potential stem cell lineages with this technique, if the labeled daughter cell is not a stem cell, it may divide a limited number of times but eventually all cells will differentiate, with such a clone being termed a transient clone. However if the labeled cell is a stem cell, this cell will generate a stem cell clone that will contain the stem cell as well as all the differentiated cells produced by the stem cell and the clone will be maintained thought out the whole lifetime of the stem cell. Both studies showed that the adult fly midgut contained mitotic cells that gave rise to clones containing the two differentiated cell types of the adult fly midgut, the ECs and the ee cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). They further showed that these were indeed stem cell clones since they contained proliferative cells as marked by expression of phospho-histone H3 (PH3) and incorporation of 5-bromodeoxyuridine [BrdU, (Micchelli and Perrimon, 2006)]. Lineage labeling analysis showed that stem cell clones contained both ECs and ee cells, which indicates that the ISCs are multipotent, with an individual ISC being able to produce both cell types. Analysis of the type of clones produced showed that approximately 50% of the clones were stem cell clones while the other 50% were transient clones containing

only one terminally differentiated daughter cell. This suggests that after mitosis, one of the daughter cells will self-renew the stem cell while the other will go on to differentiate directly, without transit amplifying divisions (Ohlstein and Spradling, 2006). Lineage analysis also suggested that the ee cells, which are usually observed in pairs, are produced sequentially by the same ISC and are not the product of a second mitosis of the stem cell daughter (Ohlstein and Spradling, 2007).

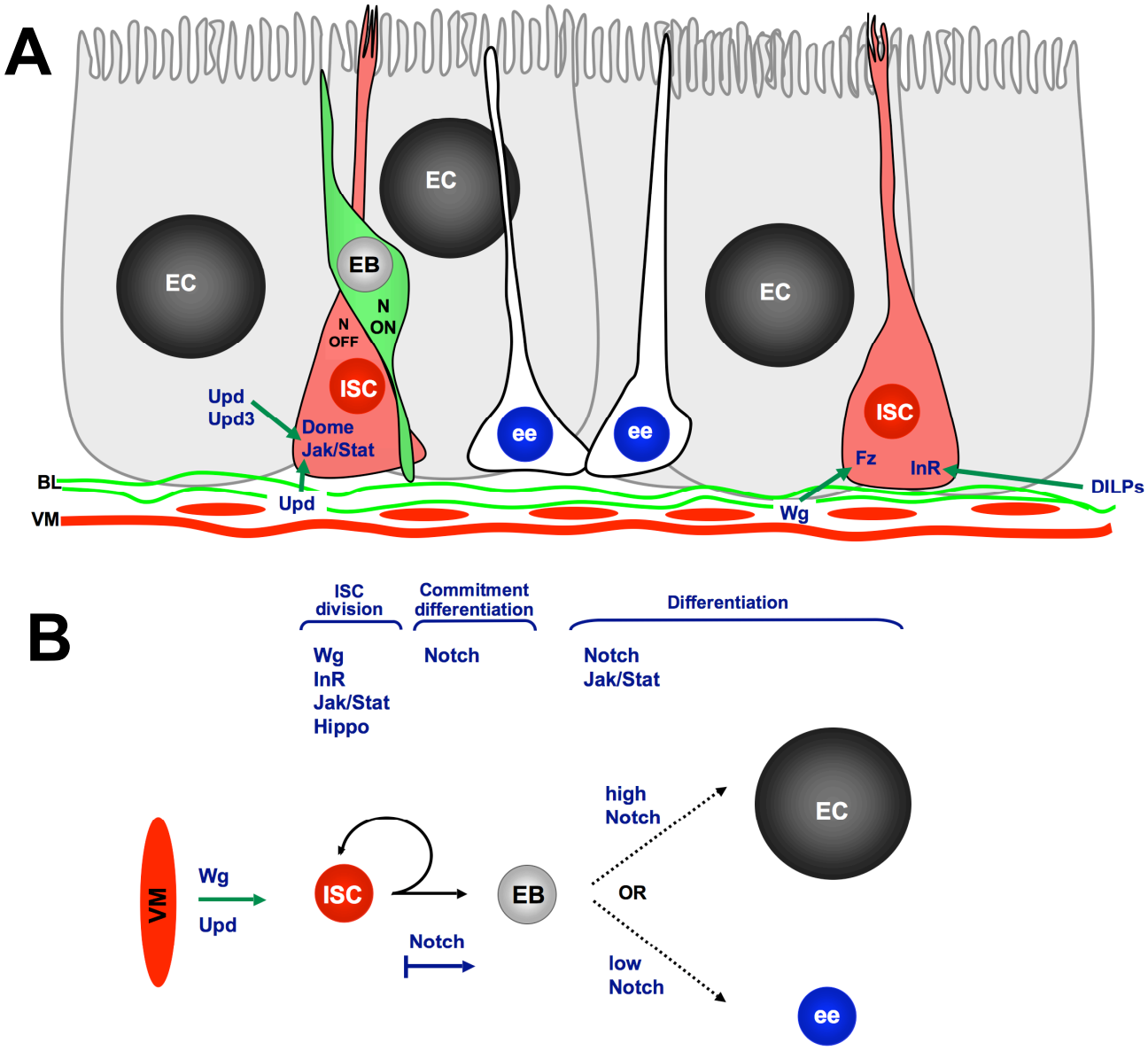


Fig. 8: **A**. Schematic representation of the different cells of the midgut epithelium: the ISCs, the EBs, the ECs and the ee cells. The midgut epithelium is resting on the basal lamima (BL) and is surrounded by two layers of visceral muscle (VM). The different mitogens that promte proliferation of the ISC are represented, with green arrows indicating proliferative stimulus. The EB has been shown to express a reporter of Notch signaling activity. **B.** Representation of the ISC lineage and of the different pathways that regulate it.

The ISCs were shown to express *esg-lacZ* and *esg-GFP*, which is used as a stem cell marker (Micchelli and Perrimon, 2006). However, *esg-lacZ* and *esg-GFP* are also expressed in pairs of small cells next to each other, which were interpreted to correspond to the stem cell and its immediate daughter cell, that will go on to differentiate but that does no yet express differentiation markers and is termed enteroblast [EB; (Micchelli and Perrimon, 2006)]. In Fig.8.B. the ISC lineage is represented: ISCs divide, approximately once a day to give rise to two daughter cells, one of the daughter cells self-renews the stem cell while the other is the undifferentiated diploid EB. The EB does not undergo any further cell divisions and will differentiate into one of the two differentiated cell types: in 67% of the cases, the EB differentiates into an EC and in 33% of the cases the EB differentiates into an ee cell (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006).

F. Role of Notch in regulating cell fate specification in the ISC lineage

Notch signaling has a key role in the lineage of both mammalian and fly ISCs. The Notch ligand Delta is expressed in small basal cells that are scattered throughout the entire posterior midgut. These cells have been identified as the ISC through lineage analysis (Ohlstein and Spradling, 2007). Furthermore, the EBs express a reporter of Notch activity, indicating that Notch is activated in these cells at some point (Micchelli and Perrimon, 2006). The role of Notch in the ISC lineage can be accessed by the generation of positively marked clones in which the cells are mutant for a component of the Notch pathway. Generation of such loss of function clones for Notch, Delta or the E3-ubiquitin ligase Neuralized (Neur), which is essential for Notch activity, all yield the same result: the clones are composed of a mass of small diploid cells (Fig.9), some of which have ISC-like properties: they are dividing cells and they express Delta (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). These results suggest that Notch activity is required in the EB cell for it to undergo differentiation.

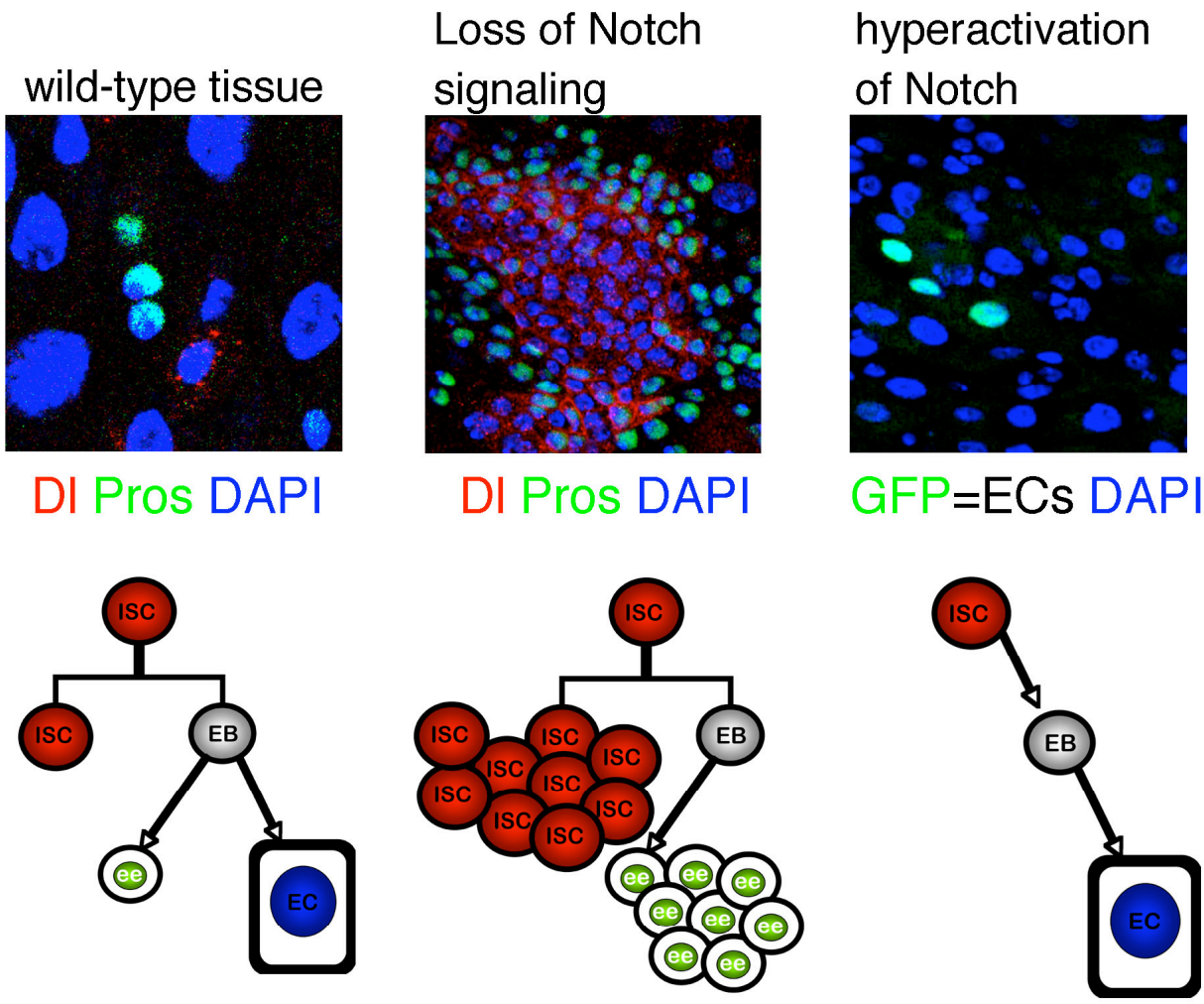


Fig. 9: Wild-type tissue, loss of function of Notch signaling and hyperactivation of Notch in the intestine. The cartoons represent the stem cell lineage in wild-type tissue and the respective interpretation of the phenotypes in terms of defects in cell fate specification in the ISC lineage when the Notch pathway is manipulated in the intestine.

When none of the ISC daughter cells activate Notch, they would both acquire the ISC fate, and become two ISC-like cells. Additionally, some of the small cells in the Notch mutant clones are Pros positive, while ECs are rarely observed in these clones (Fig.9). This was interpreted to suggest that Notch also plays a role in the decision between the EC and the ee fates, with Notch activity promoting the EC fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). These results are further confirmed by the complementary experiment: the overexpression of the activated nuclear form of Notch (NICD) in the ISC lineage results in a loss of the ISC and differentiation into ECs [Fig.9, (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007)]. While it appears that Notch signaling acitivtiy promotes differentiation and the EC fate, the role that Notch signaling plays in the fate specification of the ee cells is not clear. Ohlstein and Spradling have proposed a model in which the ISC determines the fate that the EB will adopt through differential Notch signal (Ohlstein and Spradling, 2007). In this study (Ohlstein and Spradling, 2007), the authors observed that the levels of the Delta expressed by the ISC vary. Through retrospective clonal analysis, in which the level of Delta protein in the ISC was correlated to the cell that the ISC had just produced, the authors observed that ISCs that had just produced an EC had high Delta expression while ISCs that had just produced an ee cell had low levels of Delta expression (Ohlstein and Spradling, 2007). These data, in addition to the phenotypes observed in the loss of Notch signaling mutants, led the authors to propose a model in which the ISC switches between high Delta and low Delta expression; although the signal that triggers the switch is unknown, it was speculated that it could be a feedback signal from the tissue. The ISC expressing high Delta could send a strong signal to the EB, promoting the EC fate while the ISC expressing low Delta would send a weak Notch signal to the EB, promoting the ee fate (Ohlstein and Spradling, 2007). Therefore, the EB would be able to interpret the levels of Notch signal it receives. This model could also explain the over-proliferation of ee-like cells observed in loss of Notch signaling mutants: after induction of the loss of function clone for a component of Notch signaling such as Notch, Delta or Neur, the protein of the targeted component is still present in the cells and, due to protein stability, might still be present in the mutant

ISCs, which would be able to send a weaker signal to its daughter cells, promoting their differentiation as ee cells. Once Notch signaling would be completely impaired, these mutant ee precursor cells could continue to proliferate (Ohlstein and Spradling, 2007). An alternative model has been proposed by Micchelli and colleagues, who propose that the specification of the EB that will adopt the EC fate requires Notch signaling while specification of the EB that will go on to differentiate as an ee cell is independent of Notch signaling (Beebe et al., 2010; Micchelli and Perrimon, 2006).

Notch signaling appears to be important for two cell-fate choices in the ISC lineage, being required for both the commitment to differentiate and the choice of differentiated fate. Notch has been proposed to promote multiple fates in other stem cell lineages (Mazzone et al., 2010). How exactly Notch regulates of three different fates is not entirely understood. As referred above, the level of Notch signaling could be interpreted by the stem daughter cell, but the exact mechanism for how the daughter cells perceive different levels of Notch signal, whether it be input of signal over time, the number of pulses of Notch signaling or the affinity of the NICD-CSL transcriptional complex to the different enhancer binding sites, is not known. Furthermore, data on the temporal dynamics of the activation of Notch in one of the daughter cells are lacking. We do not know how long the two daughter cells stay in contact and how long it takes to resolve their cell fates.

Recently we have shown that transcriptional repression of Notch target genes is required for the long-term maintenance of the stem cell (Bardin et al., 2010b). After division of the stem cell, both daughter cells express Delta protein and the Notch receptor (Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). In such context, it possible that both daughter cells could activate Notch and since activation of Notch signaling leads to differentiation of the ISC, mechanisms to repress Notch signaling activity could exist in the ISC to prevent its terminal differentiation. One such inhibitor of Notch signaling that could carry out this function in the ISC is Numb (Frise et al., 1996; Knoblich et al., 1995; Le Borgne, 2006). However, we observed that loss of Numb did not affect ISC proliferation, suggesting that Numb is not required for inhibiting Notch signaling in the ISC and for ISC maintenance (Bardin et al., 2010b). We next tested if transcriptional repression of Notch target genes could

be required for maintenance of the ISC. Hairless (H) acts with Suppressor of Hairless [Su(H)] as co-repressor complex to repress Notch target genes in some developmental contexts (Furriols and Bray, 2000; Koelzer and Klein, 2003; Koelzer and Klein, 2006; Morel and Schweisguth, 2000). We observed that loss of H in stem cell clones resulted in loss of ISC self-renewal, with most *H* mutant cells terminally differentiating (Bardin et al., 2010b). We further showed that H acts in combination with Su(H) to keep the expression of Notch target genes repressed in the ISC since the *H* phenotype requires *Su(H)* activity. Furthermore, we identified the *Enhancer of split complex* [*E(spl)-C*] genes as Notch target genes that are repressed in the ISC since the *H* phenotype is suppressed by loss of the genes of the *E(spl)-C*. The *E(spl)-C* is a complex of genes that are known Notch transcriptional targets (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). Loss of the *E(spl)-C* in the intestine resulted in overproliferation of ISC-like cells, suggesting that *E(spl)-C* is required for proper differentiation of the ISCs (Bardin et al., 2010b). However, while ee cells were lost in *E(spl)-C* mutants, the ECs are still present in these clones, which indicates that, while the *E(spl)-C* does appear to play a role in differentiation, other Notch target genes are also required for differentiation (Bardin et al., 2010b). Additionally, in this study, we found that Daughterless (Da, E47 in vertebrates), a basic helix-loop-helix (bHLH) transcriptional activator, is also required for ISC maintenance (Bardin et al., 2010b). In many development contexts, both in *Drosophila* and in vertebrates, the *E(spl)-C* repress the activity of Da and the bHLH transcriptional activators that act as dimmers with Da (Kageyama et al., 2007). We observed that loss of *da* in the ISC resulted in loss of the self-renewal of the stem cell and terminal differentiation (Bardin et al., 2010b). Furthermore, a promoter region that has binding sites for Da-dependent bHLH transcriptional activity fused to GFP is expressed specifically in the ISCs (Bardin et al., 2010b). We proposed that bHLH activity is required for the ISC-specific program of gene expression. Our data further suggest that the ISC-specific program is turned OFF upon activation of the Notch target genes of the *E(spl)-C* in the daughter EB cell [Fig. 10, (Bardin et al., 2010b)].

Work from the Spradling lab also supports the model that ISC maintenance requires repression of Notch target genes (Buszczak et al., 2009). In this study, the

gene *scrawny,* an ubiquitin protease that deubiquitylates histone H2B, which is important for gene silencing, was characterized . The authors observed that *scrawny* loss of function results in loss of female and male GSCs as well as loss of ISCs (Buszczak et al., 2009). Expression of Notch target genes has been proposed to require histone H2B mono-ubuitylation as well as histone H3K4 methylation (Bray et al., 2005). This suggests that loss of H2B deubiquitylation results in de-repression of Notch target genes, which would result in the loss of the ISC. To further support this model, the authors blocked Notch signaling by feeding the *scrawny* mutant flies with DAPT, and inhibitor of γ-scretase, and found that DAPT treatment suppressed the *scrawny* phenotype (Buszczak et al., 2009).

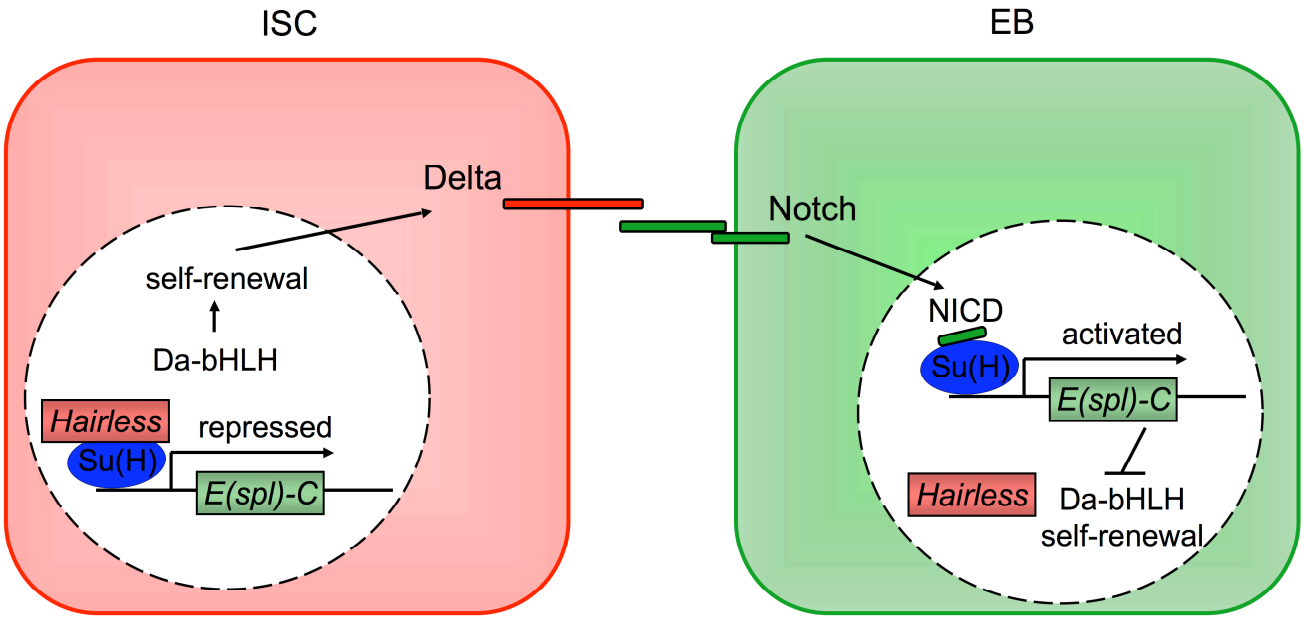


Fig. 10: Model for the transcription regulation of the ISC and EB fates. The ISC signals to the EB, promoting activation of Notch in this cell. Notch activation yields the intracellular domain of Notch (NICD) that associates with Su(H) to promote transcription of Notch target genes, including *E(spl)-C* genes. The E(spl) bHLH repressors downregulate Da-dependent bHLH activity in the EBs, which inhibits self-renewal and promotes differentiation. In the ISC, Hairless prevents expression of Notch target genes, including the *E(spl)-C* genes, which is required for maintenance of the ISC. Da-dependent bHLH activity promotes ISC fate, including the expression of Delta and the ability to self-renew.

G. Role of Wingless signaling in regulating ISC proliferation

Similar to the vertebrate intestine, paracrine Wingless (Wg, Wnt in vertebrates) signaling also appears to play an important role in regulating maintenance and self-renewal of the stem cell (Lin et al., 2008). Wg ligand binds to the Frizzled receptors, Frizzled and Frizzled 2 (Fz and Fz2) and low-density lipoprotein receptor-related protein, which leads to the stabilization of armadillo (arm, β-catetin in vertebrates) in the cytoplasm and concomitant translocation of arm to the nucleus, where it will bind to the transcription factor of the T cell factor, lymphocyte enhancer factor family (TCF/LEF, dTCF in *Drosophila*) to promote transcription of the Wg target genes. In the absence of Wg signaling, arm is targeted for proteasomal degradation through phosphorylation events: a degradation complex assembled around the scaffold protein Axin and that also contains Adenomatous polyposis coli (Apc), the kinase GSK3β (Glycogen synthase kinase3β, Shaggy in *Drosophila*) and arm phosphorylates arm, providing the signal for its ubiquitination and degradation by the proteasome. Through a not fully understood mechanism, the activation of the Fz receptor by Wg leads to the interaction of Fz with and phosphorylation of Dishevelled (Dsh). Dsh is a cytoplasmic protein that promotes the translocation of Axin from the cytoplasm to the membrane and directly inhibits the activity of the destruction complex through a Dsh–Axin interaction. The recruitment of Axin away from the destruction complex leads to the stabilization of β-catenin. (Clevers, 2006).

Xi and colleagues provide *in situ* and enhancer trap data suggesting that the Wg ligand is produced in visceral muscles that surround the posterior midgut and that Wg accumulates in the basement membrane underlying the ISCs (Lin et al., 2008). It was further shown that overexpression of Wg in the ISCs or overactivation of Wg signaling in the ISCs by overexpression of an activated form of armadillo resulted in a mild increase in proliferation of the ISC, with a mild increase in the number of ISCs and EBs (Lin et al., 2008; Lin et al., 2009). Loss of different components of Wg signaling in the ISC, such as the loss of the Wg receptors Fz and Fz2 resulted in

slower self-renewal and faster clone-turnover rate but did not result in differentiation defects, which indicates that Wg signaling is required for stem cell self-renewal but not for differentiation of the EBs (Lin et al., 2008). *wg* mutant clones were indistinguishable from wild-type clones, which argues in favor of Wg being a paracrine signal and not an autocrine one (Lin et al., 2008). Finally, these authors reported that Notch signaling is epistatic to Wg signaling since double mutants for loss of Notch and Wg signaling had the loss of Notch signaling phenotype, which supports a model in which Notch acts downstream of Wg signaling in the ISC (Lin et al., 2008). Work by Micchelli and colleagues ads further support to the role of Wg signaling in promoting stem cell self-renewal (Lee et al., 2009b). These authors investigated the role of the gene *Adenomatous polyposis coli* (*Apc*) in the posterior midgut and observed that Apc is required for regulation of ISC proliferation (Lee et al., 2009b). *Apc* is a conserved gene that was first identified as being deleted in patients with familial adenomatous polyposis, being a frequently mutated gene in many colorectal cancers (Radtke and Clevers, 2005; Sancho et al., 2004). Apc is a tumor suppressor gene, required for the degradation of the Wg signaling negative regulator armadillo (arm) thus loss of Apc results in overactivation of Wg signaling (Sancho et al., 2004). Loss of *Apc* in the fly ISC results in increase of the ISC proliferation rate without affecting the production of differentiated cells (Lee et al., 2009b). This results in alterations in the midgut histology such as multilayering and tissue hyperplasia (Lee et al., 2009b). The hyperplasia induced by loss of *Apc* function could be suppressed by reduction in the level of Wg signaling in the ISC, further supporting the role for Wg in regulating stem cell self-renewal in the fly midgut [Fig.8, (Lee et al., 2009b)].

The visceral muscle has been proposed to act as niche, with the secreted Wg being required for maintenance and self-renewal of the stem cell (Lin et al., 2008; Lin et al., 2009). This is further supported by the observation that the stem cell divides with an approximate 30° angle in relation to the basement membrane and that after division the basal-most cell renews the stem cell (Ohlstein and Spradling, 2007).

H. Role of Jak/Stat Signaling on ISC proliferation and in differentiation

The Jak/Stat ligands in *Drosophila* are three leptin-like (IL-6 family) cytokines called Unpaireds (Upd, Upd2 and Upd3) that bind the receptor Domeless (an IL-6R type receptor), which activates Hopscotch, the *Drosophila* Janus kinase (Jak) and promotes the translocation of the transcription factor Stat92E to the nucleus, where it will activate the Stat92E transcriptional targets (Arbouzova and Zeidler, 2006). Reporters for Jak/Stat signaling activity showed that Jak/Stat signaling is activated in the ISC and the EB (Beebe et al., 2010; Buchon et al., 2009a; Jiang et al., 2009; Lin et al., 2009; Liu et al., 2010). Nuclear Stat92E staining, a hallmark of activation of the Jak/Stat pathway was also observed in the ISCs and EBs (Jiang et al., 2009; Liu et al., 2010). Reporter lines for the expression of Upd and Upd3 are expressed in the midgut epithelium (Beebe et al., 2010; Liu et al., 2010). In contrast, Xi and colleagues reported that Upd is expressed not in the midgut epithelium but in the visceral muscle, although the different groups used different Upd expression reporter lines (Lin et al., 2009). Upd mediated signaling was shown to be a powerful mitogen as its overexpression in the midgut or the hyperactivation of the Jak/Stat pathway, resulted in an increase in ISC proliferation and increased number of all cell types (Beebe et al., 2010; Jiang et al., 2009; Liu et al., 2010). Loss of function analysis for different components of the Jak/Stat pathway in the ISC resulted in clones composed only of *esgLacZ*+ ISCs and EBs but no differentiated ECs or ee cells (Beebe et al., 2010; Jiang et al., 2009; Liu et al., 2010). These data suggest that Jak/Stat is required for differentiation of the EB but not for basal rates of ISC self-renewal (Beebe et al., 2010; Buchon et al., 2009a; Jiang et al., 2009; Liu et al., 2010). Globally, these data suggest that Jak/Stat plays two roles in the intestine, acting as a mitogen that promotes ISC proliferation (more on this function is discussed below) as well as being required in EBs for differentiation (Fig.8).

Reports on the genetic relationship of Jak/Stat and Notch signaling differ. Some results suggest that Jak/Stat is required downstream of or in parallel to Notch signaling (Beebe et al., 2010; Lin et al., 2009) while others suggest that Jak/Stat functions upstream of Notch signaling (Liu et al., 2010). Clones double mutant for components of Notch and Jak/Stat signaling have an overabundance of ISC-like cells, like loss of Notch signaling mutants, but not a significant overabundance of ee-like cells, which is different from the loss of Notch signaling (Beebe et al., 2010; Lin et al., 2009). This is consistent with Jak/Stat signaling not being required for basal self-renewal of the stem cells. It is also consistent with the requirement of Jak/Stat for ee differentiation, as the ee-like cells cannot be produced in the double mutants (Beebe et al., 2010; Lin et al., 2009). The overexpression of activated nuclear Notch (NICD) in Jak/Stat mutants has the same phenotype as the loss of Jak/Stat alone, which is the production of only small cells and no differentiated ECs (Beebe et al., 2010). This further suggests that Jak/Stat signaling is required for the Notch-dependent production of ECs. However, contradictory results were found by Hou and colleagues for the genetic interaction of Notch signaling and Jak/Stat, with Jak/Stat signaling not being required for the NICD-induced differentiation into ECs (Liu et al., 2010). Also, these authors observed that in the Notch and Jak/Stat double mutants, the clones failed to grow, having the loss of Jak/Stat signaling phenotype (Liu et al., 2010). The differences in the genetic interactions experiments might be explained by the difference in fly lines used (the different studies used different NICD constructs, for example) as well as differences in the clone induction protocol and time-points analyzed. To resolve these discrepancies further studies on the interaction between the two pathways are required. Both Notch and Jak/Stat signaling appear to be required at different steps of the ISC lineage, with the interaction between the two pathways being potentially very complex. Furthermore, the role of Jak/Stat signaling on basal proliferation rates of the stem cell is not entirely understood. It is clear, however, that Jak/Stat is required for ISC proliferation in response to damage as well as to the commensal bacteria of the midgut (see below). Therefore, some of the different result reported might differ due to the experimental conditions of each lab.

Xi and colleagues further tested the interaction of Jak/Stat signaling with Wg signaling (Lin et al., 2009). The increase in ISC proliferation observed when Upd is overexpressed in the midgut is enhanced by overexpression of Wg, suggesting that Wg and Jak/Stat signaling work together to promote ISC self-renewal (Lin et al., 2009). Furthermore, these authors observed that Upd was produced by the surrounding visceral muscle, which led to a model in which both Upd and Wg are paracrine mitogens secreted from the muscle niche (Lin et al., 2009). Although it is possible that multiple tissues provide Upd to the ISC, to resolve this question further data on the source of Upd cytokines are required.

I. Aging in the midgut

The fly midgut is also an excellent model to study aging of an organ. The fly midgut has been described to change with age: as the flies age, the cell-size and shape of the epithelial cells start to change, with an increase of the nuclear to cytoplasmic ratio (Salomon and Jackson, 2008). Such histological changes could be classed as dysplasias (Salomon and Jackson, 2008). In vertebrates, dysplasia has been frequently found to be an indicative of an early neoplastic process. Furthermore, it was reported that aging flies develop spontaneous tumors, with around 1,3% of 5-week old guts harboring a tumor (Salomon and Jackson, 2008).

Other age-related alterations in the midgut have been reported by Jasper and colleagues and Yoo and colleagues (Biteau et al., 2008; Choi et al., 2008). Aging flies were found to have an increase in the number of *esg-lacZ*+ as well increase in total density of cells (Biteau et al., 2008; Choi et al., 2008). However, many of these *esg-lacZ*+ cells were morphologically atypical, since they were polyploid but did not express Ferritin 1 heavy chain homologue, a protein expressed in the ECs of young guts (Biteau et al., 2008). This was proposed to be a consequence of the rapid proliferation followed by defective differentiation, since a rapid increase in daughter-cell number or age-related stress might impair differentiation. Alternatively, a rapid proliferation rate could result in perdurance of the βGal protein in the daughter cells

that have started to but have not yet fully differentiated. These changes correlated with a disorganization of the epithelial apico-basal polarity and a physiological deterioration of the mucosa (Biteau et al., 2008). These defects also correlated with an increased expression of Delta as well as an increased Notch signaling activity, as monitored with a reporter for Notch signaling activity (Biteau et al., 2008). Oxidative stress is a common aging agent and the induction of oxidative stress in the gut, resulted in a similar phenotype as observed in the aging guts (Biteau et al., 2008). Jun N-terminal kinase (JNK) signaling is activated in response to multiple environmental stresses, including oxidative stress and therefore the authors tested whether activation of JNK signaling was provoking the age-related alterations to the gut. Old midguts had high levels of JNK signaling activation, as monitored with a reporter, and reducing JNK activity resulted in reduction of the age-related alterations (Biteau et al., 2008). Overactivation of JNK signaling led to premature aging and death of the flies, which suggests that JNK is activated in the ISC as a response to oxidative stress, with the side-effect of increasing the proliferation of the ISC, which leads to tissue deterioration (Biteau et al., 2008). The observation that Notch signaling and JNK appear to both regulate stem cell proliferation but with apparent opposite effects led the authors to propose the following model: when JNK activity is moderately increased in the gut, JNK signaling promotes the expression of cytoprotective genes as well as ISC proliferation. However, as the fly ages, situations of chronic stress increase and JNK signaling is also considerably increased, which promotes excessive ISC proliferation. This excessive proliferation results in extra Delta positive cells that cannot properly signal to each other and activate Notch properly, with correct differentiation being compromised. This leads to an accumulation of cells that still express ISC markers, that have also started to differentiate but that cannot do it properly (Biteau et al., 2008).

PDGF/VEGF and MAPK signaling have also been implicated in the response to aging in the fly midgut (Choi et al., 2008; Park et al., 2009). PDGF-and VEGF-related factor 2 (Pvf2) signaling was found to contribute to the aging-related increase in proliferation and a reporter for Pvf2 is expressed in the *esg-GFP*+ ISCs and EBs (Choi et al., 2008). Also, a reporter for D-p38b, one of the two fly p38 MAPKs, is

expressed in ISCs and daughter cells and the number of cells *D-p38b-lacZ+* increases with aging. D-p38b MAPK activity is required for age-dependent increase in ISC proliferation and the concomitant differentiation defects reported above (Biteau et al., 2008; Park et al., 2009). Yoo and colleagues also showed that D-p38b MAPK activity is required for the increase in *esg-GFP+* cells observed when oxidative stress is induced in the midgut and that D-p38b MAPK signaling is downstream of Pvf2 signaling (Park et al., 2009). It would be interesting to know the epistasis of D-p38b MAPK and Pvf2 signaling in relation to JNK signaling, which is also required for the response to oxidative stress in the gut, and whether these different pathways act in parallel to mediate the response of the ISC to aging and oxidative stress. This increase in ISC proliferation due to chronic oxidative stress could lead to dysplasia and potentially increase the predisposition of the ISC to form spontaneous tumors, being an interesting model to study aging and tumorogenesis.

J. The ISCs respond to damage

The intestinal epithelium is in direct contact with the external environment, and is therefore a first line of defense from ingested pathogens that can cause damage to the midgut epithelium. Like other tissues that are in direct contact with the external environment, such as the skin and airways in mammals, it is a rapidly turning over tissue that replenishes the old and damaged cells quickly in order to maintain tissue homeostasis. Recent studies have shown that the midgut epithelium responds to damage to re-establish tissue homeostasis and have elucidated some of the cell signaling events that are behind this response (Amcheslavsky et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Buchon et al., 2009b; Chatterjee and Ip, 2009; Cronin et al., 2009; Jiang et al., 2009). Damage by chemicals, oxidative stress or bacterial infection, can induce proliferation of the ISC to replenish the damaged gut tissue. I will now describe the current evidence for this in more detail.

J.1. Chemical damage to the midgut

Injuring the midgut with chemical agents promotes stem cell proliferation (Amcheslavsky et al., 2009). Damaging the midgut with Dextran Sulfate Sodium DSS, a polysaccharide that damages the intestine of experimental mammalian models, induced proliferation of the ISC, but the extra daughter cells produced accumulated as EBs and did not differentiate (Amcheslavsky et al., 2009). Interestingly, DSS does not damage the midgut epithelium but instead disrupts the basement membrane, which suggests that damage to the basement membrane induces stem cell self-renewal but not differentiation (Amcheslavsky et al., 2009). Another tissue damaging agent, bleomycin, also induced ISC proliferation (Amcheslavsky et al., 2009). Bleomycin treatment induced DNA damage in most ECs but not in the more basal ISCs and EBs (Amcheslavsky et al., 2009). Unlike the DSS induced ISC self-renewal, bleomycin induced an increase in ISC proliferation and concomitant differentiation, since EBs did not accumulate and new ECs were produced (Amcheslavsky et al., 2009). This damage-induced proliferation is dependent on apoptosis as the expression of the caspase inhibitor p35 in the ECs inhibits partially the bleomycin-induced proliferation. In addition, cell death mediated by expression of the apoptotic inducers Reaper and Hid in the ECs resulted in increased ISC proliferation (Amcheslavsky et al., 2009). Finally, insulin receptor (InR) signaling activity in the ISC is required for the damage-induced proliferation (Amcheslavsky et al., 2009). InR signaling was required specifically in the ISC and not the ECs to promote ISC proliferation and the median neurosecretory cells in the brain were shown to be the source of the insulin-like peptides [DILPs, (Amcheslavsky et al., 2009)]. However, the authors did not distinguish between InR signaling functioning to promote ISC proliferation in response to damage or between an alternative role for InR signaling as permissive signaling required in the ISC for self-renewal.

J.2. Oxidative Stress

Induction of oxidative stress in the midgut by feeding the flies with paraquat, a compound that induces the production of reactive oxygen species (ROS), results in a increase in ISC proliferation (Biteau et al., 2008; Buchon et al., 2009a; Chatterjee and Ip, 2009). The ISC responds to the oxidative stress, at least partially, through activation of JNK, with activation of JNK signaling in the ISC also resulting in increased number of *esg-GFP*+ ISCs and EBs (Biteau et al., 2008). However, other studies reported that in the case of infection of the gut with pathogens that induce production of ROS (see below), the increase in proliferation reported does not appear to be a response to the ROS *per se* but a consequence of the damage induced by the oxidative stress and the infection (Buchon et al., 2009a).

J.3. Damage induced by apoptosis

Several studies have documented a proliferative response of ISCs in response to apoptosis of epithelial cells (Amcheslavsky et al., 2009; Jiang et al., 2009). Induction of apoptosis of the ECs triggered self-renewal of the ISCs to repair the damaged gut, with the gut returning to normal histology one month after the damage had been induced (Amcheslavsky et al., 2009; Jiang et al., 2009). Interestingly, the ECs that are not destroyed as a consequence of the damage also appear to react to the damage, undergoing endoreplication to increase their cell size and therefore contributing to the maintenance and the integrity of the tissue (Jiang et al., 2009). Activation of JNK signaling in the gut induced ISC proliferation, with this signal activating ISC proliferation in parallel and independently of the apoptosis-induced proliferation (Biteau et al., 2008; Jiang et al., 2009). Jak/Stat signaling was shown to mediate both the JNK- and the apoptosis-induced ISC proliferation (Jiang et al., 2009). Upon induction of apoptosis in the ECs or activation of JNK signaling in these cells, the expression of the fly cytokines Upd, Upd2 and Upd3 increased several fold

in the midgut and Jak/Stat signaling activity was shown to be essential for the induced ISC proliferation as mutants for components of the Jak/Stat pathway had considerably less proliferation in response to both stimuli (Jiang et al., 2009).

J.4. Infection-induced damage

The intestinal epithelium a forms a barrier from ingested pathogens that can damage the midgut epithelium and then enter the hemolymph, where they can cause systemic infection. The peritrophic membrane, the chitinous membrane that surrounds the food, protects the epithelium from microorganisms and the midgut also produces antimicrobial peptides and ROS to attack (Lemaitre and Hoffmann, 2007).

Infection with *Pseudomonas entomophila* and *Erwinia carotovora 15* (*Ecc15*), both gram-negative bacteria, was shown to kill the ECs and activate JNK and Jak/Stat signaling, promoting ISC proliferation (Buchon et al., 2009b; Jiang et al., 2009; Vodovar et al., 2005). As rapidly as 2 hours after infection there was an increase in expression in the JNK target genes, the three Upd cytokines, Jak/Stat target genes as well as Delta and 4 hours after infection there was already an increase in number of mitosis (Jiang et al., 2009). As a result of the infection, JNK signaling was activated in the ECs, expression of *Upd* was induced in small cells, probably ISCs and EBs as well as early ECs and *Upd3* was expressed mostly in ECs (Buchon et al., 2009a; Buchon et al., 2009b; Cronin et al., 2009; Jiang et al., 2009). Jak/Stat signaling activity was observed in both small cells as well as large ECs. (Buchon et al., 2009a; Buchon et al., 2009b; Jiang et al., 2009). Since these reporters were only observed to be active in the ISCs and EBs when the tissue is unchallenged, their expression in the ECs could be due to protein perdurance in a epithelium that is being rapidly renewed. Jak/Stat signaling was essential for the infection-induced proliferation and regeneration of the midgut as well as survival after infection, with reduction of *Upd3* specifically in the ECs, through RNAi, reducing the ISC proliferation in response to infection (Buchon et al., 2009a; Buchon et al., 2009b;

Cronin et al., 2009; Jiang et al., 2009). While JNK signaling was found to be dispensable for the increase in ISC proliferation in response to *P. entomophila* infection (Jiang et al., 2009), JNK signaling is required for the *Ecc15*-induced proliferation (Buchon et al., 2009a; Jiang et al., 2009). In spite of the reported increase in the number of Delta+ cells upon infection, clonal analysis suggests that the number of stem cells per clones is the same as in control clones (Buchon et al., 2009b; Chatterjee and Ip, 2009; Jiang et al., 2009). Upon infection, the rate of ISC proliferation increases but the number of ISCs in the posterior midgut does not, while the number of EBs and the production of differentiated cells increase to repair the damaged epithelium (Chatterjee and Ip, 2009). Together, these data suggest that, in response to damage, the number of stem cells does not increase by symmetric divisions that produce two stem cells.

Unlike *Ecc15*, *P. entomophila* is an entomopathogenic bacterium that is lethal to the flies at high dose (Vodovar et al., 2005). High dose of the virulent *P. entomophila* induced damage so severe that the epithelium could not self-renew, despite the infection having activated all the responsive pathways, while a less virulent form of *P. entomophila* induced epithelial self-renewal (Buchon et al., 2009a). This observation led to the hypothesis that it is the damage to the gut and not the immune response that induces the stem cell proliferation that will restore tissue homeostasis (Buchon et al., 2009a). Therefore, the virulence of *P. entomophila* infection could induce such damage to the midgut epithelium that would activate proliferation of the ISC even in JNK mutant flies, bypassing the requirement for JNK signaling (Buchon et al., 2009a; Jiang et al., 2009). This is also consistent with the observation that JNK signaling and apoptosis induce ISC proliferation independently [Fig.11, (Jiang et al., 2009)].

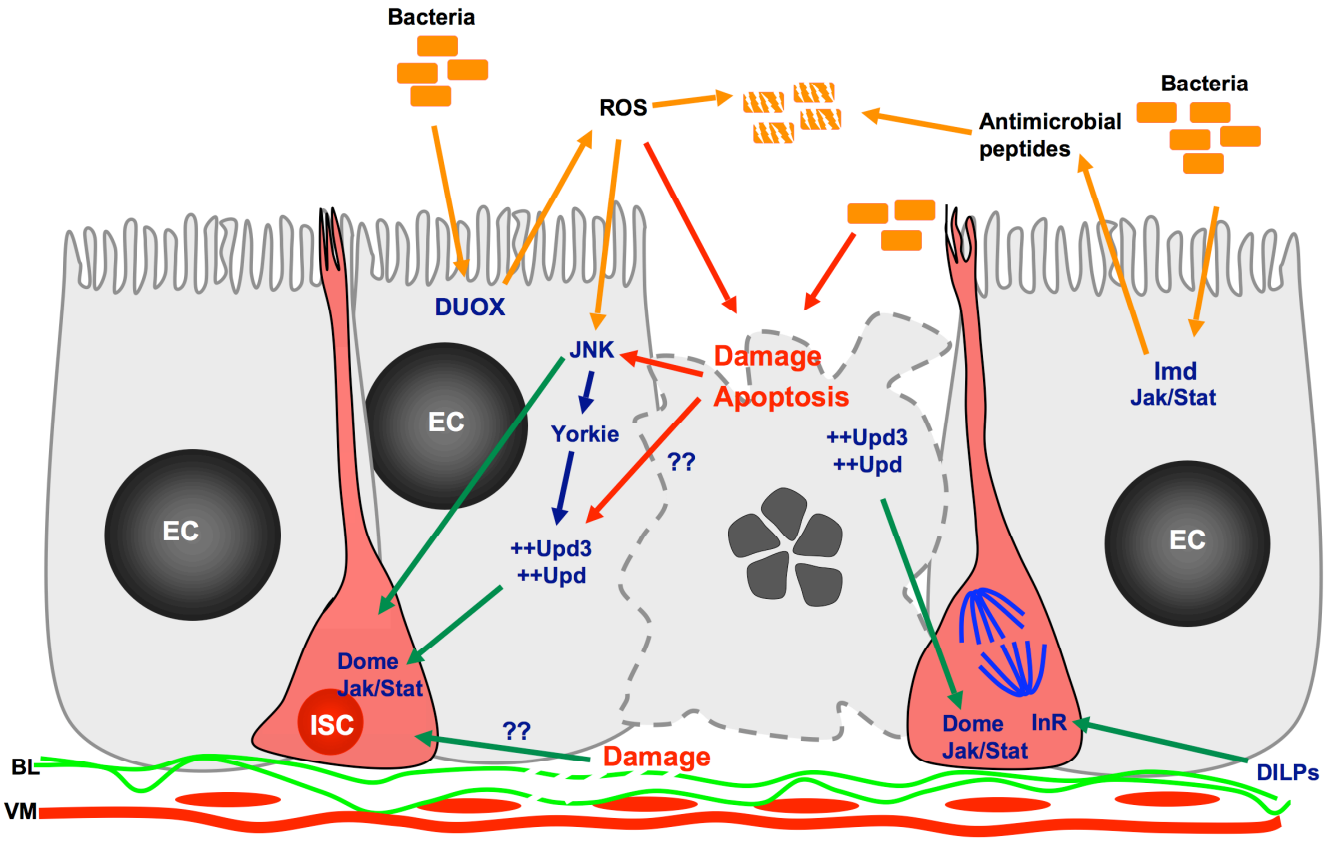


Fig. 11: Response of the midgut epithelium to infection and damage. When the gut is infected with pathogens the ECs produce anti-microbial peptides in an Imd- and Jak/Stat-dependent maner. Also, the dual oxidase DUOX produces reactive oxygen species (ROS) in response to the pathogens. Both the ROS and the toxins produced by the pathogens induce damage and apoptosis. JNK signaling is activated in response to the damage and to the oxidative stress and promotes proliferation of the ISCs, either directly or by promoting the production of Upd cytokines in the ECs, in a Yorkie-dependent maner. Both damage and apoptosis can also directly promote Upd production in the ECs. Upd cytokines, particularly Upd3, activate Jak/Stat signaling in the ISCs, inducing ISC proliferation. Damage to the basal lamina (BL) also promotes stem cell proliferation. Finally, ISC proliferation in response to damage is also dependent on activation of the InR in the ISC by the DILPs produced in the median neurosecretory cells in the brain.

To screen for genes that alter the susceptibility of the flies to pathogens, RNAi knock-down was performed on flies that were fed with the gram-negative bacterium *Serratia marcescens* (Cronin et al., 2009). This is a systemic approach, since the genes were knocked-down in the whole fly, using a collection of RNAi lines targeting approximately 10.000 genes [which corresponds to 78% of the *Drosophila* genome; (Cronin et al., 2009)]. During the secondary screen, the candidates isolated in the primary screen were knocked-down in the gut using an EC specific driver (Cronin et al., 2009). This screen found that reduction of Jak/Stat signaling in the gut increased the survival of the infected flies (Cronin et al., 2009). Paradoxically, while this study also found that Jak/Stat signaling in the ISC was required for the infection-induced proliferation that renews the damaged epithelium, the activation of Jak/Stat signaling in response to *S. marescens* is not beneficial since the activation of Jak/Stat signaling increases the lethality of the pathogen (Cronin et al., 2009). A possible explanation for this apparent contradiction is that, since *S. marescens* must cross the intestinal epithelium very rapidly to infect the hemolymph and cause a systemic infection that will kill the fly, a rapidly proliferating epithelium might actually be easier to cross (Nehme et al., 2007). Therefore Jak/Stat mediated proliferation would facilitate the infection of the hemolymph by *S. marescens*.

To summarize this complex canvas of signaling molecules: upon infection, the immune response of the gut produces ROS as antimicrobial agents. The ROS might activate JNK signaling. However, more critical for the renewal of the intestinal epithelium is that the ROS induce oxidative stress that, along with the bacterial virulent agents, induce damage to the gut. The damage to the ECs appears to activate the expression and secretion of the *Upd* cytokines, particularly *Upd3*, that activate proliferation of the ISCs to renew the gut epithelium (Fig. 11).

These studies of the midgut epithelium's response to damage, show that the midgut epithelium responds very quickly to the injuries, re-establishing homeostasis in a relatively short period of time. With a pulse-chase experiment where all the stem cells as well as all the subsequently produced daughter cells are labeled through a heat-shock-induced recombination event, Edgar and colleagues were able to follow

the complete turnover of the midgut posterior epithelium. The epithelium of the posterior midgut of control females is entirely turned-over in 12 days (Jiang et al., 2009). Flies that had been infected with *P. entomophila* during two days and then were allowed to recover for two days had completely renewed the midgut epithelium in those two days (Jiang et al., 2009). This impressive ability of the midgut to re-establish tissue homeostasis was further shown by the observation that the effects on tissue histology, such as tissue hyperplasia, by both JNK- and Jak/Stat-induced hyperproliferation were reverted in two weeks after the induction of proliferation (Jiang et al., 2009).

K. Role of Hippo signaling in ISC proliferation in response to damage

A recent study found that the Hippo pathway also promotes the proliferation of the ISCs (Staley and Irvine, 2010). The Hippo pathway regulates growth in a wide range of developmental contexts and loss of Hippo pathway signaling has been associated with multiple cancers (Oh and Irvine). Yorkie (and its mammalian homologues Yak and Taz) is a transcriptional co-activator that promotes transcription of the target genes of the Hippo pathway (Oh and Irvine). Hyperactivation of Yorkie in the ECs either through loss of *Warts*, a negative regulator of Yorkie, or overexpression of activated of Yorkie resulted in an increase in ISC proliferation with increase in Dl+ cells, increase in *esgLacZ*+ cells as well as hyperplasia (Staley and Irvine, 2010). The observed phenotype was interpreted as an increase of proliferation rate, with the increase in Dl+ and *esgLacZ*+ cells resulting from an accumulation of EBs that have not yet differentiated (Staley and Irvine, 2010). Yorkie activity was suggested to be specifically required in ECs, since increase of Yorkie activity in stem cells had no effect while the increase in activity in the ECs promoted cell non-autonomous proliferation of the ISCs (Staley and Irvine, 2010). Reduction of Yorkie activity by RNAi did not result in any major proliferation defects, which suggests that Yorkie is not required for basal proliferation of the ISC, with Warts functioning to keep

Yorkie repressed in the ECs. Activation of Yorkie in the ECs led to a dramatic increase of expression of the Upd cytokines in the intestine (Staley and Irvine, 2010). Also, the proliferation induced by the activation of Yorkie was dependent on Jak/Stat signaling in the ISCs (Staley and Irvine, 2010). Finally, Yorkie activation, as measured by detection of Yorkie protein in the nucleus of ECs, was found to occur downstream of activation of JNK signaling in the ECs while depletion of JNK activity in the ECs does not repress the overproliferation induced by activation of Yorkie in the ECs (Staley and Irvine, 2010). These data suggest that Hippo pathway is normally kept off in the ECs and that activation of JNK signaling in response to damage leads to Hippo signaling in the ECs, with nuclear Yorkie activity promoting the transcription of the Upd cytokines [Fig.11, (Staley and Irvine, 2010)].

L. Synergy between infection and dysplasia

There is increasing data suggesting that many cancers have, at their origin, cancer stem cells and it has been speculated that alteration in the proliferation rate of the ISCs are at the beginning of the oncogenic transformation in mammalian intestine (van Es and Clevers, 2005). Furthermore, bacterial infection has been shown to cause chronic inflammation, while inflammation has been associated with oncogenic transformation (Selgrad et al., 2008). To investigate whether there is link between infection and neoplastic transformation in *Drosophila* midgut, the effect of oral infection with *Pseudomonas aeruginosa,* a human opportunistic pathogen, on flies carrying latent oncogenic mutations was studied (Apidianakis et al., 2009). Infection with virulent forms of *P. aeruginosa* induced ISC proliferation, which led to tissue hyperplasia, with the width of the posterior midgut increasing considerably (Apidianakis et al., 2009). This effect is reversible after the bacteria were cleared from the gut, similarly to what has been reported for infection with other *Pseudomonas spp.* (Apidianakis and Rahme, 2009; Jiang et al., 2009). Interestingly, when the challenged flies were genetically modified and carried either a mutation in the tumor suppressor gene *discs large* or extra copies of the oncogene *Ras1*, the

response to infection was compromised and the epithelium showed dysplastic-like histology: increase in *esg-GFP*+ cells in the gut, multilayering of the epithelium and alterations to the apico-basal polarity (Apidianakis and Rahme, 2009). Dysplasias are a set of histological changes that have been associated with preneoplastic transformation in the vertebrate intestine (Salomon and Jackson, 2008). Importantly, these alteration of the gut histology were not completely reversed when the bacteria was cleared from the gut, suggesting that synergy between infection and genetic pre-conditioning to cancer can lead to permanent and long-term histological modifications in the fly intestine (Apidianakis and Rahme, 2009).

M. The gut innate immune response and the role of commensal bacteria

All metazoan intestinal tracks are in constant contact with a great number of microorganisms, both the food-borne microorganisms as well as the commensal microbiota. For example, the human intestine is estimated to be colonized by ~10^{14} organisms of more than 500 prokaryotic species. The intestine of *Drosophila* is not an exception and the relationship between its commensal bacteria and the innate immune response that keeps it under control and prevents infection by pathogenic microorganisms has been extensively studied (Lee, 2008; Ryu et al., 2010). The commensal microorganisms that inhabit the gut provide many advantages to the host organism, such as ecological out-competition of potentially hazardous pathogens that could try to colonize the gut. To ensure that mutualism between the two species occurs, the gut immune response must not attack the commensal microbiota aggressively, limiting its growth at the same time. It is not the purpose of this introduction to explain innate gut immunity and mutualism in *Drosophila* in depth. I will briefly overview the gut innate immune response in flies and mention some recent data that is relevant for the understanding of how the commensal bacteria contribute to ISC proliferation and how ISC proliferation is part of the innate immune response.

One essential component of the gut's innate immune response is the production of reactive oxygen species (ROS) by the redox system. In response to ingestion of pathogenic microorganisms the dual oxidase DUOX produces ROS to attack the pathogens (Ha et al., 2005a). After the pathogens have been cleared, an immune-regulated catalase eliminates the ROS, therefore protecting the tissue as well as the commensal microorganisms from oxidative stress (Ha et al., 2009b; Ha et al., 2005b). Flies mutant for *phospholipase C-β*, which is required for DUOX activity, have a shorter life span because they are unable to control the propagation of the essential nutritional yeast *Saccharomyces cerevisae*, which illustrates how the DUOX activity has to be precisely regulated (Ha et al., 2009a).

The other essential component of the gut innate immune response is the immune deficiency (Imd) pathway that, through the activation of the NF-κB-like protein Relish (Rel), promotes production of antibacterial peptides that have an essential role in defense against ROS-resistant pathogens (Ferrandon et al., 1998; Lemaitre and Hoffmann, 2007). Interestingly, a novel antimicrobial peptide called Drosomycin-like 3 has recently found to be induced independently of Imd response, in response to Jak/Stat signaling, which indicates that Jak/Stat is also contributes to the gut immune response (Buchon et al., 2009b).

As mentioned in section J, the proliferation rate of the ISCs increases in response to infection by several pathogenic bacteria, to rapidly renew the damaged epithelium lining. This rapid epithelium turnover is dependent on Jak/Stat signaling and appears, at least in part, to be dependent on JNK signaling as well. This rapid, Jak/Stat-dependent epithelial turnover is required for survival from infection (Apidianakis and Rahme, 2009; Buchon et al., 2009a; Chatterjee and Ip, 2009; Jiang et al., 2009). Therefore, the ISCs participate in the innate immune response of the gut as well, since their self-renewal activity to maintain the integrity of the gut epithelium increases resistance to pathogenic infection.

Finally, the commensal bacteria have also been shown to play a role in regulating the basal self-renewal of the ISCs (Buchon et al., 2009a). Using flies that were raised in axenic cultures (cultures entirely free of any microorganisms), Lemaitre and colleagues investigated the impact of the commensal bacteria on

proliferation of the ISC and found that the number of mitotic ISCs as well as epithelial turnover was reduced in the intestines of axenically raised flies (Buchon et al., 2009a). This reduction in ISC self-renewal is independent of an ability of the axenic flies to respond to self-renewal stimuli as the re-introduction of the commensal bacteria or the infection with *Ecc15* triggered stem cell self-renewal as in the non-axenically raised flies. In axenic intestines, expression of the cytokine *Upd3* as well as other components of the Jak/Stat, the JNK and the Imd pathways were reduced in comparison with the basal expression levels in non-axenic control flies. Furthermore, in flies mutant for *Rel*, an essential component of the Imd pathway, the guts contained 10 times more commensal bacteria and had higher number of mitotic ISCs. If *Rel* mutant flies were raised in axenic conditions, the number of mitotic ISCs was the same as in control axenic flies, which indicates that it is the commensal microbiota and not the Imd signaling that is regulating the proliferation of the ISCs (Buchon et al., 2009a). These data suggest that the commensal bacteria promote the basal self-renewal of the ISC through Jak/Stat and JNK signaling.

The intestinal track is a not only a complex tissue that is maintained by rapidly renewing stem cells and that has to respond quickly to injuries in order to maintain tissue homeostasis but it is also a complex ecological environment. The metazoan and its commensal microbiota have a symbiotic relationship that has co-evolved over that last 1 to 2 billion years (Lee, 2008; Ryu et al., 2010). Furthermore, these microorganisms play important roles in the development of the gut. Understanding how the commensal bacteria interplay with the gut epithelium both to maintain homeostasis and in disease is essential to better understand how tissue homeostasis is maintain and lost in the intestine.

N. Mammalian models of Stem Cells

Virtually all mammalian adult tissues are maintained by stem cells and Notch signaling has been shown to play essential in potentially all of these adult stem cells (Ables et al.; Blanpain et al., 2006; Lowell et al., 2006; Mazzone et al., 2010; Morrisey and Hogan, 2010; Ohishi et al., 2003; Sancho et al., 2004). For simplicity, I will focus on the vertebrate intestinal stem cells and very briefly overview a few adult stem cells models that have been shown to be regulated by Notch signaling, such as the skin and the developing airways.

N.1. The Intestine

The mammalian intestinal tract is lined by a thin monolayer epithelium whose cells carry out the essential functions of digestion and absorption of nutrients. This tissue is in direct contact with the external environment and its cells are subject to external aggressions. Therefore, the intestinal epithelium is quickly turned-over, with cells being renewed every 5 days, and is constantly replenished by dividing stem cells. Anatomically, the mammalian intestinal tract can be divided into two different segments, the small intestine and the large intestine or colon. The area of the epithelium of the small intestine is increased by finger-like protrusions called villi as well as invaginations of the epithelium known as crypts of Lieberkühn (Fig.12). The large intestine lacks villi and the crypts are more deeply invaginated. The mammalian intestine epithelium lining arises, like the fly midgut, from the endoderm during development (Stainier, 2005). The intestinal epithelium is a single layer epithelium, also known as the mucosa. The mucosa is in contact with the subjacent connective tissue or stroma that contains blood and lymph vessels, cells of the immune system as well as nerve fibers. Below the stroma lie several layers of visceral muscle (Sancho et al., 2004). For simplicity, I will describe in further detail the stem cells that

renew the small intestine and the different pathways that regulate the stem cell self renewal the differentiation steps in the lineage.

The dividing cells that replenish the epithelium of the small intestine are confined to the crypt. The crypts harbor the stem cells, which are localized near the base of the crypts [more on the localization of the stem cells below, (Cheng and Leblond, 1974a; Cheng and Leblond, 1974b)]. The stem cells divide once a day and give rise to cells that will continue to divide, transit-amplifying cells (Cheng and Leblond, 1974a; Cheng and Leblond, 1974b). However, since these cells are more committed to differentiate, they are also termed committed precursors or progenitors. The transit-amplifying cells divide another 4 or 5 times in the crypts before they terminally differentiate. The dividing cells migrate upwards through the crypts and then into the villi, where no further divisions occur and where the multiple types of differentiated cells of the epithelium are localized. The differentiated cells continue to migrate towards the tip of the villi, where they undergo apoptosis and are shed into the lumen (Cheng and Leblond, 1974a; Cheng and Leblond, 1974b; Hall et al., 1994). The differentiated cells of the small intestine are of four types: enterocytes, goblet, enteroendocrine and Paneth cells. Enterocytes are absorptive cells and constitute most of the epithelial cells of the intestine. The other cell types are all secretory cells. The goblet cells are mucous secreting cells and the enteroendocrine cells secrete peptide hormones. Paneth cells, unlike the other differentiated cells, are localized at the crypt bottoms and secrete antimicrobial peptides that may provide protection for the stem cells against bacterial infection, playing therefore a role in innate immunity (Cunliffe and Mahida, 2004). Upon differentiation, the Paneth cells migrate towards the crypt bottom (Fig.12).

Although the stem cells are known to be localized at the bottom of the crypts, the exact localization of the stem cell pool has been controversial for many years. In a series of articles, Cheng and Leblond proposed that the stem cells are the small, undifferentiated cells intercalated amongst the Paneth cells at the base of the crypt, which were termed crypt base columnar cells [CBCs, (Cheng and Leblond, 1974a; Cheng and Leblond, 1974b)]. These cells were characterized through electronic microscopy and found to give rise to all the differentiated cell types in the intestine

through lineage analysis. The CBCs were observed to lie at the base of the crypt, at positions 1-4 (position 1 being the basal most cell of the crypt, counting each cell going upwards as the following position), with this region being termed the stem cell zone (Cheng and Leblond, 1974a). Recent work from Clevers and colleagues has identified the gene Leucine-rich-repeat-containing G-protein coupled receptor 5 (Lgr5) as being expressed specifically in the CBCs (Barker et al., 2007). Lgr5 was identified as a target of Wnt pathway (the role of Wnt signaling in the mammalian intestine is addressed below), is an orphan receptor and was shown to be strongly expressed in the cells at the position 1-4 at base of the crypt that are intercalated with the Paneth cells. The Lgr5 positive cells were also shown to be dividing, with a cell-cycle of about 24h (Barker et al., 2007). Lineage tracing analysis, marking with βGal the lineage of Lgr5+ cells, found that these Lgr5+ cells give rise to all the differentiated cell types of the epithelium, forming a βGal+ column from the bottom of the crypt to the tip of the villus and that these are long-lived stem cells, as they are maintained up to 6 months after induction (Barker et al., 2007; van der Flier and Clevers, 2009). Further evidence for the Lgr5+ cells being the stem cells of the intestine came from another study from the Clevers lab, where single sorted Lgr5+ were able to generate, in *in vitro* cultures, structures that resemble crypt-villus organoids (Sato et al., 2009). These structures had an analogous structure to the intestinal crypts and villi and the four types of differentiated were present (Sato et al., 2009). Therefore, this study indicates that Lgr5+ cells can function as stem cells *in vitro* and are likely to be the stem cell population *in vivo*. Also, the study shows that crypt-villus structures are self-organizing structures that can form in the absence of the non-epithelial tissue, suggesting that the proliferation, differentiation and spatial organization of the crypt-villus structure does not require a physical mesenchymal niche (Sato et al., 2009). Although the function of Lgr5 in the intestine is not known, the Lgr5 expressing cells have all the characteristics of *bona fide* stem cells.

An alternative localization of the stem cells has been proposed by Potten and others, with the stem cells being localized at the cell position 4, immediately above the highest localized Paneth cells. Studies that involved labeling dividing cells with tritiated thymidine and following its progeny led to the extrapolation that all the cells in

the crypt were derived from cells at position 4 in the crypt (Cairnie et al., 1965; Potten, 1998; Qiu et al., 1994). Furthermore, the cells at position 4 were found to retain radioactive tritiated thymidine after several rounds of division, suggesting that these are label retaining stem cells, which is considered a hallmark of long-lived stem cells (Potten et al., 2002). The cells at position 4 could retain label if they are slow cycling or quiescent stem cells. Alternatively, the stem cells at position 4 could be retaining always the same strand of DNA (Potten et al., 2009). This would be in accordance with the immortal strand hypothesis that has been proposed by Cairns in 1975 and that postulates that stem cells may retain the original template strand of DNA to protect the stem cell from errors occurring during DNA replication, which would compromise its genome (Cairns, 1975). If the stem cells are the cells at position 4, then the dividing CBCs at positions 1-4 would be the daughter cells of these stem cells. Recent work by Sangiorgi and Capecchi supports the model of the stem cells at position 4 (Sangiorgi and Capecchi, 2008). *LacZ* expression driven by a Cre insertion in Bmi1, a gene of the Polycomb Repressing Complex 1, which is required for maintenance of chromatin silencing, was shown to be expressed specifically in the cells at position 4 (Sangiorgi and Capecchi, 2008). Also, lineage tracing analysis following the progeny of these Bmi1+ cells revealed that the Bmi1+ cells give rise to all the differentiated cells in the intestine, similarly to what has been observed for the Lgr5+ cells (Sangiorgi and Capecchi, 2008). The Bmi1+ cells were observed to be actively cycling cells and not a quiescent population and the clones of the progeny of the Bmi1 expressing cells where long-lived, with clones still being detected in the intestine 1 year after induction (Sangiorgi and Capecchi, 2008). Whether the cycling Bmi1+ cells are label retaining cells has not yet been addressed.

The Lgr5+ cells and the Bmi1+ cells both share functional characteristics of stem cells: they are actively cycling cells that generate long-lived clones that contain all the cell types of the small intestine (Barker et al., 2009; Barker et al., 2007; Sangiorgi and Capecchi, 2008). However, they appear to correspond to two different populations. One model proposes that the Lgr5+ and the Bmi1+ cells correspond to two different populations of stem cells. Alternatively, both the Lgr5+ and the Bmi1+ cells could correspond to two different phases of the same stem cell population. The

cells in position 4 have been suggested to have quiescent features, and therefore could correspond to a quiescent pool of stem cells while the Lgr5+ cells could correspond to the active stem cells (Lee et al., 2009a). Lgr5 reporter can be observed to be weakly expressed in cells at position 4 and it has been reported that 10% of the Lgr5+ where observed at position 4 (Barker et al., 2007; van der Flier and Clevers, 2009). Therefore, the expression of Lgr5 could be dynamic in the stem cells. The stem cells could express at one moment Bmi1 and then start expressing Lgr5; this difference in expression could be a consequence of the position of the cells relative to a mesenchymal niche surrounding the crypt, with certain signals promoting the change in position and gene expression in the stem cells. Finally, the Bmi1+ cells at position 4 could correspond to the true stem cells, producing both the transit-amplifying cells that move upwards and the Lgr5+ cells that move downwards to give rise to the Paneth cells. Evidence for this model comes from the observation that CBCs cells can have early Paneth granules, suggesting that they are undergoing differentiation into Paneth cells (Leblond, 1976). Since 10% of the Lgr5 expressing cells are localized at position 4, these could correspond to stem cell daughters that are committing to becoming Paneth cells (Barker et al., 2007). These Lgr5+ cells at position 4 could also be Bmi1+ (which has not been yet tested) and they could correspond to the cells that generate the long-lived stem cell clones in the Lgr5 lineage tracing experiment. However, the Lgr5+ cells are cycling about every 24h hours, which appears to be too fast for them to be merely the progenitors of the Paneth cells, since these take 2-3 weeks to turnover (Cairnie, 1970). To sort out this apparent contradiction and address the question of whether these are two separate populations of stem cells further experiments have to be done, such as visualization the expression of both markers in a same crypt, better characterization of the expression of both markers over time and further lineage analysis. It is important to notice that the cells in the crypt are able to regenerate the epithelium in cases where extensive damage has been caused to the tissue. Early transit amplifying cells are capable of functioning as stem cells if these are killed by irradiation (Ijiri and Potten, 1984; Potten et al., 2009). These data suggest that the stem cell population in the intestine is not static but highly dynamic, with the committed precursors being able to

replace the stem cells, similarly to what has been observed in other stem cell models, such as the *Drosophila* male germ stem cell (Cheng et al., 2008).

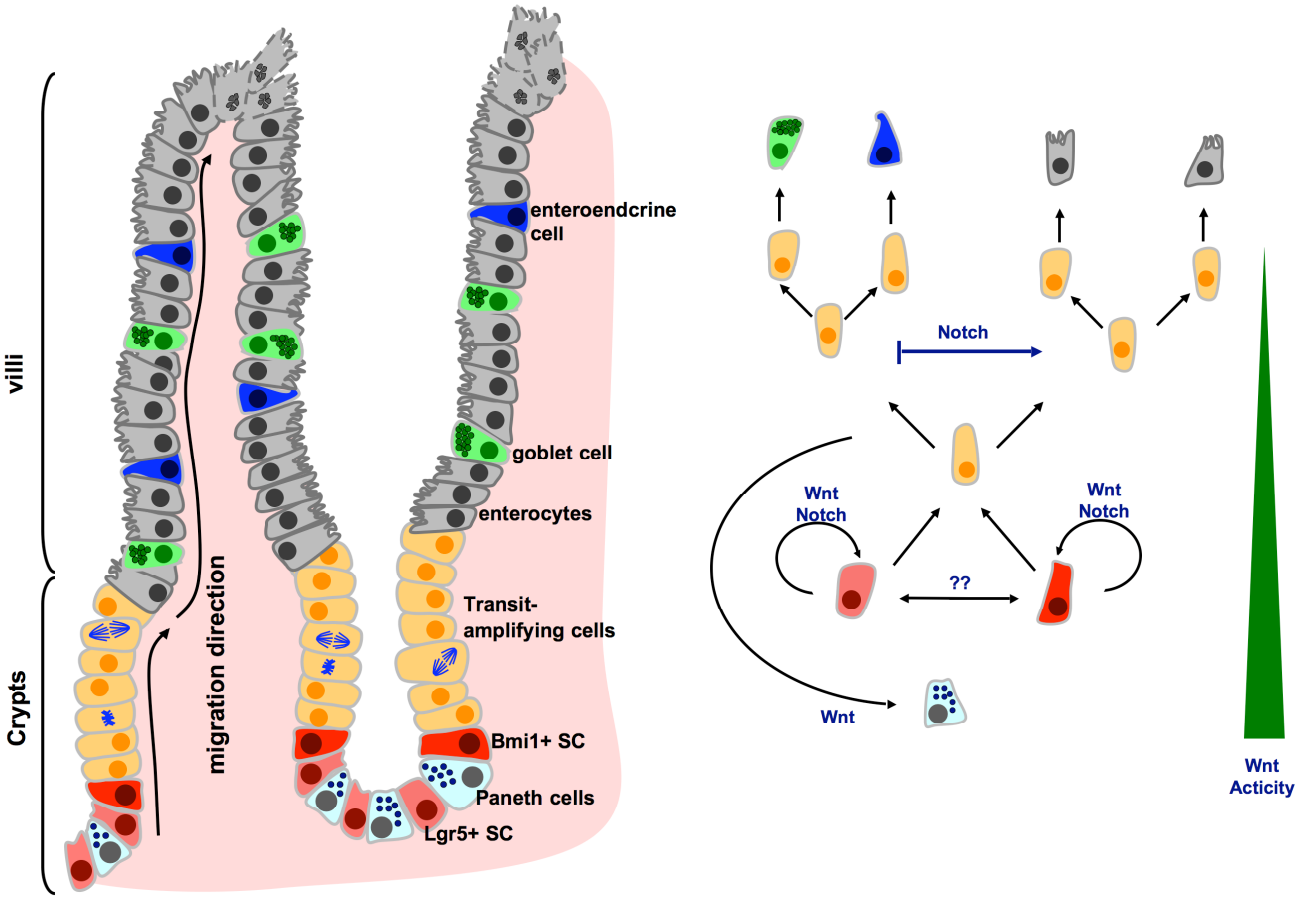


Fig. 12: Structure of the mammalian small intestine epithelium with the multiple cell type represented and the stem cell lineage. Both the Lgr5+ cells and the Bmi1+ cells have been proposed to be the stem cell responsible for the self-renewal of the intestinal epithelium and it is possible that both correspond to the stem cells, that can alternate between both states and localizations. Wnt signaling promotes proliferation of the stem cells and the transit-amplifying cells and functions as a gradient, with the high Wnt signaling at the bottom of the crypts promoting differentiation of the Paneth cells. Notch signaling is required for proliferation, promotes the absorptive fate and inhibits the secretory fates.

Wnt signaling is a major player in regulation of the stem cells in the intestine, being the main driving force of proliferation of the stem cells and the transit-amplifying cells. Microarray studies identified the Wnt target genes expressed in the intestine and showed that these target genes are expressed in the proliferative cells in the crypts as well as in neoplastic proliferative cells in colorectal cancers (Kosinski et al., 2007; Stappenbeck et al., 2003). The expression pattern of the Wnt target genes suggests that there is a gradient of Wnt activity, with the highest activity at the base of the crypt (Gregorieff et al., 2005). Studies that tested the loss of Wnt signaling in the intestine by removing Tcf4, the transcription factor that mediates expression of the Wnt target genes, by conditionally removing β-catenin from the epithelium or by expression of a secreted inhibitor of extracellular Wnt, showed that loss of Wnt signaling activity in the crypt resulted in loss of proliferation in the crypts (Fevr et al., 2007; Ireland et al., 2004; Korinek et al., 1998; Kuhnert et al., 2004; Pinto et al., 2003). Differentiation into enterocytes does not appear to be compromised, with these cells still present in the villi, while the secretory lineages are absent in intestines in which Wnt activity was lost (Fevr et al., 2007; Ireland et al., 2004; Korinek et al., 1998; Kuhnert et al., 2004; Pinto et al., 2003). As expected, the activation of Wnt through overexpression of a Wnt agonist or in mutants for *Apc,* a negative regulator of Wnt signaling, results in hyperproliferation in the crypt compartment (Andreu et al., 2005; Kim et al., 2005; Sansom et al., 2004). Besides its role as the key mitogen in the crypts, Wnt signaling plays two other roles in the crypts. Wnt signaling was shown to be required for the differentiation of the Paneth cells at the bottom of the crypts (van Es et al., 2005a). Thirdly, Wnt signaling also regulates cell position through the expression of the Ephrin B ligands and receptor (Batlle et al., 2002). Ephrin molecules and their receptors are used throughout development to organize cell migration and maintenance of tissue boundaries. Disruption of the *EphB2* and *EphB3* results in intermingling of proliferating cells with differentiated cells and the Paneth cells fail to migrate to the base of the crypt (Batlle et al., 2002). There is evidence that Wnt functions as a paracrine signal in the gut, with the Wnt pathway being activated in a non-autonomous way (Kuhnert et al.,

2004; Pinto et al., 2003). Wnt activity in the crypt cells, as measured by nuclear localization of β-catenin, is higher at the base of the crypts (Batlle et al., 2002) and multiple Wnts have been shown to be expressed in the epithelial cells in base of the crypt (Gregorieff et al., 2005).

BMP signaling also plays an important role in the coordination of proliferation and differentiation in the intestinal epithelium. The mesenchymal cells localized in the underlying stroma have been proposed to function as a niche for the stem cells in the crypts (Mills and Gordon, 2001). The mesenchymal cells in the villi express BMP ligands, while the BMP receptor is expressed in the epithelium. BMP signal acts as a negative regulator of crypts, with loss of BMP signaling resulting in hyperproliferation in the crypts and ectopic crypt formation (Haramis et al., 2004; Hardwick et al., 2004; He et al., 2004). Expression of Noggin and Gremlin, two inhibitors of BMP, in the stroma of the crypts inhibits BMP signal in the crypts and promotes proliferation (He et al., 2004; Kosinski et al., 2007). Other growth factors and cytokines have been proposed to be expressed by the mesenchymal cells to promote stem cell maintenance and proliferation in the crypts (Powell et al., 1999). As mentioned above, single Lgr5+ cells are capable of generating crypt- and villi-like structures *in vitro* and in the absence of mesenchymal cells (Sato et al., 2009). However, in this study, the authors supplied the cells with a plethora of growth factors, cytokines as well as Noggin; therefore while the mesenchymal cells do not need to be physically present, the components they secrete are still required as components of the niche (Sato et al., 2009).

Notch signaling has also been shown to play essential functions in the intestinal epithelium. Loss of Notch signaling in the intestine, either by pharmacological inhibition of γ-secretase, knock-out of the CSL mouse protein or double knock-out of both Notch1 and Notch2, results in a loss of proliferation in the crypts, with all the cells being converted into goblet cells (Milano et al., 2004; Riccio et al., 2008; van Es et al., 2005b; Wong et al., 2004). Over activation of the Notch pathway by overexpression of NICD results in an increase in proliferation in the crypts, an increase in enterocytes, loss of goblet cells and a decrease in Paneth and enteroendocrine cells (Fre et al., 2005; Stanger et al., 2005). Notch and Wnt appear

to have a cooperative affect in regulating proliferation in the intestine since the pharmacological inhibition of γ-secretase blocks overproliferation in *Apc* mutants while hyperactivation of Notch signaling in $Tcf4^{-/-}$ knock-out mice does not result in increased proliferation, with no dividing cells present in the crypts (van Es et al., 2005b; Fre et al., 2009). Therefore, both Notch and Wnt require the other pathway to be activated in order to promote proliferation. A reporter for Notch1 proteolysis labels long-lived lineages in he mammalian intestine, which suggests that Notch signaling is active in the stem cells (Vooijs et al., 2007) with Notch signaling being required for stem cell proliferation. One possible model for how Notch regulates the stem cells lineage is that the transit-amplifying commitment to either a secretory lineage or a absorptive lineage occurs by lateral inhibition, with the precursors committing to secretory fates and inhibiting its neighbors from differentiating in the same way by activating Notch pathway in them. These Notch ON precursors would then commit to the secretory lineage (Fig.12). The Notch pathway regulates fate, at least partially, by the Hes1 (hairy and enhancer of split 1, homologue to the fly Enhancer of split genes) target gene, that promotes enterocytes fate and inhibits enteroendocrine and goblet cells fate (Jensen et al., 2000). Hes1 is bHLH protein that will in turn repress other bHLH proteins. In the intestine. Hes1 represses Math1 (mouse atonal homologue 1), which is required for the commitment toward the secretory lineage while cells that lack Math1 differentiate as enterocytes (Yang et al., 2001). Downstream of Math1, other transcription factors are required for specification of the different secretory fates: Gfi1 promotes goblet and Paneth cell fates and Neurogenin3, another bHLH protein, promotes enteroendocrine fate (Jenny et al., 2002; Shroyer et al., 2005). As mentioned above, specification of the Paneth cells also requires Wnt activity (van Es et al., 2005a). How exactly Notch activity is regulated in the intestine is not understood. While, both Notch1 and Notch2 are expressed in epithelial cells in the crypts, which cells express the Notch ligands is not known (Riccio et al., 2008). Notch signaling is required for proliferation and appears to be ON in proliferative cells that form long-livid lineages but it is not clear whether these are stem cells. Notch signaling is also required to mediate commitment to different differentiation lineage, with lateral inhibition being a possible mechanism

promoting this segregation of lineages. How exactly when does this segregation occur is not known. Although Notch signaling appears to play the opposite role in the fly intestine, where it appears to inhibit self-renewal, Notch signaling seems to be playing a similar role in terms of fate specification of the differentiated cells in both flies and mammals. As mentioned above, Notch signaling activity promotes enterocyte fate at the expense of the secretory cells in both systems.

Mutations in the Wnt pathway resulting in pathway hyperactivation, such as mutations in *Apc* or *axin2* are responsible for the initiation of the great majority of colorectal cancer (Fodde and Brabletz, 2007; Liu et al., 2000; Miyaki et al., 1994; Miyoshi et al., 1992; Powell et al., 1992). Several mouse models of colorectal cancer have been developed to study neoplastic transformation in the intestine at a cellular and molecular level (Radtke et al., 2006; van der Flier and Clevers, 2009). The mammalian small intestine is also a powerful model to study the cancer stem cell hypothesis. Work from Clevers and colleagues has shown that deletion of *Apc* in Lgr5+ cells results in transformation of these cells, with defects detected as early as 3 days after induction and the formation microadenomas and neoplastic transformation of the intestine occurring within 3-5 weeks (Barker et al., 2009).

N.2. The Skin epidermis

The skin epidermis is a fast renewing epithelium that functions as a barrier from environmental aggressions such as external microorganisms or dehydration. Homeostasis of adult epidermis is maintained by basal cells that self-renew as well as produce cells that undergo a complex differentiation program, moving outwards in a columnar manner. First the basal cells move into the spinous layer, where they start expressing genes that promote the formation of a robust cytoskeleton network that strengthens cell-cell junctions, providing resistance against mechanical stress. From the spinous layer the cells move into the granular layer where more structural proteins are deposited beneath the plasma membrane that will participate in interaction with lipid bilayers and the stratum corneum cells to render the skin

waterproof. The last layer is the stratum corneum and when the cells reach this layer they undergo terminal differentiation that will lead to their death. They serve as a barrier from microorganisms and are eventually shed and replaced by new cells from below (Blanpain and Fuchs, 2009). Notch signaling was initially reported to be required for differentiation in the epidermis, with loss of Notch1 in the epidermis resulting in hyperplasia of the epidermis and loss of the expression of differentiation markers (Rangarajan et al., 2001). This loss of Notch signaling phenotype appeared to be homologous to the loss of Notch signaling phenotype in the fly intestine, suggesting homologies between the two adult stem cells. However, careful analysis by Fuchs and colleagues showed that loss of *RBP-J*, the mammalian homologue of Su(H), which results in loss of all Notch signaling, resulted not in hyperplasia but in defect of commitment from the basal to spinous cell fates (Blanpain et al., 2006). Furthermore, these authors argue that the reported hyperplasia in the *Notch1*$^{-/-}$ knock-outs was a probable a consequence of the postnatal *Notch1*$^{-/-}$ knock-out mice having a compromised epidermis, which was shown to trigger hyperproliferation as a secondary response (Blanpain et al., 2006). These authors suggested that Notch signaling activity promotes the commitment from basal fate to spinous fate, with expression of Notch target genes promoting the expression of genes that will promote the spinous fate (Blanpain et al., 2006). This role is analogous to the role of Notch signaling in the fly ISC lineage, where it also appears to promote differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007).

N.3. The Epithelium of the Lung

The mammalian respiratory system is composed of the trachea and the lungs, that arise from the foregut during development, being, like the intestinal epithelium, a tissue of endodermal origin that develops through interaction between the endoderm and the mesoderm (Morrisey and Hogan, 2010). The lungs carry out the essential function of gas exchange having, for this effect, a branched tree-like tubular system.

Like the intestine, the lungs are exposed to the external environment, including airborne pathogens and potential risk of dehydration. To surmount these problems, the lungs have a large array of specialized epithelial cells lining the airways that secrete and clear mucous, participate in innate immunity, maintain surface tension and carry out fluid and electrolyte transport. This is a very sophisticated tissue and I will not go into the description of its development but will only briefly mention the role of Notch in the specification of some fates in the epithelial lineage. During the pseudoglandular stage in development of the lungs, primary buds will generate a complex tree-like structure ending in thousands of terminal tubules. During this stage, multipotent progenitors of the epithelium divide to self-renew as well as generate the bronchiolar progenitors. Notch signaling has been shown to be required for the specification of the bronchiolar lineage, with loss of Notch signaling resulting in an increase in the multipotent progenitors [Fig.13, (Tsao et al., 2008)].

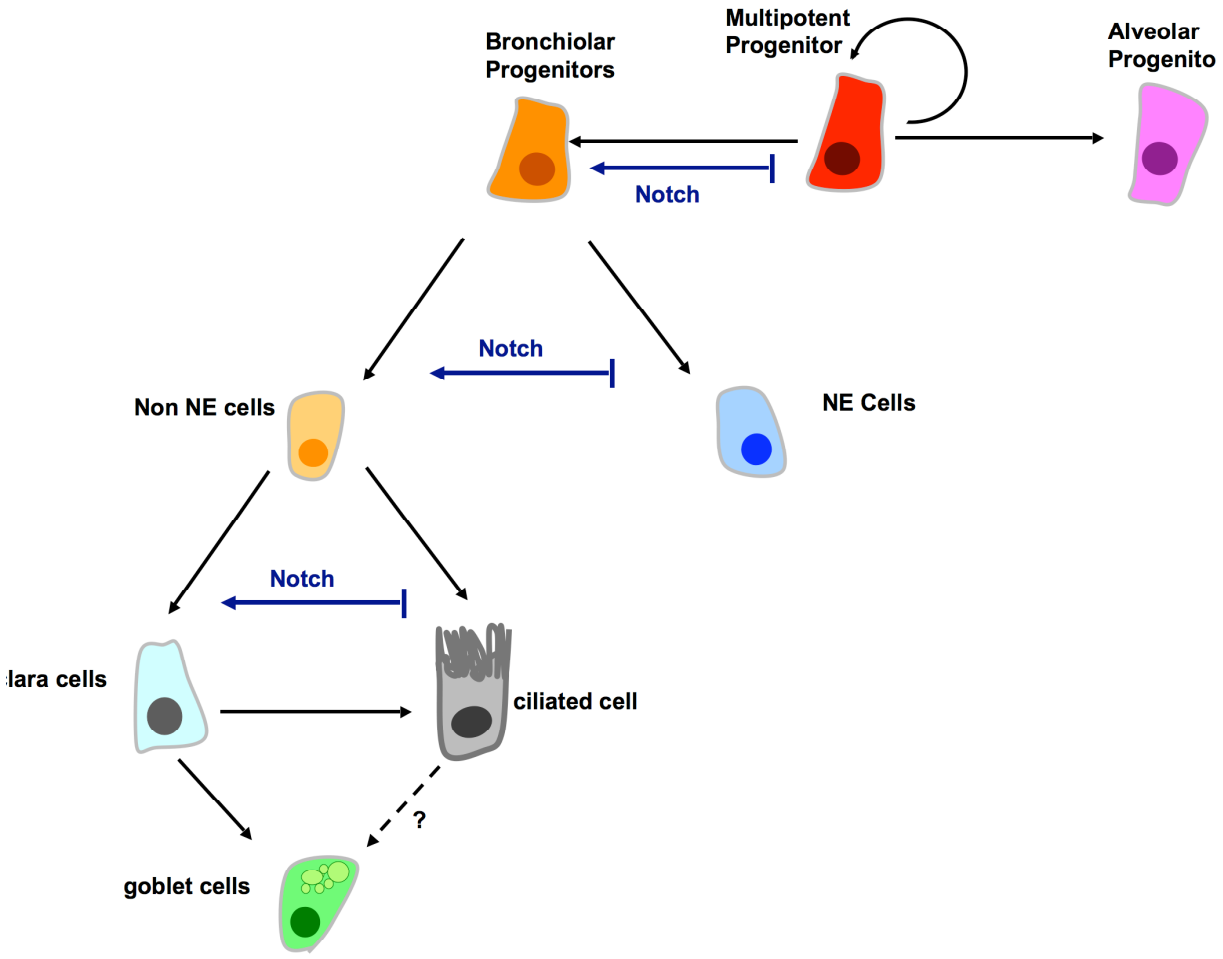


Fig. 13: Outline of lineage of the multipotent progenitors and the bronchiolar progenitors. The multiple cell fate choice that are mediated by Notch signaling are represented.

The first lineage decision in the bronchiolar lineage is between neuroendocrine (NE) or non-neuroendocrine lineage (Fig.13). Again, this decision appears to be mediated by Notch signaling, with mutants for the Notch effector *Hes1* resulting in an increase in NE cells and a reduction in the number of non-NE cells [Fig.13, (Ito et al., 2000)]. The non-NE cells undergo another Notch-dependent lineage decision between the secretory lineage and the ciliated cell lineage. Conditional loss of Notch signaling analysis showed that Notch signaling promotes the clara cells, which are secretory cells, and antagonizes the ciliated cell fate [Fig.13, (Tsao et al., 2009)]. The Clara cell population has also been shown to give rise to ciliated cells and to mucous producing goblet cells [Fig.13, (Morrisey and Hogan, 2010)]. Notch signaling appears to be playing multiple cell fate decision in the epithelial lineage of the airways and it would be very interesting to understand how these multiple decisions are mediated. It would also be interesting to understand whether Notch signaling mediates multiple fate decisions in the same lineage through different levels of Notch signaling activity, as it has been suggested for the fly intestine.

Chapter II

Materials and Methods

Immunofluorescence of Drosophila Intestinal cells

Material

PBS

PBS-50% Glycerol

PBT(0.1% Triton)

4% PFA

PBS-EGTA(50mM)

Formaldehyde solution 37-40%

Heptane

Methanol

I.a) Fixation 2h with PFA 4%

1. Use aged female flies older than 3 days at 25°C, that have had fresh yeasted tubes each day (or two). Have males in tube so females are laying eggs normally.

2. Put to sleep on CO_2 pad. Transfer 1 female to glass dish of PBS.

3. Carefully tear abdomen and remove intestines. Can try to gently straighten it out so that it is no longer coiled. Can keep associated ovaries to use as internal control in IF.

4. Place directly into covered dish of 4% PFA. Keep covered with glass and in a box to avoid fumes from PFA.

5. Dissect over 20 min as many as possible, then place into fume hood and allow to continue fixing for 1h50min (so 2h average).

6. Remove PFA and rinse with PBT 1X (0,1% Triton-X 100).

I.b) Fixation 15min with 4% Formaldehyde and Methanol (adapted from Embryo Protocols)

1. Prepare vials for fixation containing 3ml of PBS-EGTA(50mM) + 300µl Formaldehyde solution 37-40% in interface with 3ml Heptane.

2. Use aged female flies older than 3 days at 25 degrees that have had fresh yeasted tubes each day (or two). Have males in tube so females are laying eggs normally. Put to sleep on CO_2 pad. Transfer 1 female to glass dish of PBS.

3. Carefully tear abdomen and remove intestines making sure to leave the ovaries and some cuticle attached. Can try to gently straighten out the intestine so that it is no longer coiled. The ovaries and attached cuticle keep the intestines afloat at the interface between the 4% formaldehyde and the heptane.

4. Place dissected intestine into fresh PBS. Dissect over 5 min as many intestines as possible.

5. Place all the intestines at the same time in the fixation vial, dropping them at the interface between the 4% formaldehyde and the heptane. Fix for 15 min with the vials in vertical position and with mild agitation.

6. Remove the 4% formaldehyde and add methanol. The intestines should drop into the methanol phase. Remove all the heptane and let the intestines fix in methanol for 5 min (up to 15 min).

7. Re-hydrate the intestines with PBT 1X (0,1% Triton-X 100), transferring them progressively from 25%PBT-75%methanol to 100% PBT. Rinse in PBT.

II. IF

1. Trim intestines and try to cut ends so that they are open and waste can exit. At this step, you can either leave the intestines relatively long, or trim so you have just the posterior midgut (first large loop).

2. Place into 50% glycerol for 30-60 min until they sink and are equilibrated.

3. Transfer back into PBT- waste will now start to exit and they equilibrate. Let sit 10-30 min.

4. Rinse well with PBT to clean out waste yeast, etc.

5. Add 1st primary Ab for at least 4 hours at RT or (better) ON at 4 degrees.

12. Wash 3X 20 min with PBT.
Note: it is practically impossible to remove all primary Ab from the guts, even after the washes and so the 2nd primary Ab is likely to be contaminated with the 1st primary Ab. If you have a precious Ab you should always use it first and also keep track if an Ab aliquot has been used after another Ab.

13. Add either 2nd primary for at least 4 hours at RT or ON at 4 degrees OR secondary Ab for 3-4 hours.

14. Wash 3X 10-20 min, adding DAPI in the last wash for at least 5 min.

15. Equilibrate in 50% glycerol for at least 30 min (longer is better since shape changes as equilibrating).

16. Mount on slide with 55µl of mounting medium. Allow at least 1h to equilibrate prior to adding cover slip.

Mounting Medium

Material

40ml Glycerol 99,5% spectrophotometric grade (Sigma 191612), 80%final

1ml PBS 10x, 0,2x final

2g n-proplygallate, m/v 4% final

9ml H_2O

Protocol

1. Mix all the reagents together in a 50ml falcon, use a sterile pipette to break down the clumps of n-propylgallate powder.

2. Cover falcon with paper to protect from the light and put the mix rotating over night at 4°C or until all the n- propylgallate powder is fully dissolved.

3. Keep at -20°C.

RNA probes Synthesis

Material

1μg linearized plasmid

DIG-NTP RNA labeling mix 10x OR Biotin-NTP RNA labeling mix 10x OR Fluorescein-NTP RNA labeling mix 10x (all from Roche), depending on which type of probe being synthesized

10x Transcription Buffer

DEPC H_2O

RNAse inhibitor (50u/μl, Roche)

DTT 50mM

RNA polymerase (SP6, T7 or T3)

Stop Solution (NaAc at 0,2M, pH=6)

LiCl 4M

2x carbonate buffer (120mM Na_2CO_3, 80 mM $NaHCO_3$, pH=10,2)

tRNA 20mg/ml

absolute Ethanol

Protocol

1. In an eppendorf on ice, add the following:
    1. 1μg linearized plasmid
    2. DEPC H_2O (to a final volume of 20μl)
    3. 2μl 10x transcription buffer
    4. 10x DIG/Biotin/Fluorescein RNA labeling mix
    5. 2μl RNAse inhibitor (50u/μl, Roche)
    6. 2μl DTT 50mM
    7. 2μl RNA polymerase (SP6, T7 or T3)

mix, spin-down and incubate for 2h at 37°C.

2. Add 50μl 2x carbonate buffer and incubate at 60°C for 10min precisely.

3. Place tube on ice and add 100μl of stop solution.

4. Add 20μl of LiCl 4M, 10μl tRNA 20mg/ml and 600μl of ethanol. Mix and incubate at -20°C for at least 15 min.

5. Centrifuge at max speed for 20 min at 4°C.

6. Remove the supernatant ethanol and wash pellet 70% ethanol (prepared with DEPC H_2O).

7. Remove supernatant and re-suspend RNA in 150μl of DEPC H_2O. Keep probe at -80°C.

In situ hybridization on Drosophila Intestines

Alkaline Phosphatase and Tyramide amplification

Material

PBS, PBS-50% Glycerol, PBT(0.1% Triton), PBT (0.3% Triton), PBTween (0.1%), PBTween (0.1%) in dH_2O DEPC ,

TNT (0.1MTris-HCl pH7.5, 0.15M NaCl, 0.1% Tween 20)

TNB (0.1MTris-HCl pH7.5, 0.15M NaCl, 0.5% Blocking Reagent)

Blocking Reagent (Perkin-Elmer no°FP1020) is to be added slowly to the buffer while stirring and heated to approximately 60°C until everything dissolves and stored in aliquots at -20°C.

Glycine 2mg/ml in PBTween

Proteinase K 10mg/ml in PBTween diluted from a 50μg/ml stock.

HYB buffer (For 100ml: 50ml of Formamide; 25ml of SSC 20x in dH2O DEPC; 24 ml Tris HCL pH=6,8; 1ml Tween 10% in dH_2O DEPC; 100μl heparin at 50mg/ml and 100μl yeast RNA 10mg/ml)

Alkaline Phosphatase staining Buffer (100mM NaCl; 50mM $MgCl_2$; 100mM Tris pH=9.5 and 0.1% Tween20)

Protocol for fluorescent *in situ*, using tyramide amplification

Day 1

1. Dissect intestines and put immediately in 4%PFA (fix approximately 2h, in groups of about 20-30). Rinse in PBT (0.1% Triton)

2. Cut off narrow ends and trim of ovaries. Place in PBS-50% glycerol for at least 30 min to overnight (4°C if ON).

3. Place back into PBT(0.1% Triton) and allow pressure to clean out yeast. Leave 10 min and do several washes being sure to remove sedimented yeast from bottom of glass dish.

4. Wash into PBT(0.3% Triton) 3 x 5-10 min.

5. Place in eppendorf tubes in PBTween (0.1%) about 10-20 per tube per probe.

6. Remove liquid and add in 1ml Proteinase K 10micrograms/ml in PBTween. Invert gently to mix. Stagger reactions by 1-2 minutes. Digest 4min. and remove proteinase K during last 30s and add in 1ml glycine 2mg/ml in PBTween at 4min and place on ice. Rinse 1x in glycine 2mg/ml in PBTween.

7. Rinse 2x in PBTween

8. Post-fix in 4%PFA 20min RT rotating very slowly.

9. Rinse 5X in PBTween (last 2x in PBTween DEPC).

10. Add in 50% HYB for 5min

11. Remove sup and add in 100% HYB and pre-hybridize for 1hr at the desired temp (Normally use 55°C, but higher may be more specific for certain RNA probes).

12. Add 1μl of probe in 300μl of HYB and heat in 85°C block for 5 min. Transfer to oven and allow to cool to approximately 55°C.

13. Remove HYB and add probe to intestines. Leave ON.

Day 2:

The washes in HYB buffer are done at 55°C.

14. 1x HYB 20min

15 1x 50% HYB 20min

16. 4x PBTween 20min (last time at RT)

17. Rinse into TNT 2x. Block with TNB for 1h rotating.

18. Dilute anti-DIG POD antibody in TNB 1/2000 final. Add to intestines and incubate ON at 4°C lying down on rocking platform propped up on by a pipette tip.

Day 3:

19. Rinse 3x 20min in TNT

20. Do tyramide reaction for 6min. (Dilute 1μl of Cy3 tyramide in 50μl reaction buffer with kit; each intestine needs about 150μl). Stagger reactions by a couple minutes.

21. Rinse 3x 10min in TNT.

22. Add PBT and DAPI for 10 min.

23. Use a cut p1000 pipette tip to move into glass dish. Add in PBS 50% glycerol for 30min to ON.

24. Put in 55μl of mounting medium on slide without adding cover slip. Leave for at least 2hr.

25. Place cover slip on and seal.

Protocol for *in situ*, with alkaline phosphatase reaction to develop.

Day 1

Day 1 of the protocol is the same for both *in situ* protocols. Follow protocol above

Day 2:

The washes in HYB buffer are done at 55°C.

14. 1x HYB 20min.

15 1x 50% HYB 20min.

16. 4x PBTween (last time at RT)

17. Dilute anti-DIG AP antibody in PBTween at 1/2000 final. Add to intestines and incubate at room temperature for 2h, rotating slowly.

18. Wash 3x 20 min in PBTween.

20. Wash 3x 5min in alkaline phosphatase staining buffer.

21. .Use a cut p1000 pipette tip to move intestines into a glass dish. For each glass dish, remove the staining buffer and add 400μl of staining buffer +3,5μl BCIP and 4,5μl NBT. Follow the formation of the blue precipitate. Usually the staining will take 20min to 2h to develop but may take longer. In case of a staining that takes several hours, add fresh staining buffer with BCIP and NBT every couple hours and put at 4°C if the reaction continues ON.

22. Stop reaction by rinsing several times with PBTween.

23. Use a cut p1000 pipette tip to move back into eppendorf tubes. Dehydrate intestines by transferring them into ethanol 50%, 70%, 95% and 4 times in 100% Keep ON at 4°C.

Day 3:

24. Rehydrate intestines by transferring them into ethanol, 95%, 70%, 50%and 4 times in PBTween.

25. Use a cut p1000 pipette tip to move into glass dish. Add PBS-50% glycerol for 30min to ON.

26. Put in 55μl of mounting medium on slide without adding cover slip. Leave for at least 2hr.

27. Place cover slip on and seal.

Double Fluorescent *In situ* hybridization on Drosophila Intestines

Tyramide amplification

Material

PBS, PBS-50% Glycerol, PBT (0.1% Triton), PBTween (0.1% Tween), PBTween (0.1% Tween) in dH_2O DEPC, PBS-50% Glycerol

PBS-EGTA(50mM), Formaldehyde solution 37-40%, Methanol, Absolute Ethanol

Xylene solvent

Proteinase K 10mg/ml in PBTween diluted from a 50μg/ml stock.

HYB buffer (For 100ml: 50ml of Formamide; 25ml of SSC 20x in dH2O DEPC; 24 ml Tris HCL pH=6,8; 1ml Tween 10% in dH_2O DEPC; 100μl heparin at 50mg/ml and 100μl yeast RNA 10mg/ml)

10% H_2O_2 solution

TNT (0.1MTris-HCl pH7.5, 0.15M NaCl, 0.1% Tween 20)

TNB (0.1MTris-HCl pH7.5, 0.15M NaCl, 0.5% Blocking Reagent)

Blocking Reagent (Perkin-Elmer no°FP1020) is to be added slowly to the buffer while stirring and heated to approximately 60°C until everything dissolves and stored in aliquots at -20°C.

Protocol

Day 1

1. Dissect intestines in PBS and transfer to fresh PBS. When all intestines are dissected, transfer intestines into a vial with 4% formaldehyde in PBS-EGTA and fix for 30 min while agitating mildly. Rinse 3x in methanol.

2. Re-hydrate the intestines with PBT (0,1% Triton), transferring them progressively from 25%PBT-75%methanol to 100% PBT. Rinse in PBT (0.1% Triton).

3. Cut off narrow ends and trim of ovaries. Place in PBS-50%glycerol for at least 30 min to overnight (4°C if ON).

4. Place back into PBT and allow pressure to clean out yeast. Leave 10 min and do several washes being sure to remove sedimented yeast from bottom of glass dish.

5. Place intestines in eppendorf tubes, about 10-20 per tube per probe and progressively dehydrate into methanol 100%.

6. Rinse 2x in ethanol and then do 3x 5min washes in ethanol with tube lying down horizontally.

7. Wash in 50/50 xylem/ethanol for 60min. Wash with tube lying down horizontally.

8. Rinse 2x in ethanol and then do 3x 5min washes in ethanol with tube lying down horizontally.

9. Wash 2x 5min in methanol with tube lying down horizontally.

10. Fix for 5min in 50/50 methanol/PBTween+5% formaldehyde with tube lying down horizontally.

11. Fix for 30min in PBTween+5% formaldehyde. Fix rotating slowly. Make 2x the amount you will need for this step.

12. Rinse 2x in PBTween and then do 3x 10min washes in PBTween. Wash rotating slowly.

13. Remove liquid and add in 1ml Proteinase K 8μg/ml in PBTween. Invert gently to mix. Stagger reactions by 1-2 minutes. Digest for 8min and remove proteinase K during last 30s. Rinse 2x quickly in PBTween and place on ice until all reactions are rinsed.

14. Rinse 3x in PBTween

15. Post-fix in PBTween+5% formaldehyde, ON at 4°C while rotating very slowly.

Day 2

16. Rinse 2x in PBTween and then do 3x 10min washes in PBTween, rotating slowly (last 2x in PBTween DEPC).

17. Wash in 50/50 HYB/PBTween for 10min. Start temperature block to 55°C.

18. Wash in 100% HYB 3x 10min.

19. Pre-hybridize in HYB for 3h at 55°C.

20. Add 1μl of each probe (1 DIG-labeled probe and 1 biotin- or fluorescein-labeled probe) in 300μl of HYB and heat in 85°C block for 5 min. Transfer to block and allow to cool to approximately 55°C.

21. Remove HYB and add probes to intestines. Hybridize for 18-24h.

Day 3:

The washes in HYB buffer are done at 55°C.

22. Rinse 1x in HYB and then wash 1h in HYB.

23 Wash in HYB for 6x 20min. During last wash, bring tubes to RT.

24. Do the following washes for 15min each, at RT:
75/25 HYB/PBTween
50/50 HYB/PBTween
25/75 HYB/PBTween

25. Rinse into PBTween 2x and then wash 3x in PBTween for 10min, rotating slowly.

26. Rinse into TNT 2x. Block with TNB for 1h, rotating.

27. Dilute anti-DIG-POD antibody in TNB (1/2000 final). Add to intestines and incubate ON at 4°C, rotating slowly.

Day 4:

28. Wash 3x 20min in TNT rotating.

29. Do tyramide reaction for 6min. (Dilute 1μl of Cy3 tyramide in 50μl reaction buffer with kit; each intestine needs about 150μl). Stagger reactions by a couple minutes.

30. Rinse 3x 10min in TNT.

31. Quenching of peroxidase: rinse into PBTween 2x and then incubate with 1% H_2O_2 in PBTween for 20min rotating.

32. Wash 3x 5min with PBTween rotating.

33. Rinse into TNT 2x. Block with TNB for 1h, rotating.

34. Dilute anti-Fluorescein-POD antibody in TNB (1/100-1/500 final, requires some optimization) OR Streptavidin-POD in TNB (1/2000 final) depending on probe used. Add to intestines and incubate ON at 4°C, rotating slowly.

Day 5:

35. Wash 3x 20min in TNT rotating.

36. Do tyramide reaction for 6min. (Dilute 1μl of Cy2 tyramide in 50μl reaction buffer with kit; each intestine needs about 150μl). Stagger reactions by a couple minutes.

37. Rinse 3x 10min in TNT.

38. Add PBT and DAPI for 10 min.

39. Use a cut p1000 pipette tip to move intestines into glass dish. Add in PBS 50% glycerol for 30min to ON.

40. Put in 55μl of mounting medium on slide without adding cover slip. Leave for at least 2hr.

41. Place cover slip on and seal.

EMS Mutagenesis

Material

Males FRT arm to mutagenize

EMS (Sigma M0880-1G)

1% sucrose-0.1M Tris pH 7.5 solution filter-sterilized (0.5 g sucrose + 5ml Tris pH=7.5 in 50ml dH_20)

4 tubes for mutagenesis (approximately 50mm diameter and 10cm high)

Whatman filters for EMS solution (4X 20 filters)

2L of 5% Sodium thiosulfate solution (STS) to inactivate EMS

Waste containers, gloves, syringes (10ml), needles (21G)

Collect Males

1. Empty bottles of isogenized FRT stock (FRT40A) Friday night and collect 4 X 60 males on Monday morning so males will be age 3-5 days on Thursday. Place in tubes at 18°C.

2. Thursday morning transfer males to empty tubes at 25°C for at least 6 hours to "dry out"

Collect Females

1. Monday to Friday collect virgin females (4 X 100) of stock to mate with mutagenized males (yw hsflp tubGAL4 UAS-GFPnls; if/Cyo)

Preparation for Mutagenesis

1. Starve males at 25°C in empty tubes at least 6 hours

2. Place 20 Whatman filters in each tube for mutagenesis- may have to trim filters, should fit nicely in bottom without too much of a gap.

3. Prepare a 1% sucrose solution, 0,5g sucrose in 50ml dH_2O, filtered. Aliquot 5 ml in

each of 4x 50ml falcon tubes.

4. Make 2,5L of 5% sodium thiosulfate in large beaker 3L with plenty of room on top- put 300ml into a squirt bottle for decontamination if needed.

5. Prepare the hood for mutagenesis. Clean, change paper, place a yellow bag into yellow box for solid waste. Place clear plastic box with EMS supplies in hood: a smaller box that contains EMS, syringes, needles, pipette for EMS, pipette tips.

EMS Mutagenesis

Use 2 pairs of glove, a lab coat, and eye protection. Work under fume hood. Place note on fume hood that EMS is being used.

1. Place 4 X 5ml of 1% sucrose-Tris solution in 50 ml falcon into styrofoam rack, 1 falcon for each pot of flies to be mutagenized.

2. Add correct amount of EMS to the first tube with pipetteman for EMS. Do not add EMS to all falcon tubes at once, do pot by pot.

EMS Sigma M 0080 density 1,17g/ml. FW 124.2 g/mol, therefore:
35 mM = 18.58μl
30 mM = 15.92μl
25 mM = 13.26μl
20 mM = 10.61μl

3. Mix EMS and sucrose solution with 10ml syringe taking care to suck up all solution. Squirt vigorously at least 3 times.

4. Add liquid to 1 pot on filters. Filters should be thoroughly wet without excess liquid on sides.

5. Add males to pot with funnel and screw cap on.

6. Suck up 5% STS into syringe and place in beaker with STS to inactivate ON. Place tips in STS.

7. Repeat for 3 other pots.

8. Place gloves, paper, etc. in solid waste container.

9. Leave males in EMS pots ON (around 16h).

The following morning:
10. Transfer males to normal fly tubes for 2 hours. Allow them to clean themselves and expel EMS from digestive tract.

11. Transfer males again to fresh tubes. Place other tubes in EMS solid waste.

12. Mate treated males to virgin females. 9 males X 15 virgin females in 26 tubes.

13. Transfer crosses to new tubes every day for 4 days, dispose of adults on day 5.

Screen Protocol

The fly stocks indicated in the protocol are represented in Fig. 14.

1. Isogenize FRT 40 Males (Males P): generate a FRT 40A stalk from a single 2nd chromosome to prevent that random mutations spontaneously occurring in the stock might be selected in the screen.

2. At beginning of week 1, collect virgins with both second chromosomes marked (Virgins P). Collect up to 4 x 100 virgins. At the same time, collect 4 x 60 males of the isogenized FRT 40A (Males P).

3. On Thursday, treat the Males (Males P) with EMS (see **EMS Mutagenesis** protocol).

4. On Friday, cross EMS treated Males (Males P) in batches of 9 males to 15-20 virgins (Virgins P) in a total of 26 crosses. Flip crosses every day during 4 days and through away adults on the 5th day. Keep crosses at 25˚C. **Note:** the first round of EMS was termed EMS1 (all the rounds were numbered).

5. F1: One generation later, at the beginning of week 3, pick virgins of the MARCM stock for 40A (Virgins F1). Virgins are picked 3 times a day; put 4 virgins per tube. Continue to pick virgins during the whole 3rd week (also, starting to pick virgins during the weekend before might be helpful). **Note:** the MARCM 40A stock must be flipped every 3 days during the whole screen, as it must be kept thoroughly amplified. The generation time of these flies is of approximately 12-13 days and the stock always yields less adults that the number of pupae would indicate. Finally, it does not appreciate being put at 18˚C. Typically, we would flip 20-40 tubes every 2/3 days. For a set of single crosses, 4x700 virgins are needed. Each tube would yield

approximately 50 virgins and this flipping scheme allows for approximately 60 tubes to be yielding virgins during the week and for there to be enough adults coming out at the end of the week to start the following generation for the subsequent EMS round.

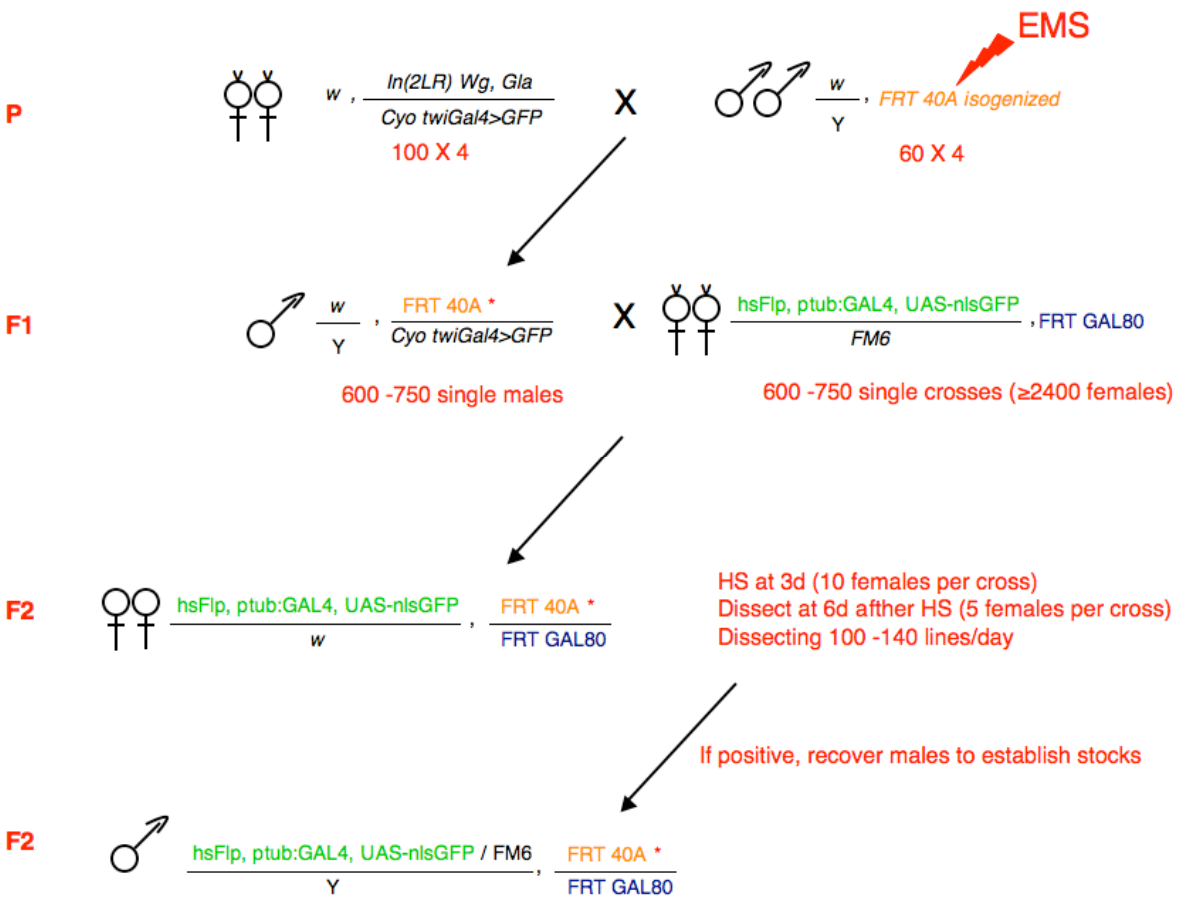


Fig.14: Outline of the crossing scheme for the genetic screen.

6. On Wednesday of the 3rd Week, start the single crosses: pick single males (Males F1) that are FRT 40A mutagenized over *CyO* balancer and add each male to a tube that contains 4 virgins (Virgins F1). Start 100-150 single crosses per day, depending on the virgin yield. Start single crosses from Wednesday to Saturday/Sunday, again depending on when the virgins come out. Ideally, 700 crosses should be started until Sunday. Put the Crosses at 25˚C.

7. During Week 4 the crosses will develop. At the end of week 4, when most of the larvae have pupated, add dry yeast to the tubes. This yeast will be available for the females to eat when they eclose (adding it too early, would dry out the medium and would kill the larvae).

F2: On Tuesday of the Week 5, start sorting the females that will be screened (Females F2). Select 10-15 females of the appropriate genotype to generate MARCM clones that are not virgins (should be 3 days old). Put females in tube without yeast and add dry yeast to tubes. At this point label both the original cross tube and the tube with the females (Females F2) with the same number. Add also a 2-3 males (Males F2) to the tubes (they will both fertilize the females, as egg laying might drive food uptake, and possibly be used to establish a stock in case the tube has clones with a phenotype). Put original crosses at 18˚C. **Note:** tubes where labeled with the number of the EMS round, followed by a letter for the day of the week (A-E corresponds to Tuesday-Sunday) followed by the number of that tube. Tubes sorted on Tuesday of week 5 would be numbered 1-A-1 to 1-A-100, for example.

8. Put tubes with sorted females for heat shock (Females F2) directly in plastic racks for heat shock. A large water bath could carry 2 and half plastic racks full of

tubes (50 tubes). As soon as 50 tubes have been sorted, start the heat shock for those 50 tubes. Heat shock 45' at 36,5˚C. After heat shock, put another plastic rack on top of the tubes and invert the tubes and tap them slightly to release any adults that might have been stuck in the yeast. Tape the two plastic racks together and put the tubes, inverted, at 25˚C. The following morning, repeat the heat shock for 45' at 36,5˚C (the two heat shocks are approximately 18h apart). After the second heat shock, remove tubes and put them, inverted in a large rack. Re-invert them a few hours later. Proceed in the exact same way for the next set of 50 tubes. Keep heat shocked flies at 25˚C.

9. On Monday of the 6th week, start screening. Prepare a fresh batch of PBS plus DAPI (1 µg/ml). Dissect 5 heat shocked females per tube in PBS + DAPI (we have observed that in intestines dissected in PBS with DAPI, the large polyploid ECs will uptake DAPI, whish serves as a good indicative of the histological state of the dissected intestine) and mount these intestines on a slide with drop of PBS + DAPI and cover with a cover slip. Keep some of the ovaries attached to intestines, as these will both serve as a control for the generation of clones and will prevent the intestines from being squashed by the cover slip. After dissecting, quickly, females from up to 4 tubes, analyze the intestines under the fluorescent microscope.

10. Analysis of the clones: all intestines of each cross where scored and the intestine was scanned along its anterior-posterior axis but bigger attention was paid to the posterior midgut. First it was observed whether the intestines had scorable clones (clones with more that 3 cell, ideally at least 5 cells per clone). We observed that around 20% of the intestines don't have scorable clones, with this number remaining constant throughout the whole screen. If the intestines have at least one scorable clone, they were considered. Then, it was determined whether the clones where wild-

type in composition, by counting the ratio of small to large cells. If clones had an increase in proportion of small cells, it was noted, with these lines being marked for re-screen. Although the screen was designed to isolate mutants that altered the proliferation of small cells, if a line had a striking, strong and consistent EC defect that affected only the GFP positive ECs but not the surrounding wild-type ECs, it was also kept for re-screen. The intestines where screened under a 20x non-immersion objective and if clones appeared to be potentially mutant, they would be further observed with a 40x or 63x immersion objective.

11. For the lines with potential proliferation phenotype, the remaining females (if any) are recovered for re-test at a later time point and males (Males F2) are recovered from the original tube at 18˚C to establish a stock of the second chromosome.

Note: The screening protocol described above is for a complete round, from mutagenesis to the screen. The role protocol takes 6 weeks to complete and is represented in Fig. 15. Fly pushing is done every other week (week 1, week 3 and week 5 for EMS 1). All the following EMS treatment where carried out every other week, on odd numbered weeks (EMS 2 on week 3, EMS 3 on week 5, etc.). Week 5 on the schedule represents a typical fly pushing week, with one EMS treatment and the fly pushing for the F1 and F2 generations of the precedent EMS treatments. Even numbered weeks are screening weeks (screening for EMS 1 on week 6, screening for EMS 2 on week 8, etc). Therefore, once started, the screen went on uninterrupted until the last EMS treatment (EMS 11).

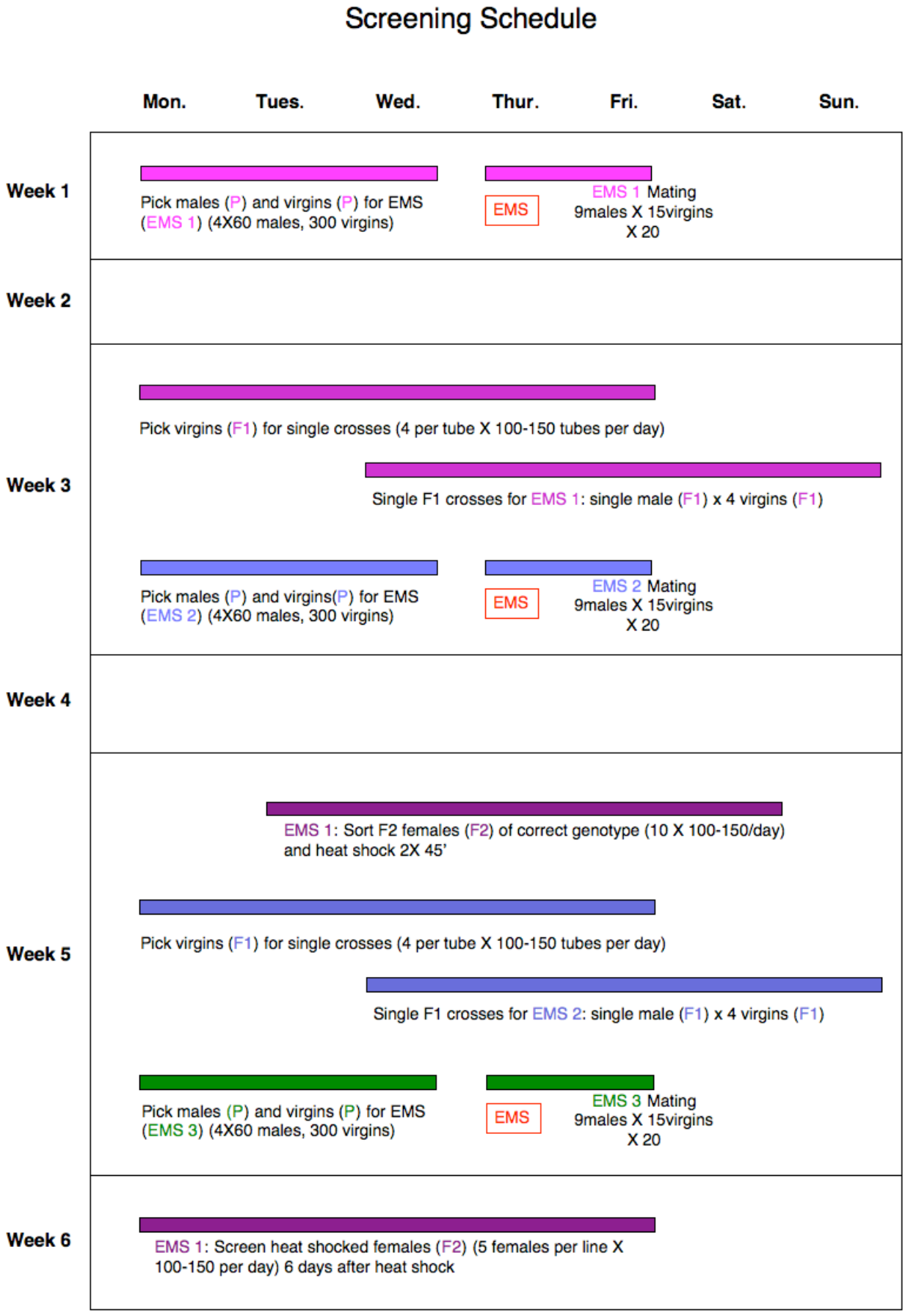


Fig.15: Outline of screen schedule for the first 6 weeks of screening.

Chapter III

A Genetic Screen for Cell Fate Specification Defects in the adult Intestinal Stem Cell Lineage

I. Introduction of the Genetic Screen for Cell Fate Specification Defects in the adult Intestinal Stem Cell Lineage

One of the strengths of *Drosophila* as a model organism is the ability to utilize it for forward genetics screens to identify genes involved in a process of interest. Notably, the Nobel-Prize-winning screen carried out by Christiane Nüsslein-Volhard and Eric Wieschaus for mutations that affect the patterning of the fly embryo identified most of the genes responsible for producing positional information in the embryo (Nusslein-Volhard and Wieschaus, 1980).

The Intestinal Stem Cell (ISC) of the adult fly midgut is a recently identified stem cell model (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) and many of the key regulators of the ISC and its lineage are unknown, such as what maintains stem cell identity and promotes stem cell proliferation, how is the adoption of different fates regulated and does the surrounding differentiated cells as well as the other tissues regulate the stem cell? Notch signaling activity is known to play a role in the ISC lineage: loss of Notch signaling results in overabundance of small stem cell-like cells, which express the Notch ligand Delta, as well as an overabundance of small cells that express the enteroendocrine fate marker Prospero (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). However, how Notch signaling is regulated in the gut is not entirely understood. Furthermore, it is also unknown which other pathways are involved in the regulation of the ISC lineage

and what role could they be playing. A genetic screen is an ideal unbiased approach to try to identify such regulators of the ISCs.

A. Technique for Generating Mosaic Clones in the Gut

Most genes involved in cell-cell communication or regulation of cell physiology in adult tissues also play key roles during embryonic development and are often lethal when removed in the embryo. One way to overcome the embryonic lethality and study the role of these genes in later processes is to study their loss of function specifically in adult tissues. We generate such loss of function mutant clones specifically in the adult intestine through the Mosaic Analysis with a Repressible Cell Marker or MARCM technique (Lee and Luo, 2001). Mosaic analysis consists of the generation of homozygous mutant cells in an heterozygous background by mitotic recombination in the heterozygous precursors. It is based on the Flp/FRT system, which has been adapted from yeast. The yeast site-specific recombinase (Flp) is expressed under control of a heat shock promoter, and can promote mitotic exchange between homologous chromosomes that contain FLP Recombination Target (FRT) sequences [Fig.16, (Chou and Perrimon, 1996; Xu and Rubin, 1993)]. Therefore, one can analyze the loss of one gene in homozygous mutant cells in an otherwise heterozygous, phenotypically wild-type organism. The MARCM technique combines the Flp/FRT system for generating mosaics with the GAL4-UAS system for directed gene expression, also adapted from yeast (Brand and Perrimon, 1993). The yeast transcription activator Gal4 is expressed under the control of any chosen promoter and will bind specifically to the upstream activating sequence (UAS), inducing the expression of a desired gene, as well as the expression of green fluorescent protein (GFP) which is used as a marker [Fig.16, (Brand and Perrimon, 1993)]. Finally, the yeast Gal80 protein is an inhibitor of Gal4 (Fig.16). The MARCM technique combines these genetic tools in the following way: Gal4 and Gal80 are both expressed under the ubiquitous tubulin (tub) promoter and are therefore expressed in all cells of the organism. Gal4 is present in all the cells but does not

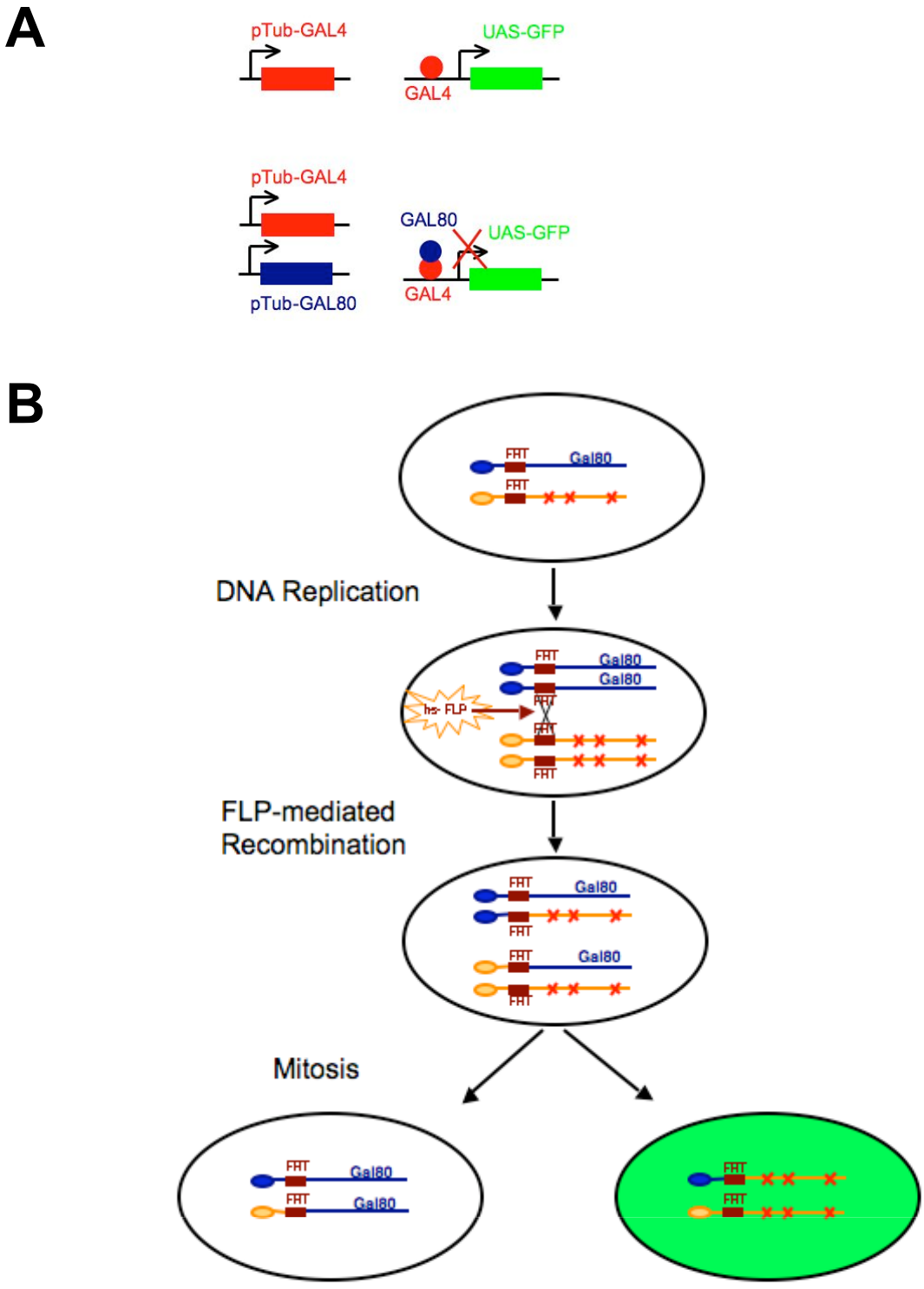


Fig. 16: **A** The UAS-GAL4 technique for targeted gene expression, combined with Gal80 **B** The MARCM technique for generating positively marked GFP clones.

activate expression of its targets through binding to the UAS upstream of the nuclear GFP (nlsGFP) since Gal80 binds and inhibits Gal4 in all cells (therefore, the organism is GFP negative). A tub-Gal80 construct is inserted on a wild-type chromosome arm that contains an FRT sequence. The other chromosome arm also contains the same FRT sequence and can be either wild-type or contain a mutation for a gene(s) of interest. If the FLP recombinase is expressed, by heat shock induced expression, in mitotic cells it can promote recombination between of the FLP sequences and generate mitotic exchange between the homologous chromosomes. After mitosis, one daughter cell will contain two chromosome arms that contain the tub-Gal80. The other daughter cell will be homozygous for the chromosome arm that contains the mutated gene and has concomitantly lost the tub-Gal80. This cell will be homozygous for the mutation in the gene of interest (or wild-type) and will be GFP positive since it no longer has Gal80 repressing Gal4 mediated expression of GFP [hence the name Mosaic Analysis with a Cell Repressible Marker; Fig.16.B., (Lee and Luo, 2001)].

Fig.17 shows a wild-type, GFP positive clone in an unmarked wild-type intestine as well as a GFP positive clone mutant for loss of Notch signaling in unmarked wild-type tissue. As mentioned above, the loss of Notch signaling results in overabundance of small stem cell-like cells as well as an overabundance of small cells that express Prospero (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). The difference in the composition of the clone is striking, with the overabundance of small cells in the loss of Notch signaling clones being very obvious. This is one of the phenotypes potential phenotypes affecting proliferation of the stem cell that we can screen for. The MARCM technique has the added advantage that potentially mutant clones can be scored for their phenotype with-out fixing the tissue, since GFP can be observed with a epifluorescent microscope, on unfixed tissue (Fig.17).

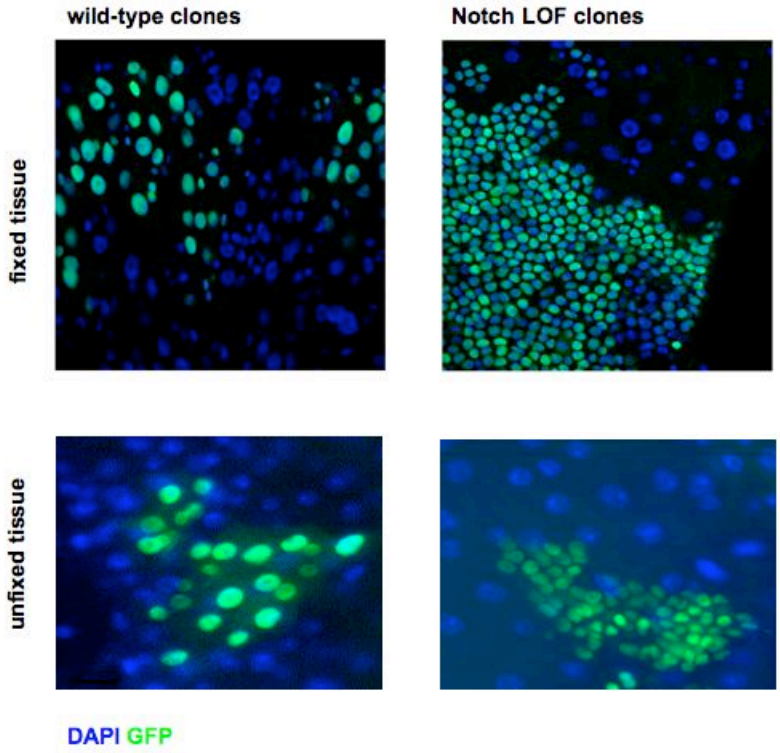


Fig. 17: Wild-type and loss of Notch signaling positively marked clones in both fixed and unfixed tissue.

B. Screen Outline

Genetic screens are a powerful way to identify the genes that are required for a biological event. By introducing random mutations in the genome, we can screen for genes that, when carrying mutations, perturb the biological process of interest. This is an un-biased way to identify genes that contribute for the process of interest.

We decided to use ethyl methane sulphonate (EMS) as a mutagen. EMS is a alkylating agent that induces mostly point mutations, which can disrupt the gene by introducing missense or nonsense mutations, but may also produce small deletions and other rearrangements (Greenspan, 2004; St Johnston, 2002). EMS introduces dose-dependent point mutations: for example, a dose of 25mM of EMS will induce on average, one lethal per chromosome arm or one hit per 1000 chromosome arms per locus. However, the rate at which each individual gene can be mutated depends on the size of its coding regions as well as the number of critical amino acids that it has. Although EMS will induce random mutations in all the chromosomes of a given cell, when using the Flp/FRT system, only the chromosome arm that contains the FRT is analyzed in the homozygous mutant cells produced by recombination. This has the advantage of allowing to screen for lethal mutations that could kill the homozygous individuals but the disadvantage that only one chromosome arm can be screened at a time. For this screen we decided to screen chromosome arm 2L because all the reagents for this chromosome arm were immediately available as a gift from the lab of Jean-René Huynh.

Since mutant clones generated in the fly intestine are not easily observed through the adult cuticle, it is necessary to dissect the flies in order to score the clones regarding their cell composition. Therefore, we designed an F2 screen, in which each individual mutagenized male that has a unique set of random mutations and is crossed to a stock that allows the generation of MARCM clones, allowing us to dissect some of the progeny of that cross for flies with mutant clones in their

intestines and to recover other flies with the same chromosome arm to establish a stock in case of a positive result.

The outline of the screen is the following (Fig.18):

• Males of the parental generation (P) that have the FRT inserted at the 40A chromosomal region of an isogenized chromosome arm 2L are fed EMS (see Material and Methods for protocol). The mutagen will induce random mutations in the entire genome of all cells, including the mature sperm and the gonial stem cells.

• EMS treated males (P) are crossed to virgin females (P) that have dominant markers on each chromosome 2 (*Wg,Gla* give a characteristic small eye and *CyO* yields characteristically curled up wings) that allows us to follow the 2nd chromosome during the crosses. The males are crossed in batch of 9 to 15-20 virgin females to allow each male to mate at least once with a virgin female.

• One generation later, F1, single males are picked. These males have one chromosome 2 that was mutagenized over a *CyO* chromosome. Each male has a unique set of random mutations.

• Each single male (F1) is crossed with 4 virgins (F1) of the stock that contains all the constructs to generate the MARCM clones on 2L (MARCM stock): on the X chromosome they have the heat shock inducible Flp recombinase, the tubulin driven Gal4 activator and the UAS-nlsGFP and on the 2nd, the FRT at the 40A location and tubulin driven Gal80 construct distal to the FRT.

• One generation later, F2, 3-5 day old females of the appropriate genotype to generate MARCM clones are selected as well as 5 males (F2) that can be used to recover the mutated chromosome 2L that are kept in the same tube. 10-15 females per cross are heat shocked (HS) to induce Flp mediated recombination in mitotic cells.

• 6 days after HS, 5 females (F2) per cross are dissected in PBS+DAPI and the clones are analyzed in unfixed, DAPI-stained tissue under a fluorescent microscope. If the clones do not appear wild-type, the remaining females are kept to be retested at a later time-point and males (F2) are used to recover the mutated chromosome.

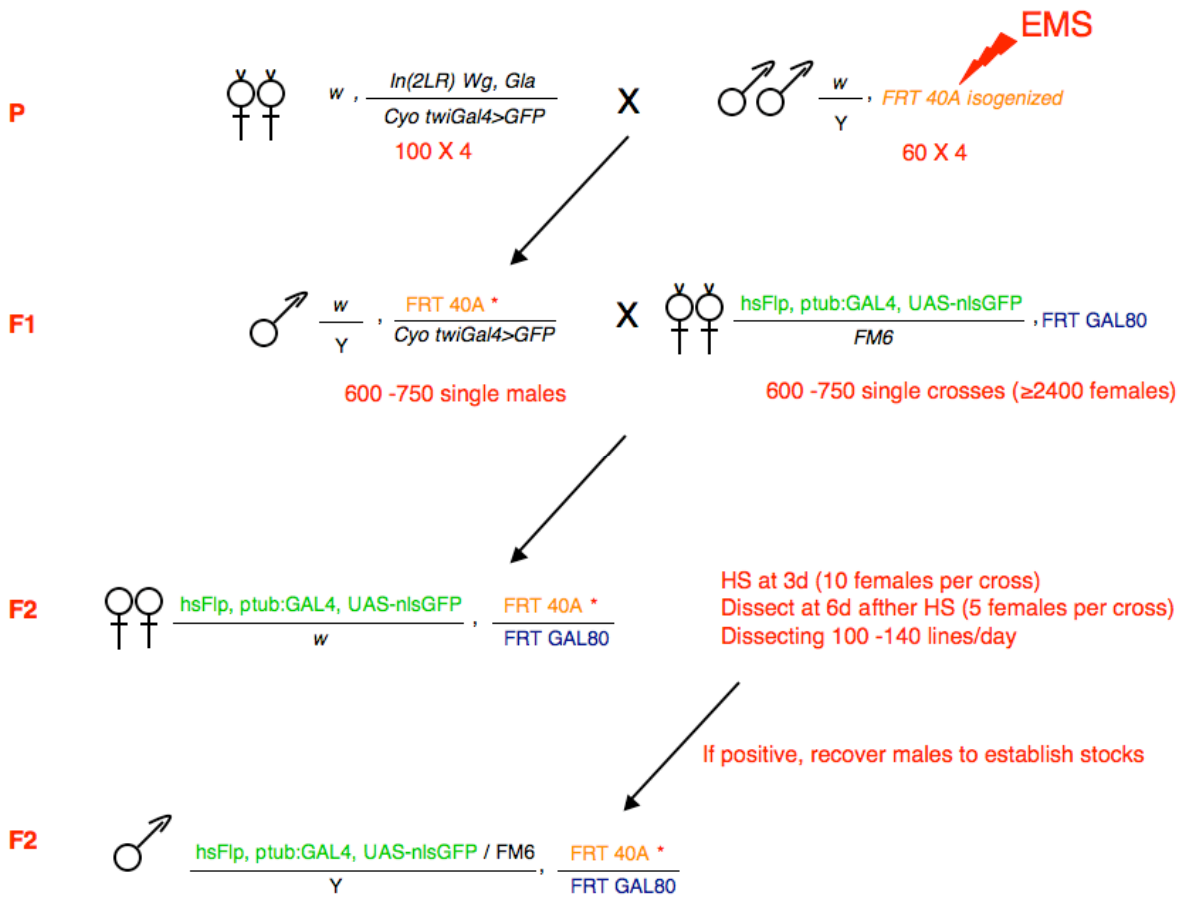


Fig.18 : Outline of the crossing scheme for the genetic screen.

C. Screening Schedule

As mentioned before, we screened F2 generation flies. This implies that three generations of flies must be dealt with. From the collection of the first virgins until the screening of the mutant, six weeks are spanned (Fig.19). In the shown screening schedule, the time for each step is depicted (Fig.19, each generation represented corresponds to the genotypes represented in the crossing scheme in Fig.18). One complete round of EMS treatment (EMS1) is represented in magenta (darker shades for successive generations). Generally, for one full round of EMS, flies are picked and crossed on odd weeks (week1, week3 and week5), with the generation time of 10 days spanning the even week. At the same time, the adults are screened on even weeks (the first round of screening occurred on week 6, the schedule continued so that EMS2 was screened on week 8, EMS3 on week 10 etc.) Week 5 on the schedule represents a typical ‘fly pushing’ week: one round of EMS starts, and the F1 and F2 flies of the previous EMS rounds are picked, crossed, sorted and heat shocked (for more information on the screen, see Material and Methods).

Fig. 19: Outline of screen schedule for the first 6 weeks of screening.

II. The Screen

A. Primary screen:

The primary screen consisted of 11 rounds of EMS treatment, during which a total of 5310 lines where screened. However, not all of these lines had clones that could be scored. Consequently, we screened a total of **4238** of lines with scorable clones, which corresponds to 79,8% of the screened lines. Indeed, throughout the EMS screen, approximately 20% of the lines screened consistently did not have scorable clones, which could be due to the mutagenesis introducing mutations that are incompatible with cell viability, to the nutritional and physiological state of the flies or to the limit of the technique for generating MARCM clones in such high throughput conditions. These different causes are not mutually exclusive and could all contribute to reduce the efficiency of the clone induction.

Of these 4238, **89** had a mild to strong phenotype on proliferation and were kept for re-screening and secondary screen, which corresponds to 2.1% of the lines scored.

B. Testing the Percentage of Lethals on Chromosome II

The males that were mutagenized were fed 30mM of EMS, which is predicted to induce, on average, at least one lethal mutation per chromosome. To test the

percentage of lethals on the 2nd chromosome, we back-crossed 130 males (F2 from Fig.18), that have one mutagenized FRT 40A chromosome arm and one FRT 40A tub-Gal80 chromosome, to virgins of a stock with a second chromosome *CyO* balancer and established about 100 stocks FRT 40A mutagenized balanced with the *CyO* chromosome. Of these, we scored how many contained FRT 40A homozygous flies after one generation by scoring non-*CyO* flies. These correspond to lines in which the 2nd chromosome carries no lethal mutations. We observed that 64,7% of the stocks carried lethal mutations on chromosome 2, which is within the expected rate for this dosage of EMS treatment (Greenspan, 2004).

C. Re-testing selected lines

Of the 89 lines kept after the primary screen we were able to establish stock for 88 of these lines (for the one line that we were not able to recover, there were not males left in the original tube). These 88 lines were crossed to the stock to generate MARCM clones and these clones were re-screened, without fixing them, at two time points: 1 week after heat shock and 2 weeks after heat shock. Of the 88 lines, 18 had wild type looking clones at both time points and were not kept. The others where put into four categories (see table 1):

- **Type I - 'Notch LOF-like'**: clones composed of only small cells (or mostly small cells and very rare large ECs) that resemble the loss of function (LOF) of components of the Notch pathway. 16 lines had this phenotype.
- **Type II – 'Overabundance of small cells'**: clones that have a strong overabundance of small cells but in which there are still large ECs present. 15 lines had this phenotype.
- **Type III - 'Mild overabundance of small cells'**: clones that have a mild increase in small cells. 50 lines had this phenotype.

Type IV – 'EC phenotype': some lines had a very strong EC phenotype, which consisted of many single ECs, with normal or altered morphology. 7 lines had this phenotype.

Type I: 'Notch Loss of function'-like						
Mutant	**Phenotype**	**Secondary**	**Phenotype Strength**	**Complementation**	**Mapping**	**obs**
2-E-57	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
2-E-84	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
3-E-7	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
4-A-41	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
5-E-95	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
6-A-103	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
6-B-97	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
6-E-89	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
7-E-42	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
8-A-64	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
8-C-41	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
8-C-66	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
9-B-21	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
9-B-87	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
10-D-117	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
11-F-25	"Notch LOF"	Only small cell - Dl and Pros cells	+++	Kuzbanian	Kuzbanian	
Type II: Overabundance of small cells						
Mutant	**Phenotype**	**Secondary**	**Phenotype Strength**	**Complementation**	**Mapping**	**obs**
6-A-100	Extra Sm Cells	Extra Delta cells	++		ongoing	rare homozygous flies with Macrochaete defects
8-A-9	Extra Sm Cells	Extra Delta cells	+++	Fails compl. 11-C-87	Gmd	
8-A-43	Extra Sm Cells	Extra Delta cells	++			possible phenotype on Prospero cells
8-D-18	Extra Sm Cells	Extra Delta cells	++			
9-C-95	Extra Sm Cells	Extra Dl cells,Delta upregulated	+++	Fails compl. 9-C-95	Spen	Delta very strongly upregulated
9-E-34	Extra Sm Cells	Extra Dl cells,Delta upregulated	+++	Fails compl. 9-E-34	Spen	Delta very strongly upregulated
10-B-8	Extra Sm Cells	Extra Delta cells	++			homozygous non lethal
10-B-46	Extra Sm Cells	Extra Delta cells	++			
10-D-26	Extra Sm Cells	Extra Delta cells	+++		Kismet	
10-D-32	Extra Sm Cells	inconclusive, re-do				homozygous non lethal
10-E-24	Extra Sm Cells	Extra Delta cells	+++	Fails to compl. 6-B-16 7-E-15 & 8-D-70	ongoing	
11-B-94	Extra Sm Cells	Extra Delta cells	+++			
11-C-87	Extra Sm Cells	Extra Delta cells	+++	Fails compl. 8-A-9	Gmd	
11-C-169	Extra Sm Cells	Extra Delta & Prospero cells	+++		ongoing	increase in prospero cells
11-D-59	Extra Sm Cells	Extra Delta cells	+			
Type IV: EC phenotype						
Mutant	**Phenotype**	**Secondary**	**Phenotype Strength**	**Complementation**	**Mapping**	**obs**
2-A-43	EC phenotype	only ECs, ECs with abnormal morphology	+++		ial	ECs express Delta
3-B-10	EC phenotype	ECs with abnormal morphology	+			
5-C-53	EC phenotype	only ECs	+++	Fails compl. 8-C-97	ongoing	
7-A-113	EC phenotype	ECs with abnormal morphology	++			
7-B-75	EC phenotype	ECs with abnormal morphology	+			
8-C-97	EC phenotype	only ECs	+++	Fails compl. 5-C-53	ongoing	
9-B-20	EC phenotype	only ECs,ECs with	++			

Table 1. Summary of the mutants isolated in the screen with strong phenotypes.

Mutants lines that were considered to have a mild increase in small cells (type III) do not have a very strong phenotype but where kept since they could be weaker alleles of other mutants, as complementation groups with at least two alleles of a gene are more easily mapped (see below). During the screen, in 5 different lines the same event was observed once and only once: one of the screened intestines would have wild-type clones or clones with more small cells and one only clone that had a very strong overabundance of small cells and no large cells (a 'Notch LOF-like' clone). For each of these lines this single mutant clone was only observed only once, even after re-screening, which suggests that these were probably spontaneous mutations, maybe on other chromosomes, related to EMS-induced mosaicism. All of these lines, with the exception of 6-A-100 (see below), did not have a strong phenotype during re-screening but were kept.

D. The Secondary Screen

The lines with the strongest phenotypes (type I, II and IV mutants) were put through a secondary screen. For the secondary screen, we dissected flies at 1 week or 2 weeks after heat shock (depending on which time point gave a stronger phenotype during the re-screen). Intestines were fixed and then stained with anti-Delta antibody (marks the ISCs) and anti-Prospero (marks the enteroendocrine cells). Both proteins are expressed in small cells and were used to see which population of small cells are affected in the type I and type II mutants, compared to wild-type clones (Fig.20).

All the mutants of the **type I** group (phenotype like the loss of Notch signaling) where found to have an overabundance of Delta+ cells as well as an overabundance of Pros+ cells, which corresponds to the phenotype observed in the loss of Notch signaling (Fig.21 has one example of these mutants;).

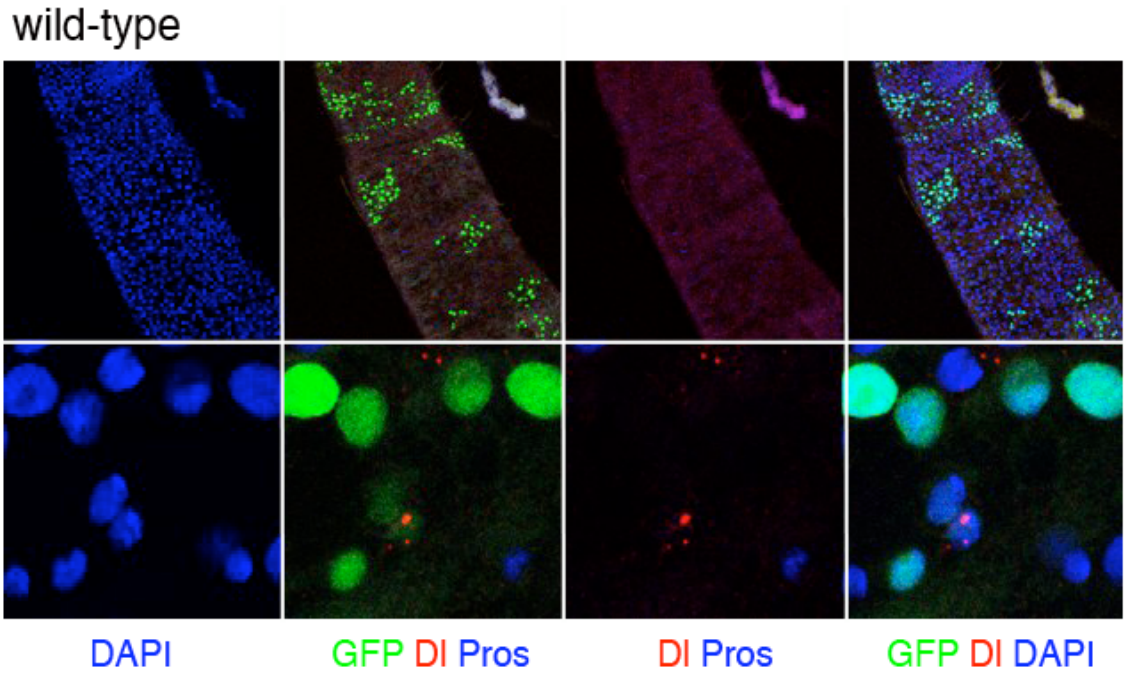


Fig.20: Wild-type clones – control for the secondary screen.

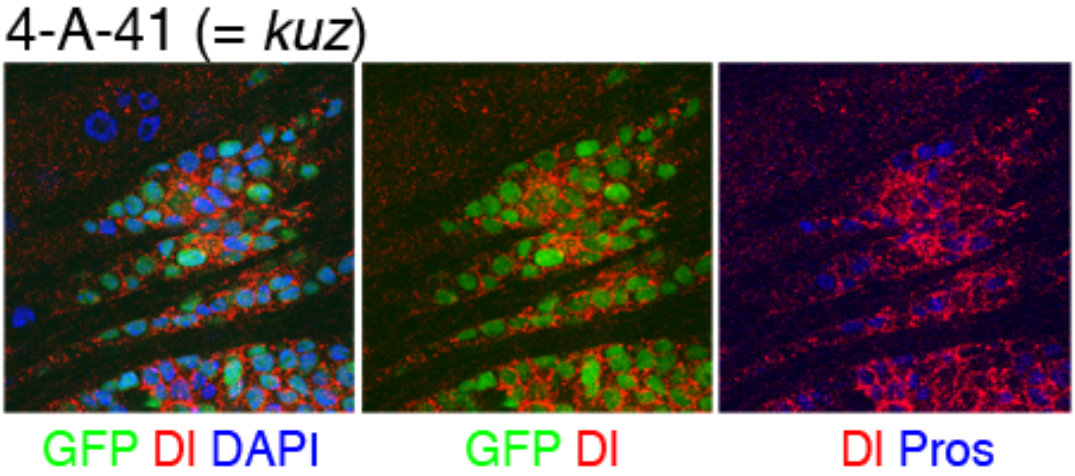


Fig.21: 4-A-41 (*kuz)* mutant clones.

Most of the mutants of **type II** where found to have an overabundance of Delta+ cells, although the phenotype was stronger in some lines than others. In Fig. 22 there is an example of a candidate, 11-D-59, which has a mild increase in the number of Delta+ cells while in Fig. 23 there is an example of a mutant in which there is a strong increase in the number of Delta+ cells, which will be better described below. Only one line, 11-C-169 (Fig. 24) had an increase in the number of Delta+ cells as well as overabundance of Pros+ cells. Therefore, in the type II mutants we identified two subtypes, one in which there is an overabundance of Delta+ cells and another in which there is an overabundance of Delta+ and Pros+ cells.

In mutants of **type IV**, the phenotypes observed varied between lines, with some lines displaying only ECs of normal morphology, while in others the EC morphology is altered, either in isolated ECs or in clones. A better description of some of these lines follows in the paragraphs below. The results are summarized in table 1

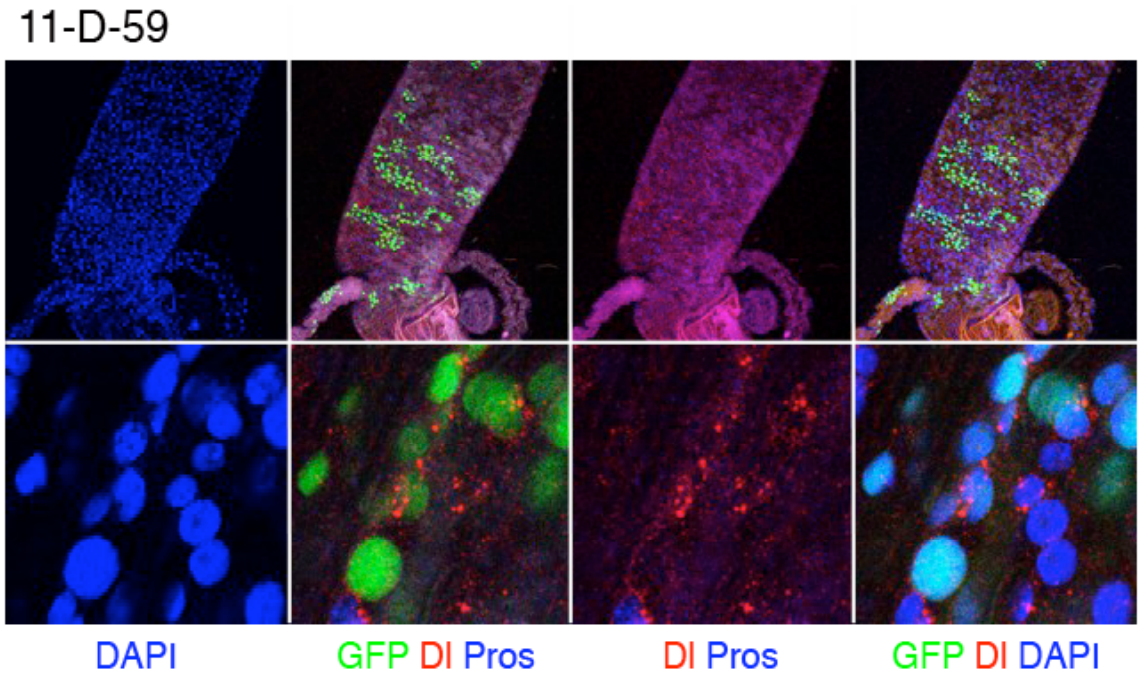


Fig 22. 11-D-59 mutant clones.

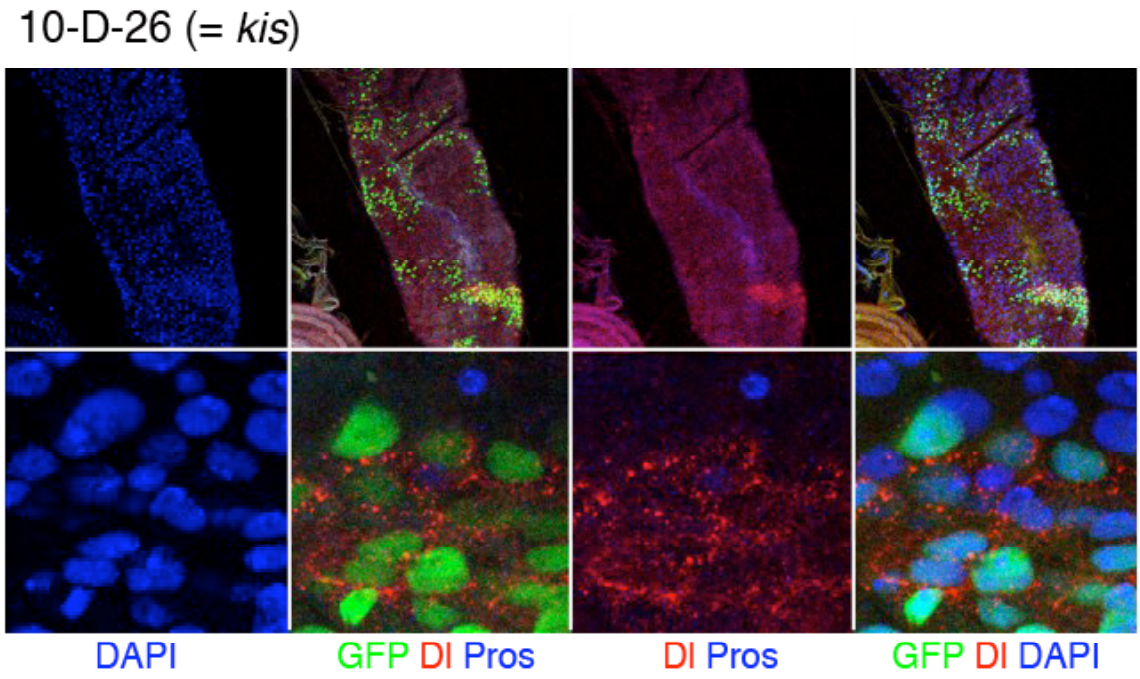


Fig.23: 10-D-26 mutant clones.

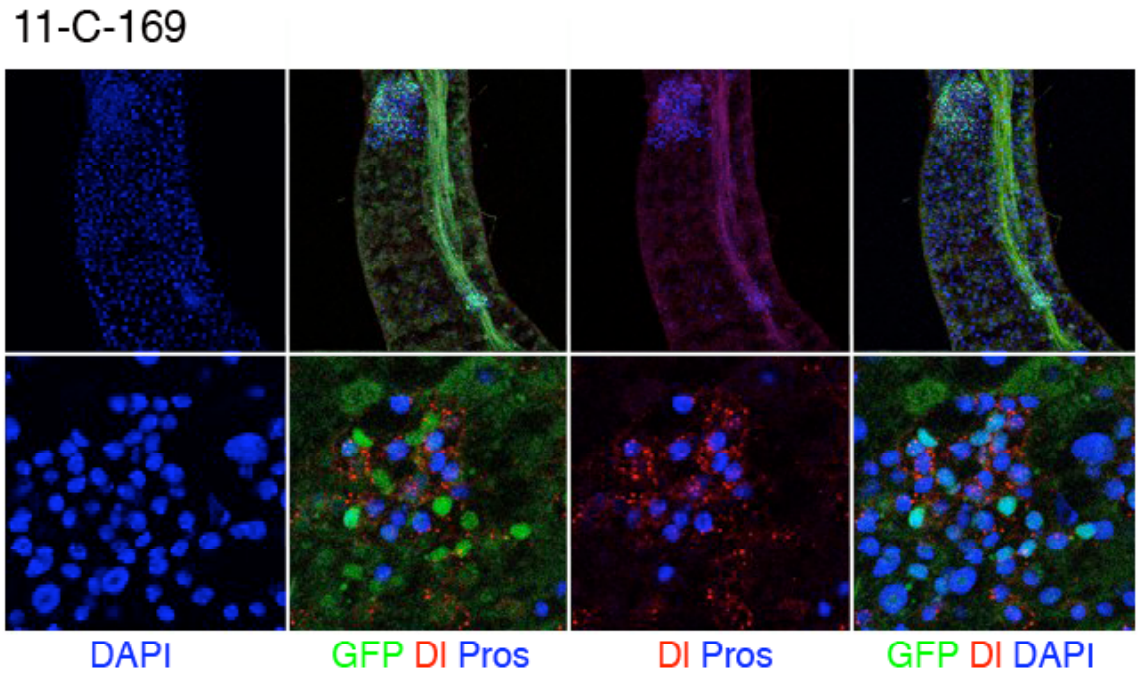


Fig.24: 11-C-169 mutant clones.

E. Complementation analysis: principle

The objective of the complementation test is to identify whether two fly lines containing lethal mutations both have a lethal mutation in the same loci. To test this, two lines containing lethal heterozygous mutation are crossed with each other and the outcome of the cross is analyzed. In order to maintain a lethal mutation in a stock, the chromosome that carries the lethal mutation is kept heterozygous with a balancer chromosome. A balancer chromosome is a genetically engineered chromosome that has 3 main characteristics: it is lethal and therefore cannot be homozygous in the stock, it cannot recombine with its chromosome since it has multiple chromosomal inversions and it has visual markers that allow it to be identified in the crosses and the stock. The two lethal mutations are kept with balancer chromosomes and are crossed to each other (Fig. 25). In the next generation each lethal mutation is recovered with the balancer chromosome. If the two chromosomes tested have the same lethal locus, then no flies containing both chromosomes are recovered, since these flies are transheterozygous for the same lethal mutation. In this case, these two fly lines containing a lethal mutation fail to complement each other (Fig. 25.A).

However, if the two lethal mutations are in different loci, then flies carrying the two chromosomes being tested are recovered (transheterozygous flies) and these two lines complement each other (see Fig.25.B). The complementation test allows us to identify whether mutations in known genes were recovered from the screen and is also utilized to identify complementation groups of mutants, that is, mutants that have the same lethal loci. This allows us to correlate the observed phenotype for one gene with its lethality, which is particularly important for mapping the mutations by lethality (more on mapping below).

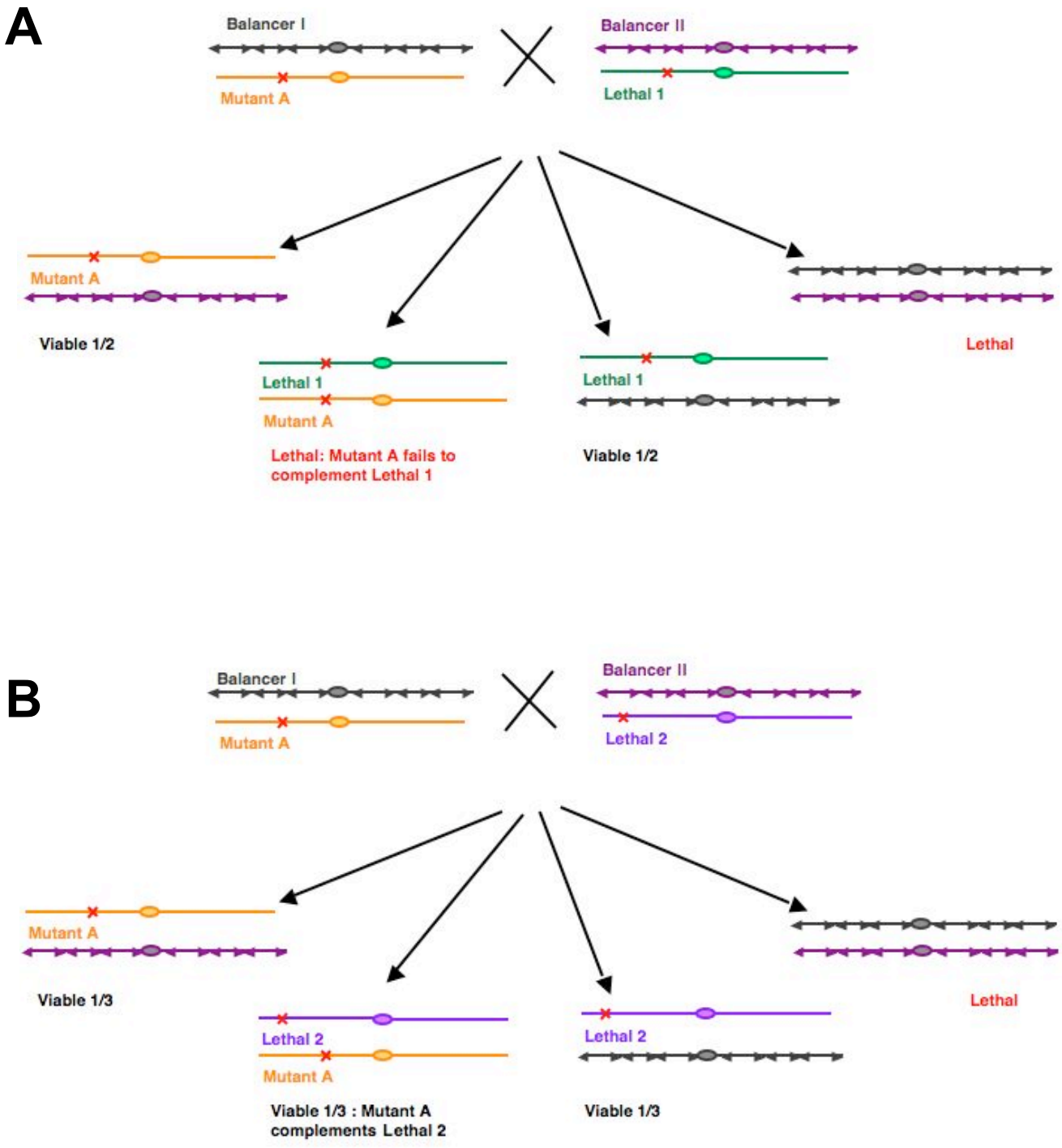


Fig.25: Scheme of complementation crosses: **A** a complementation cross between two lethal loci that fail to complement. **B** a complementation cross between two lethal loci that complement each other.

F. Complementation analysis to known components of the Notch Pathway on 2L

We first tested whether the mutants that result in an overabundance of small, stem cell-like cells (type I and II) could be mutations in known components of the Notch signaling pathway on chromosome 2L. We started by crossing all the lines of type I and type II mutants to flies carrying mutations in the two known components of the canonical Notch pathway on 2L, *Suppressor of Hairless* (*Su(H)*$^{\Delta 47}$) and *kuzbanian* (*kuz*^{ES24}). We observed that while none of the lines from the screen failed to complement *Su(H)*$^{\Delta 47}$, which indicates that we did not isolate any new alleles of *Su(H)*, all of the lines that were identified as having type I phenotype failed to complement *kuz*^{ES24}, which means that we have isolated 16 alleles *kuz*, all of which had the expected loss of Notch signaling phenotype. Therefore, we were indeed able to isolate, during the screen, mutations in genes predicted to give a strong phenotype. However, it is surprising that we did not isolate any mutations in *Su(H).* This could be explained by the fact that the screen was not carried out until saturation. Moreover, the difference between the numbers of mutations that were isolated in *kuz* versus *Su(H)* could be explained by a higher number of critical amino acids in *kuz,* rendering it more vulnerable to random mutagenesis. Importantly, all of the mutants of the type II complemented *Su(H)*$^{\Delta 47}$ and *kuz*^{ES24}, which indicates that they do not have mutations in these genes.

We than crossed the type II mutants to other genes on 2L that have been shown to play a role in Notch signaling in some contexts but that are not essential in the Notch pathway: *echinoid* (*ed*^{k01102}), *Suppressor of deltex* [*Su(dx)*^{KG02902}], *big brain* (*bib*^{1}) and *lethal (2) giant larvae* [*l(2)gl*^{4}]. We observed that all the type II mutants complemented these genes and therefore, do not have mutations in these genes.

The EC phenotype in type IV mutants could correspond to a hyper activation of the Notch pathway similar to what is observed upon overexpression of activated nuclear Notch in the ISC (Micchelli and Perrimon, 2006). Therefore we crossed these

mutant lines with genes on 2L known to promote the hyperactivation of the Notch pathway in some contexts: *numb* (*numb*^{2}), *daughterless* (*da*^{3}) and *lethal (2) giant discs 1* [*l(2)gd1*^{d7}]. We observed that all the type IV mutants complemented these genes and therefore, do not have mutations in the corresponding genes.

G. Complementation analysis amongst lines with similar phenotypes

To establish complementation groups, we then crossed lines with a similar phenotype to each other: we crossed all the type II mutants to each other and all the type IV mutants to each other. The crossing scheme is represented in figures 26 and 27 respectively.

From the complementation analysis of the type II mutants (Fig. 26) we identified two complementation groups: 8-A-9 and 11-C-87 fail to complement each other and 9-C-95 and 9-E-34 also fail to complement each other.

From the complementation analysis of the type IV mutants (Fig. 27) we found that 5-C-53 and 8-C-97 fail to complement each other.

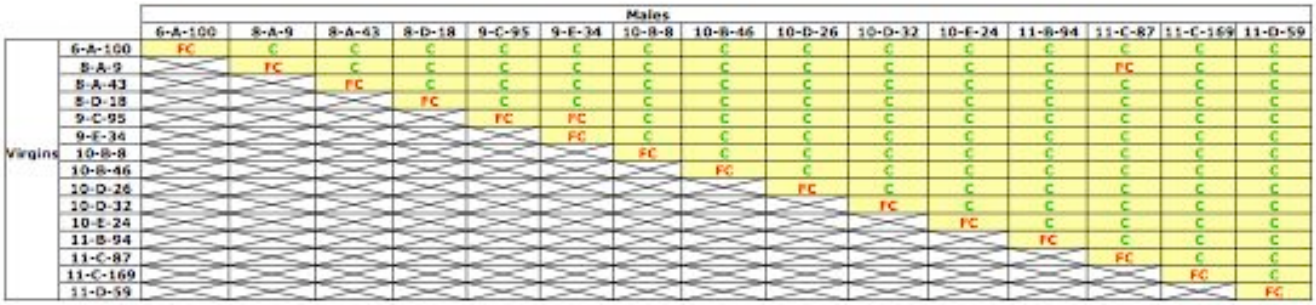


Fig.26: Scheme of complementation crosses between mutants that have an increase in ISC-like cells. Yellow boxes represent crosses.

		Males						
		2-A-43	3-B-10	5-C-53	7-A-113	7-B-75	8-C-97	9-B-20
Virgins	2-A-43	FC	C	C	C	C	C	C
	3-B-10		FC	C	C	C	C	C
	5-C-53			FC	C	C	FC	C
	7-A-113				FC	C	C	C
	7-B-75					FC	C	C
	8-C-97						FC	C
	9-B-20							FC

Fig.27: Scheme of complementation crosses between mutants that have an increase EC phenotypes. Yellow boxes represent crosses.

H. Complementation analysis *en masse*

Since we still had many candidates with strong phenotypes of both type II and type IV mutants for which only had one allele, we decided to do complementation analysis *en masse*, testing with all the lines in order to try to identify other alleles of our candidates amongst the type III lines, which would be very useful for mapping (see below). In order to identify all complementation groups, we decided to cross all lines to each other. The crossing scheme, represented in Fig. 28, represents the approximately 2500 crosses that were started to cross all lines to each other. These crosses, yielded initially, a total of 7 new complementation groups, although one of these was not confirmed when the cross was repeated (the results are summarized in table 2). In some crosses, the two lines did complement each other but the different progeny were not present in Mendelian ratios, with only very few transheterozygotes present. This could indicate a genetic interaction between the two mutated genes. Alternatively, one or both of the fly lines might have multiple mutations that make it less viable and therefore less present in the progeny of the cross. This seems to be the case for some fly lines in whose progeny the transheterozygotes are systematically present in non-Mendelian ratios. These fly lines are represented as generally weak lines in table 2.

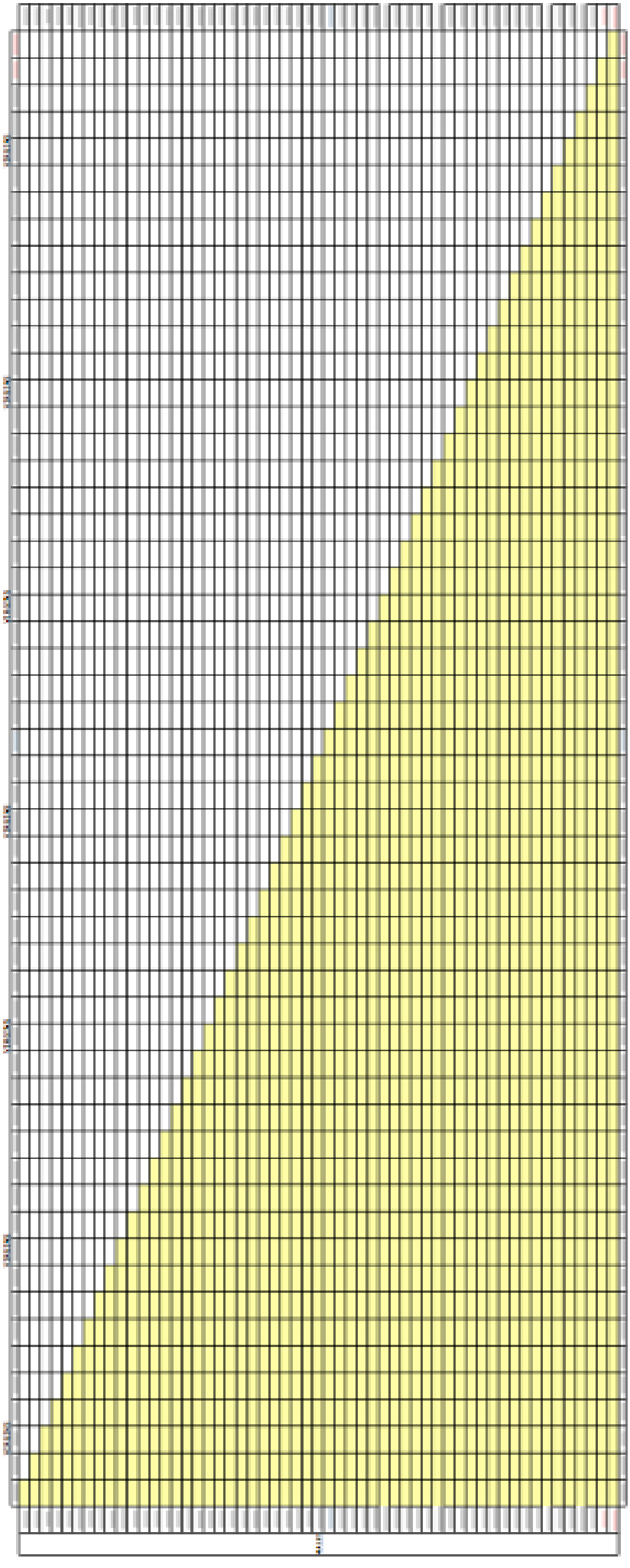


Fig.28: Scheme of complementation crosses *en masse* between all mutants. Yellow boxes represent crosses.

	Crosses that to Fail to complement	Crosses with Progeny in non-Mendelian ratios	Generally Weak lines	obs:
1	3-B-9 x 7-E-68 0:160			confirmed by F2
2	5-C-53 x 8-C-97			confirms previous result
3	6-B-16 x 8-D-70 0:130			
	6-B-16 x 10-E-24 0:76			
	6-B-16 x 8-B-49 0:89			6-B-16 X 7-E-15 didn't take
		8-B-49 x 8-C-97 5:112	**8-B-49**	8-B-49 X 6-B-16 complements
		8-B-49 x 9-C-95 3:113		8-B-49 X 8-D-70 complements
		8-B-49 x 9-E-34 8:90		8-B-49 X10-E-24 complements
		8-B-49 x 10-B-8 1:67		
				7-E-15 x 8-D-70 complements
	7-E-15 x 10-E-24 0:130			
	8-D-70 x 10-E-24 0:29			confirmed by F2
	COMMENTS:potential complementation group of 6-B-16, 8-D-70, 10-E-24 and 7-E-15. 8-D-70 stock can be rarely homozygous viable. 8-B-49 does not seem to belong to this complementation group.			
4	7-A-39 x 11-D-107 0:39			
		7-C-42 x 9-C-95 2:52		9-C-95 is generally weak line
3	7-E-15 x 8-B-49 0:79			7-E-15 fails to complement both 8-B-49 and10-E-24
	7-E-15 x 10-E-24 0:130			Howevern, these 2 lines do not FC.
5	8-A-9 x 11-C-87			result confirmed
		8-A-43 x 9-C-95 5:47		
		8-A-43 x 10-D-32 2:41		
		8-A-43 x 10-D-67 0:47		8-A-43 x 10-D-67 complements
6	8-B-49 x 7-E-15 0:181	8-B-49 x 8-C-97 5:112	**8-B-49**	
		8-B-49 x 9-C-95 3:113		
		8-B-49 x 9-E-34 8:90		
		8-B-49 x 10-B-8 1:67		
	8-C-97 x 10-E-46 0:30			8-C-97 x 10-B-46 complements
		8-B-49 x 8-C-97 5:112		
3	8-D-70 x 6-B-16 0:130			8-D-70 stock rarely homozygous viable
	8-D-70 x 10-E-24 0:29			7-E-15 x 8-D-70 complements
				8-B-49 X 8-D-70 complements
				for more info,see 6-B-16 above
7	9-C-95 x 9-E-34 0:69			9-C-95 and 9-E-34 as confirmed complementation group
		9-C-95 x 7-C-42 2:52		
		9-C-95 x 10-B-27 1:41	**9-C-95**	
		9-C-95 x 10-B-46 0:31		
		9-C-95 x 10-B-95 2:55		
		9-C-95 x 10-C-112 1:70		
		9-C-95 x 10-D-26 1:65		
		9-C-95 x 10-D-32 2:73		
		9-C-95 x 10-D-109 0:30		
		9-C-95 x 11-D-21 3:31		
7	9-E-34 x 9-C-95 0:69			confirmed
		10-B-27 x 10-B-46 1:79	**10-B-27**	
		10-B-27 x 10-D-99 1:89		
		10-B-27 x 10-D-109 2:70		
		10-B-27 x 10-E-24 1:50		
		10-B-27 x 10-E-46 2:84		
		10-B-27 x 11-C-169 4:63		
	10-B-46 x 10-E-46 0:85			10-B-46 & 10-E-46 are probably the same line,there was a mix up in the labels
8	10-B-73 x 11-C-169 0:28			10-B-73 x 11-C-169 Complements
		10-C-16 x 10-D-109 1:30	**10-C-16**	
		10-C-16 x 10-E-46 1:34		
		10-C-16 x 11-C-169 1:169		
		10-C-16 x 11-D-21 0:50		
		10-C-16 x 11-D-59 1:50		
		10-C-16 x 11-D-107 1:46		
		10-C-16 x 2-F-2 0:71		
		10-D-26 x 10-C-112 0:12	**10-D-26**	10-D-26 x 10-C-112 complements
		10-D-26 x 10-D-67 2:50		
		10-D-26 x 10-D-109 2:50		
		10-D-26 x 10-E-24 1:84		
		9-C-95 x 10-D-26 1:65		
		10-D-109 x10-E-24 1:135	**10-D-109**	
		10-D-26 x 10-D-109 2:50		
		10-C-16 x 10-D-109 1:30		
		9-C-95 x 10-D-109 0:30		
		10-D-109 X 10-B-8 3:80		
		10-D-109 X 10-B-27 2:70		
3	10-E-24 x 6-B-16 0:76			10-E-24 fails to complement 6-B-16, 8-D70
	10-E-24 x 8-D-70 0:29			and 7-E-15. However, it complements 8-B-49
	10-E-24 x 7-E-15 0:130			
		10-E-24 x 10-F-12 2:73		
9	10-F-17 x 11-D-21 0:83			
5	11-C-87 x 8-A-9			confirmed
8	11-C-169 x 10-B-73 0:28			COMPLEMENTS when repeated

Table 2: Summary of the results of complementation analysis

I. Deficiency mapping: Principle

To map a gene to the approximate genomic region we use the collection of Exelixis deficiencies for the chromosome arm 2L, that together uncover around 90% of chromosome arm 2L (Parks et al., 2004; Thibault et al., 2004), and use deficiencies from the Szeged and Bloomington collections to complement the missing genomic regions (Fig. 29). Each deficiency line consists of a fly line carrying a chromosome arm that lacks a small portion of a genomic region. A small deficiency removes 2 to 3 genes, while in large deficiencies can affect 100 or more genes. Most of the deficiencies we used for mapping removed around 20 genes.

Our deficiency mapping was based on lethality and was used to map lethal mutants. The basis of deficiency mapping is the following (see Fig. 30): we cross a mutant A with all the deficiencies that uncover chromosome 2L. If a lethal mutation is in a region that is removed in the deficiency ('Deficiency x' in Fig. 30) then those flies are lethal and no transheterozygotes are recovered from the cross. However, if the lethal mutation is not uncovered by the deficiency ('Deficiency y' in Fig. 30) then the transheterozygous flies are not lethal and are recovered in the F1. Since an EMS protocol, on average, induces 1.5 lethal mutations per chromosome, there may be more than one lethal mutation on one given chromosome arm. How do we know which is the lethal mutation responsible for the phenotype? In such situations, having several alleles for the same mutant (having a complementation group with more than one allele) is critical, since this correlates the lethal mutation with the observed phenotype. Therefore, after crossing a line carrying a lethal mutation with the collection of deficiencies, then the other allele of the complementation group is crossed to the deficiencies over which the first allele was lethal. The outcome of this cross should indicate the region that fails to complement both alleles.

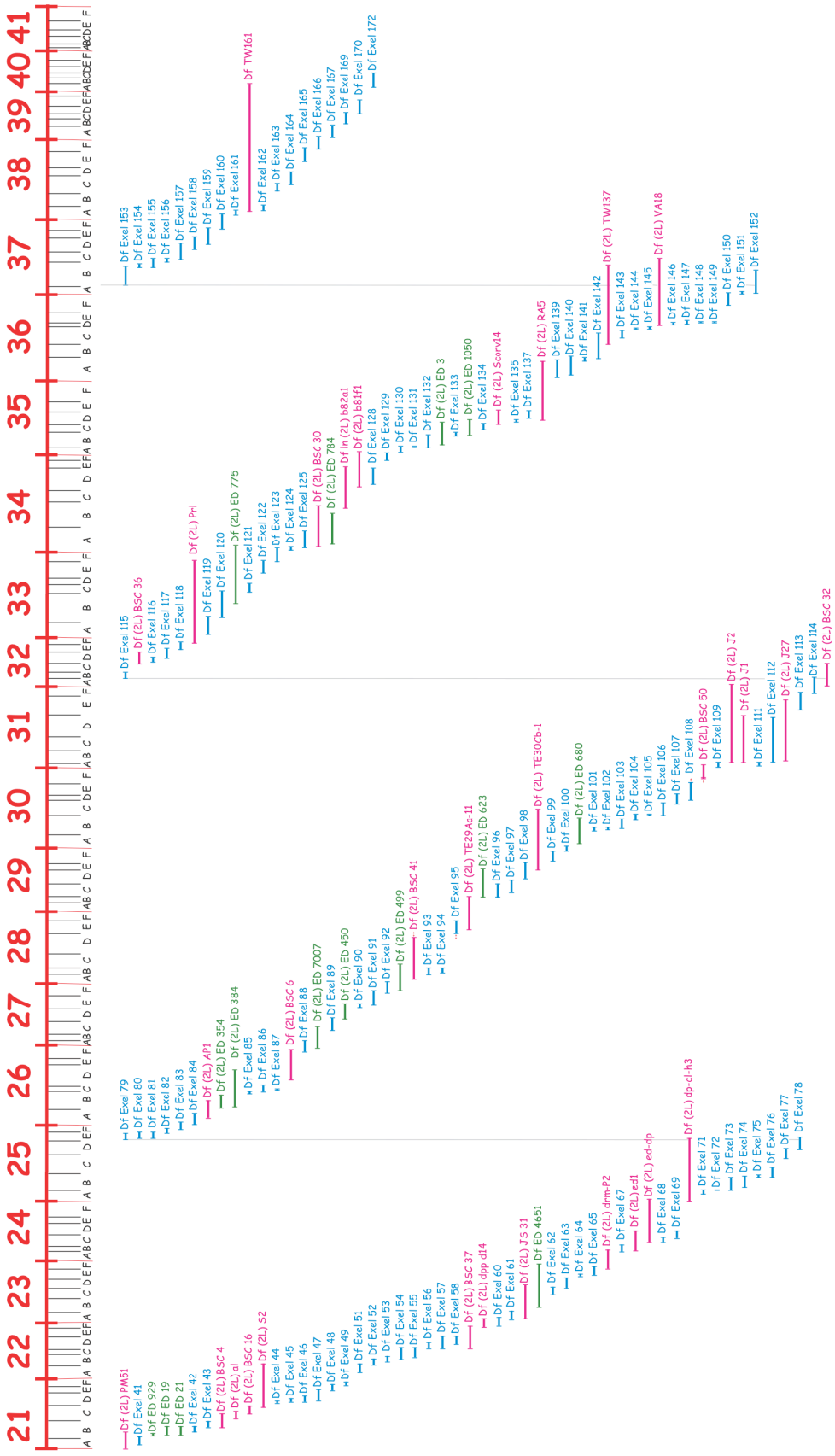


Fig.29: Collection of deficiencies tested for deficiency mapping of 2L.

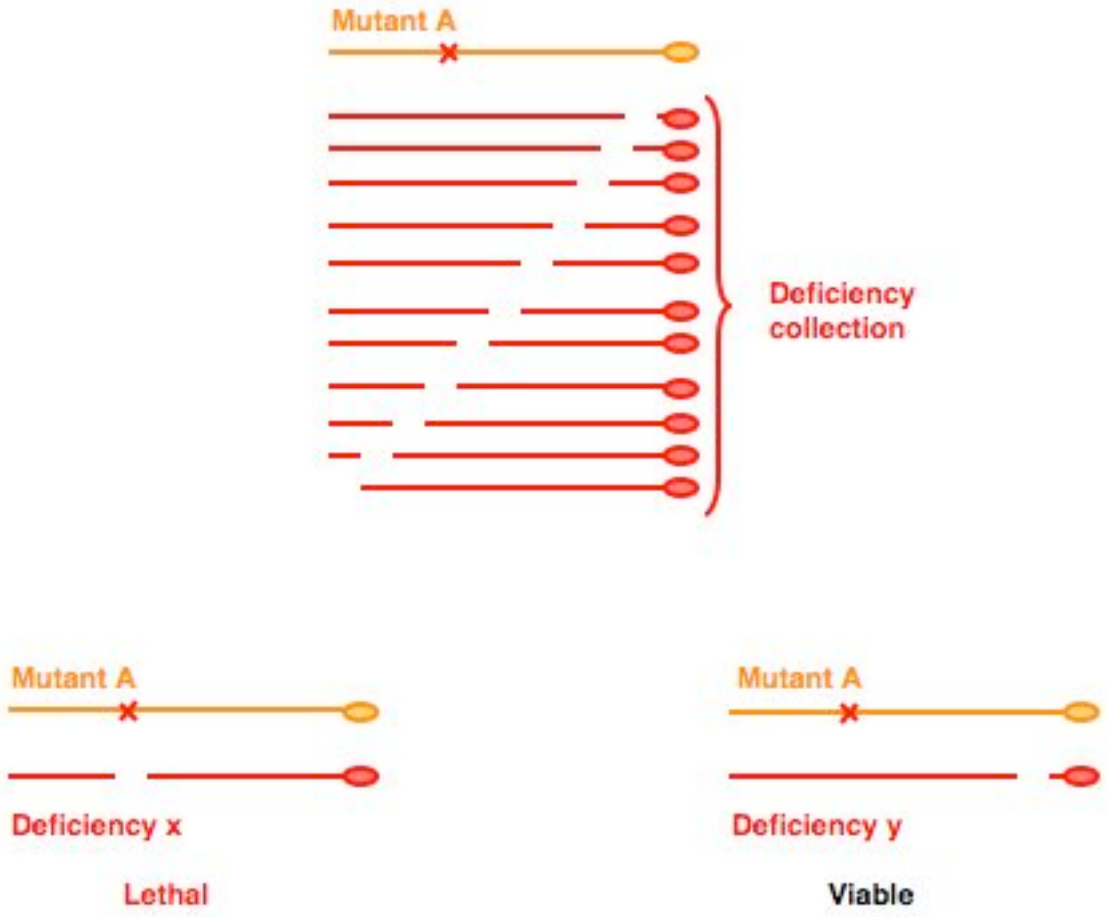


Fig.30: Cartoon representing the principle of deficiency mapping.

J. Deficiency mapping of Mutants with an overabundance of Delta+ cells

The group of mutants with an overabundance of ISC-like cells was the one with the most complementation groups (12) and several of these were chosen to map through deficiency mapping. All the complementation groups with more than one allele and some with only one allele were put through deficiency mapping.

J.1. Mapping of 8-A-9 & 11-C-87

The complementation group that comprised of the alleles 8-A-9 and 11-C-87 had a strong increase in density Delta+ cells. Interestingly, the differentiated Pros+ enteroendocrine (ee) cells as well as the large polyploid enterocytes (ECs) do not appear to be affected, still being present in the clones and in wild-type-like proportions, unlike what is seen in the mutants for the loss of function of the Notch pathway (Fig.31.A)

First, 8-A-9 was crossed to the whole collection of deficiencies and it was lethal over four different regions of the chromosome arm. The other allele, 11-C-87, was than crossed to those same four regions and it was found to only have a lethal in one of these regions. The region is represented in Fig. 23.B. Both 8-A-9 and 11-C-87 were lethal over three different deficiencies: Df(2L)Exel8012, Df(2L)Exel7021 and Df(2L)Exel8013. The overlap between the three deficiencies corresponds to two genes: *GDP-mannose 4,6-dehydratase (Gmd)* and *CG3792*, which suggests that the most likely lethal gene responsible for the phenotype is one of these two (Fig.31.B).

There are known and well characterized null alleles of *Gmd*, therefore two of these were crossed to 8-A-9 and 11-C-87. We found that both 8-A-9 and 11-C-87 failed to complement Gmd^{1} and Gmd^{H78}, which confirmed that Gmd is the mutated gene in this complementation group. We further confirmed that clones of Gmd^{H78}, a

characterized null allele of *Gmd* (Sasamura et al., 2007), have the same phenotype as 8-A-9 and 11-C-87. Finally, we sequenced the coding region of both mutants isolated in the screen and found that Gmd^{8A9} contains an S177F mutation adjacent to the catalytic T178 and Gmd^{11C87} contains an E332K mutation. The role of *Gmd* in regulating the ISC lineage will be further addressed in the next chapter.

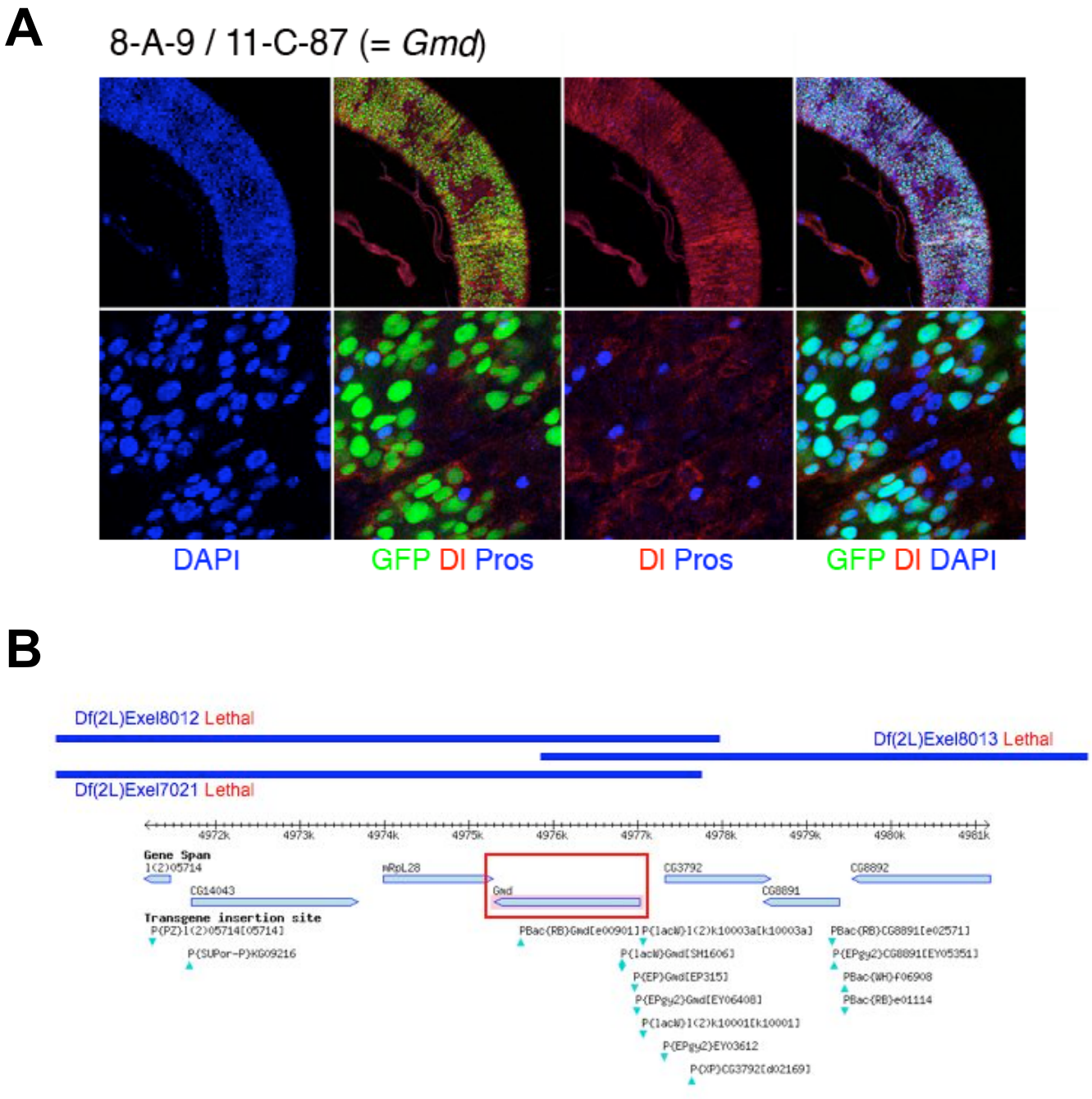


Fig. 31: **A** Mutant clones of 11-C-87. **B** Deficiency mapping of 8-A-9 and 11-C-87.

J.2. Mapping of 9-C-95 & 9-E-34

The complementation group comprised of the alleles 9-C-95 and 9-E-34 had a strong increase in density Delta+ cells as well as defects in localization of Delta protein, which is very strongly expressed and localized at the plasma membrane in many of the mutant cells. Furthermore, the differentiated Pros+ ee cells as well as the large polyploid ECs do not appear to be affected, still being present in the clones, although this has not yet been characterized in detail (Fig.32.A).

First, 9-C-95 was crossed to the entire collection of deficiencies and it was found to have a lethal in two different regions of the chromosome arm. The other allele, 9-E-34, was then crossed to those two regions and it was found to have a lethal in one of the regions, which is represented in Fig.32.B. Both 9-C-95 and 9-E-35 had a lethal in two different deficiencies that overlap partially, Df(2L)ED292 and Df(2L)ED21. The overlap between the two deficiencies only has one gene in common removed: the gene *split ends* (*spen).*

There are known and well characterized alleles of *spen*, therefore two of these were crossed to 9-C-95 and 9-E-35. We found that both 9-C-95 and 9-E-35 failed to complement $spen^{3}$ and $spen^{5}$, which confirms that *spen* is the mutated gene in this complementation group. We also found that $spen^{5}$ mutant clones have the same phenotype in the intestine as $spen^{9\text{-}C\text{-}95}$ and $spen^{9\text{-}E\text{-}34}$.

Spen is a nuclear protein, containing three N-terminal RNA binding motifs (RRM) and a C-terminal SPOC (Spen paralog and ortholog C-terminal) domain. Spen has been shown to interact with different signaling pathways in *Drosophila* such as Notch, Wingless, and EGFR (Rebay et al., 2000) (Chang et al., 2008; Chen and Rebay, 2000; Doroquez et al., 2007; Kuang et al., 2000). However, the molecular function of Spen is not understood. One human homologue of Spen, SHARP (SMRT/HDAC1-associated repressor protein) has been shown to associate with transcriptional repression complexes (Sanchez-Pulido et al., 2004) and SHARP protein has been shown to be upregulated in human colon and ovarian carcinomas (Feng et al., 2007). Another human homologue of Spen, the OTT1 gene has been

found to be translocated in some in some acute megakaryocytic leukemias (Mercher et al., 2001). Recently, plant homologue of Spen, FPA, has been shown to regulate alternative polyadenylation of mRNA transcripts, which affects transcript stability and can regulate gene silencing (Hornyik et al., 2010). Therefore, Spen could be regulating transcription or mRNA stability in the ISC lineage, potentially regulating several signaling pathways in the fly intestine.

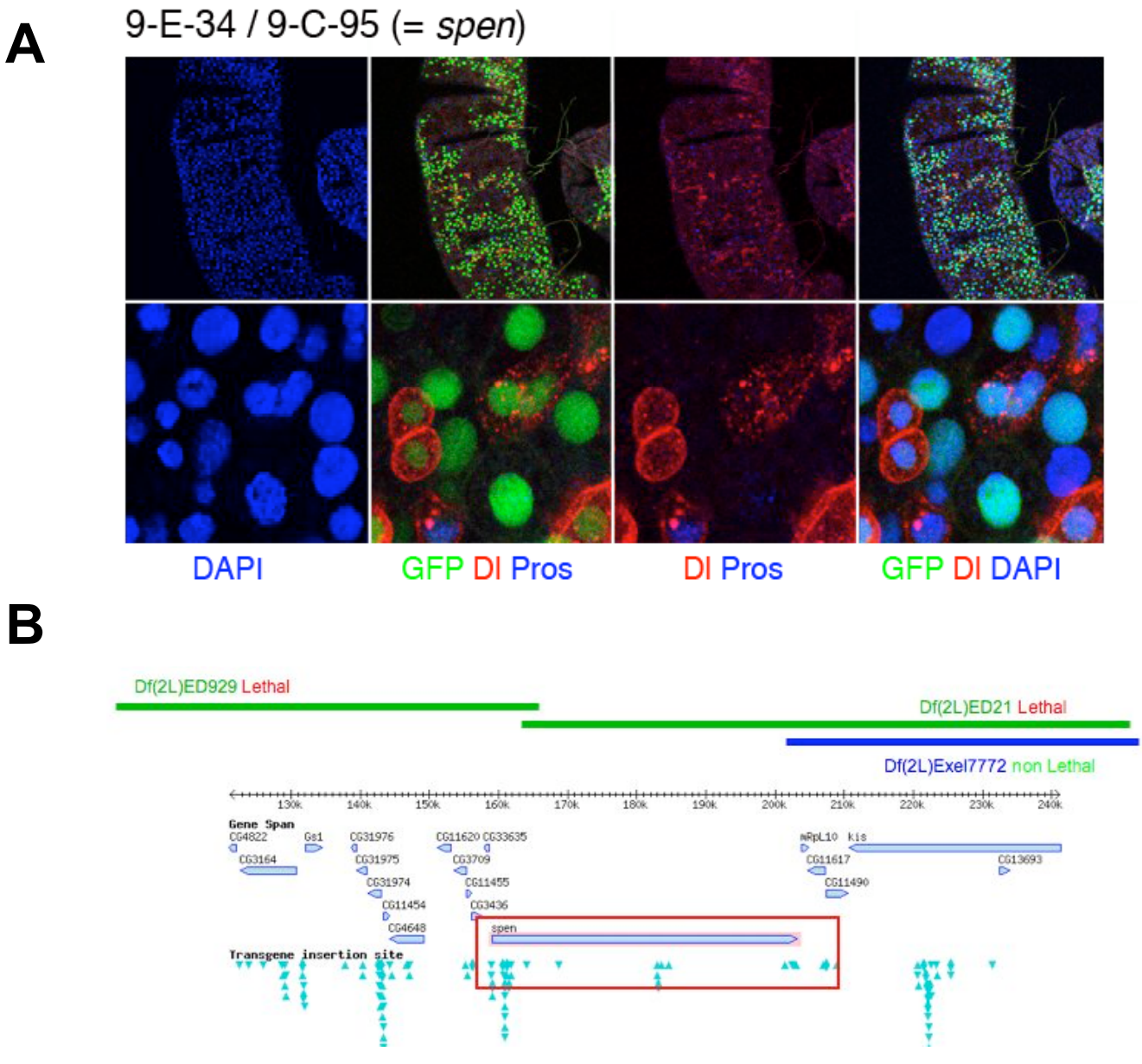


Fig.32: **A** Mutant clones of 9-C-95. **B** deficiency mapping of 9-C-95 and 9-E-34

J.3. Mapping of 10-D-26

The mutant 10-D-26 has a strong increase in density Delta+ cells. Furthermore, the differentiated Pros+ ee cells as well as the large polyploid ECs do not appear to be affected, still being present in the clones (Fig.33.A). Since we only isolated one allele of 10-D-26 from the screen, deficiency mapping by lethality might be problematic. We, nevertheless, mapped it through deficiency mapping.

10-D-26 was crossed to the entire collection of deficiencies and it had a lethal in only one region of the chromosome arm (Fig. 33.B). 10-D-26 had a lethal in region of the deficiency Df(2L)Exel7002 but was viable with the deficiency Df(2L)ED21, which covers the same region as Df(2L)Exel7002. Occasionally, the limits of deficiencies and the genes removed are not precisely mapped. Therefore, we decided to test whether the known lethal genes in region uncovered by the Df(2L)Exel7002, would fail to complement 10-D-26. We tested the available lethals and found that 10-D-26 fails to complement an allele of the gene kismet (kis^{1}). We tested other alleles of *kis*, kis^{EC1} and kis^{LM57}, and found that 11-D-26 failed to complement both. Finally, we generated clones of kis^{1}, kis^{EC1} and kis^{LM57} in the intestine and observed that they have the same phenotype as 10-D-26.

Kis is a protein with an ATPase domain, two chromodomains (that are found in CHD ATPases and that meditate protein-protein interaction or protein-RNA interaction) and a BRK domain, a domain of unknown function, that is also present in brahma, another TrxG protein and its homologues (Srinivasan et al., 2005). Kis has been shown to counteract Polycomb group (PcG) repressive activity in some developmental contexts; consistent with a function as antagonistic of PcG, Kis has been shown to negatively regulate histone H3 lysine 27 methylation, which is repressive chromatin (Daubresse et al., 1999; Srinivasan et al., 2005). More recently, Kis has been found to promote early elongation by RNA pol II (Srinivasan et al., 2008). The human homologue of Kis, CHD7, has been shown to be mutated in patients with CHARGE syndrome, a complex condition that affects many organs such as the central nervous system, the heart, the urinary tract and also causes

hearing defects and mental retardation (Aramaki et al., 2006; Lalani et al., 2006). The *kis* loss of function clones result in an increase of Delta+ cells in the intestine, which can either be extra stem cells but could also be EBs that have not yet activated its differentiation program. One possible model for its role in the intestine is that Kis, through the regulation of transcription by RNA polymerase II, could promote differentiation of the EB by promoting the transcription of the differentiation program.

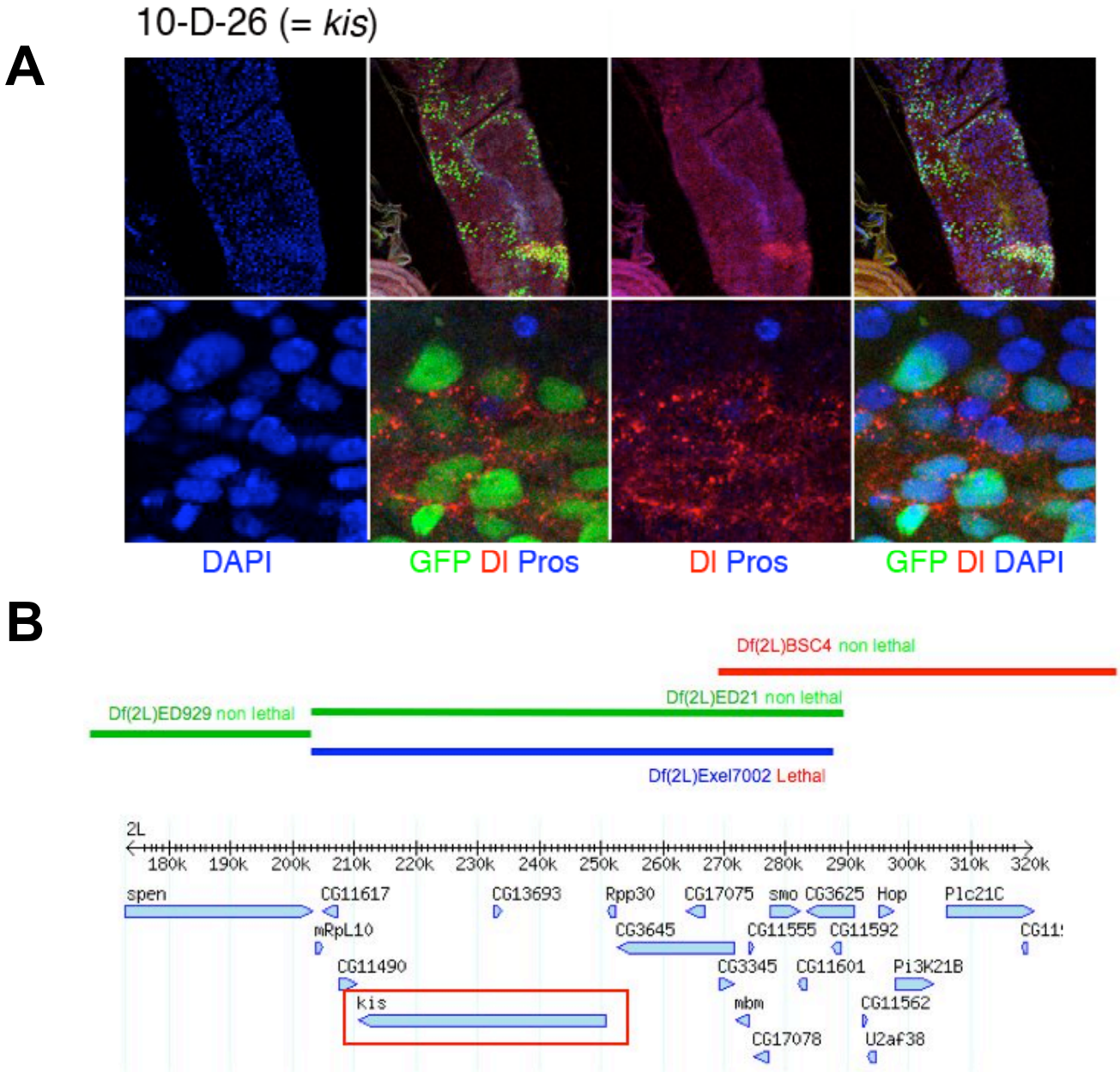


Fig.33: **A** Mutant clones of 10-D-26. **B** deficiency mapping of 10-D-26.

J.4. Mapping of 6-B-16, 7-E-15, 8-D-70 and 10-E-24

The complementation group comprised of mutants 6-B-16, 7-E-15, 8-D-70 and 10-E-24 is problematic because 7-E-15 also fails to complement 8-B-49 while none of the other lines, 6-B-16, 8-D-70 or 10-E-24, failed to complement 8-B-49. One possibility for this inconsistency is that 7-E-15 has two different lethals on 2L, one that fails to complement 6-B-16, 8-D-70 and 10-E-24 and another lethal that fails to complement 8-D-49. 10-E-24 clones have a very strong increase in density of Delta+ positive cells and is therefore an interesting candidate to be mapped. The other lines have a weaker phenotype.

10-E-24 was found to contain lethal mutations in two regions of 2L: the region of the deficiency Df(2L)Exel6277 and the region of the deficiency Df(2L)Exel 8036 (Fig.34). Closer inspection of the region uncovered by Df(2L)Exel6277 showed that one gene that is removed from this region is *anterior pharynx defective 1* (*aph1*), a component of the γ-secretase complex, which is required for S3 cleavage of Notch receptor and the production of the activated form of nuclear Notch, NICD (Schweisguth, 2004a). We had overlooked this gene, which should have been included in the group of genes to test directly by complementation (see section F.). Since 10-E-24 has a very strong phenotype, this is the most likely mutated gene. Complementation analysis with known alleles of aph1 should confirm this hypothesis. However, we found that the other three lines, 6-B-16, 7-E-15 and 8-D-70 were not lethal in the region of Df(2L)Exel6277 but were all lethal in the region of Df(2L)Exel 8036. Therefore, all these four lines probably have one lethal in common. If 10-E-24 is shown to not have a mutation in the *aph1* gene, than the region Df(2L)Exel 8036 should be analyzed more carefully, to identify a potential lethal in this region that is associated with the strong phenotype seen in 10-E-24 and the milder phenotypes seen in 6-B-16, 7-E-15 and 8-D-70.

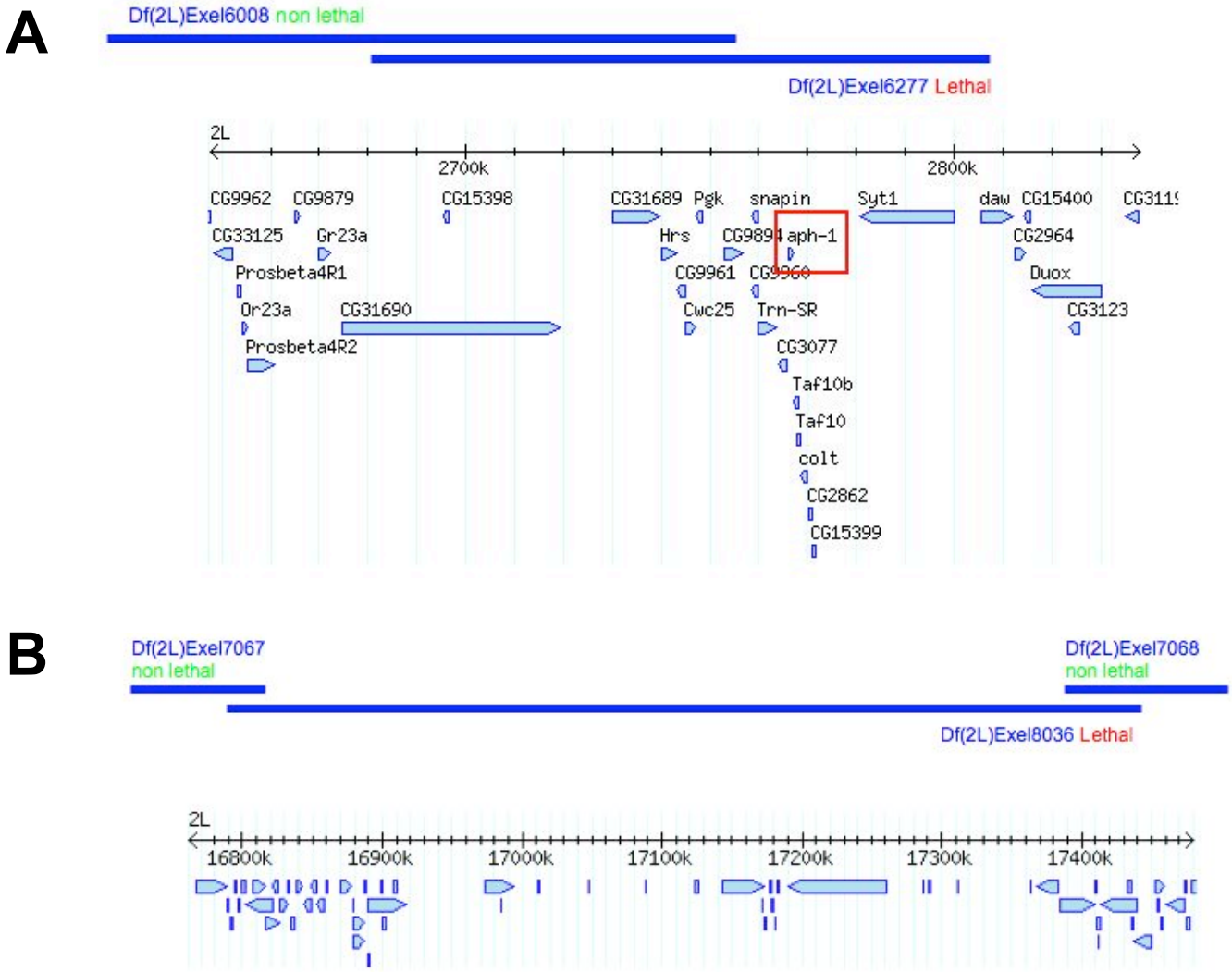


Fig.34: Deficiency mapping of the complementation group 6-B-16, 7-E-15, 8-D-70 and 10-E-24 **A** Region over which only 10-E-24 is lethal. **B** genomic region in which all the alleles are lethal.

J.5. Mapping of 7-E-15 and 8-B-49

As mention above, 7-E-15 fails to complement both the lines in the complementation group comprised of 6-B-16, 8-D-70 and 10-E-24 and 8-B-49. However, 8-B-49 complements 6-B-16, 8-D-70 and 10-E-24. To solve this genetic puzzle, we crossed both 7-E-15 and 8-B-49 to the deficiency kit and found that both contain a lethal mutation in the same region, uncovered by the deficiencies Df(2L)Exel6002 and Df(2L)Exel 7005 (Fig. 35). However, we found that 7-E-15 is lethal in the region of the deficiency Df(2L)Exel 8036, as described above, but 8-B-49 is not lethal in this region. This resolves the issue of the two complementation groups, with 7-E-15 having two distinct lethal loci, which results in it failing to complement the fly lines in both groups. Cleaning the chromosome arm 2L of 7-E-15 to generate two new chromosomes, one with each lethal mutation would be important to understand if both play a role in the intestine. The region uncovered by the deficiencies Df(2L)Exel6002 and Df(2L)Exel 7005 has 14 possible genes. Testing lethals in this region would be the next step towards identifying the lethal gene.

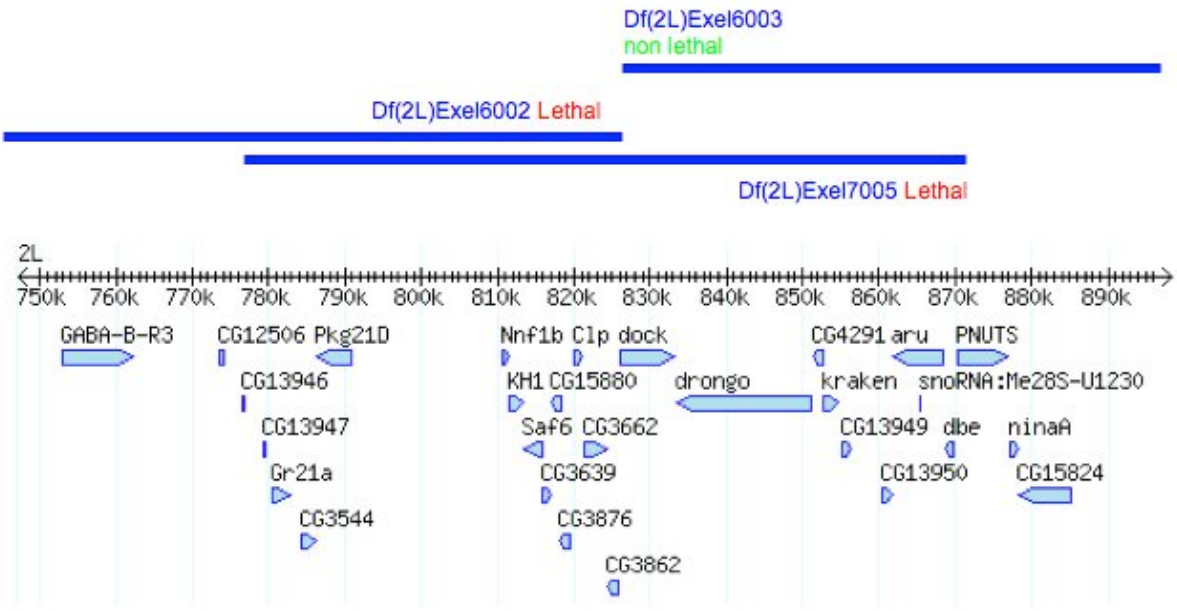


Fig.35: Deficiency mapping of the complementation group 7-E-15 and 8-B-49.

J.6. Mapping of 6-A-100

The mutant 6-A-100 has a strong increase in density Delta+ cells (fig.36.A). Furthermore, although it is mostly lethal, it yields rare homozygous escaper flies that have a extra macrochaete bristles on the adult flies' notum. Since it has rare escaper flies, it is not ideal to map by lethality. However, the homozygous macrochaete phenotype, if caused by the same mutation that gives the ISC phenotype, could be used for mapping: even if 6-A-100 is not lethal over a deficiency that uncovers the mutated gene, the rare homozygous escapers might have the macrochaete phenotype.

6-A-100 was crossed to the deficiency kit and observed that there were no regions in which 6-A-100 had a lethal mutation. However, there were two regions, removed by the deficiencies Df(2L)Exel7070 and Df(2L)Exel8038, with which 6-A-100 transheterozygous flies had a macrochaete phenotype (Fig.36.B-C). 6-A-100 appears to have a lethal in region of the large deficiency Df(2L)VA18 but we observed that most chromosomes are lethal over this deficiency, that is essentially haploinsufficient. 6-A-100 also has a machrochaete phenotype when combined with the deficiency Df(2L)dpp[d14], which is a very large deficiency that removes potentially a large number of genes (its limits are not precisely mapped). Since the interaction of 6-A-100 with both deficiencies was not very strong, we put the mapping of this gene on stand by. One strategy to pursue its mapping would be the generation of another allele by EMS induced mutagenesis to screen for new alleles (Greenspan, 2004; St Johnston, 2002).

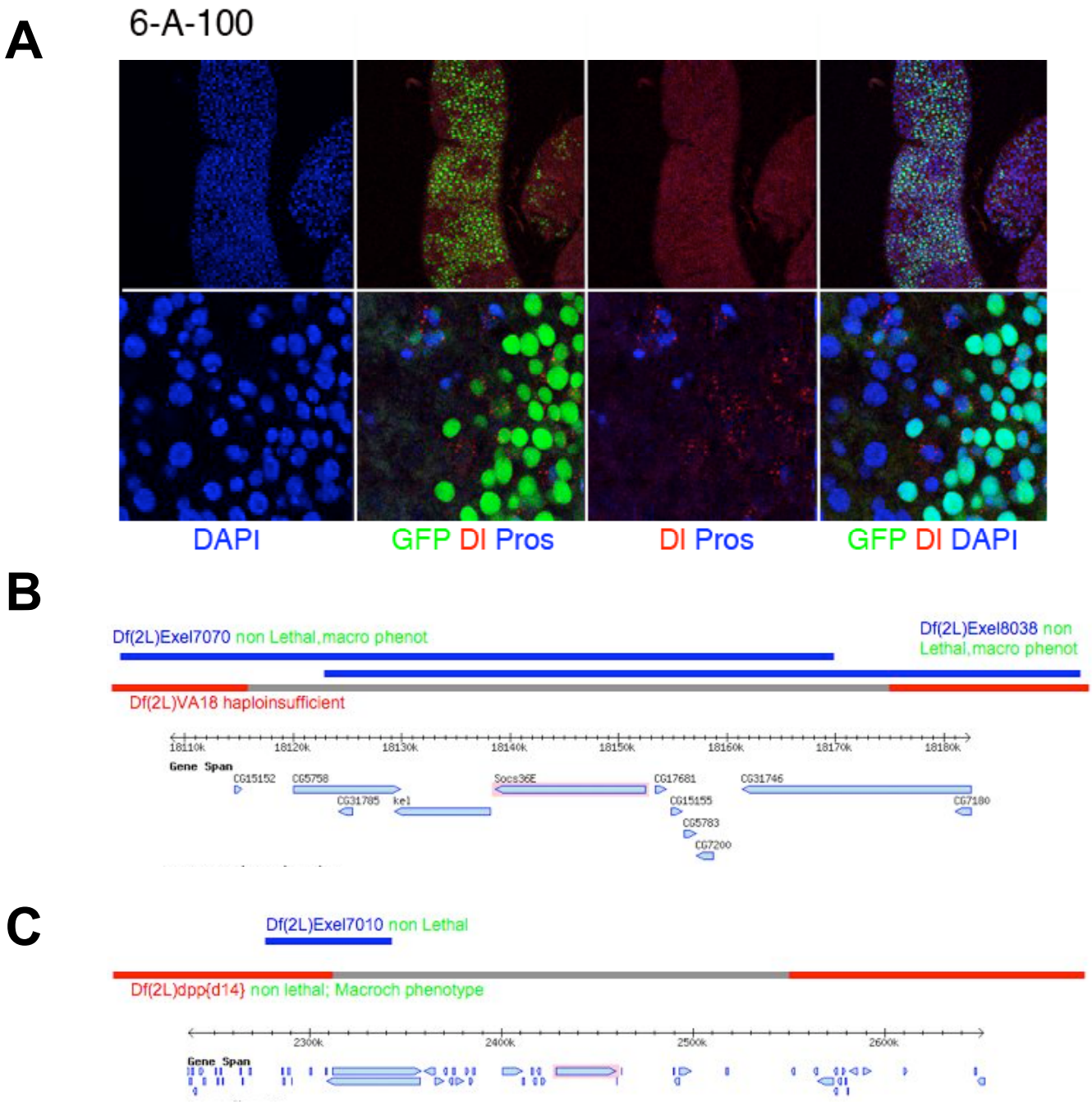


Fig.36: **A** Mutant clones of 6-A-100. **B** & **C** deficiency mapping of 6-A-100.

J.7. Mapping of 8-A-43

The mutant 8-A-43 has an increase in density Delta+ cells. However, since we only isolated one allele of 8-A-43 from the screen, deficiency mapping by lethality might be problematic.

8-A-43 was crossed to the entire collection of deficiencies and did not find any region over which it was lethal. Further retesting should be done. Furthermore, the collection of deficiencies used only uncovers approximately 90% of 2L. Since more deficiencies exist in stock collections, it might be possible to find deficiencies that cover the missing genomic regions in 2L. Finally, new alleles of 8-A-43 could be generated, and used to for mapping with molecularly defined P-elements (see below).

J.8. Mapping of 10-B-46

The mapping of the mutant 10-B-46 was similar to the mapping of 8-A-43, described above. 10-B-46 has an increase in density Delta+ cells and, as 8-A-43, we have only one allele of 10-B-46 from the screen.

After crossing 10-B-46 to the collection of deficiencies, we did not find any region in which it contained a lethal mutation. Similar strategy as the one described for 8-A-43 (above) could be implemented for the mapping of 10-B-46.

J.9. Mapping of 11-B-94

The mutant 11-B-94 has a very strong increase in the number of Delta+ cells. Interestingly, while large polyploid ECs are still present in the mutant clones, Pros+ cells appear to not be present in the clones (Fig. 37.A). Further investigation is

necessary to better describe this potentially very interesting novel phenotype. However, we have only one allele of 11-B-94, which can make mapping difficult.

After crossing 11-B-94 to the collection of deficiencies, we found that it contained lethal mutations in two regions: the region of the large Bloomington deficiency Df(2L)JS 31, that removes more than 60 genes, and the region of the deficiency Df(2L)ED623 (Fig.37.B). The next step is to test whether available lethal genes in the region fail to complement 11-B-94. If none of the available defined lethal mutations fail to complement 11-B-94, then new alleles in the gene mutated in11-B-94 should be generated by EMS mutagenesis and subsequent mapping with molecularly defined P-elements (see below).

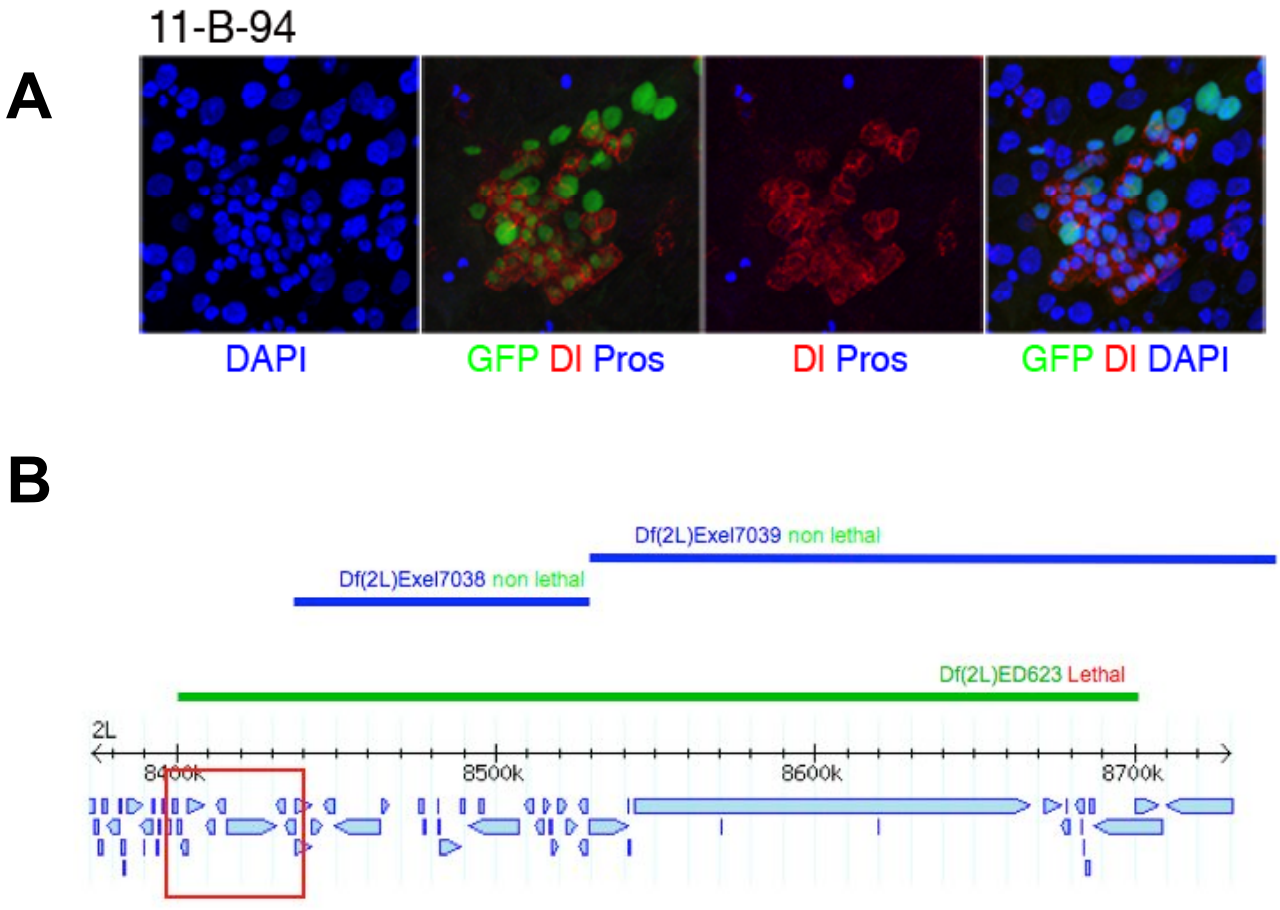


Fig.37: **A** Mutant clones of 11-B-94. **B** deficiency mapping of 11-B-94.

K. Deficiency mapping of Mutants with an overabundance of Pros cells

Of the 15 complementation groups of the type II phenotype (increase in density of small cells) only one had an increase in Pros+ cells: 11-C-169.

K.1. Mapping of 11-C-169

The mutant 11-C-169 has an increase in the number of Delta+ cells as well as an increase in the number of Pros+ cells (Fig.38). Further investigation is necessary to better describe this phenotype. Unfortunately, we have only one allele of 11-C-169, which can make mapping difficult.

After crossing 11-C-169 to the collection of deficiencies, we did not find any region that contained lethal mutation of 11-C-169 , which may be due to the fact that the collection of deficiencies used only uncovers approximately 90% of 2L. Since more deficiencies exist in stock collections, it might be possible to find deficiencies that remove genes in the missing genomic regions and test whether they fail to complement 11-C-169. Finally, generating a new allele of 11-C-169 is key to further map this complementation group, possibly with molecularly defined P-elements (see below).

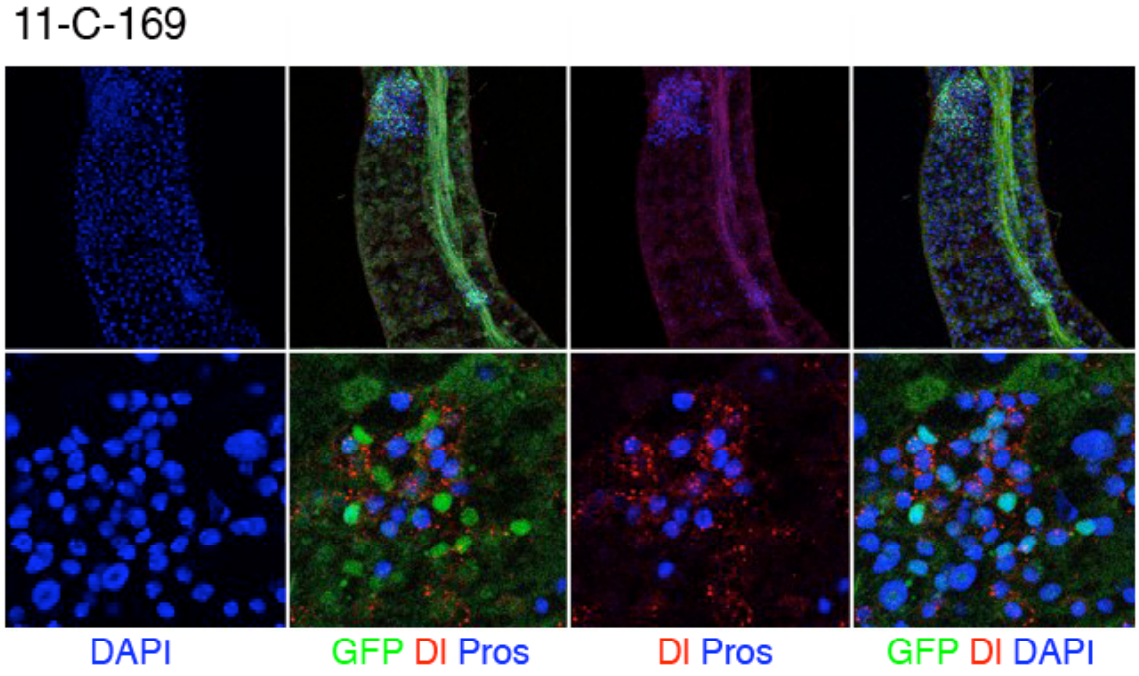


Fig.38: **A** Mutant clones of 11-C-169.

L. Deficiency mapping of Mutants with EC defects

Finally, we attempted to map some of the complementation groups that resulted in EC associated phenotypes, concentrating on those that lead to ISC loss.

L.1. Mapping of 2-A-43

The mutant 2-A-43 has a very strong phenotype: only single large polyploid cells are present and these have abnormal morphology and express Delta (Fig.39.A).

2-A-43 was crossed with the collection of deficiencies and had a lethal mutation in the region of the deficiencies Df(2L)Exel8026 and Df(2L)Exel7049 (Fig. 39.B). One of the genes in this region is IpII-aurora-like kinase (ial) that is also known as aurora-B (aurB). Although there are no alleles available for *aurB*, we were able to test whether 2-A-43 phenotype results from a mutation in the aurB gene: Juliette Mathieu and Jean-René Huynh (private communication) have identified that a mutation in the gene Survivin (svv) results in polyploid cells in the germline, the follicular epithelium, the imaginal discs epithelia, and the larval neuroblasts. Both svv and aurB belong to the Chromosomal Passenger Complex, a major regulator of mitosis that plays a role in chromosome alignment by correcting defective chromosome-spindle interactions (Adams et al., 2001). 2-A-43 phenotype in the intestine is consistent with a polyploid ISC. J. Mathieu and J.R. Huynh provided us with a full-length rescue construct for aurB ($aurB^{FL}$) and we observed that the expression of the $aurB^{FL}$ construct rescues the phenotype of 2-A-43 in the ISC (Fig. 39.A). A second, independent *aurB* allele tested by J. Mathieu and J.R. Huynh failed to complement 2-A-43. Finally, the open reading frame of 2-A-43 was sequenced and a 19 base pair (bp) deletion was identified in the activation loop of the aurB protein that causes a frame shift of the open reading frame and results in a truncation of the protein. Further investigation of the role of aurB in regulating mitosis in stem cells is being carried out in collaboration with the J. Mathieu and J.R. Huynh.

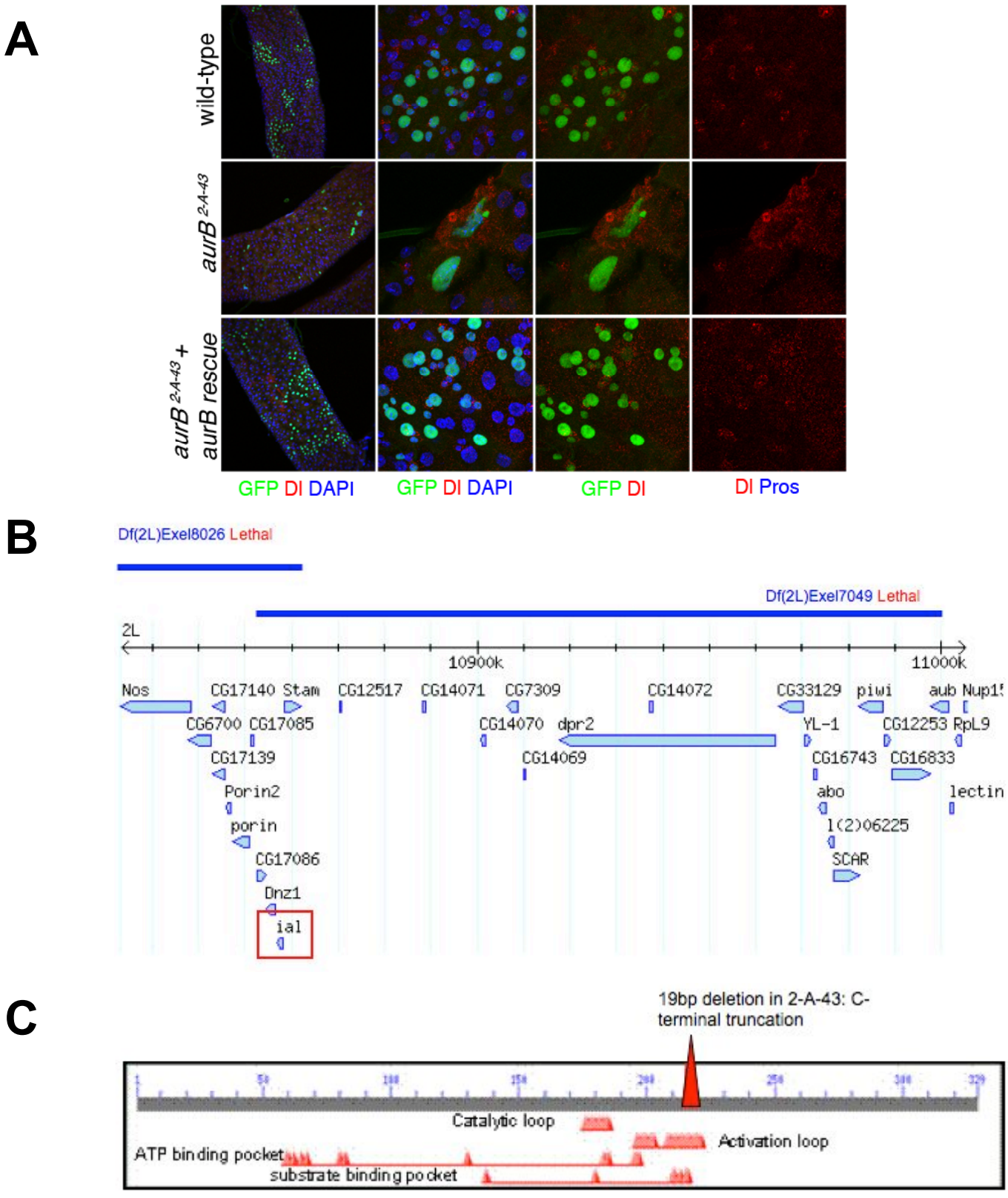


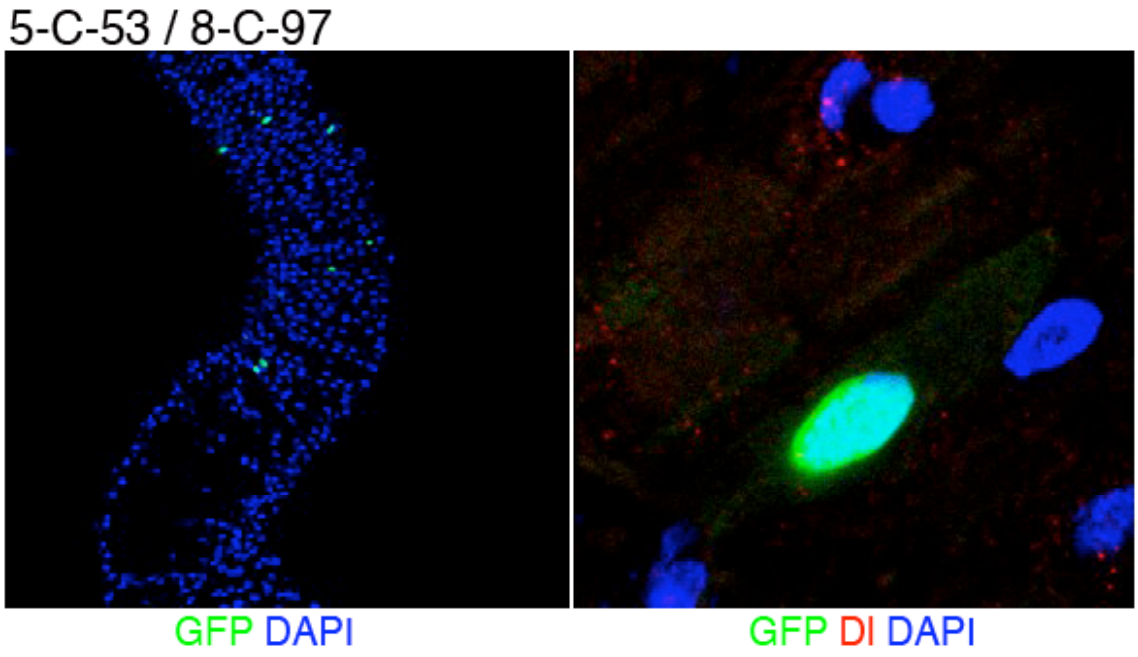
Fig.39: **A** Mutant clones of wild-type, 2-A-43 (*aurB*) and 2-A-43 + the AurB^{FL} **B** deficiency mapping of 2-A-43. **C** aurB gene and localization of the 2-A-43 defeciency.

L.2. Mapping of 5-C-53 and 8-C-97

The complementation group comprised of the alleles 5-C-53 and 8-C-97 has a strong loss of ISC phenotype, with only GFP labeled ECs present in the intestine (Fig.40.A).

First, 5-C-53 was crossed to the whole collection of deficiencies and it had lethal mutations in two different regions of the chromosome arm. The other allele, 8-C-97, was then crossed to those two regions and it was found to only have a lethal in one of these regions. The region is represented in Fig.40.B. Both 5-C-53 and 8-C-97 had a lethal in the region the deficiency Df(2L)Exel6031, which uncovers 28 genes. Available lethals in that region were tested and none failed to complement 5-C-53 or 8-C-97. To narrow down the region in which the lethal mutation is localized, smaller deficiencies, represented as Df A, Df B and Df C in Fig. 40.B., that remove part of the genes in the region of Df(2L)Exel6031 were tested. All three deficiencies were found to complement both 5-C-53 and 8-C-97. Another large deficiency, Df(2L) esc-P3-0 (Fig.40.B.), that removes all the genes in the genomic region of Df(2L)Exel6031, was tested and was also found to complement both 5-C-53 and 8-C-97. Therefore, it is not clear whether the lethal mutation in 5-C-53 and 8-C-97 is in the region that is predicted to be uncovered by Df(2L)Exel6031. Further deficiencies in this genomic region should be tested to understand whether the affected locus in 5-C53 and 8-C-97 is really in this region. It is also possible that the mutated locus is localized in one of the genomic regions that are not uncovered by any of the deficiencies in the collection, which is predicted to be about 10% of the chromosome arm. Therefore, the lethal mutation could be mapped instead with molecularly defined P-elements (see below).

A



B

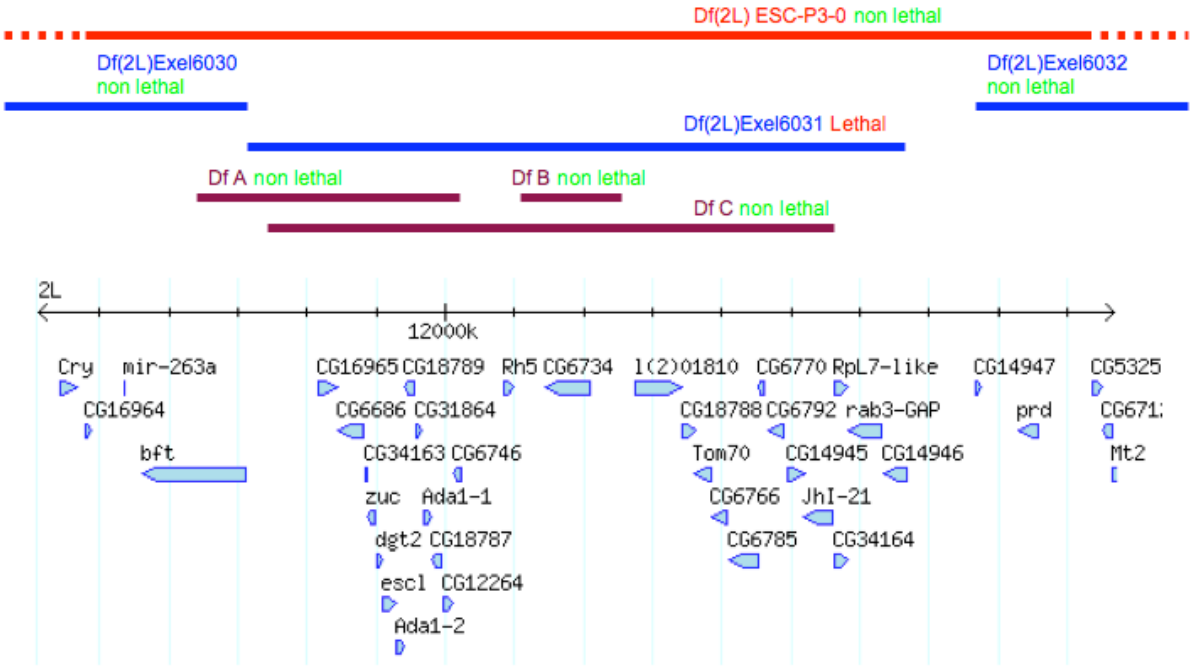


Fig.40: **A** Mutant clones of 5-C-53 and 8-C-97 **B** deficiency mapping of 5-C-53 and 8-C-97.

L.3. Mapping of 7-A-113

Clones of the mutant line 7-A-113 had abnormal EC morphology. 7-A-113 was crossed to the whole collection of deficiencies and it had a lethal mutation the region of the deficiency Df(2L)Exel7077 (Fig.41). Although we have not yet tested whether lethals in this region fail to complement 7-A-113, there is one interesting potential gene in the region: sickie (sick). The gene sick has been shown to be required for activation of the NF-κB-like protein Relish [Rel, (Foley and O'Farrell, 2004)]. The Imd pathway is part of the *Drosophila* innate immune system that responds to Gram-negative bacterial infection and promotes production of antibacterial peptide genes through the activation of Rel (Lemaitre and Hoffmann, 2007). It is tempting to speculate that the abnormal EC morphology observed in 7-A-113 could be due to the inability of these cells to respond to Gram-negative bacteria, which would attack the ECs and damage them. This hypothesis will have to be tested by further complementation tests of 7-A-113.

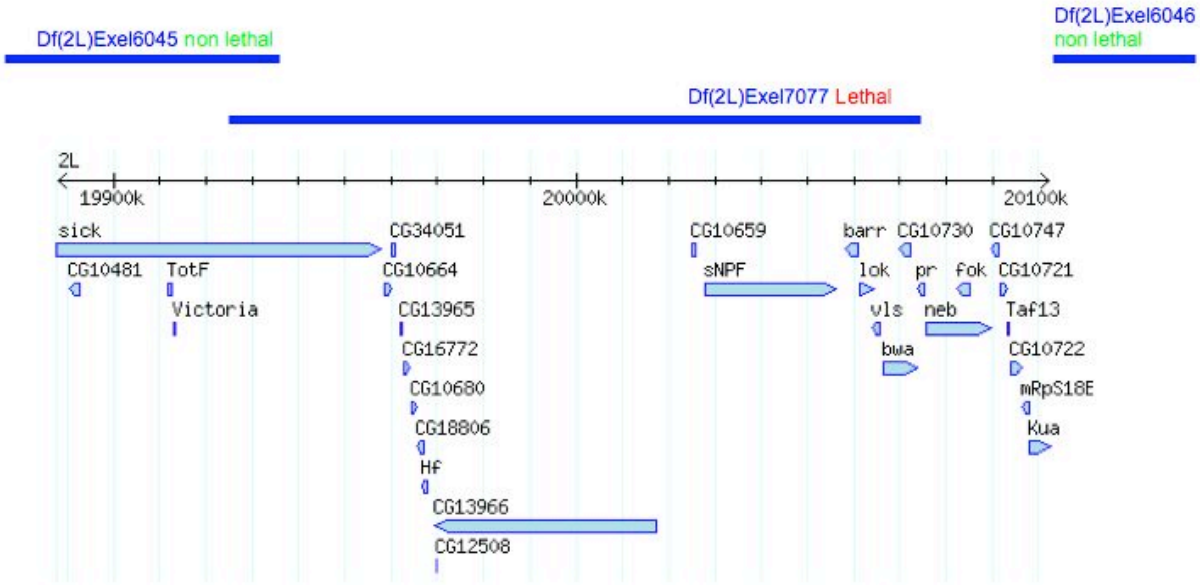


Fig.41: **D**eficiency mapping of 7-A-113.

M. Mapping with molecularly defined P-elements

For mutants that have only been mapped to their genomic region by deficiency mapping, one strategy to map the precise gene locus is to use molecularly defined P-elements, measuring the rate of recombination to map with high resolution (Zhai et al., 2003); a cartoon of how this strategy works is featured in Fig. 42. This strategy could allow the identification of the locus of our mutant candidates that are yet unmapped.

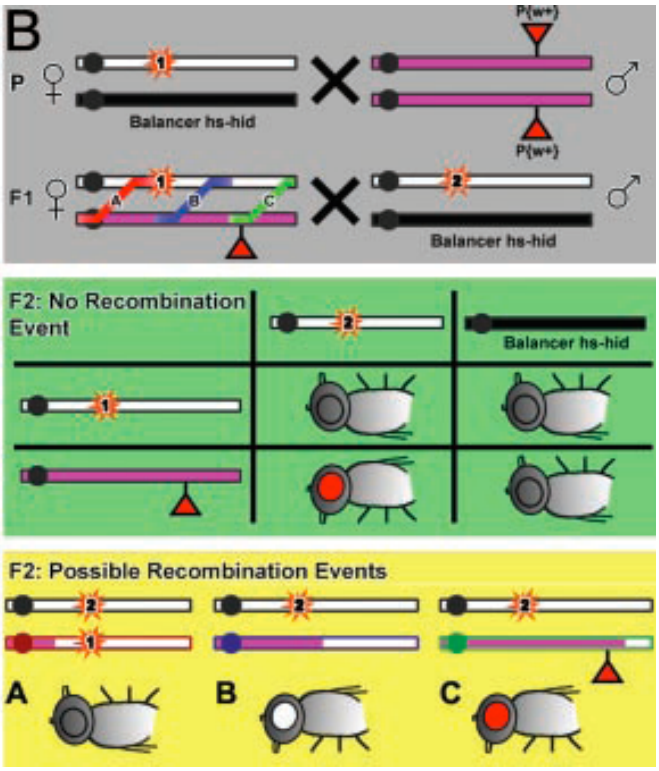


Fig.42: Cartoon, adapted from (Zhai et al., 2003), representing the strategy for mapping with molecularly mapped P-elements Mutant chromosomes are indicated by open bars, *P* insertion-containing chromosomes are in pink, and the balancer chromosomes are in black. The mutation sites (red stars) are marked with either 1 or 2 to indicate the different alleles. All flies are in a w^- background, meaning the *P* insertions are the only source of m*w*. Shown in the gray box are *P* and F1 crosses. Shown in the green box are the nonrecombinant offspring. Shown in the yellow box are the possible recombination events, which are color-coded and labeled A, B, and C, corresponding to the F1 female, where the three types of recombination events are marked likewise.

N. Concluding remarks

This screen for defects in stem cell fate specification and proliferation was quite successful. Albeit not having carried out the screen to saturation and therefore isolating many mutants with only allele, which compromises mapping, we have been able to successfully identify novel phenotypes and map many of the genes responsible for them. Many of the mutants isolated had novel phenotypes such as increase in density of Dl+ cells without affecting differentiated cells or novel EC morphology phenotypes. Four complementation groups have been mapped to the locus responsible for the phenotype, *Gmd, spen, kis and aurB*, while one complementation group, 5-C-53 and 8-C-97, has been mapped to a genomic region containing few genes. For other candidates, further retesting and possible generation of more alleles will probably be required. Since the beginning of the screen we have improved our sensitivity to detection of phenotypes. Retesting the remaining unmapped candidates will probably be useful to prioritize which mutants should be further investigated and for which effort should be put into mapping.

For the continuation of my PhD I decided to further investigate the role of *Gmd* in regulating the cell fate decisions of the ISC lineage, which is presented in the next chapter of this thesis.

Chapter IV

The role of *Gmd* in limiting the stem cell pool in the adult fly intestine

Distinct thresholds of Notch activity for commitment and differentiation of stem cells in the adult fly intestine

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Summary

Tight regulation of self-renewal and differentiation of adult stem cells ensures that tissues are properly maintained. In the *Drosophila* intestine, both commitment, i.e. exit from self-renewal, and differentiation are controlled by Notch signaling. Here we show that commitment requires high Notch activity whereas differentiation can occur with lower Notch activity. We identify the gene *GDP-mannose 4,6-dehydratase* (*Gmd*), a modulator of Notch signaling, as being required for commitment but dispensable for differentiation. Loss of *Gmd* activity leads to increased symmetric, self-renewing stem cell divisions generating ectopic stem cells, together with proper asymmetric divisions resulting in enteroendocrine and enterocyte differentiation. Our work suggests that a high-threshold requirement for Notch activity safeguards the stem cells from loss through differentiation. Commitment through high-threshold signaling may represent a general mechanism to protect tissues from stem cell loss.

Results and Discussion

Intestinal stem cells (ISCs) are multipotent progenitors that replenish the gut during the lifetime of the adult fly as well as respond to induced tissue damage to promote tissue repair (Amcheslavsky et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Buchon et al., 2009b; Jiang et al., 2009). Each ISC divides to self renew and to produce a daughter cell (enteroblast; EB) that does not divide but differentiates into one of two types of cells: an enteroendocrine cell (ee) expressing the transcription factor Prospero (Pros) or an enterocyte cell (EC) expressing another transcription factor, Pdm1, and undergoing endoreplication to become a large epithelial cell (Figure 1A; (Lee et al., 2009b; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006)). Thus the ISC divides asymmetrically with respect of the fate of its daughter cells. Whether a wild-type ISC can also divide symmetrically to produce two stem cells is unknown.

The Notch ligand Delta is specifically expressed in ISCs (Ohlstein and Spradling, 2007). Upon division of the ISC, expression of the Notch ligand Delta is progressively restricted to one of the two resulting daughter cells. This cell keeps the ISC fate and activates the Notch receptor in its sister cell, the EB, thereby promoting expression of Notch target genes important for differentiation (Bardin et al., 2010a; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Loss of Notch signaling results in ectopic number of stem cells, ectopic number of ee cells and a loss of ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Moreover, the level of Delta expression in the ISC appeared to correlate with the fate of its recently specified daughter cell. This correlation led to the model that differential Notch signaling regulated the ee versus EC fates (Ohlstein and Spradling, 2007).

It is currently not well understood how the different fates of stem cell daughters are specified in the adult *Drosophila* intestine leading to one daughter stem cell and one daughter cell that

will exit this state and differentiate. To identify genes specifically implicated in regulating stem cell numbers, we conducted an EMS-based genetic screen (Supplementary Experimental Procedures and Supplementary Table 1). We identified two alleles of the gene *Gmd* (*GDP-mannose 4, 6-dehydratase*; Figure1B) required for the synthesis of GDP-fucose. Loss of *Gmd* resulted in more cells per clone and more ISCs per clone (Figure 1 C-J, O). *Gmd* mutant clones contained an average of 9.6 cells (+/-1.4 SEM) and 5.4 (+/-0.8 SEM) Delta+ cells per clone at 5 days (5 d) after heat shock (AHS) compared to 6.9 cells (+/-0.6 SEM) and 1.7 (+/-0.2 SEM) in wild-type control clones (Figure 1O). By three weeks AHS, guts were almost entirely composed of *Gmd* mutant tissue suggesting that mutant clones had a growth advantage over with heterozygous (*Gmd*/+) and wild-type cells, consistent with an increased number of stem cells being produced (Figure S1A-C"). Interestingly, the ability of cells to differentiate was unaffected: both Pros+ ee cells and polyploid Pdm1+ ECs were found at a wild-type density (Figure 1C-J, P; Figure S2A-F"). Thus while the number of Delta+ cells was largely increased, the specification and density of differentiated cells was unaffected. In addition, the overall tissue morphology appeared largely unaffected (Figure 1E, F, I, J). This *Gmd* phenotype was distinct from those previously reported for loss of Notch signaling components which strongly affect differentiation (Bardin et al., 2010a; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007) as shown here for *Ofut1* and *Su(H)* (Figure 1K-N; Figure S1; Figure S2E-F"). Additionally, loss of canonical Notch components led to a multilayering of ISCs and ee cells that was absent in *Gmd* mutant tissue (Figure 1I, J, M, N).

We then examined the number of dividing cells detected here as Phospho Histone H3 positive (PH3+) cells. Both the frequency of clones containing at least one PH3+ cell (15.8%; n=133 clones) and of PH3+ cells within clones (0.75%; n=2906 cells) were higher in *Gmd* mutant clones than in control wild-type clones (0.8%, n=244 clones, and 0.16%, n=2540 cells,

respectively; Figure 1Q, R). These data suggested that ectopic stem cells were produced in *Gmd* mutant clones, possibly via symmetric divisions, respective to cell fate, having produced two ISCs whereas proper differentiation also occurred via asymmetric cell divisions, each having produced an ISC and a cell that differentiated.

Symmetric and asymmetric divisions within the population of *Gmd* mutant cells could arise either from a single population of stem cells capable of switching between modes of division, or from two distinct populations, one dividing symmetrically and one dividing asymmetrically. Two distinct populations might result from gradual loss of *Gmd* function or its product, D-fucose (see below), within the mutant tissue. To distinguish between these possibilities, we conducted lineage analysis within *Gmd* mutant tissue. We used recombination of the *tubulin* promoter and *lacZ* gene to trace lineages as described in (Harrison and Perrimon, 1993) within wild-type or *Gmd* mutant clones that were marked by GFP expression using the MARCM system (Lee and Luo, 1999) (Figure 2A; see Experimental Procedures). A 4 d time-point was used for analysis to allow maximal time for lineages to produce differentiated cells while avoiding caveats of lineage fusion that occurred after 4 d (Experimental Procedures). At this early time point, approximately 60-70% of the wild-type and *Gmd* lineages contained only one of the two differentiated cell types, either ee or EC (Figure 2B). Importantly, 28% of wild-type (n= 25) and *Gmd* lineages (n = 39) contained both ee and EC cells and no *Gmd* lineages contained only Delta+ ISCs (Figure 2B-F"). These data suggested that ectopic ISCs did not result from a distinct symmetrically dividing subpopulation of Delta+ ISCs but rather from ISCs that were capable of switching between asymmetric and symmetric modes of division to produce properly differentiated progeny as well as expansion of the stem cell population.

Gmd is required for the biosynthesis of D-fucose that is used as a substrate of fucosyltransferases to modify lipids, glycans, and proteins. Consistent with this, the fucose-

dependent epitope recognized by the horseradish peroxidase (HRP) antibody (Seppo et al., 2003) was lost in clones of *Gmd* in a cell autonomous manner (Figure S2G, G'). Thus, in the midgut, D-fucose production requires *Gmd,* as in other contexts, and is not provided via gap junctions from neighboring cells as proposed in the wing epithelial cells (Okajima et al., 2008; Sasamura et al., 2007).

Gmd has been shown to be required for the *O-fucosyltransferase-1* (*Ofut1*) -dependent modification of the Notch receptor (Okajima et al., 2005; Sasamura et al., 2007). Consistent with the Notch receptor being the relevant fucosylation target and with *Gmd* acting upstream of intracellular Notch activation, we found that the expression of nuclear activated Notch suppressed the formation of ectopic Delta+ ISCs in the *Gmd* mutant and resulted in differentiation into EC cells (Figure 3A, B). We next examined whether the enzymatic activity of Ofut1 was similarly required to limit the ISC pool. Ofut1 has two distinct molecular activities: a protein fucosyltransferase activity that modifies Notch EGF repeats with fucose and a chaperone function (Okajima et al., 2008; Okajima et al., 2005). The fucosyltransferase function is required for further modification by the N-acetylglucosaminyltransferase Fringe that biases Notch ligand specificity, whereas the chaperone activity is required for the proper folding and secretion of the Notch receptor, essential in all Notch signaling contexts. We tested the requirements of these two functions on specification of stem cells and differentiation. To separate these two functions, we examined $Ofut1^{4R6}$ clones (genetic null lacking both activities) and null clones in which we expressed the mutant protein OFUT1R245A that retains chaperone activity but has lost fucosyltransferase activity (Okajima et al., 2008; Okajima et al., 2005). Loss of both chaperone and fucosyltransferase functions led to an increase of Delta+ cell and Pros+ ee cells and to a loss of ECs (Figure 3C-D'). By contrast, the specific loss of fucosyltransferase activity resulted in increase in Delta+ stem cell-like cells without affecting the differentiation

of Pros+ ee cells and ECs (Figure 3E-F'). This phenotype is similar to the one associated with the loss of *Gmd*. We conclude that the role of *Gmd* in the midgut is to produce D-fucose that then serves as a substrate for Ofut1.

Work in mammalian cells has demonstrated that fucosylation of Notch increases its capacity to signal, likely through increased binding to its ligand Delta (Chen et al., 2001; Stahl et al., 2008). However, since fucosylated residues of Notch can be further modified in some developmental contexts by the glycosyltransferase Fringe, a modification that also modulates ligand binding (Bruckner et al., 2000; Moloney et al., 2000a; Panin et al., 1997), we tested the possible role of Fringe in the intestine. ISC specification and cell differentiation in *fringe* clones were indistinguishable from wild-type clones (Figure 3G-H'). Therefore, Ofut1-mediated fucosylation affects a process in the intestine that does not require *fringe*, in contrast to previously described roles of *Gmd* and *Ofut1* during *Drosophila* development (Okajima et al., 2008; Okajima et al., 2005; Sasamura et al., 2007). We found no gross change in the Notch protein expression in *Gmd* mutants (Figure S3). Thus, we propose that loss of *Gmd* likely affects Notch signaling independently of Fringe possibly by adding fucose on Notch that is not further glycosylated, thereby influencing the interaction of Notch with Delta (Stahl et al., 2008; Zhou et al., 2008).

We next assessed nuclear Notch activity in *Gmd* mutant cells using the reporter Su(H)GBE-lacZ (Furriols and Bray, 2001) and found that Notch was active in *Gmd* mutant cells (Figure S4A-C'''). The Su(H)GBE-lacZ signal was then quantified in cells with small nuclei (3-5μ) that correspond to ISCs, EBs, undifferentiated ECs and ees (Figure S4D; see also Experimental Procedures). First, we found that 5 d *Gmd* clones contained more Delta+ βGal low cells than wild-type clones (Figure S4F). These cells likely correspond to ISCs that are increased in number in *Gmd* mutant clones. Second, *Gmd* and wild-type clones contained similar percentages of Delta- βGal high cells (Figure S4E). These cells likely correspond to

differentiating EBs. This result is consistent with our finding that differentiation is unaltered in *Gmd* mutants. Third, we observed a three-fold increase in a population of Delta+ cells that were βGal high in *Gmd* mutant clones (Figure S4E). These cells co-expressed Delta, an ISC marker, and Su(H)GBE-lacZ, an EB marker, and may represent cells that have not yet accumulated high enough levels of Notch signaling to exit self-renewal and differentiate. Together, these data are consistent with *Gmd* being required to reach a critical threshold of Notch signaling within a given time window in the stem cell daughter in order for commitment to occur properly. Failure to reach the critical threshold would result in a symmetric division self-renewing both daughter cells into ISCs.

We then wanted to test the hypothesis that high Notch activity is required for the self-renewal / commitment choice whereas low Notch activity is sufficient for the ee and EC differentiation. To do so, we examined the effects of progressively reducing Notch activity in a *rumi* null mutant background in which Notch activity is temperature-dependent: at 18°C, $rumi^{44}$ cells have nearly wild-type levels of Notch activity whereas at 28°C they exhibit little if any Notch signaling activity (Acar et al., 2008). In the intestine, we found that at 18ºC, $rumi^{44}$ mutant tissues were indistinguishable from wild-type controls suggesting that ISC self-renewal and EB differentiation occurred normally (Figure 4A, A', G-I; note that 10 d clones have often fused). However, at 21ºC and 22ºC, ISC commitment but not EB differentiation was affected: $rumi^{44}$ mutant tissue had ectopic Delta+ ISC-like cells whereas the ratio of differentiated ee to EC cells was unaffected like in Gmd^{H78} tissue (Figure 4B-C', G-I). Thus, the self-renewal / commitment choice appeared to be specifically affected whereas the ee / EC differentiation choice was not detectably altered. At 23°C, many ectopic Delta+ cells and a very mild increase in the ratio of ee to EC cells were observed. Nevertheless, most ee cells appeared as pairs, as in wild-type, and ECs were normally specified (Figure 4D-D', G-I). At 25ºC, both ISC commitment and differentiation were strongly affected: a large increase in

Delta+ cells was detected and the ratio of ee to EC was strongly increased, resulting from ectopic Pros+ ee cells. At 28°C, large polyploid ECs were no longer observed (Figure 4E-I). These data strongly suggest that the self-renewal / commitment decision requires a high level of Notch activity whereas the ee / EC differentiation choice can occur properly with reduced levels of Notch activity.

Previous work based on retrospective clone analysis has suggested that levels of Delta in the ISC regulate the fate, ee or EC, of its differentiating daughter cells: ISCs with high Delta levels would produce ECs whereas ISCs with low Delta levels would generate ees. (Ohlstein and Spradling, 2007). Surprisingly, our data reveal that the self-renewal / commitment decision can be uncoupled from the ee / EC differentiation decision. We propose that EBs first receive a high, fucose-dependent, Notch commitment signal to exit self-renewal and that EB differentiation into ee or EC can occur via low Notch activity (Figure S5). It has also been proposed that the EC fate, but not the ee fate, is dependent on Notch signaling (Beebe et al., 2010; Micchelli and Perrimon, 2006). Our data are also consistent with this model that does not explain, however, how ISCs differentiate into ees. Importantly, our model proposes a simple mechanism that acts to ensure that ISCs are not lost via weak differentiation signals that may promote ee fate.

Additionally, our data reveal that *Gmd* is required to promote asymmetric cell fate acquisition of ISC daughter cells and suggest that *Gmd* mutant ISCs can switch between asymmetric and symmetric modes of division. One possible mechanism regulating this switch of division modes could be the length of the time window available for the cell fate decision to occur. The time window may correspond to a particular cell cycle phase, G1 for example. Proliferative signals coming from the surrounding tissue may influence the rate of passage to the next cell cycle phase, thereby regulating the length of this time window. If Notch levels are not high enough when proliferative signals are received, as a consequence of

the loss of *Gmd* for instance, the daughter cell may self-renew as a stem cell, undergoing a symmetric division (Figure S5). Temporal integration of differentiation signals within a window of the cell cycle has been proposed to underlie differentiation decisions in cell lines (Mummery et al., 1987), ES cells (Filipczyk et al., 2007), in mammalian neural precursors (Calegari and Huttner, 2003; Lange et al., 2009) and in the *Drosophila* male germ line (Insco et al., 2009) (reviewed in (Singh and Dalton, 2009) (Salomoni and Calegari, 2010)).

In summary, our work indicates that one strategy for fine-tuning Notch levels and altering fate acquisition is through enzymatic pathways impacting fucose levels and glycosylation events. Our work also shows that commitment of ISCs requires a high threshold Notch signal. This could represent a general strategy whereby the stem cell needs to cross a high signaling barrier for differentiation to occur, possibly providing insurance that a stem cell pool is not lost. It will be important to determine whether this model is a general feature of stem-daughter cell decisions.

Experimental Procedures

Drosophila stocks and Clonal analysis

Adult flies were kept in freshly yeasted tubes, changed every 2 d. Adults were kept at 25°C except for Figure 4 where the indicated temperatures were used. The Mosaic Analysis with Repressible Cell Marker (MARCM) technique (Lee et al., 2000) was used to positively mark mitotic clones by nuclear GFP expression, using stocks previously described (Bardin et al., 2010a). This technique was combined with the LacZ lineage labeling technique (Harrison and Perrimon, 1993) using the following genotypes: *y w P[hs-FLP] P[pTub-GAL4] P[UASnlsGFP] / yw ; X-15-29 FRT40A P[l(2)35Bg+] / X-15-33 FRT40A P[pTub-GAL80]* ("wild-type MARCM + LacZ lineage") and *y w P[hs-FLP] P[pTub-GAL4] P[UASnlsGFP] /*

yw ; X-15-29 FRT40A P[l(2)35Bg+] Gmd^{H78} / X-15-33 FRT40A P[pTub-GAL80] ("*Gmd* MARCM + LacZ lineage").

For the double labeling of clones with GFP and LacZ flies were first heat shocked 3 d after eclosion (10 minutes, 36.5°C) and were heat shocked again 5 d later (25 minutes at 36.5°C). To assess the frequency of double-labeled clones occurring with only one heat shock, both wild-type MARCM + LacZ lineage and *Gmd* MARCM + LacZ lineage flies were heat shocked once 3 d after eclosion (10 minutes, 36.5°C), and characterized at 5 d AHS. The number of single labeled clones (GFP+ only or LacZ+ only) and double labeled (GFP+ and LacZ+) clones were counted: 7.8% of wild-type clones were double positive while 12.8% of the *Gmd* MARCM + HP clones were double positive. Thus, while occurring at low frequency, double labeling of an ISC can occur with only one heat shock event. Therefore, we scored only double-labeled LacZ+ clones that where clearly inside a larger GFP+ clones. A 4 d time-point (after 2nd heat shock) was used for analysis, allowing maximal time for clones to produce differentiated cells. *Gmd* clones grown more than 4d (after 2nd heat shock) were often fused, thus we limited our analysis to 4 d time-point.

The following alleles and fly stocks were used in this study: *Gmd^{8A9}, Gmd^{11C87}* (this study), *Gmd^{H78}*(Sasamura et al., 2007), *fringe^{13}* (Irvine and Wieschaus, 1994), *Ofut1^{4R6}*, UAS-OFUT1R245A (Okajima et al., 2005), *kuzbanian^{ES24}*, (Li and Baker, 2001), *rumi^{44}*(Acar et al., 2008), *Su(H)$^{\Delta 47}$* (Morel and Schweisguth, 2000), Su(H)GBE-LacZ (Furriols and Bray, 2001).

Quantification:

The total number of cells and the Delta positive cells were quantified in Figure 1O by counting the number of GFP+ and Delta+ cells per clone at 5 d AHS, respectively, in all of the "stem cell clones", i.e. clones containing 2 or more cells, of the posterior midgut of at least

three different flies. Note that for this analysis, we excluded two very large clones that likely occurred as a result of leaky clone inductions at an earlier developmental stage. The full data set, however, of number of cells per clone is presented in Figure S1D. Phospho Histone H3 positive cells were quantified in Figure 1Q and 1R by counting the number of PH3+ cells per clone and all of the cells in the clone (for 1R) in all the clones at 5 d AHS of the posterior midgut of at least three different flies. In Figure 4G, the percentage of Delta+ cells per GFP+ cell was assessed in clonal tissue at 10 d AHS since individual clones were impossible to determine accurately due to clone fusion. At least 3 representative clonal regions were counted per midgut of three different flies.

The density of ee and ECs in both Figures 1P and 4H was quantified by counting the number of Pros+ cells, ECs and clone area using ImageJ. The nuclei were segmented and identified by applying a dynamic Otsu thresholding technique and counted with the "Nucleus counter" plugin in ImageJ. We applied this method to each clone by analyzing 2D optical sections separated by 4 μm in depth. The number of Pros+ nuclei (ee cells) and ECs (DAPI+ nuclei with diameter ≥7μm) were counted automatically. Nuclei were classified as having a diameter superior or inferior to 7μm, determined empirically as having a pixel area ≥ or <170, respectively. The clone area was then quantified by measuring the area of the clone (as marked with anti-GFP antibody) with ImageJ. The densities of ee cells, ECs, as well as the ratio between the two cell types was then determined. A 10 d time-point was used to quantify differentiation to ensure that the tissue was at homeostasis.

The same data set used in Figure 1 for *wild-type* controls and *Gmd* mutants is presented again in Figure 4 for reference purposes.

For Figure 2 B, a 4 d time-point after the second heat shock was used to allow cells within clones time to differentiate. Lineages with greater than 2 cells and containing at least one Delta+ cell were included in the analysis and the cell composition of each clone was

determined. ISCs were identified as Delta+ cells, ee cells as Pros+, ECs were identified as cells with a DAPI+ nuclei with diameter ≥7μm, and EBs as Delta- cells less than 7μm nuclear diameter.

In Figure S4D-F the pixel intensity of βGal signal (Su(H)GBE-LacZ) in absence of Notch signaling *kuzbanian*^{ES24} mutant clones (Figure S4C) was assessed using intensity profiling from Image J and we concluded that <30 βGal units corresponds to background βGal signal ("βGal low" data not shown). Figure S4E a z-test of proportions was used in which significant differences have epsilon values greater than 1.96 for 95% confidence levels. The difference in proportion of Delta- βgal high cells between *Gmd* mutant and wild-type cells was not significant (epsilon=0.22). In contrast, the difference in proportion of Delta+ βgal high was very significant (epsilon=3.00).

Immunofluorescence

Fixation protocol described by (Lin et al., 2008) was used except for Figure1 E, I , M and Sup Figure 2 where the fixation protocol described in (Bardin et al., 2010a) was used. The following antibodies were used: mouse anti-Delta (ascites, 1/2000, Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Pros (1/10; DSHB), rabbit anti-Pros (1/2000; YN Jan), goat anti-βGal (1/500; Biogenesis), rabbit anti-Pdm1 (1/1000; W. Chia), rabbit anti-GFP (1/1000, Molecular Probes), anti-HRP (1/1000; J-R Martin), rabbit anti-PH3 (1/2000; Upstate).

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Figure legends:

Figure 1: Loss of *GDP-mannose 4, 6- dehydratase* (*Gmd*) specifically increased stem cells numbers

(A) The intestinal stem cell (ISC) self-renews and produces a post-mitotic enteroblast daughter cell (EB). The EB subsequently differentiates into either an enterocyte (EC, gray) or an enteroendocrine cell (ee, blue).

(B) Structure of *Gmd* mutants isolated in the EMS screen (Gmd^{8A9} contains an S177F mutation and Gmd^{11C87} contains an E332K mutation) and the H78 deletion (Sasamura et al., 2007).

(C-E) Wild-type clones at 10 d AHS (GFP+) contained Delta+ ISCs, Pros+ ee cells and Pdm1+ ECs. Tissue organization in cross-section of a wild-type clone. Inset shows low magnification cross-section view.

(G-I) Gmd^{H78} mutant clones at 10 d (GFP+) contain increased numbers of Delta+ ISCs but normal numbers of Pros+ ee cells and Pdm1+ ECs. Tissue organization shown in cross-section (I) is like that of wild-type but contains extra small basal Delta+ cells. Inset shows low magnification cross-section view.

(K-M) Loss of canonical Notch signaling ($Ofut1^{4R6}$ mutant shown, GFP+) leads to increased numbers of Delta+ ISCs, increase numbers of Pros+ ee cells and loss of Pdm1+ ECs. Tissue organization shown in cross-section (M) is highly disrupted leading to multilayering. Inset shows low magnification cross-section view.

(F, J, N) Cartoon of cross-section of wild-type (F) Gmd^{H78} mutant (J) and canonical Notch mutant (N). ISCs are represented in red, EBs in white, ee cells in blue and ECs in gray.

(O) *Gmd*^{H78} mutant stem cell clones (5 d) contain more cells (green) and Delta+ cells (red) than wild-type stem cell clones. Data are represented as mean +/- SEM in this figure and following figures.

(P) *Gmd*^{H78} mutant tissue (10 d) contain similar densities of Pros+ ee cells (blue) and large nuclear size ECs (gray).

(Q-R) *Gmd*^{H78} clones (5 d) contain more dividing cells per clone (phosopho -Histone H3, PH3+) (Q) and a higher percent of total dividing cells than wild-type (R). Scale bars are 50 μm in C, G and K and 10μm in D, E, H, I, L and M.

Figure 2: *Gmd* ectopic stem cells are multipotent

(A) Lineage marking strategy: heat shock induced recombination created wild-type or Gmd-/- MARCM clones marked by GFP expression as well as βGAL marked lineages; a second heat shock after 5 d was used to induce a second set of recombination events. Lineages were selected for analysis in which βGAL+ GFP+ lineages were completely surrounded by GFP+ cells.

(B) Composition of *Gmd*^{H78} and wild-type βGAL+ lineages at 4 d. Lineages were scored for the cell type present. *Gmd*^{H78} and wild-type had approximately the same percent of lineages that contained both differentiated ee cells and ECs.

(C-F") Examples of βgal+ lineages in wild-type and *Gmd*^{H78} as described in (A) βgal clone within GFP clone is outlined. Both wild-type and *Gmd*^{H78} mutant ISC lineages produced ee cells and ECs however, *Gmd*^{H78} lineages also expanded the ISC pool (Delta+ cells). Scale bars are 50 μm in C and E and 10μm in D and F.

Figure 3: Ofut1 glycosyltransferase activity but not that of Fringe is required to limit the stem cell numbers

(A-B) Expression of the activated nuclear Notch intra cellular domain (NICD) in wild-type or *Gmd*^{H78} MARCM clones (GFP+) promoted differentiation into ECs (large nuclei).

(C-H') MARCM clones (GFP+, outlined) of *Ofut1*^{4R6} removing chaperone and catalytic activity (C-D'), catalytic activity alone (E-F'; *Ofut1*^{4R6} with rescued chaperone activity by expression UAS-OFUT1R245A) and *fringe*^{13} (G-H'). Reduction of Ofut1 catalytic activity alone, led to extra ISCs (Delta+) whereas differentiated cells were unaffected (ee cells, Pros+; and ECs, large nuclei). Loss of *fringe* did not affect ISCs (Delta+ cells) or differentiation (ee, Pros+; ECs large nuclei).

Scale bars are 50 μm in C, E and G and 10μm in A-B, D, F, H.

Figure 4: Modulation of Notch signaling using *rumi* mutants reveals high level signaling requirement for efficient commitment to differentiation

(A-F') Modulation of Notch activity in *rumi*^{44} clones (GFP+) at temperatures from 18°C - 28°C. Assessment of ISCs (Delta+) and differentiated cells (ee cells, Pros+; ECs large nuclei). Adults were aged an equivalent of 10 d at 25°C, note that at this time point clones were often fused.

(G) Quantification of the ISCs (Delta+ cells) as a percent of total cells in clone tissue. *Gmd*^{H78} mutant tissue at 10 d had an average of 46% ISCs compared to 22% in control clones at 25ºC. Wild-type clones as controls are shown both at 18°C and 25°C, note the percentage of Delta+ cells is slightly higher at 18°C than at 25°C. n=43 (clones), wild-type 25°C; n=13, *Gmd*^{H78}; n=11, wild-type 18°C; n=11, *rumi*^{44} 18°C; n=16, *rumi*^{44} 21°C; n=23, *rumi*^{44} 22°C; n=14, *rumi*^{44} 23°C; n=15, *rumi*^{44} 25°C; n=9, *rumi*^{44} 28°C.

(H) Quantification of the density of large nuclei ECs and Pros+ ee cells in wild-type, *Gmd*^{H78} and *rumi*^{44} mutant clones at the indicated temperatures.

(I) The ratio of ee cells to ECs in wild-type control and *rumi*^{44} mutant clones at the indicated temperatures was assessed.

For H and I: n=27 (clones), wild-type 25°C; n=19, Gmd 25°C; n=15, *rumi*^{44} 18°C; n=14, *rumi*^{44} 21°C; n=18, *rumi*^{44} 22°C; n=24, *rumi*^{44} 23°C; n=19 *rumi*^{44}, 25°C; n=9, *rumi*^{44} 28°C. Scale bars are 10μm in A-F.

Figure 1

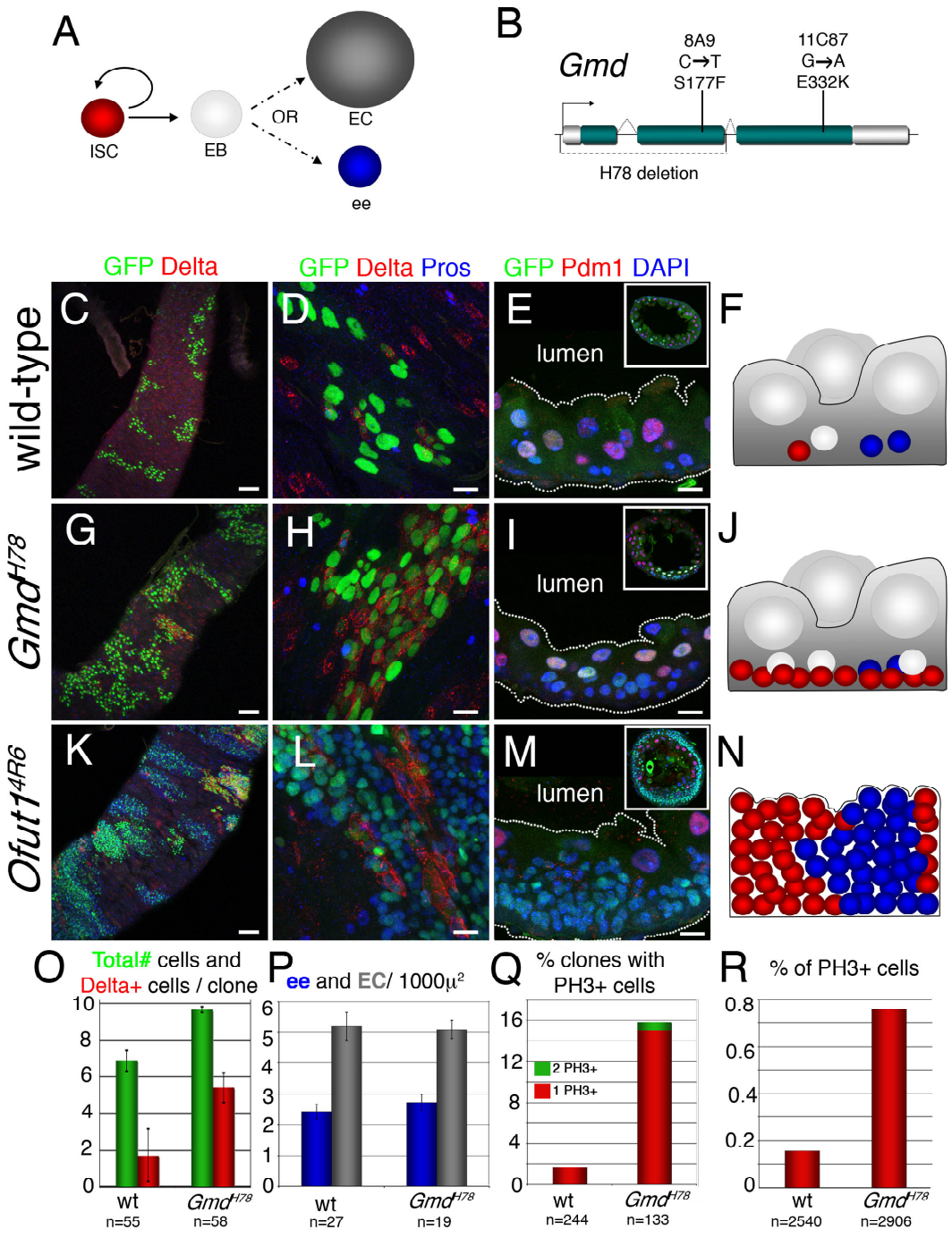
A
ISC
EB
OR
EC
ee
B
Gmd
8A9
C→T
S177F
11C87
G→A
E332K
H78 deletion
GFP Delta
GFP Delta Pros
GFP Pdm1 DAPI
wild-type
Gmd^H78
Ofut1^4R6
C
D
E
F
G
H
I
J
K
L
M
N
lumen
O
Total# cells and
Delta+ cells / clone
P
ee and EC/ 1000μ²
Q
% clones with
PH3+ cells
2 PH3+
1 PH3+
R
% of PH3+ cells
wt
n=55
Gmd^H78
n=58
wt
n=27
Gmd^H78
n=19
wt
n=244
Gmd^H78
n=133
wt
n=2540
Gmd^H78
n=2906

Figure 2

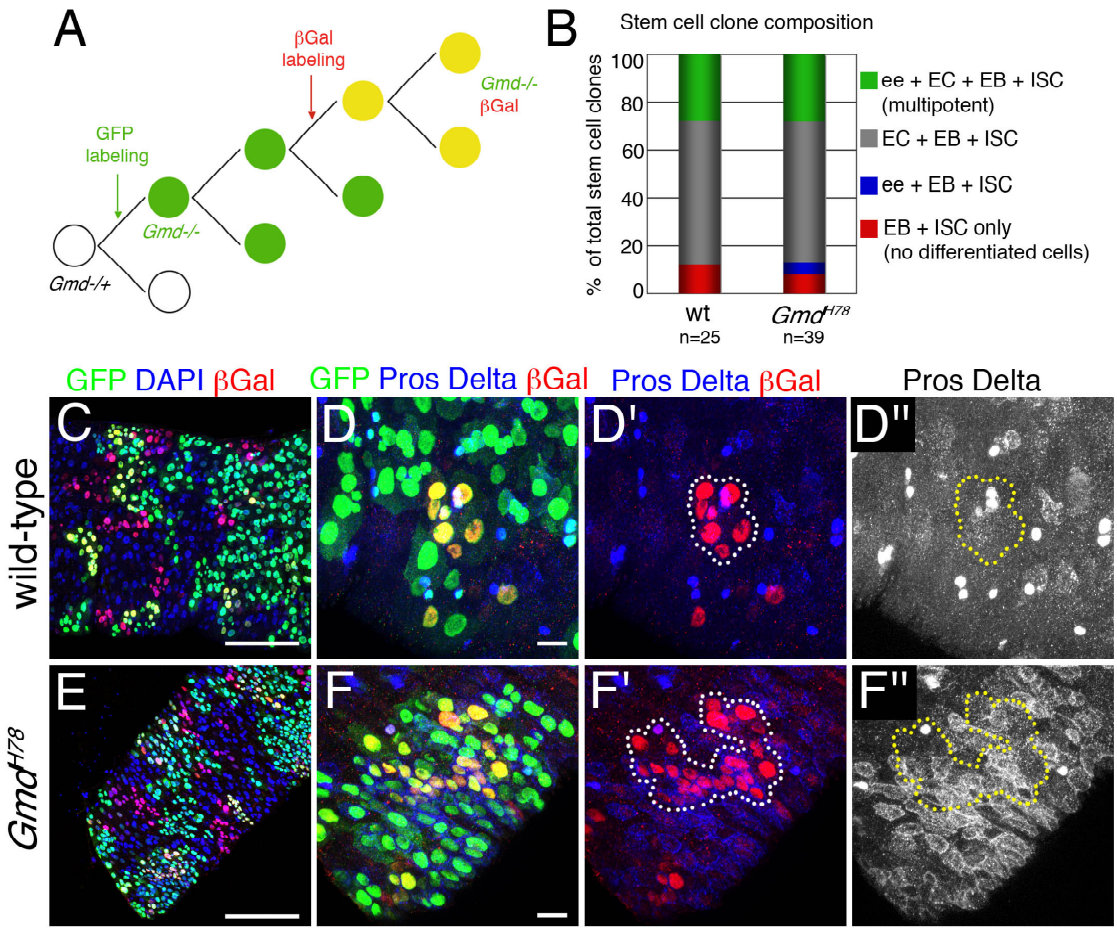
A
βGal labeling
Gmd-/- βGal
GFP labeling
Gmd-/-
Gmd-/+
B
Stem cell clone composition
% of total stem cell clones
100
80
60
40
20
0
wt
n=25
Gmd^H78
n=39
ee + EC + EB + ISC (multipotent)
EC + EB + ISC
ee + EB + ISC
EB + ISC only (no differentiated cells)
GFP DAPI βGal
GFP Pros Delta βGal
Pros Delta βGal
Pros Delta
C
D
D'
D"
E
F
F'
F"
wild-type
Gmd^H78

Figure 3

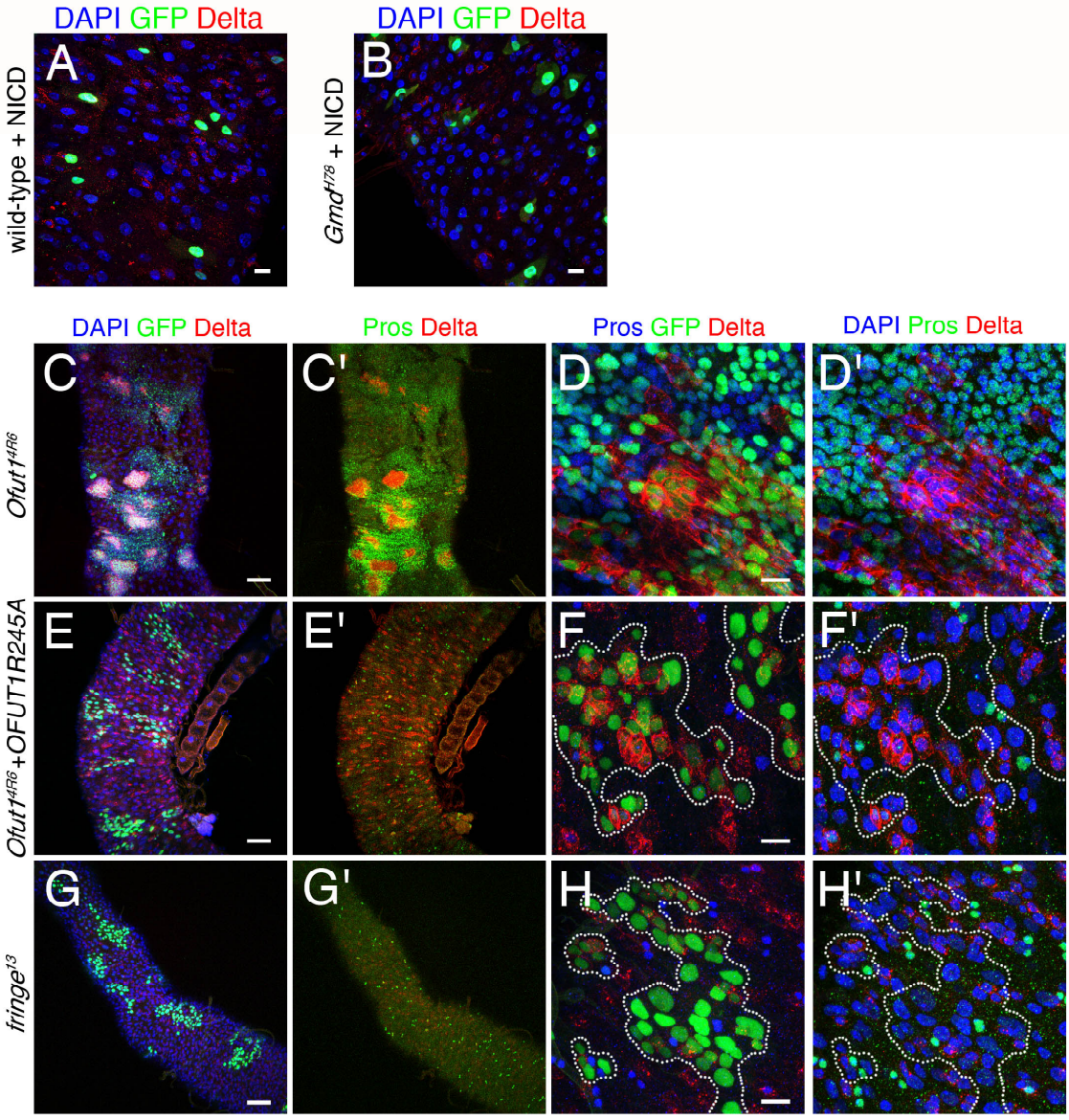
DAPI GFP Delta
DAPI GFP Delta
wild-type + NICD
Gmd^f78 + NICD
A
B
DAPI GFP Delta
Pros Delta
Pros GFP Delta
DAPI Pros Delta
Ofut1^4F6
Ofut1^4F6+OFUT1R245A
fringe^13
C
C'
D
D'
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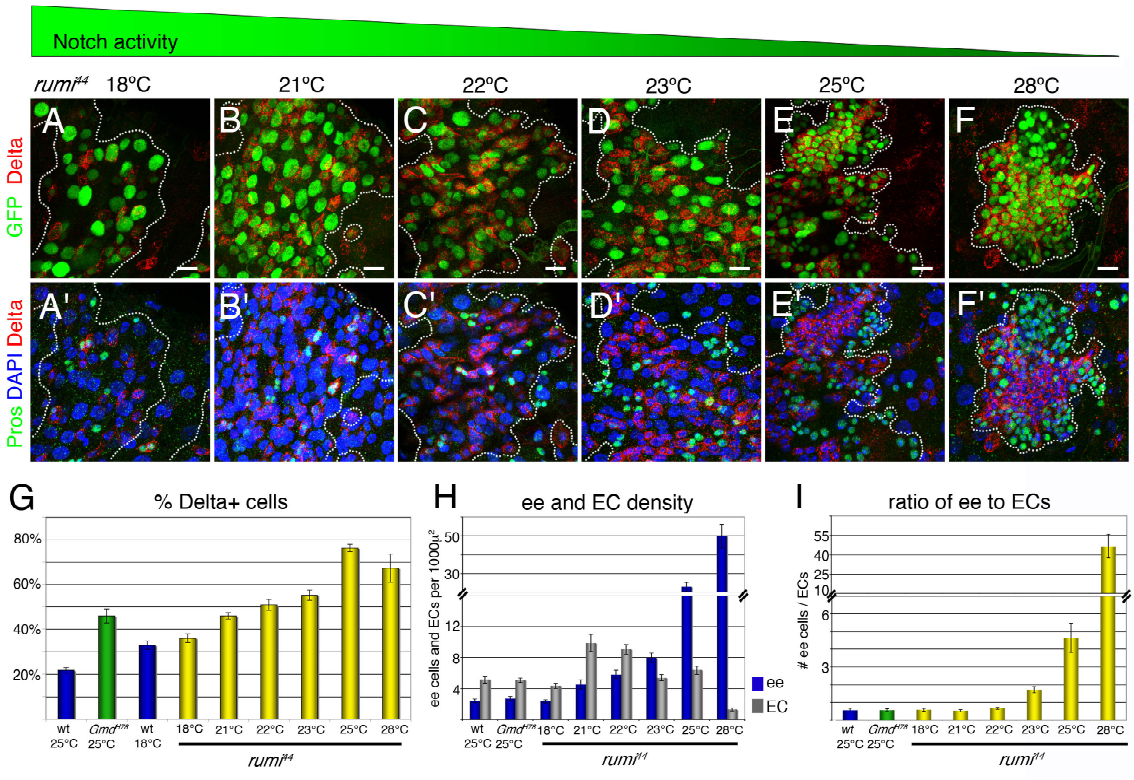
Figure 4


Notch activity
rumi#4 18°C
21°C
22°C
23°C
25°C
28°C
A
B
C
D
E
F
GFP Delta
A'
B'
C'
D'
E'
F'
Pros DAPI Delta
G
% Delta+ cells
80%
60%
40%
20%
wt 25°C
Gmd#7/+ 25°C
wt 18°C
18°C
21°C
22°C
23°C
25°C
28°C
rumi#4
H
ee and EC density
ee cells and ECs per 1000µ²
50
30
12
8
4
ee
EC
rumi#1
I
ratio of ee to ECs
ee cells / ECs
55
40
25
10
6
3
rumi#1

Supplementary Figures

Figure S1: Characterization of *Gmd*^{H78} mutant clones

(A-C''') MARCM clones of wild-type, *Gmd*^{H78} and *Su(H)*$^{\Delta 47}$. Clones at 3 weeks AHS (outlined; many clones are fused at this time point). *Gmd*^{H78} mutant tissue had a dramatic increase in the number of ISCs (Delta+) compared to wild-type tissue, although differentiated cells were still properly specified (ee cells, Pros+). In addition, *Gmd*^{H78} mutant tissue comprised a majority of the gut tissue. Loss of canonical Notch signaling components (*Su(H)*) led to increased numbers of ISCs, increased numbers of ee cells and loss of ECs cells. Scale bars are 50 μm in A-C.

(D) Quantification of clone size at 5d analyzed as in Fig. 1O. We excluded from the analysis in Fig. 1O two outlying data points (in red) of the wild-type clones that correspond to two very large clones that likely resulted from early recombination events occurring during development due to leaky flipase expression.

Figure S2: Differentiation of enteroendocrine cells and enterocytes is unaffected in *Gmd*^{H78} mutant clones.

(A-F'') 10 d AHS MARCM clones (GFP+) of wild-type, *Gmd*^{H78} and *Su(H)*$^{\Delta 47}$ mutants. *Gmd*^{H78} mutant clones have properly specified ee cells (Pros+) and ECs (Pdm1+) like wild-type clones (A-B'') as opposed to loss of canonical Notch signaling (*Su(H)* shown E-F'') that have a large increase in ee cells and a loss of ECs.

(G-G') *Gmd*^{H78} mutant clones (GFP+) lack reactivity for the fucosylation-dependent epitope recognized by anti-horse radish peroxidase antibodies (HRP), in a cell-autonomous manner, indicating that fucose is absent in *Gmd*^{H78} mutant tissue and is not provided via gap junctions from neighboring cells.

Scale bars are 50 μm in A, C, E and 10μm in B, D, F, G.

Figure S3: Notch localization in *Gmd* mutants

(A-B") Wild-type and Gmd^{H78} mutant cells (GFP+) both express Notch. No gross defects in Notch localization were detected. Scale bars are 10μm in A-B.

Figure S4: Notch reporter activation in Gmd^{H78} mutant clones

(A-C"') The Notch signaling transcriptional reporter (Su(H)-GBE-LacZ; βGal+) was activated in cells of wild-type and *Gmd* mutant clones, but not that of the canonical Notch signaling component *kuzbanian (kuz)*. Clones marked by nuclear GFP.

(D) The relative % of the total population of small cells (3-5μ) that are Delta-βGal high (dark blue), and Delta- βGal low (light blue), Delta+ βGal high (yellow) and Delta+ βGal low (red) in wild-type and Gmd^{H78} mutant.

(E) The difference in the % of Delta- that are βgal high (dark blue) between wild-type and Gmd^{H78} was not significant (n.s.) whereas the difference in the % of the Delta+ cells that are βgal high (yellow) between wild-type and Gmd^{H78} was very significant.

(F) The number of Delta+ βgal low cells (red) per clone was greater in Gmd^{H78} than in wild-type clones. In addition, Gmd^{H78} contained greater numbers of Delta+ βgal high (yellow) per clone.

Scale bars are 10μm in A-C.

Figure S5: Model for high-threshold signal to exit self-renewal

A high-threshold Notch signaling barrier needs to be crossed in order for ISCs (red) to exit the self-renewing state and become committed (white). Inability to reach this high threshold either by loss of *Gmd* or reduction of Notch signaling levels results in an increase in the number of symmetric ISC divisions producing two ISCs. Attaining threshold level signaling

likely must be achieved within a given time frame or cell cycle state. However, differentiation of committed EBs into ee (blue) or EC (gray) cells can occur with lower levels of Notch signaling and does not require *Gmd*. A requirement for high-threshold signaling may ensure that the stem cell pool is protected and not lost through weak differentiation signals.

Supplementary Experimental Procedures

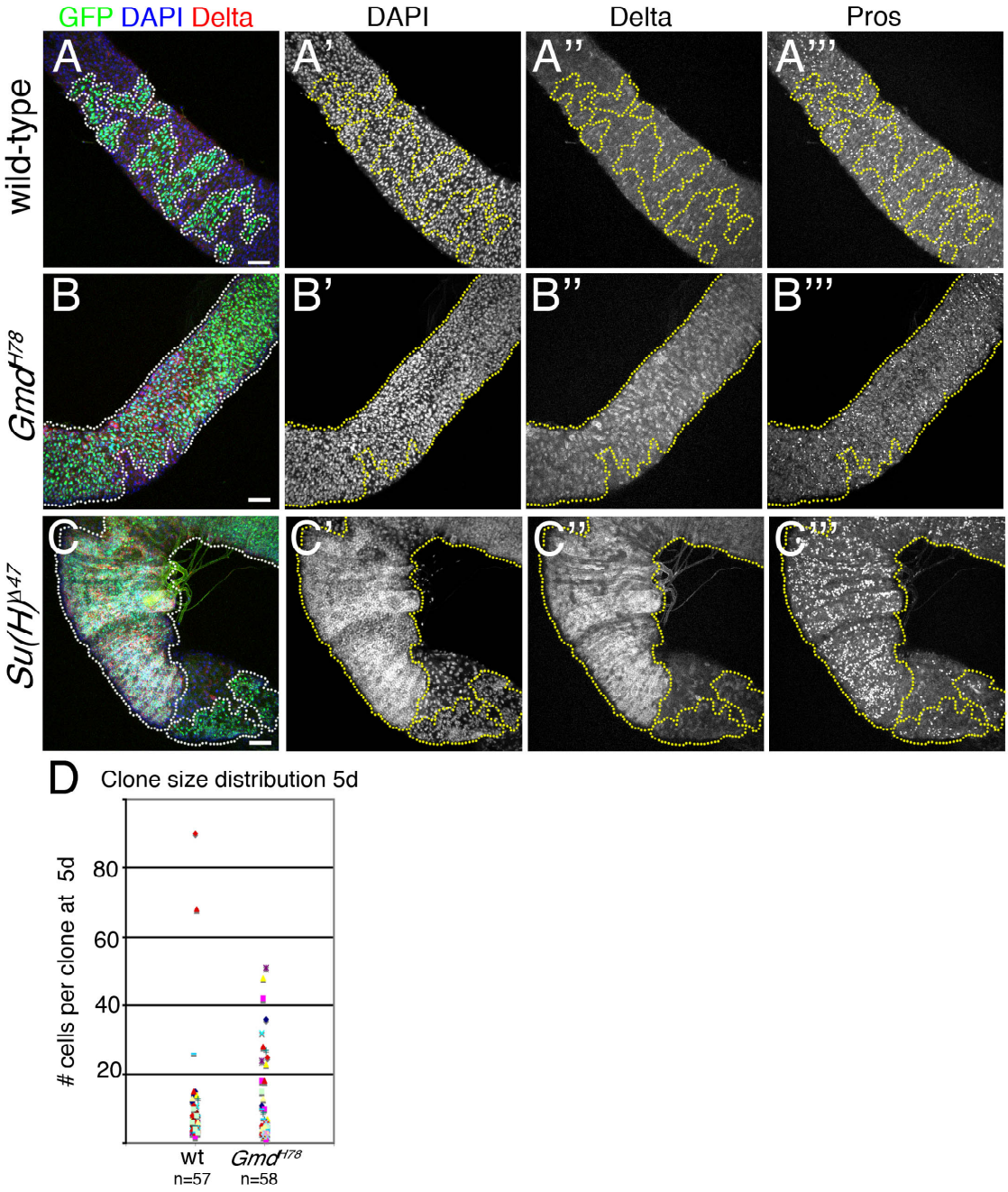
EMS screen

We used the chemical mutagen ethyl-methanesulfonate to induce germline mutations in w; FRT40A males using standard methods. w; FRT40A * (where * indicates the potential mutation) were then crossed in batch to w; In(2LR) *Gla* /Cyo *twistGal4 UAS-GFP* virgin females. F1 males of the genotype w; FRT40A */ Cyo *twistGal4 UAS-GFP* were then individually crossed with 5 virgin females of the genotype: *yw hsFLP tub-GAL4 UAS-nlsGFP*; FRT40A *tub-GAL80*. Dry yeast was added to tube containing pupae and at approximately 3 d post-eclosion, F2 adults of the correct genotype were sorted. Adults of the genotype *yw hsFLP tub-GAL4 UAS-nlsGFP*; FRT40A */ FRT40A *tub-GAL80* were transferred to new vials containing dry yeast and heat shocked 2X, 45 minutes with a 18 hour resting period between heat shocks. After 1 week, 3-5 adult females were dissected, stained with DAPI and screened visually using an epifluorescent microscope. Lines were tallied only if more than one gut contained clones. We identified lines producing an increased number of diploid nuclei (potentially ISCs) and a loss of diploid nuclei (potential ISC loss). To recover mutagenized chromosomes, males of the genotype *w*/Y or /*yw hsFLP tub-GAL4 UAS-nlsGFP*/Y ; FRT40A */ FRT40A *tub-GAL80* were backcrossed to virgin females w; In(2LR)

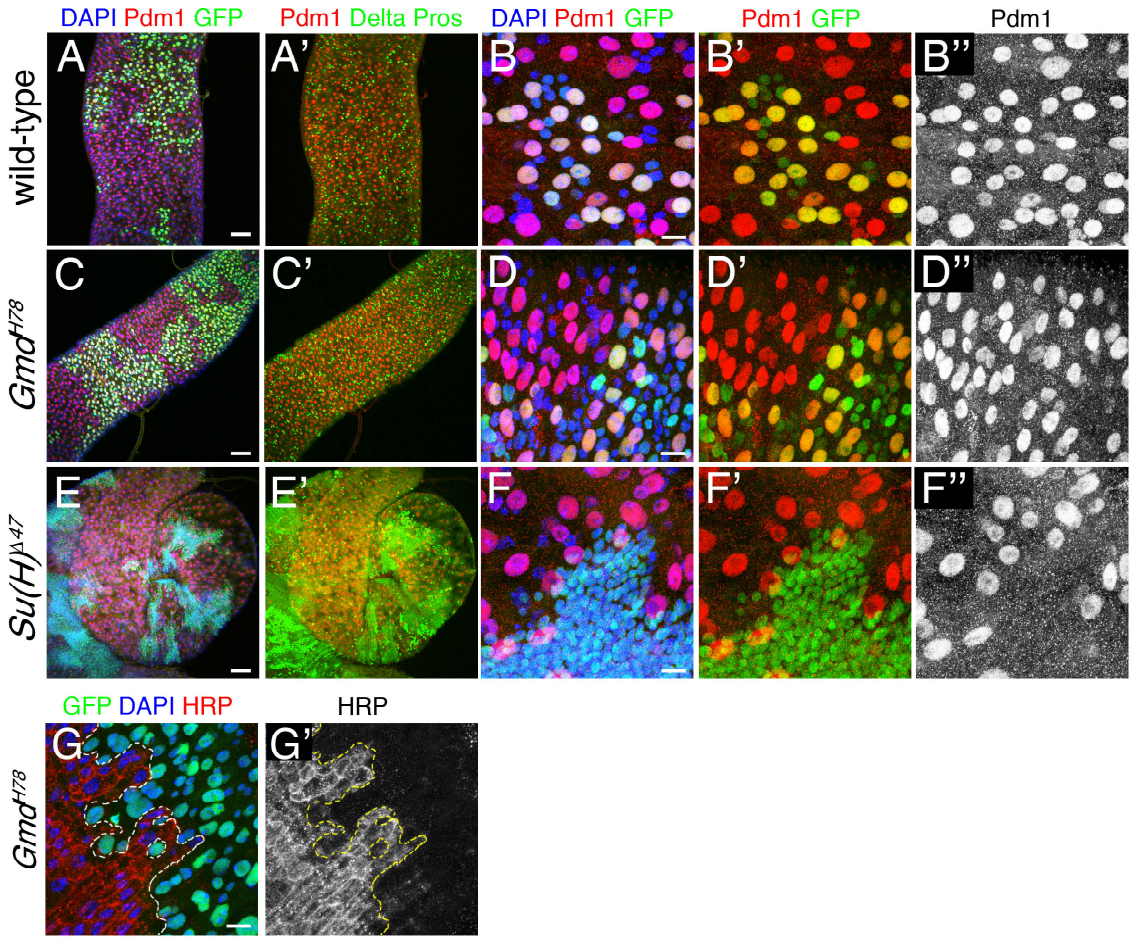
Gla /Cyo *twistGal4 UAS-GFP*. Established stocks were then recrossed and screened by immunofluorescence using anti-Delta (ISCs) and anti-Pros (enteroendocrine cells).

Supplementary Table 1: Summary of EMS screen for ectopic ISCs and ISC loss			
Lines screened: 4238			
Phenotypic categories:	ectopic ISCs	ectopic enteroendocrine cells	ISC loss
Complementation groups			

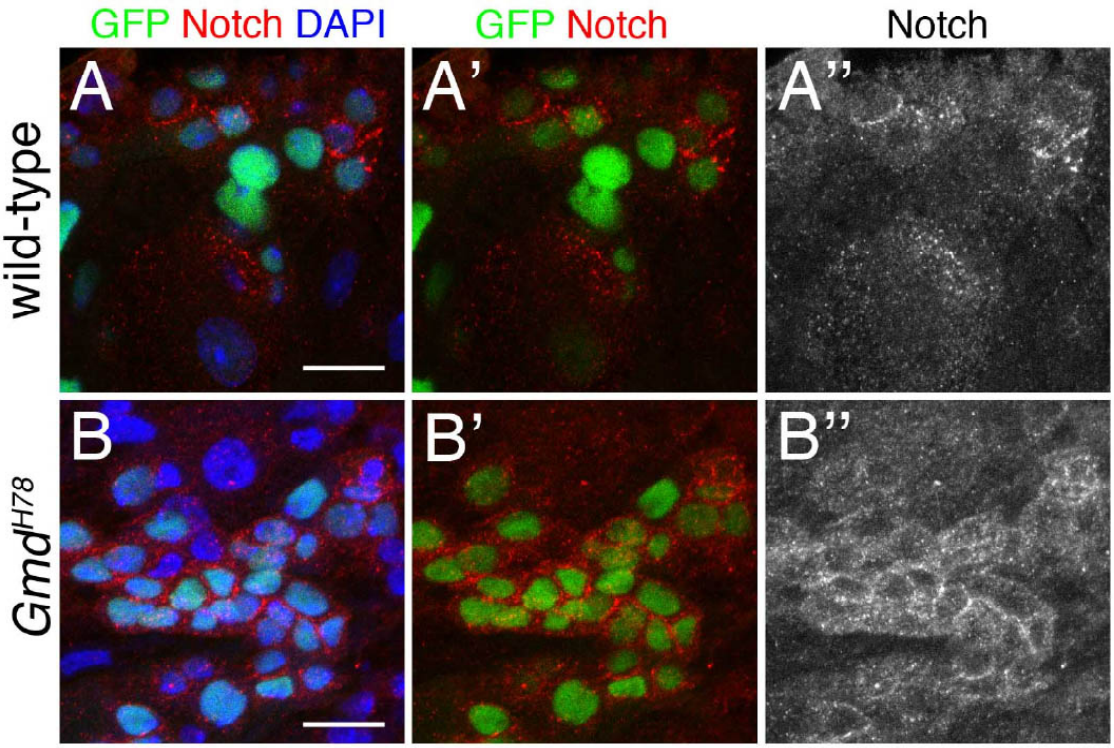
Supplementary Figure 1



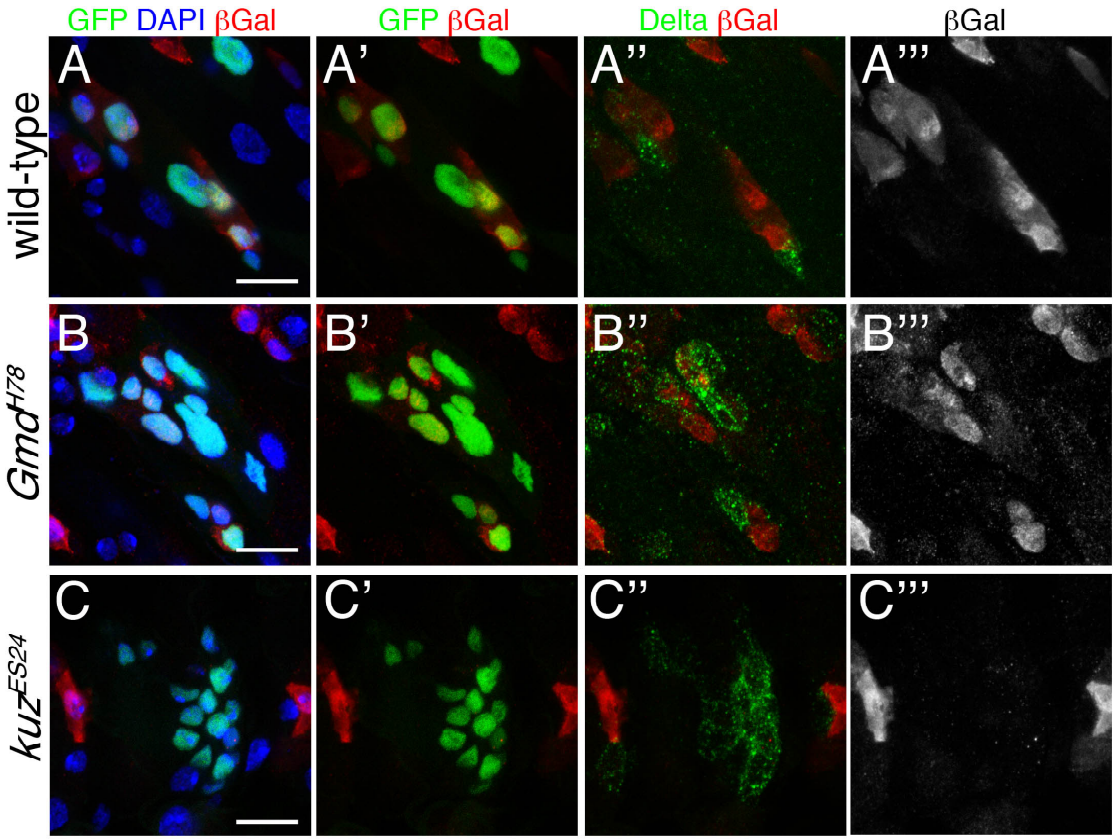
Supplementary Figure 2

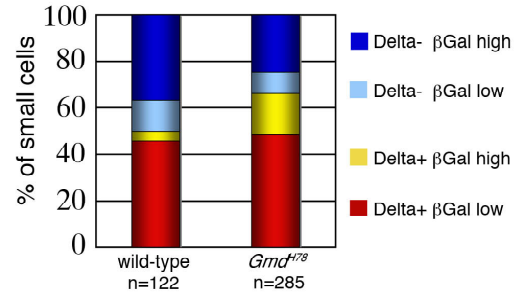


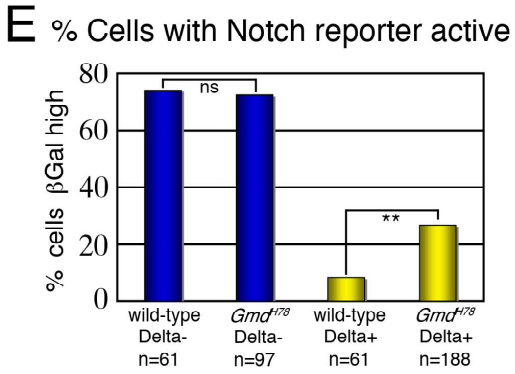
Supplementary Figure 3

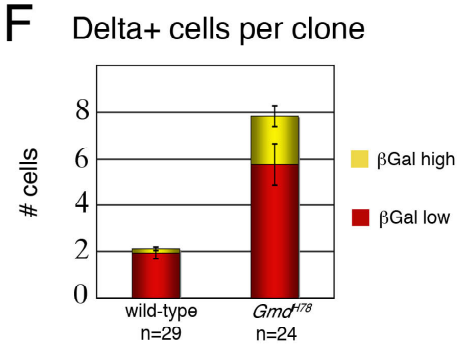


Supplementary Figure 4

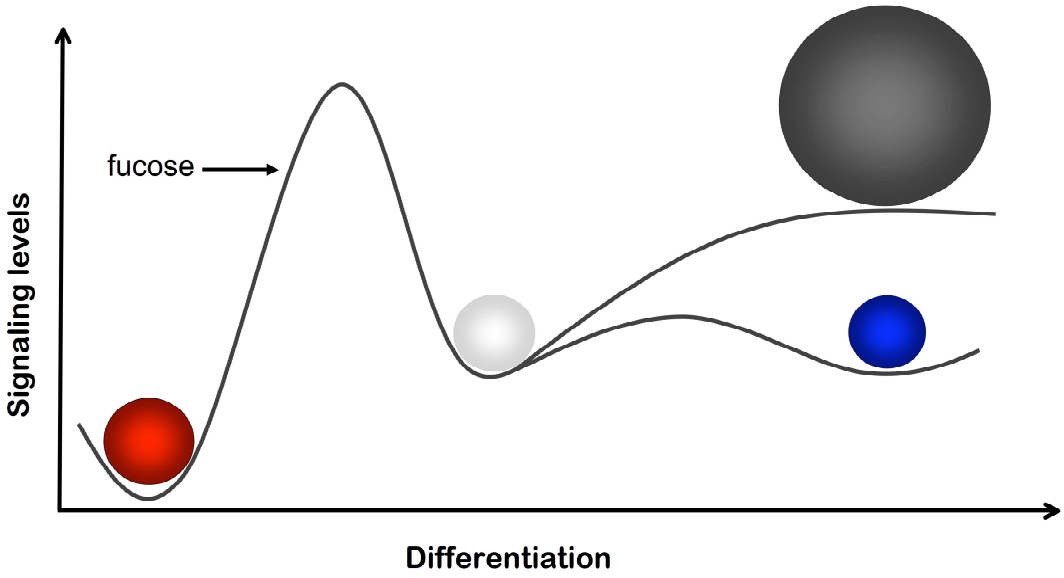








Supplementary Figure 5



Chapter V

General Discussion

General Discussion

In this general discussion I will first discuss the screen for regulators of the ISC lineage described in Chapter III as well as the potential role for some of the genes identified in screen. Then, I will discuss the role of *Gmd* in the regulating the cell fate decisions of the stem cell lineage and its potential implications for our understanding of how the ISC lineage is regulated to maintain tissue homeostasis.

I. Analysis of the Screen

We decided to do an EMS-induced F2 generation screen to identify novel regulators of the ISC lineage. This is a very time consuming screen and it is worth discussing whether it was indeed successful.

A. The design of the screen

EMS was chosen as the mutagen because in induces random point mutations [or, more infrequently, small deletions, (St Johnston, 2002)]. X-rays radiation can alternatively be used as mutagen. However, x-ray induced mutagenesis is less efficient than EMS in inducing mutations and, most importantly, x-rays frequently induce deletions or chromosome rearrangements, which may affect more than one

locus. Another strategy would have been to screen for mutations caused by lethal P-element insertions. P-element mutations have the advantage of being very easy to identify, since the P-element can be used as a tag for identification of the gene disrupted through PCR. Also, P-elements can induce different types of mutations from the ones induced through EMS mutagenesis The collection of P-element insertions available at the Berkeley *Drosophila* Genome Project contains insertions in approximately 25% of the essential genes and can be screened (Spradling et al., 1999). However, most genes are predicted to be cold spots for P-elements. This inefficiency of P-element as mutagens does not allow the identification of all the genes involved in a process. Furthermore, to perform a screen for lethal P-elements in the adult fly would imply that the P-elements would have to be recombined to FRT to test the function of the gene in clones. Mutagenesis based on the mobilization of *piggy*Bac transposons has been published as an attractive alternative (Hacker et al., 2003). Unlike the mobilization of P-elements, *piggy*Bac elements have been described to not share hotspots of integration with the P-elements, therefore allowing them to be used to generate a different spectrum of mutations in the genome. Furthermore, *piggy*Bac elements can be mobilized on FRT bearing chromosomes. Mutant loci generated by *piggy*Bac insertions have the added advantage of being easy to identify by PCR. The most time consuming step is the generation the collection of lethals induced by *piggy*Bac mobilization, on chromosomes with FRT. However, it has been recently published that *piggy*Bac mutagenesis generates considerably less mutants affecting a process than EMS-induced mutagenesis and was suggested that the *piggy*Bac hotspots probably overlap more with the P-element hotspots than what had been previously published (Mathieu et al., 2007). Any chosen mutagenesis method has its advantages and disadvantages. Considering this recent evidence, I believe that the choice of EMS as a mutagen was the best.

The biggest disadvantage of using EMS as a mutagen is the mapping of the mutated loci. In the light of the mapping data presented in this thesis, I believe that this is only a partial handicap: some mutants might be easily mapped, especially if two alleles are identified in the screen, by deficiency mapping while others might reveal to be more problematic and require a higher investment in time to map.

Complementation groups with at least two alleles have been reported to be quite easy to map to the locus using molecularly mapped P-elements (Zhai et al., 2003). Since I did not map any of the mutants with this technique, I cannot argue whether or not it is indeed efficient and reliable for mapping a mutant with <50 kb accuracy. Another way to identify the mutated locus that has already been mapped to a large genomic region by deficiency mapping is to generate a duplication of that region through the P[acmen] technique of BAC transgenesis that allows the insertion of large DNA fragments into flies (Venken et al., 2006). Through recombineering, the BAC can be mutated in bacteria, which can be used to generate duplications of said region where only one or a few genes are removed and test whether these duplications fail to complement the lethal mutation being mapped (Venken et al., 2006). Alternatively, to map a mutation in a large genome region, smaller deficiencies of the region can frequently be generated using P-elements. This, in combination with increasingly more efficient and less-expensive DNA sequencing can be used to identify the mutated locus. The classical technique for mapping is mapping by meiotic recombination between visible genetic markers. This technique is very time-consuming and error-prone since many of the markers are far apart and only give an estimate of position of the mutation. Also, the position of many of the visible markers has only been inferred by cytological and genetic experiments, making it very hard to align the cytological and genetic maps with the genome sequence (Chen et al., 1998). Recently, single-nucleotide polymorphism maps have been established for the *Drosophila* genome, which allows for rapid meiotic mapping to regions smaller than 50 kb (Berger et al., 2001; Martin et al., 2001). This technique could be an alternative for mapping the mutant lines for which deficiency mapping has been unsuccessful.

An alternative screen would have been to do an RNAi screen, using a tissue specific driver. Such screens have been proven efficient to identify many genes affecting a process (Mummery-Widmer et al., 2009). A disadvantage of doing such a screen is that the genes identified by RNAi have to subsequently be analyzed as genetic mutations, which necessitates generating mutants for many of the identified genes. Also, when driving expression of the RNAi constructs in all stem cells, using

an ISC specific driver such as *esg-Gal4*, there is no wild-type tissue that can be used as an internal control. Therefore, the observed effect might not be specific to the knock-downed gene but instead be a consequence of stressed induced by the knock-down of the gene or the RNAi construct itself. This is particularly problematic since we know that stress to intestinal epithelium induces stem cell proliferation (Biteau et al., 2008; Buchon et al., 2009a; Chatterjee and Ip, 2009). This problem could be avoided by expressing the RNAi constructs in positively marked clones, but the amount of crosses required would be equivalent to a F2 genetic screen. Finally, when using RNAi constructs, there is a problem of off-target effects, with many small RNAs being able to down-regulate multiple genes (Ma et al., 2006b). Therefore an RNAi screen is very efficient to identify most of the genes involved in one process but imposes a bottleneck at the characterization of individual genes affecting a process.

One key aspect of a screen is whether the screen has been carried out to saturation, meaning, whether all the genes affecting a process have been identified, with ideally at least two alleles of each mutated locus having been isolated. In the classic screen paper, C. Nüsslein-Volhard, E. Wieschaus and H. Kluding (Nusslein-Volhard, 1984) describe a large-scale screen for mutants on the 2nd chromosome, affecting embryonic patterning. 5764 lines with an EMS treated, balanced 2nd chromosome where screened for patterning phenotypes in homozygous embryos. They argue that the screen was carried out to saturation and report an interesting observation for the rate of discovery of new loci (Fig.43). It was observed that “after scoring the first 25% of all lines, at least one mutant in more than 50% of the finally identified loci had been found, while in the last 25% of the lines only three new loci (5%) were found. The frequency of total mutants per lines scored was constant throughout the screen. Thus, at the end of the screen alleles of previously isolated mutants were predominantly found.” Therefore, if the F2 screen described here had been carried to near saturation it might not have yielded many more new mutants than the ones identified. In such a time consuming screen as the one described, a compromise between screening for second alleles and the actual number of mutants isolated is critical.

We have not generated novel alleles for complementation groups containing only one allele through EMS mutagenesis. However, this strategy where flies are treated with EMS and screened, by complementation crosses, for novel alleles of previously isolated mutants, might prove to be a more efficient way of obtaining complementation groups with multiple alleles than screening until near saturation.

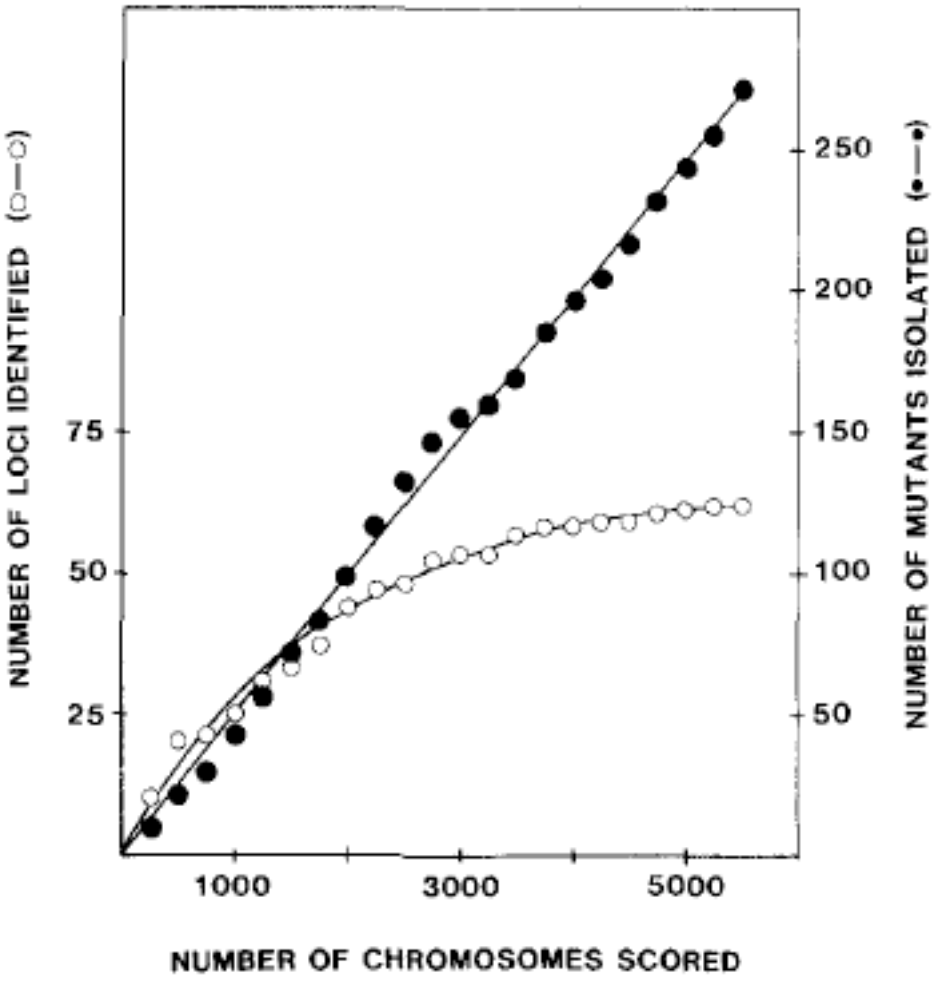


Fig.43 The frequency of isolation of new loci compared to the total number of mutants isolated in the screen for embryonic lethals on the second chromosome affecting embryonic patterning (Nusslein-Volhard, 1984).

Finally, one may question if the screen described here could have been done as an F1 screen instead. In an F1 screen, single adults are screened and crossed to make stocks if they have a phenotype. In order to carry out an F1 screen, the phenotype screened for has to be visible without dissecting the fly. F1 screens are advantageous as they allow the screening of large number of flies. In order to do an F1 screen for genes affecting the adult ISCs, the clones must be scored inside living adults, through the cuticle. Considering that many of the candidates we isolated in the screen had effects on cell composition of the clones that were only observable under an epifluorescence microscope, I find it highly unlikely that such a screen could have been done by imaging through the cuticle. If developments in microscopy produce a high resolution, tissue-penetrating macroscope that would allow for precise scoring of cell types in a clone, through the cuticle, than a much faster F1 screen would be possible. Alternatively, the F1 adults could have been crossed first to establish a stock and then dissected to score the clones in the intestine. This would have been almost as time-consuming as an F2 screen and would have the disadvantage that only one intestine *per* stock could be scored. Given the variability in clone induction in the intestine, this would have been a considerable disadvantage, with many potential mutants going unscored.

B. Brief analysis of genes isolated in the screen

During the screen we recovered 16 mutant alleles of the canonical component of the Notch pathway *kuz*. However, no alleles of *Su(H)* were isolated, which further demonstrates that this screen has not been carried to saturation. The open reading from of *kuz* open is about 5530 nucleotides long while the open reading frame of *Su(H)* is 2943 nucleotide long, which can partially explain the difference in recovered alleles. However, this striking difference between the number of *kuz* alleles identified and the absence of alleles of *Su(H)* identified also suggests that *kuz* is mores sensitive to point mutations. *kuz* has approximately 2 times more amino acids than

Su(H); if the probability of any amino acid in either gene being mutated by EMS is the same, then, theoretically, we should have recovered approximately 2 time more mutants for *kuz* than *Su(H).* However, our data suggests that *kuz* is at least 16 times more likely to be altered by EMS in a way that will yield more loss of function alleles. Since the difference in amino acid number does not account for this sensitivity to EMS mutagenesis, it is likely that *kuz* has a higher number of crucial amino acids, that is, has a higher number of amino acids that are absolutely required for the protein function and that, when mutated, yield a protein that is not functional.

The most interesting group of mutants isolated in the screen are the ones that have an overabundance of ISC-like cells but in which differentiation does not seem to affected in a dramatic way. This type of phenotype is novel and was not easily identified in the first rounds of screening. In a future screen, it is likely that lines with this type of phenotype could be more easily identified from the beginning. One of the genes isolated, *Gmd*, uncouples the proliferation of the stem cell from the specification of the differentiated fates, and will be discussed further down in this discussion (part II).

Mutant clones for *split ends (spen)* in the intestine not only have a strong increase in number of Delta+ cells, but Delta protein localization appears to be altered, with Delta being present at the membrane at high levels. Preliminary data suggest that differentiated cells are not affected in *spen* mutant clones. Spen is a nuclear protein that contains 3 N-terminal RNA binding motifs (RRM) and a C-terminal SPOC (Spen paralog and ortholog C-terminal) domain. However, the molecular function of Spen is not fully understood. One mammalian homologue of Spen is the SHARP (SMRT/HDAC1-associated repressor protein) protein, that has been shown to associate with transcriptional repression complexes (Sanchez-Pulido et al., 2004). SHARP has been shown to be upregulated in colon and ovarian carcinomas (Feng et al., 2007). Another human homologue of Spen, OTT1, has been found to be translocated in some in some acute megakaryocytic leukemias (Mercher et al., 2001). More recently, the plant homologue of Spen, FPA, has been shown to

regulate alternative polyadenylation of mRNA transcripts, which affects transcript stability and regulates gene silencing (Hornyik et al., 2010). Together, these data suggests that Spen could be regulating transcription or mRNA stability in the ISC lineage, potentially regulating several signaling pathways in the fly intestine. Spen has been shown to interact with different signaling pathways in *Drosophila* such as Notch, Wingless, and EGFR (Chang et al., 2008; Chen and Rebay, 2000; Doroquez et al., 2007; Kuang et al., 2000; Rebay et al., 2000) and it is interesting to speculate whether it could function in the intestine as a common hub between different pathways, integrating input from more than one signaling pathway. Understanding how Spen functions to limit the production of ISCs could help elucidate the role of its human homologues.

Another gene that was identified by its increase in density of Delta+ cells phenotype is *kismet* (*kis*). Kis is thritorax group (TrxG) protein with an ATPase domain (related to the one found in chromatin remodeling factors), two chromodomains (that are found in CHD ATPases and that meditate protein-protein interaction or protein-RNA interaction) and a BRK domain [domain of unknown function, that is also present in brahma, another TrxG protein and its homologues, (Srinivasan et al., 2005)]. Kis has been shown to counteract Polycomb group (PcG) repressive activity in some developmental contexts; consistent with a function as antagonistic of PcG, Kis has been shown to negatively regulate histone H3 lysine 27 methylation, which is repressive chromatin (Daubresse et al., 1999; Srinivasan et al., 2005). Preliminary data suggest that other members of the TrxG are not required in the intestine, as mutant clones generated for the components of the TrxG *osa, brm, trx* and *Snr1* (Pietersen and van Lohuizen, 2008) had no detectable phenotype. However, better analysis of mutant clones for these mutants should be done before they are ruled out. More recently, Kis has been found to promote early elongation by RNA polymerase II (Srinivasan et al., 2008). In the midgut, *kis* loss of function results in an increase of Delta+, which can either be extra stem cells but could also be EBs that have not yet activated its differentiation program. The observation that Kis plays a role in early transcription by RNA polymerase II in *Drosophila* suggests that Kis

could play a role in pausing of the polymerase II downstream of promoters. Pol II stalling has been proposed to be a mechanism that entices a dynamic and rapid response in gene transcription as a response to extracellular signaling molecules. This suggests a model in which Kis could promote differentiation in the EB by promoting the fast transcription of the differentiation program. Kis could play a role in keeping the targets of the differentiation program in a 'poised-to-go' state, antagonizing histone H3 lysine 27 methylation (repressive chromatin) and promoting pol II stalling. Once the differentiation genes are targeted in the EB, they could be quickly transcribed to promote differentiation. The next steps would be accessing the state of pol II in the ISC lineage, as well as identify the genes regulated by Kis. Identifying these genes would not only be informative on the role of Kis but might give information on the differentiation program too.

II. Differential Notch Signaling in regulating Cells fate decisions in the Intestine: Insights from the study of *Gmd*

In Chapter IV, I present evidence that *Gmd* loss of function uncouples the decision between self-renewal of the ISC vs. commitment to differentiate from the specification of the differentiated fates. I showed that commitment to differentiation requires high Notch activity while differentiation can occur with lower levels of Notch signaling. *Gmd*, a gene identified in the screen described in chapter III, is a known modulator of Notch signaling and was shown to be required for the commitment to differentiate but not for the adoption of the differentiated fate. *Gmd* is required for the biosynthesis of fucose in cells and I have cited published work suggesting also that high levels of Notch signaling is dependent on fucosylation of the Notch receptor but not further elongation of the fucose residues. I showed that loss of *Gmd* leads to an increase in the stem population due to an increase in symmetric divisions in respect to fate, where the stem cell produces two stem cells, instead of asymmetric divisions in respect to fate, where the stem cell produces one stem cell and one cell committed to differentiate. This data led us to propose that high threshold Notch signaling activity is required for the commitment of the ISC daughter cell to differentiation, which could function as a mechanism to protect the tissue from terminal differentiation of the stem cell and therefore its loss. In this discussion I will address some specific questions that remain open related to role of Gmd in the intestine, address what *Gmd* data could be telling us about how cell fate decisions occur in the intestine as well as other questions and possibilities that arouse from this study.

A. Role of Fucosylation in modulating Notch signaling

The work presented in chapter IV is the first described requirement for Gmd in a Fringe-independent context in *Drosophila.* I did not address directly the fucosylation of Notch in the intestine but I did present evidence that Notch is the relevant target of Gmd, since the nuclear Notch activity represses the *Gmd* phenotype. However, this repression could be independent of Notch signaling. There are good reasons to suspect that it is indeed Notch signaling that is affected in *Gmd*. First, the loss of the *O*-fucosyltransferase activity of Ofut1 has the same phenotype as loss of *Gmd* and Ofut1 has only been shown to be required in Notch signaling (Okajima and Irvine, 2002; Okajima et al., 2008; Okajima et al., 2005; Sasamura et al., 2003). This suggests that the role of Gmd is to provide fucose as a substrate for Ofut1. While, Ofut1 has also been shown to modify Delta, the loss of *Ofut1* does not affect the signaling activity of Delta (Panin et al., 2002). Ofut1 could, however, have additional substrates. Second, the *Gmd* phenotype in the intestine is consistent with a reduction of the Notch signaling activity and can be reproduced with the reduction of Notch signaling activity in *rumi* mutants, that also affects Notch signaling. However, it is not possible to exclude that other fucosylated proteins are affected in the intestine and contribute to the *Gmd* phenotype. Glycoshingolipids are also fucosylated molecules and it is known that Notch signaling can be regulated by glycoshingolipids (Barrows et al., 2007; Goode et al., 1996; Goode et al., 1992; Hamel et al., 2010; Katic et al., 2005; Muller et al., 2002; Wandall et al., 2003). We do not know whether the biosynthesis of glycoshingolipids is affected in *Gmd* mutant intestines, which could also contribute to the observed phenotype. Finally, fucosylated glycans have also been shown to be required in many developmental contexts and we cannot exclude that they are also contributing to the *Gmd* phenotype (Becker and Lowe, 2003; Ohata et al., 2009). The ideal experiment to prove that loss of *O*-fucosylation of Notch is responsible for the phenotype observed in *Gmd* would be to test, in the context of loss of *Notch,* the effect of the expression of a full-length Notch protein in which the

EGF repeats that are substrates for *O*-fucosylation are mutated. This experiment would indicate whether the loss of fucosylated Notch alone could affect the decision between self-renewal or differentiation. However, this is a technically challenging experiment since multiple EGF repeats are subtracts for *O*-fucosylation and the mutation of single EGF repeats does not dramatically affect binding to the ligands *in vitro*, while mutation of multiple EGF repeats affects the structure of the extracellular domain of Notch (Xu et al., 2005). These questions could be partially addressed biochemically. Accessing the fucosylation of the Notch receptor could be carried out by immunoprecipitating Notch (and Delta) protein from wild-type and *Gmd* mutant guts where most of the gut is mutant tissue and test, using a lectin that recognizes *O*-linked fucose, whether Notch and Delta are *O*-fucosylated in the intestine and whether there is a difference in *Gmd* mutants (Ishikawa et al., 2005). Finally, co-immunoprecipitation studies of Notch could address whether the co-immunoprecipitation of Delta with Notch is affected *Gmd* mutants, indicating whether the interaction between the receptor and the ligand is affected when Notch is not *O*-fucosylated.

There is good evidence that mouse Notch1 expressed in *Gmd* mutant Chinese hamster cells binds less efficiently to both mouse Delta1 and Jagged1, resulting in weaker activation of Notch in this cell model (Chen et al., 2001; Moloney et al., 2000a; Stahl et al., 2008). The same has been observed in ES cells where *Pofut1* activity is diminished (Stahl et al., 2008). However, in *Drosophila* the specific role of *O-fucosylation*, without further elongation of the sugar chain, in the interaction of Notch receptor with its ligands has not been yet addressed. To test this, cell-based binding essays where the interaction of *O*-fucosylated and non-*O*-fucosylated Notch with Delta could be measured and compared, similarly to what has been done to test the effect of Fringe-dependent modification of Notch (Xu et al., 2007).

Finally, *O*-fucosylation of Notch has been proposed to affect its localization and trafficking in *Drosophila* (Sasaki et al., 2007; Sasamura et al., 2007). I have shown that no defects in Notch localization have been found in fixed *Gmd* clones. However, this is subject to the resolution/detection methods and trafficking could not be directly analyzed. The trafficking and localization of Notch could further tested by looking at

Notch::GFP fusion, assessing its localization and trafficking in *Gmd* mutant and wild-type tissue through live imaging. Since there are no live imaging protocols developed to image the adult fly intestine, this is yet a challenging experiment that would require some time to develop the protocols for culturing intestines long enough to image. Considering the potential of *Drosophila* as model organism to study cells *in vivo*, developing live imaging in the intestine is an investment that is likely to be fruitful.

B. Maintenance of tissue homeostasis in *Gmd* mutants

One very interesting observation of the study of the *Gmd* phenotype is that, in spite of the considerable increase in proliferative ISC-like cells, the tissue architecture does not appear grossly altered. In mutants for the loss of Notch signaling I observed multilayering of small diploid cells that obstruct the lumen. It has also been reported that in other genetic contexts, where there is an increase in proliferation rate, tissue hyperplasia occurs (Amcheslavsky et al., 2009; Apidianakis and Rahme, 2009; Jiang et al., 2009; Lee et al., 2009b; Staley and Irvine, 2010). In these cases, the increase in proliferation rate appears to result in an excess production of EBs that pile up before there is time to differentiate. However, this does not seem to occur in *Gmd* mutant tissue. In *Gmd* there are more cells in the clones, with more ISCs being produced, but the number of EBs per clone seems to be equivalent to wild-type and density of differentiated cells is also like in wild-type. Therefore, *Gmd* mutant ISC-like cells appear to produce continuously cells that will differentiate and maintain the density of differentiated cells like in wild-type. It is tempting to speculate that the differentiated cells, particularly the ECs, are the cells that maintain the tissue architecture, responding to cues from the tissue, such as the production of new differentiated cells and also cell death. Differentiated cells would therefore be the ones regulating the length and diameter of the gut. In an experiment where the ISCs cells were killed by expressing pro-apoptotic proteins in them, the

tissue integrity was maintained, with the remaining ECs having grown in size and increased their polyploidy (Jiang et al., 2009). In the same way that the ECs can compensate the lack of new ECs being produced, the ECs could, hypothetically, sense an increase in production of cells in the *Gmd* mutant clones and feedback to the epithelium to increase the tissue size. It is not clear if the muscle layer would be remodeled to increase its length or would just rearrange itself around the epithelium. One empirical observation that we have made is that intestines with large *Gmd* clones appear to be longer than wild-type intestines. In contrast, intestines of under-fed flies also seem to be smaller than the intestine of well-fed flies. Therefore, it would be very interesting to measure tissue length and weight in *Gmd* and other experimental contexts and see whether there is indeed a difference. It would also be very interesting to test whether the differentiated cells are indeed the ones regulating the length and diameter of the gut. Alternatively, the rate of ISC proliferation, a general sensor of nutritional state or a caloric sensor that measures food absorption could be regulating tissue homeostasis instead.

The *rumi* phenotype in the intestine and the fact that its 'severity' is dependent on the temperature at which the flies are reared is also potentially interesting to study tissue homeostasis. Is there hyperplasia in *rumi* mutants and at what temperature does it commence? Furthermore, it would be interesting to vary the temperature at which *rumi* mutants are reared: if and overabundance of ISC-like cells are produced and then the flies are moved to the permissive temperature, what happens to the extra ISCs? Do they differentiate or do they die by apoptosis?

C. Differential Notch signaling and cells fate specification

Previous work by Ohlstein and Spradling consisting of retrospective clone analysis and correlation between the level of Delta protein in the ISC and the cell that the ISC had just produced led these authors to propose a model in which the ISC determines the fate that the EB will adopt through differential Notch signaling (Ohlstein and Spradling, 2007). In this model, the ISC switches between high Delta

and low Delta expression. The ISC expressing high Delta could send a strong signal to the EB, that would adopt the EC fate while the ISC expressing low Delta would send a weak Notch signal to the EB, that would adopt the ee fate (Ohlstein and Spradling, 2007). Therefore, the EB would be able to interpret the levels of Notch signal it receives and both cell fate decisions, to commit to differentiate and the differentiated fate adopted, would be made during the same signaling event. Surprisingly, the data presented in chapter IV indicates that the decision between self-renewal or commitment, i.e. exit from self-renewal, can be uncoupled from the decision between adoption of EC or ee cell fates. This data led us to propose a model in which the exit from self-renewal of the daughter cell requires a high-level, fucose-dependent Notch signaling event (Fig.44). This requirement for high-level signaling could function as protective mechanism, preventing the ISC daughter cells from differentiating prematurely in response to noisy Notch signaling activity. Furthermore, in a previous study we have shown that the transcriptional repression of Notch target genes is required to prevent loss of the ISC, which further supports the model that noisy Notch activity could lead to terminal differentiation of the stem cell (Bardin et al., 2010). Requirement for high-threshold signaling for commitment to differentiation could be a general mechanism employed by stem cells to prevent premature differentiation.

This is model is not incompatible with the model proposed by Ohlstein and Spradling for the differentiation of the ECs and ee cells: the committed EB could receive different levels of Notch signaling that would determine which fate it would adapt (Fig.44.A).

Micchelli and colleagues have proposed that the specification of the EB that will adopt the EC fate requires Notch signaling while specification of the EB that will go on to differentiate as an ee cell occurs in a Notch-independent manner through a, however, unknown mechanism (Beebe et al., 2010; Micchelli and Perrimon, 2006). The evidence for the specification of the ee cell in a Notch-independent way is the production of ee-like cells in mutant clones for loss of Notch signaling (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). However, we have preliminary data of the expression of the reporter for Notch

signaling activity, *Su(H)GBE-LacZ* (Furriols and Bray, 2001) in a percentage of the ee cells, which suggests that these cells had active Notch signaling at some point in their specification. The fact that not all ee cells express this reporter can be explained by the stability of βGal in cells. To address whether the future ee cells have active Notch signaling at any point in there specification we could perform lineage labeling analysis using a *Su(H)GBE* promoter, to permanently mark all the cells that have had Notch signaling activity. According to our model, in order for the daughter cell to commit to exit from self-renewal, it would have to experience high levels of Notch signaling. The specification of the EC cell would require further Notch signaling, albeit a lower lever of Notch signaling, while the specification of the ee cell could occur in a Notch-independent manner or might even require active repression of Notch signaling (Fig.44.B). Therefore, our model is not completely incompatible with the model proposed by Micchelli and colleagues.

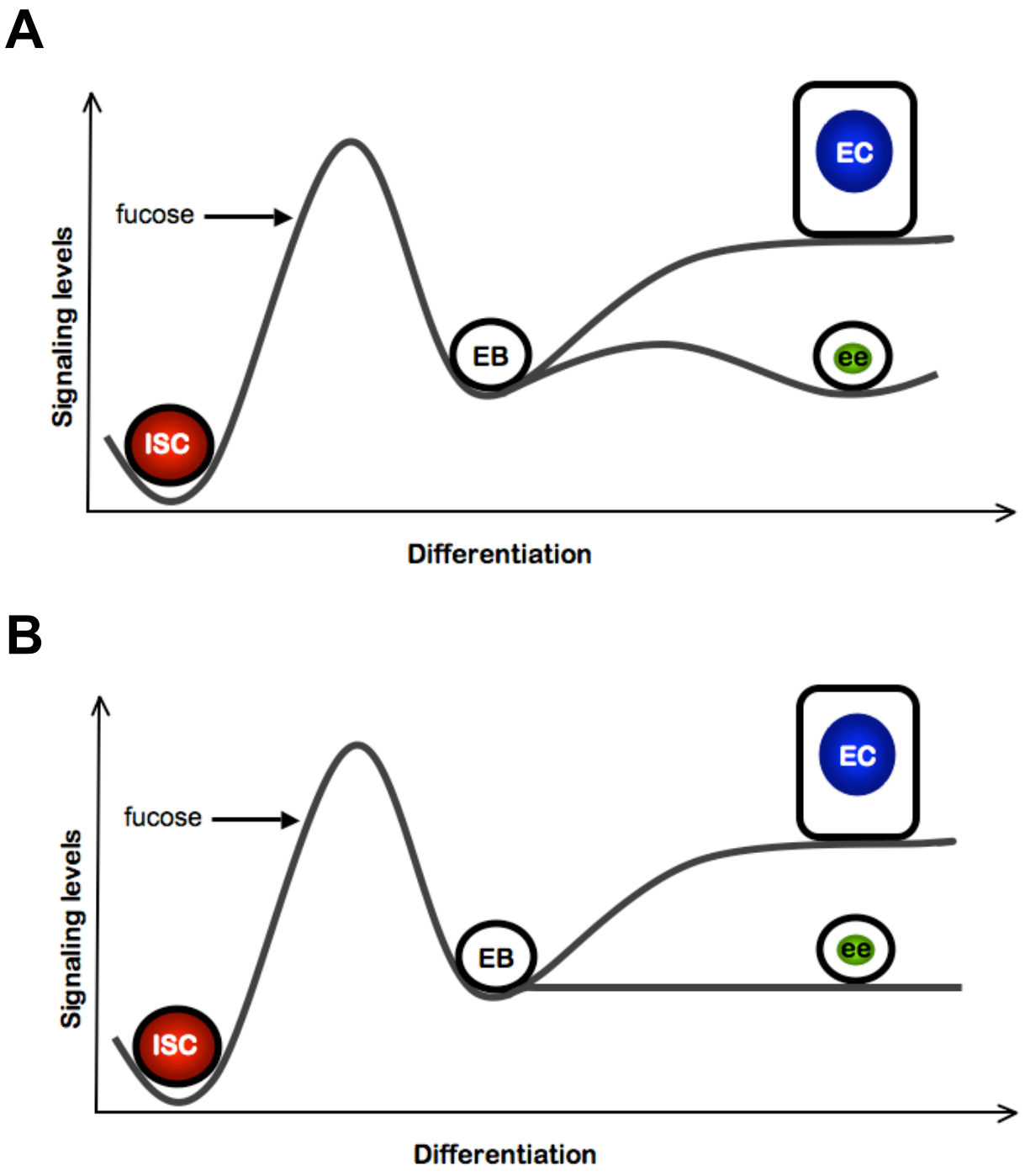


Fig. 44: In both **A** and **B**, the ISC daughter cell requires high threshold, fucose-dependent Notch signal to exit the stem cell state and commit to differentiation, becoming an EB. In **A**, the fate of the EB is dependent of differential Notch activity, with high level Notch signaling in the EB specifying the EC and lower level Notch signaling specifying the ee cell. In **B**, the specification of the EC fate requires Notch signaling while the specification of the ee fate is independent of Notch.

D. Dynamics of Notch signaling in the Intestine

In the Intestine, different cells fate decisions appear to require different levels of Notch signaling. However, how the fate of the daughter cells is specified in function of the levels of Notch received may occur in different ways. Three models could explain how the daughter cells interpret Notch signaling to determine the level of signaling received. In the first model (Fig.45.A.) the EB could receive two different pulses of Notch signaling: the first pulse would have to be strong Notch signal to specify the EB and the second pulse would specify an EC or an ee cell, depending on the level of Notch signaling of that pulse. A second possible model (Fig.45.B) is that the EB measures the total Notch signal it receives. An initial level of Notch signal would be required for the daughter cell to commit to the EB fate and then the EB would continue to receive Notch signaling from the ISC. The total level received could then be interpreted by the EB to determine which fate to adopt. A third possible model (Fig.45.C) is that the EB interprets the signal received over time. After initial high level Notch signal that specifies the EB, the specification of the ECs and ee cells depends on the duration of the Notch signal. These models are not mutually exclusive, being possible that different factors play into how the fates are specified.

To test the different models, Notch signaling activity should be monitored over time in the EBs. There is an available reporter for Notch signaling activity in flies, the *Su(H)GBE-LacZ* construct (Furriols and Bray, 2001). However, due to βGal perdurance this reporter is not adequate to look at the dynamic activation of Notch in the intestine. A better reporter would be the promoter Su(H)GBE driving the expression of a fast folding and fast degrading fluorescent protein such as the YFP-based Venus protein. Similar reporters have been developed to monitor activation of Notch signaling during somitogenesis (Aulehla et al., 2008). Again, to monitor Notch transcriptional activity *in vivo* will require the development of live imaging in order to observe the activation of Notch in single cell over time. However, analysis in fixed tissue with markers of cell fate could also generate correlative data on the dynamics of Notch signaling in the intestine.

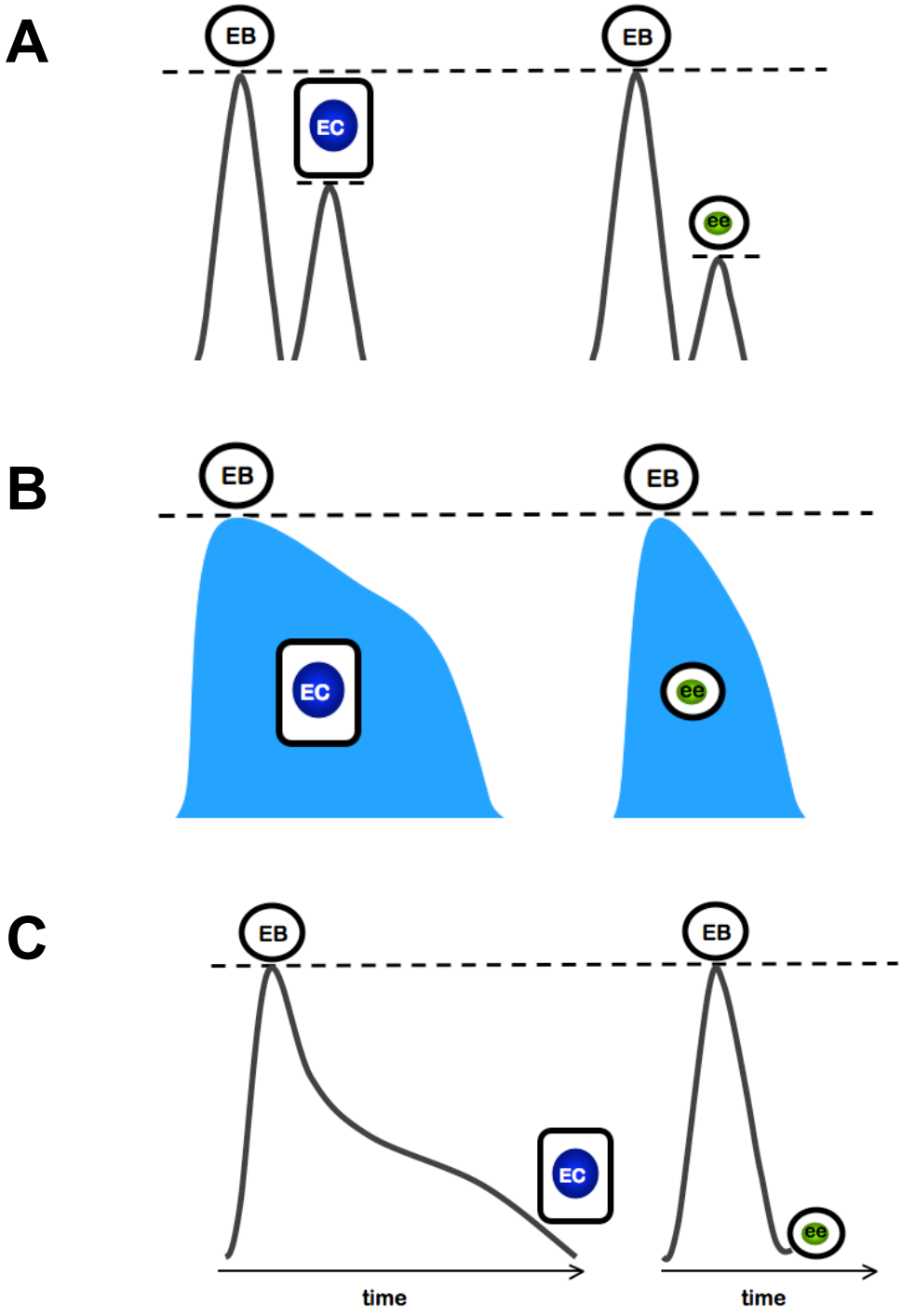


Fig. 45: Models for how different levels of Notch signaling specify the two differentiated cell fates. **A** The EB receive two pulses of Notch signaling: the first pulse is a strong pulse of Notch signal to specify the EB and the second pulse specifies the EC or the ee cell, depending on the level of Notch signaling of that pulse. **B** The EB measures the total Notch signal it receives. After the initial Notch signal required for commitment to the EB fate, the EB would continue to receive Notch signaling, with the total signal received being interpreted by the EB to determine which fate to adopt. **C** The EB interprets the signal received over time, with the specification of the ECs and ee cells depending on the duration of the Notch signal. These 3 models are not mutually exclusive.

E. Identifying the Notch target genes

Considering that once the Notch receptor is activated the NICD translocates directly to the nucleus to associate to the CSL-Mam transcription activation complex, it could potentially be at the level of the Notch target genes that high Notch signal is integrated. The genes required for specifying the EB fate / repressing the ISC fate might be activated as the Notch target genes that promote differentiation but may have different enhancers that determine the affinity of the NICD-CSL-Mam complex to the promoter region. Alternatively, the enhancers of the two groups of target genes are not very different but the differentiation target genes require more signal input over time, maybe to buildup enough of the factors that will determine the differentiated fate. These hypotheses are not mutually exclusive. Additionally, one could speculate that a group of transcriptional targets could be facilitators of Notch signaling activity, being either co-activators or chromatin remodeling proteins that could render the transcription of the Notch target genes that specify the differentiated fates more sensitive to a lower level of Notch signal. High Notch signaling could lead to the opening of the chromatin and then subsequent Notch signaling events could specify differentiated cell fate, even if the signaling activity is lower.

To understand how Notch is specifying the different cell fates in the intestine it is critical to identify the tissue specific Notch target genes. Microarray analysis to identify the genes that are up or down in loss of Notch signaling mutants could be compared to the same analysis of the genes that are up or down in *Gmd* mutants to identify the different Notch target genes. However, in this experiment it would not be possible to distinguish the direct target genes from the ones that are downstream of fate. Therefore, direct identification of the NICD-CSL target genes would be a alternative strategy. Chromatin immunoprecipitation (ChIP) with anti-Su(H) antibodies has allowed the identification of regions targeted by Su(H) in Notch-activated cells (Krejci et al., 2009). Similar experiments could be carried out in the intestine to identify the tissue specific Notch target genes. The expression of these target genes

could then be tested by *in situ* hybridization or immunofluorescence and the role of the potential target genes could be further tested by genetic analysis.

F. Chromatin configuration

Chromatin is a highly organized structure in which DNA is efficiently packed, regulating the transcription of the DNA and creating an additional level of regulation for gene transcription (Loden and van Steensel, 2005). Several proteins have been characterized as participating in chromatin remodeling complexes (Becker and Horz, 2002; Bouazoune and Brehm, 2006). In *Drosophila,* chromatin remodeling complexes have been shown to act synergistically with signaling pathways to regulate stem cell maintenance and differentiation (Cherry and Matunis, 2010; Xi and Xie, 2005). The state of the chromatin in the stem cell daughter cells could be, at least partially, the reason why high levels of Notch signaling are required for the exit from the stem cell state and commitment to differentiation. There is some evidence that Notch target genes that promote differentiation might be kept in a repressed chromatin state. First, Hairless is required to repress Notch target gene in the ISC and Hairless protein has been shown to recruit, in association with Su(H), the co-repressors CtBP, Groucho and the Histone H3/H4 chaperone Asf1, promoting gene silencing through repressive chromatin (Bang and Posakony, 1992; Barolo et al., 2002; Brou et al., 1994; Castro et al., 2005; Goodfellow et al., 2007; Morel et al., 2001; Nagel et al., 2005). Second, Scrawny, an ubiquitin protease that deubiquitylates histone H2B, which is important for gene silencing, is also required for maintenance of the ISC (Buszczak et al., 2009). This suggests that loss of H2B deubiquitylation results in de-repression of Notch target genes, which leads to the loss of the ISC.

To test this hypothesis, one could lower the level of chromatin repression in heterozygous mutant contexts and test the phenotype of *Gmd* clones in these contexts. If, in *Gmd* mutants, the low level Notch signaling activity was confronted with less repressed chromatin due to loss of one copy of a chromatin remodeling

protein, Notch signaling activity in the daughter cells would have to overcome a lower barrier and commitment to differentiation could occur more frequently. Therefore in a state of less repressed chromatin in the daughter cells, low levels of Notch signaling would be able to resolve the decision between self-renewal or commitment more frequently, which would suppress the *Gmd* phenotype. First, testing *Gmd* clones in *Hairless*, *asf1* and *scrawny* mutant heterozygous flies could indicate whether the loss of transcriptional repression and repressed chromatin could suppress the *Gmd* phenotype. Also, there are multiple chromatin remodeling proteins in *Drosophila* that could be tested. Identification of a the protein(s) involved in chromatin remodeling that are repressing the expression of differentiation genes in the intestine could lead to better understanding of how chromatin is regulated in stem cells and during differentiation.

G. Cell Cycle and cell fate decisions

Early studies of cell differentiation *in vitro* showed that cells preferentially enter differentiation when in G1 phase of the cell cycle (Jonk et al., 1992; Lange and Calegari, 2010; Mummery et al., 1987; Singh and Dalton, 2009). These studies found that a short G1 reduces the window in which a cell can be responsive to differentiation cues. If differentiation relies on reaching a threshold of inductive signal, on an accumulation of signal over time or in an opening of repressive chromatin, the time that the cells have to achieve this is directly dependent on the length of the G1 phase. Furthermore, the length of the G1 would determine the competence for that cell to differentiate. Consistent with this model, cell differentiation has been associated with lengthening of the cell cycle, especially G1 (Lange and Calegari, 2010). There are many correlative studies that suggest that the length of G1 controls the differentiation competency of a precursor cell but this hypothesis has not yet been fully demonstrated (Lange and Calegari, 2010). In the *Drosophila* male germ line, switch from proliferation to differentiation is dependent on the accumulation of the protein Bag of Marbles and the number of divisions that occur before

differentiation correlates with the length of the cell cycle and the time it takes to accumulate Bag of Marbles: if the cell cycle is faster, more divisions can occur before the critical level of Bag of Marbles is reached (Insco et al., 2009). It is possible that differentiation in the intestine could also be dependent on the length of the cell cycle in stem cell daughter cells: after division both cells signal to each other and eventually one of these cells starts to 'lose', becoming the Notch ON cell while the other becomes the signal-sending Notch OFF cell. The Notch ON cell has a limited amount of time to transcribe the Notch target genes and accumulate differentiation factors that will promote the EB fate before a mitogenic signal from the intestine (Wg, Upd, etc) will promote the re-entry of the cells in the cell-cycle. If the pre-EB has already accumulated enough factors that promote differentiation, it will not re-enter the cell cycle but will go on to differentiate (Fig.46.a). However, manipulation of the cell cycle in the ISCs could speed up the cell cycle. In such a situation, if neither of the daughter cells has yet accumulated enough differentiation factors, then both will re-enter the cell cycle (Fig.46.b). This would result in symmetric division, in respect to fate, with one ISC producing two ISCs. The tools to manipulate cell cycle length in *Drosophila* are available and it would definitely be interesting to test this hypothesis. Furthermore, in *Gmd* mutants, the limiting factor appears to be the accumulation, through low level of Notch signaling, of differentiation factors in the available time window of G1. Lengthening of G1 and/or the whole cell cycle could provide enough time for the differentiation factors to accumulate through low Notch signaling and the *Gmd* phenotype could be suppressed. There is some evidence that Notch-dependent cell fate choices occur preferentially during a particular phase of the cell cycle: the daughter-cells of the SOP were shown to be more prone to respond to Notch signaling during S-phase (Remaud et al., 2008). Regulation of the ISC-daughters' fate by the length of the cell-cycle length is a very tantalizing hypothesis that should be tested.

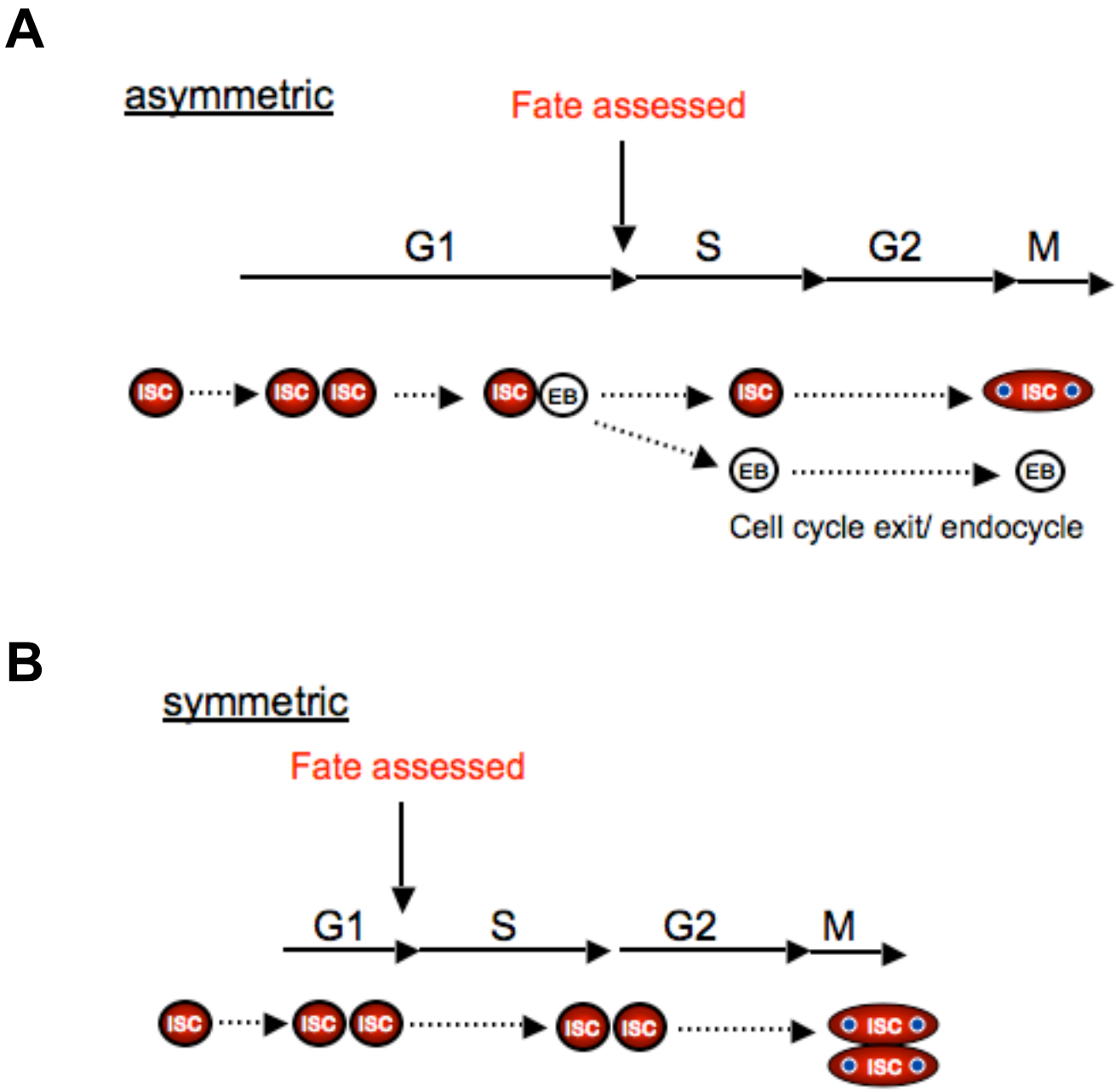


Fig. 46: After division of the ISC, both cells signal to each other, with one becoming the Notch ON cell while the other becomes Notch OFF cell. The Notch ON cell has a limited amount of time to accumulate differentiation factors that will promote the re-entry of the cells in the S phase of the cell cycle. **A** If the pre-EB has already accumulated enough factors that promote differentiation, it will not re-enter the cell cycle but will go on to differentiate. **B** If the G1 phase is shorter, at G1 to S phase transition, when cell-fate is assessed, if neither of the daughter cells has accumulated enough differentiation factors to commit to differentiation, then both cells could hypothetically become ISCs.

H. Symmetrical division of the ISC

All studies that investigated the proliferation of ISCs have indicated that ISCs divide, at least in the great majority of the time, asymmetrically in respect to the fate of its daughter cells, producing one ISC and one EB (Ohlstein and Spradling, 2006). The *Gmd* phenotype raises the possibility that ISC symmetric divisions in respect to fate of the daughter cells decisions could occur in wild-type intestines. Also, the rapid increase in Delta+ cells observed when proliferation of the ISCs is stimulated raises the possibility that symmetric divisions are occurring in these contexts (Beebe et al., 2010; Buchon et al., 2009a; Cronin et al., 2009; Jiang et al., 2009; Lin et al., 2009; Liu et al., 2010; Staley and Irvine, 2010). The first step to test this hypothesis would be to investigate the lineages of stem cell daughters and assess whether two daughter cells can become two ISCs, as a product of a symmetric division. In the lineage labeling experiments that we have done so far, only one daughter cell is marked after division; therefore sister cells are not marked and only the lineage of one of the cells is labeled. Recently, a technique to label both daughter cells has been developed, the twin spot generator (Griffin et al., 2009). Following twin spot clones in the intestine would allow for the identification of divisions that produce two ISC clones. It is possible that these do not occur when the tissue is at homeostasis. Generating twin spot clones in contexts where the gut is damaged should also test the effect of damage on the type of ISC division.

If ISC symmetric divisions do occur, it would be interesting to assay whether the ISC regulates the rate of symmetric division by modulating negatively Notch signaling activity and therefore prevent Notch activation in both daughter cells, promoting adoption of the ISC fate by both cells. Alternatively, the ISC could regulate symmetric cell divisions through regulation of the cell cycle length.

I. Quiescence

The dramatic increase in Delta+ cells and proliferative cells observed in contexts where proliferation of the ISC is stimulated by damage or infection also raises the possibility that quiescent stem cells are present in the intestine (Beebe et al., 2010; Buchon et al., 2009a; Cronin et al., 2009; Jiang et al., 2009; Lin et al., 2009; Liu et al., 2010; Staley and Irvine, 2010). It has been proposed that many tissues harbor quiescent and active stem cells, with the first being slow cycling cells that are more resistant to damage while the active stem cells would be cycling more frequently and replenishing periodically the cells that turnover (Li and Clevers, 2010). A general characteristic of the slow cycling quiescent stem cells is their label retaining properties. A pulse labeling experiment were all the ISCs are labeled during larval stage, followed by a chase to identify whether all the labeled stem cells are actively cycling is a possible experiment to address this question. Furthermore, if there are indeed quiescent stem cells in the intestine, it would be interesting to test their response to damage, induced by infection or toxins, as well as to direct stimulation of ISC proliferation by expressed mitogens, in order to see if these stimuli activate the quiescent stem cells. These experiments are likely to elucidate how stem cells respond to stimuli and regulate themselves in order to maintain proper tissue homeostasis.

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Appendix

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PLoS one

Overexpression of Partner of Numb Induces Asymmetric Distribution of the PI4P 5-Kinase Skittles in Mitotic Sensory Organ Precursor Cells in *Drosophila*

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Abstract

Unequal segregation of cell fate determinants at mitosis is a conserved mechanism whereby cell fate diversity can be generated during development. In *Drosophila*, each sensory organ precursor cell (SOP) divides asymmetrically to produce an anterior pIIb and a posterior pIIa cell. The Par6-aPKC complex localizes at the posterior pole of dividing SOPs and directs the actin-dependent localization of the cell fate determinants Numb, Partner of Numb (Pon) and Neuralized at the opposite pole. The plasma membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) regulates the plasma membrane localization and activity of various proteins, including several actin regulators, thereby modulating actin-based processes. Here, we have examined the distribution of PIP2 and of the PIP2-producing kinase Skittles (Sktl) in mitotic SOPs. Our analysis indicates that both Sktl and PIP2 reporters are uniformly distributed in mitotic SOPs. In the course of this study, we have observed that overexpression of full-length Pon or its localization domain (LD) fused to the Red Fluorescent Protein (RFP::Pon^{LD}) results in asymmetric distribution of Sktl and PIP2 reporters in dividing SOPs. Our observation that Pon overexpression alters polar protein distribution is relevant because RFP::Pon^{LD} is often used as a polarity marker in dividing progenitors.

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Introduction

Ontogenesis of complex multicellular organisms involves the generation of different cell types. One mechanism by which cell fate diversity can be achieved is asymmetric cell division. During asymmetric cell division, progenitor cell polarity directs the orientation of the mitotic spindle and the asymmetric localization of cell fate regulators, thereby ensuring unequal segregation of these regulators [1,2].

Each mechanosensory bristle located on the notum of *Drosophila* is composed of four different cells. These cells originate from a single sensory organ precursor cell (SOP) via a fixed lineage comprising four stereotyped asymmetric cell divisions [3]. The SOP first divides within the plane of the epithelium to generate a posterior pIIa cell and an anterior pIIb cell [4]. Two regulators of Notch receptor signaling, Numb and Neuralized (Neur), localize at the anterior cortex of dividing SOPs and segregate into the anterior daughter cell [5,6]. Numb inhibits Notch whereas Neur positively regulates the signaling activity of the Notch ligand Delta. This therefore leads to Notch inhibition in the anterior cell that adopts the pIIb fate and Notch activation in the posterior cell that becomes pIIa.

By analogy with the role of atypical Protein Kinase C (aPKC) in neuroblasts [7], asymmetric localization of Numb and Neur at the anterior cortex of dividing SOPs is thought to depend on the kinase activity of aPKC, which localizes at the posterior pole. In brief, aPKC phosphorylates and inhibits Lethal (2) giant larvae (Lgl) at the posterior cortex, such that active nonphosphorylated Lgl protein is restricted to the anterior cortex where it promotes the cortical localization of Numb and Neur [7,8]. Direct phosphorylation of Numb by aPKC may further contribute to restricting the localization of Numb to the anterior cortex [9]. Additionally, drug studies have shown that depolymerization of microfilaments prevents cortical localization of Numb and Neur in dividing SOPs, indicating that actin plays an essential role in their polar distribution [6,10]. Lastly, anterior localization of Numb probably also involves its interaction with Partner of Numb (Pon). The Pon protein contains an N-terminal Numb-interacting domain, a central coiled-coil domain, and a C-terminal localization domain (LD) that is sufficient for its asymmetric localization in neuroblasts and SOPs. Pon colocalizes with Numb and is required, at least in neuroblasts, for its asymmetric localization [11,12].

Phosphatidylinositol 4,5-bisphosphate (PIP2) is a phospholipid present at the inner leaflet of the plasma membrane that has a wide range of proposed functions [13]. PIP2 directly interacts with several actin regulators [14] as well as with proteins known to be involved in the process of asymmetric division, including Par3 [15], Neuralized [16] and Numb [17]. PIP2 is mostly produced by

type I (PIP5KIs) phosphatidylinositol phosphate 5-kinases that use phosphatidylinositol 4-phosphate (PI(4)P) as a substrate [13]. The *Drosophila* genome encodes three predicted PIP5KIs: PIP5K59B, CG17471 and *skittles (sktl)* [18,19]. Recently, the *sktl* gene has been shown to play a critical role in PIP2 synthesis in the oocyte (Gervais et al., unpublished). Interestingly, the *sktl* gene appears to be expressed in SOPs [18]. To begin studying the potential role of PIP2 in asymmetric cell division, we have examined here the localization of Sktl and PIP2 reporters in dividing SOPs. Our analysis indicates that PIP2 and Sktl are distributed at the cortex of dividing SOPs with no clear sign of planar asymmetry. However, in the course of these experiments, we have observed that an increased accumulation of Pon, which is known to accumulate at the anterior cortex in mitotic SOPs, resulted in the posterior localization of Sktl. We discuss here the practical implications and possible biological significance of this overexpression phenotype.

Results

Analysis of PIP2 distribution in mitotic SOPs

The dynamics of PIP2 distribution can be followed in live cells using PIP2 reporters consisting of a PIP2-interacting domain fused to a fluorescent protein [20]. In this study, we have used the Pleckstrin Homology (PH) domain of the phospholipase Cδ1 fused to GFP (PH::GFP) [21] and the Epsin N-Terminal Homology domain of *Drosophila* Liquid facets (Lqf) [22] also fused to GFP

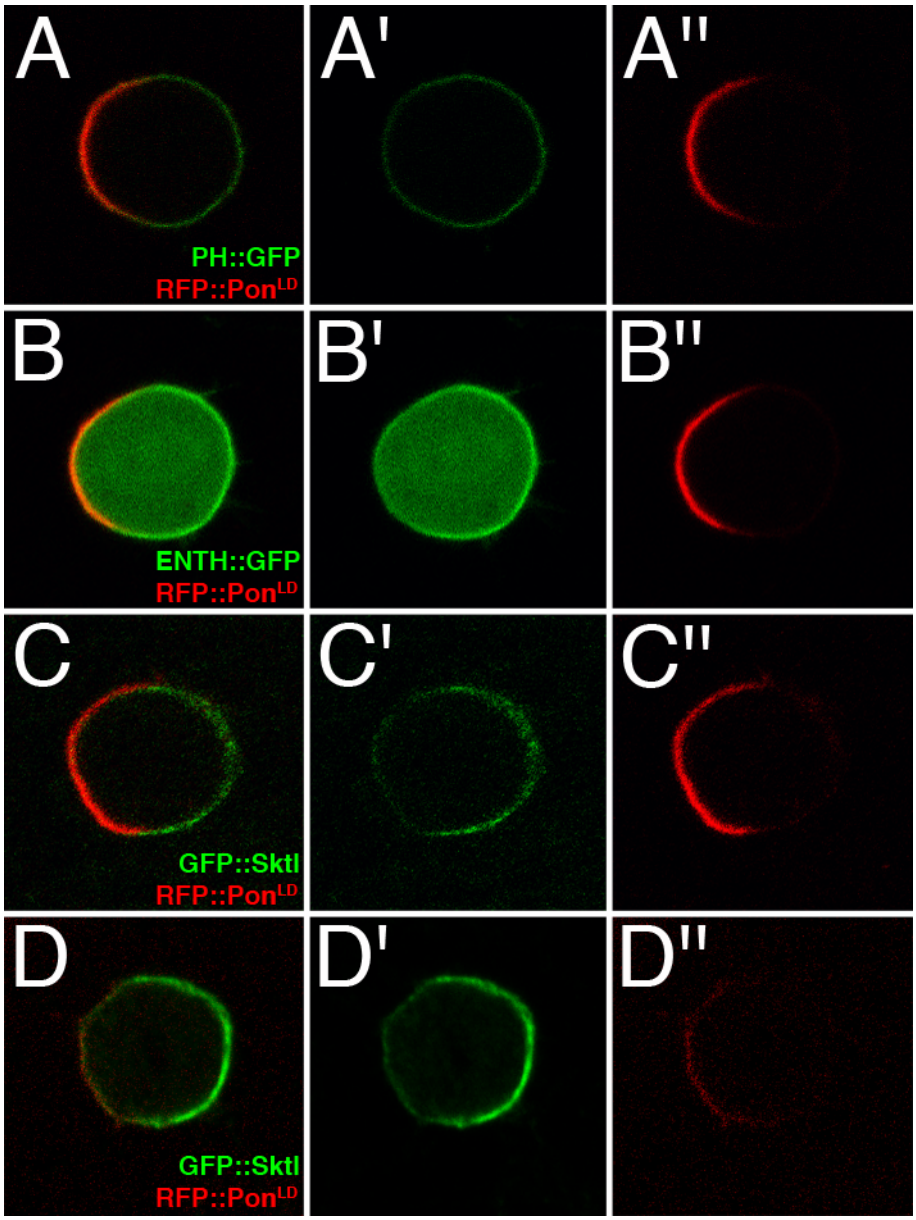

Figure 1. PIP2 reporters and GFP::Sktl localized asymmetrically in dividing SOPs expressing RFP::Pon^{LD}. The distribution of PH::GFP (A,A′), ENTH::GFP (B,B′) and GFP::Sktl (C,C′) was examined by live imaging (A–C″) or by antibody staining (anti-GFP in green in D–D″) in dividing SOPs expressing RFP::Pon^{LD} (A″,B″,C″,D″). Both PIP2 reporters and GFP::Sktl (C; 83%; n = 18) localize at the posterior pole of dividing SOPs at prometaphase when co-expressed with RFP::Pon^{LD}. All transgenes were expressed under the control of neur^{P72}Gal4 Gal80^{ts}. Anterior is on the right in this and all other figures.
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(ENTH::GFP). These two PIP2 reporters were specifically expressed in SOPs using the UAS/GAL4 system [23] and their localization was monitored in living pupae. A fusion protein consisting of the Red Fluorescent Protein (RFP) fused to the C-terminal localization domain of Pon (RFP::Pon^{LD}) [11,24] was co-expressed with the PIP2 reporters to reveal SOP polarity. When co-expressed with RFP::Pon^{LD}, PH::GFP was found to localize at the cell cortex and appeared to be slightly enriched at the posterior cortex (Fig. 1A–A″). ENTH::GFP was found to localize at higher levels at the posterior cortex (Fig. 1B–B″). ENTH::GFP was also found in the cytoplasm, perharps reflecting a lower affinity of the ENTH for PIP2 relative to the PH domain [25]. To test whether the posterior accumulation of these reporters correlated with the localization of the PIP2-producing kinase Sktl, we next examined the distribution of Sktl using a functional GFP::Sktl fusion protein (Gervais et al, unpublished). Similarly to PH::GFP and ENTH::GFP, GFP::Sktl cortical localization was concentrated at the posterior cortex, i.e. opposite to RFP::Pon^{LD} (Fig. 1C–D″; 83% n = 18).

In the experiments described above, RFP::Pon^{LD} reporter was used simply as an internal control for asymmetry. However, we observed unexpectedly that when SOPs did not co-express RFP::Pon^{LD} the localization of PH::GFP, ENTH::GFP and GFP::Sktl was no longer polarized. PH::GFP (Fig. 2A) and ENTH::GFP (Fig. 2B) localized uniformly at the cell cortex. Similarly, GFP::Sktl localized uniformly at the cortex of dividing SOPs, both in living (Fig. 2C; 100% n = 20) and fixed cells (Fig. 2D–D′). Two conclusions can be drawn. First, PIP2 reporters and GFP::Sktl are distributed uniformly along the a–p polarity axis of wild-type SOPs at mitosis. Second, expression of RFP::Pon^{LD} has the ability to alter the distribution of PIP2 in dividing SOPs, possibly by altering the distribution of the PIP2-producing enzyme Sktl. Finally, the defective distribution of PIP2 seen upon RFP::Pon^{LD} expression may reveal a novel activity of Pon that is distinct from its known Numb-binding activity.

Pon overexpression alters the distribution of Sktl

To test whether the activity seen with the C-terminal LD domain of Pon indeed reveals a novel activity of Pon, a tagged version of full-length Pon was overexpressed in dividing SOPs. While expression of Pon did not detectably alter the localization of PH::GFP (Fig. 3A) or ENTH::GFP (Fig. 3B), it resulted in the posterior localization of GFP::Sktl. This posterior accumulation was seen both in living (Fig. 3C; 56%, n = 9) and in fixed SOPs (Fig. 3D′; 55% n = 11). The difference in results with Pon and RFP::Pon^{LD} (compare Figs 2A,B and 3A,B) may be due to higher level of RFP::Pon^{LD} expression and/or activity. This difference suggests that the asymmetric distribution of PIP2, as monitored using the PH::GFP and ENTH::GFP reporters, does not necessarily correlate with those of GFP::Sktl and that GFP::Sktl may be more sensitive to the overexpression of Pon. Moreover, this effect of Pon appeared to be specific since overexpression of Numb or Miranda (Mira), two proteins that colocalize with Pon at the anterior cortex of dividing SOPs [11,26] (our unpublished data), had no significant effect on the spatial localization of GFP::Sktl in dividing SOPs (Fig. 3E,F; Numb: 100%, n = 14; Mira: 100% n = 9; see quantification in Fig. 3G). We conclude that increasing the levels of Pon at the anterior pole of dividing SOPs prevents GFP::Sktl from localizing uniformly at the cell cortex.

We next examined whether expression of Pon can also perturb the localization of endogenous Sktl in SOPs. We therefore analyzed the distribution of Sktl in fixed nota using an anti-Sktl antibody that specifically recognize Sktl on fixed tissues (Gervais et al., unpublished). Sktl was detected at the apical cell cortex of all cells in the notum. We did not detect an increased accumulation of Sktl in SOPs. Sktl was found to accumulate uniformly at the cell cortex in dividing SOPs (Fig. 4A′; 100% n = 15). However, Sktl preferentially localized at the posterior cortex upon RFP::Pon^{LD} expression in SOPs (Fig. 4B′; 69% n = 32). Thus, overexpression of Pon modifies the spatial localization of endogenous Sktl during SOP asymmetric cell division (Fig. 4B′). As the mechanism

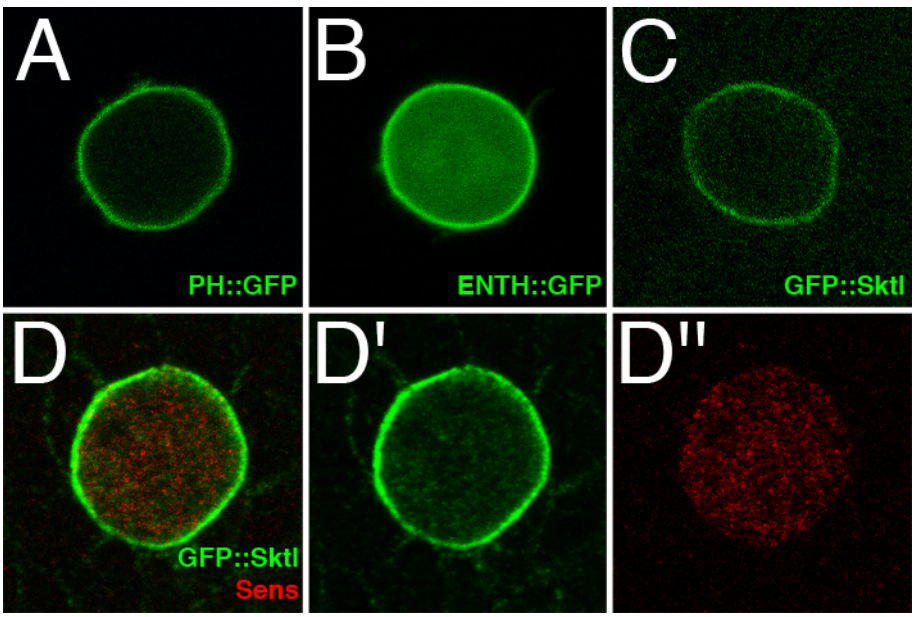

Figure 2. PIP2 Reporters and GFP::Sktl localized uniformly in SOPs in the absence of RFP::Pon^{LD}. The distribution of PH::GFP (A), ENTH::GFP (B) and GFP::Sktl (C–D″) was examined by live imaging (A–C) or by antibody staining (anti-GFP in green and anti-Senseless in red in D–D″) in dividing SOPs at prometaphase. When RFP::Pon^{LD} was not co-expressed in SOPs, the PIP2 reporters and GFP::Sktl (panel C; 100%; n = 20) localized uniformly. All transgenes were expressed under the control of neur^{P72}Gal4Gal80^{ts}.
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whereby Pon and Sktl localize at the cell cortex are not known, how Pon may prevent GFP::Sktl accumulation at the anterior cortex is unclear.

Discussion

We have shown that PIP2 and the PIP2-producing kinase Sktl are distributed at the cortex of dividing SOPs with no asymmetry within the plane of the epithelium. Whether PIP2 and Sktl regulate SOP polarization and/or polar localization of cell fate determinants in dividing SOPs remains to be further studied. Of note, we have not been able to analyze the phenotypes associated with a loss of *sktl* activity in the notum since *sktl* mutant clones fail to grow in wing imaginal discs (C.P., unpublished results).

We have also shown that overexpression of Pon can alter the spatial distribution of PIP2 binding proteins and of endogenous Sktl in dividing SOPs. This unexpected observation raises at least two questions and should also serve as a note of caution for studies using Pon as a marker of polarity.

Multiple mechanisms may explain how Pon overexpression alters the distribution of Sktl. One hypothesis is that both proteins compete for transport machinery components and/or cortical anchoring sites. Endogenous Pon would not affect the transport and/or anchoring of Sktl but overexpression of Pon could saturate the system. Also, the posterior pole accumulation of the PIP2-binding proteins PH::GFP and ENTH::GFP in Pon-overexpressing cells can be interpreted to suggest that overexpression of Pon results either in lower PIP2 concentration at the anterior pole or in higher concentration of competing PIP2-interacting proteins. It would also be interesting to examine whether the localization of the PIP2-producing kinase is itself regulated by PIP2 levels.

How may endogenous Pon regulate protein distribution at the cortex of asymmetrically dividing progenitors? A known function of Pon is to recruit Numb, most likely via direct protein-protein interaction [11,12]. In this case, Pon plays a positive role by recruiting specific proteins at one pole of the dividing cell. However, our observation that Sktl accumulates at the posterior cortex when Pon is overexpressed raises the hypothesis that

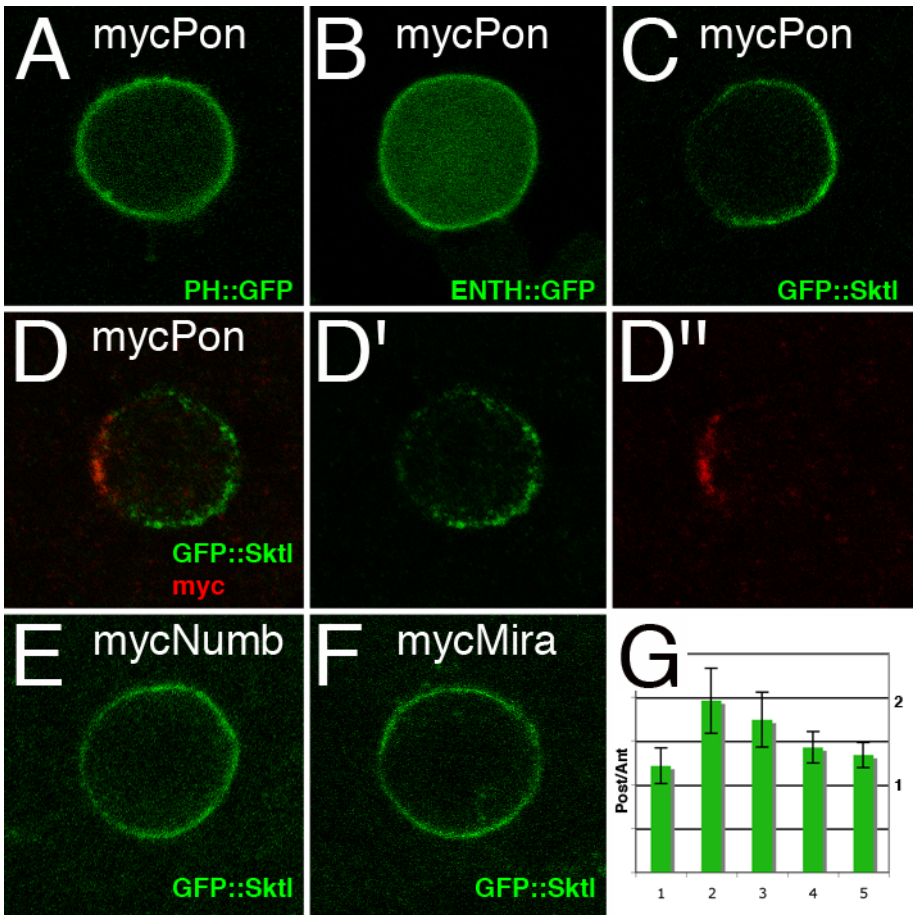

Figure 3. GFP::Sktl localized at the posterior pole upon Pon overexpression. Distribution of PH::GFP (A), ENTH::GFP (B) and GFP::Sktl (C–F) in SOPs co-expressing Myc-tagged versions of full-length Pon (mycPon; A–D″), Numb (mycNumb; E) or Miranda (mycMira; F). Pon, Numb and Mira localized at the anterior pole of dividing SOPs at prometaphase (anti-Myc in red in D″; data not shown). Overexpression of Pon did not detectably modify the localization of PH::GFP (A), ENTH::GFP (B) in living SOPs. However, Pon overexpression resulted in the posterior accumulation of GFP::Sktl in live SOPs (panel C; 56%; n = 9) as well as in fixed cells (D,D′). Overexpression of Numb (E; 100%; n = 14) or Mira (F; 100%; n = 9) did not change the distribution of GFP::Sktl in live SOPs. All transgenes were expressed under the control of neur^{P72}Gal4Gal80^{ts}. (G) Quantification of the relative GFP signal intensity measured at the posterior *vs* anterior cortex of dividing SOPs at metaphase. GFP::Sktl was expressed in combination with the following constructs: 1. none (as in Fig. 2C); 2. RFP::Pon^{LD} (as in Fig. 1C′); 3. mycPon (as in Fig. 3C); 4. mycNumb (as in Fig. 3E); 5. mycMira (as in Fig. 3F). Ratio values are (mean+/− standart deviation): 1.2 +/− 0.2; 2.0 +/− 0.4; 1.7 +/− 0.3; 1.4 +/− 0.2; 1.3 +/− 0.1. Expression of RFP::Pon^{LD} and mycPon, but not mycNumb nor mycMira, alters the distribution of GFP::Sktl in a statistically significant manner.
doi:10.1371/journal.pone.0003072.g003

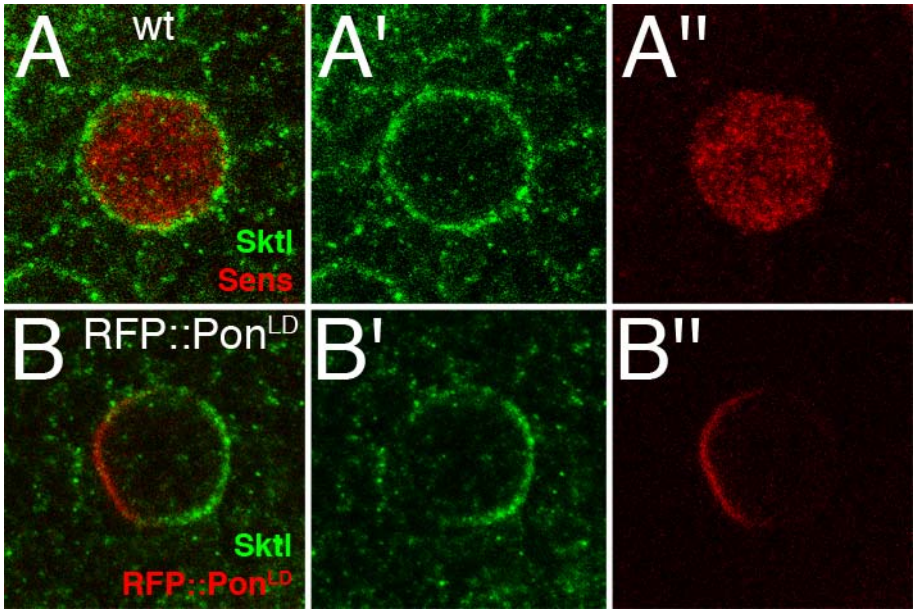

Figure 4. Endogenous Sktl localized uniformly in wild-type SOPs but accumulated at the posterior pole upon RFP::Pon^{LD} overexpression. Localization of endogenous Sktl (anti-Sktl in green) in wild-type (wt; A–A″) and RFP::Pon^{LD}-expressing SOPs (B–B″). RFP::Pon^{LD} was expressed under the control of neur^{P72}Gal4Gal80^{ts}. Sktl localized uniformly in wt mitotic SOPs (A,A′; 100%; n = 15), which were identified by Sens staining (red in A,A″). When RFP::Pon^{LD} was overexpressed in SOPs, Sktl localized asymmetrically at the posterior pole (B,B′; 69%; n = 32).
doi:10.1371/journal.pone.0003072.g004

endogenous Pon may also normally exclude proteins from the membrane domain where it localizes.

Finally, our observation that Pon and RFP::Pon^{LD} alter the distribution of both PIP2 reporters and Sktl is relevant since both RFP::Pon^{LD} and GFP::Pon^{LD} have often been used to study asymmetric cell division of neural precursor cells in *Drosophila* [see: [8,11,26]] including detailed quantitative aspects of asymmetric protein localization [27]. As accumulation of free PIP2-binding sites at the posterior pole of RFP::Pon^{LD}-expressing cells is likely to cause subtle deviations from the wild-type, our observation should serve as a note of caution when interpreting studies that use RFP::Pon^{LD} as a marker.

Materials and Methods

The following transgenes were expressed using neur^{P72}Gal4 [26] combined with a pTub-GAL80^{ts} transgene: ENTH::GFP [22,28], PH::GFP [21], RFP::Pon^{LD} [24], Sktl::GFP (Gervais, in press), mycPon [11], mycMira [29], mycNumb [30].

Live GFP imaging was carried out as described in [26]. All images were acquired on SP2 and SP2AOBS confocal microscopes. Quantification of GFP signal intensity was performed using the plot profiling function of ImageJ 1.32.

Pupal nota were dissected from staged pupae and processed as previously described [3]. Primary antibodies were: rabbit anti-Sktl (Gervais et al. unpublished; 1:500), guinea-pig anti-Senseless (Sens; gift from H. Bellen; 1:3000), rabbit anti-GFP (Molecular Probes; 1:1000) and mouse anti-Myc (9E10; DSHB; 1:500). Cy3- and Cy5-coupled secondary antibodies were from Jackson's Laboratories and Alexa-488 coupled secondary antibodies were from Molecular Probes.

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Author Contributions

Conceived and designed the experiments: CNLRP FS. Performed the experiments: CNLRP. Analyzed the data: CNLRP. Contributed reagents/materials/analysis tools: LG EO JF AG. Wrote the paper: CNLRP FS.

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Transcriptional control of stem cell maintenance in the *Drosophila* intestine

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SUMMARY

Adult stem cells maintain tissue homeostasis by controlling the proper balance of stem cell self-renewal and differentiation. The adult midgut of *Drosophila* contains multipotent intestinal stem cells (ISCs) that self-renew and produce differentiated progeny. Control of ISC identity and maintenance is poorly understood. Here we find that transcriptional repression of Notch target genes by a Hairless-Suppressor of Hairless complex is required for ISC maintenance, and identify genes of the *Enhancer of split* complex [*E(spl)-C*] as the major targets of this repression. In addition, we find that the bHLH transcription factor Daughterless is essential to maintain ISC identity and that bHLH binding sites promote ISC-specific enhancer activity. We propose that Daughterless-dependent bHLH activity is important for the ISC fate and that E(spl)-C factors inhibit this activity to promote differentiation.

KEY WORDS: *Drosophila*, Notch signaling, Adult stem cells

INTRODUCTION

The homeostasis of adult tissues is controlled through the renewal of differentiated cells by adult stem cells or committed progenitors. A detailed understanding of how stem cells are controlled in their in vivo environment has proved challenging owing to the requirement for precise genetic manipulation and in vivo lineage-labeling techniques. The posterior midgut of the adult *Drosophila* intestine is a simple model system in which to understand how adult stem cells are maintained. The posterior midgut epithelium contains intestinal stem cells (ISCs) distributed throughout the tissue that self-renew and produce differentiated cells during the adult lifetime (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Upon cell division, each ISC produces one daughter cell that retains the ISC fate and one enteroblast (EB) that differentiates into either an absorptive enterocyte (EC) or a secretory enteroendocrine cell (ee) (Ohlstein and Spradling, 2006) (see Fig. 1N). This simple cell lineage greatly facilitates the analysis of fate decisions using precise lineage labeling and genetic manipulation of the ISC and its progeny.

Recent studies have begun to address the control mechanisms of stem cell self-renewal and proliferation. Self-renewal of ISCs is influenced by Wingless (Wg) (Lee et al., 2009; Lin et al., 2008). Wg, however, is not strictly required to maintain ISC identity, as ISCs are still detected in the absence of Wg signaling (Lin et al., 2008). Additionally, ISC proliferation is modulated by Insulin and Jak/Stat signaling, at least in response to intestinal damage (Amcheslavsky et al., 2009; Buchon et al., 2009; Jiang et al., 2009; Lin et al., 2010). Despite these significant recent advances, the signals and transcriptional programs that control ISC identity and maintenance are unknown. One important clue, however, has come from studies on the role of Notch in this lineage that showed that differentiation of ISC progeny requires Notch signaling and that forced expression of activated Notch results in the differentiation of ISCs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007).

In this study, we find that ISC maintenance requires transcriptional repression of Notch target genes by Hairless. We identify the *Enhancer of split* complex [*E(spl)-C*] genes as the key Notch targets that need to be repressed by Hairless to ensure ISC maintenance, while being upregulated in EBs to promote differentiation. Additionally, Daughterless (Da), a basic helix-loop-helix (bHLH) transcriptional activator, is also essential for ISC maintenance and bHLH E-box binding sites are required for expression of an ISC-specific enhancer. We propose a model in which Da and E(spl)-C factors act antagonistically to regulate maintenance of the ISC fate versus EB differentiation.

MATERIALS AND METHODS

Drosophila stocks and clone analysis

Drosophila stocks and crosses were kept at 18-25°C. Adults were aged at 25°C unless stated otherwise. Clones were induced in 3- to 6-day-old flies and analyzed in well-fed females. MARCM clones (Lee and Luo, 2001) were generated using the X chromosome *y w P[hs-FLP] P[pTub-GAL4] P[UAS-nlsGFP]* combined with FRT *P[pTub-GAL80]* chromosomes on the second and third chromosomes. MARCM clones on the X chromosome were induced with *hsflp122 P[pTub-GAL80] FRT19A; P[pAct-GAL4] P[UASGFP]* (Lin et al., 2008). The following mutant alleles were used to generate recombinant lines and for experiments: H^{E31}, H^{1}, H^{2}, $neur^{IF65}$, *P[gro+] Df(3R)* $gro^{32.2}$, *P[gro+]* on II, *P[l(2)35Bg+] Su(H)*$^{\Delta 47}$ [a small deletion of *Su(H)* with a genomic rescue construct of the neighboring gene (Morel and Schweisguth, 2000)], $numb^{2}$, $numb^{15}$, da^{10}, da^{3}, *Df(1)*sc^{B57}, $amos^{1}$, and ato^{1}. Control wild-type MARCM clones were generated using *FRT82B P[w+]90B*, *FRT40A* and *y w FRT19A* chromosomes. We generated the *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ deletion by FLP-mediated recombination using lines XPd08311 and RBe00084 (Exelixis). This deficiency removes a genomic region containing the coding sequences (CDSs) of the *E(spl)-C* genes *mδ*, *mγ*, *mβ*, *mα*, *m1*, *m2*, *m3*, *m4*, *m5* and *m6*. It also removes promoter sequence from *m7*, but leaves the coding sequence intact. The *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ deficiency does not remove *m8* or *groucho*. The entire *E(spl)-C* and *groucho* sequences are removed by *Df(3R)*$gro^{32.2}$ and *groucho* is rescued by *P[gro+]*.

We have adapted the MARCM technique to induce positively marked *Su(H) H* double-mutant clones in *y w P[hs-FLP] P[pTub-GAL4] P[UAS-nlsGFP]; FRT40A P[l(2)35Bg+] Su(H)*$^{\Delta 47}$ / *FRT40A P[pTub-GAL80];*

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FRT82B P[w+]90B H^{E31} / FRT82B P[pTub-GAL80] flies, in which GFP is expressed only upon recombination at both *FRT40A* and *FRT82B* sites. A two heat-shock protocol was used to alleviate residual Su(H) protein. The first heat shock creates three types of clones: unmarked $Su(H)^{\Delta 47}$ mutant clones that grow into large clones, unmarked H^{E31} mutant clones that do not proliferate, and marked double-mutant clones that do not proliferate; these double-mutant clones behave as *H* single-mutant clones (data not shown), probably owing to residual levels of Su(H) protein. A second heat shock, applied 5 days later, creates the three types of clones described above as well as a fourth type: marked double-mutant clones produced by a single recombination event at the *FRT82B* occurring in $Su(H)^{\Delta 47}$ mutant cells.

The *esg-GAL4 UAS-GFP; pTub-GAL80^{ts}* driver (Jiang et al., 2009) was used to ectopically express *UAS-scute* and *UAS-asense* in adults raised at 18°C and shifted to 29° C at 3 days of adulthood. The MARCM system was used to overexpress *UAS-Hairless* and *UAS-m7-VP16* (Jimenez and Ish-Horowicz, 1997). *E(spl)-mβ1.5-lacZ* (Cooper et al., 2000), *EE4-lacZ* (Culi and Modolell, 1998; Giagtzoglou et al., 2005) and *Su(H)-GBE-lacZ* (Furriols and Bray, 2001) flies were used.

The wild-type *mira-promoter-GFP* transgene consists of PCR-amplified genomic sequence flanking the *mira* CDS up to adjacent genes (for details, see Fig. S6 in the supplementary material), with the *mira* CDS replaced by nuclear-targeted *mGFP6* sequence (Haseloff et al., 1999). The resulting product was cloned with *Not*I/*Spe*I into the *Not*I/*Xba*I sites of the pattB vector (Bischof et al., 2007). The E-box-mutated version was generated by PCR amplifying seven individual regions (the E-box sequence CAGCTG was mutated to CAAATG within the primer sequences). All PCR was performed using Phusion polymerase (Finnzymes). The *mira-promoter-GFP* and *miraΔEbox-promoter-GFP* constructs were sequenced and injected into ϕX-22A flies (Bischof et al., 2007) to allow site-specific integration of the promoter constructs.

Immunostaining and in situ hybridization

Adult female intestines were dissected in PBS and fixed for 2 hours at room temperature (RT) in 4% paraformaldehyde. Intestines were rinsed in PBT (PBS containing 0.1% Triton X-100), trimmed and incubated for at least 30 minutes in PBS containing 50% glycerol, then equilibrated in PBT to osmotically clean the lumen. For Fig. S1 in the supplementary material and Fig. 6A, intestines were fixed for 15 minutes in 4% formaldehyde/heptane, dehydrated in methanol and rehydrated in PBT as described (Lin et al., 2008). Primary antibody incubations were either overnight at 4°C or 3-5 hours at RT. Secondary antibodies were incubated 3-5 hours at RT. DAPI (1 μg/ml) was added to the final wash. Intestines were mounted in 4% N-propyl-galate, 80% glycerol and imaged on Leica SP2 and SPE confocal microscopes. Images are selected layers of confocal stacks. For Fig. 4B,C, intestines were processed as previously described for embryos (Bardin and Schweisguth, 2006). The following antibodies were used: mouse anti-Delta [C594-9B, developed by the laboratory of S. Artavanis-Tsakonas (Harvard Medical School, Boston, MA, USA); 1:2000, DSHB], guinea pig anti-Hairless A (A. Preiss, Institute of Genetik, University of Hohenheim, Stuttgart, Germany; 1:500), mouse anti-Pros [MR1A, developed by the lab of C. Doe (University of Oregon, Eugene, OR, USA); 1:10, DSHB], rabbit anti-Pros (Y.-N. Jan, University of California, San Francisco, CA, USA; 1:2000), rabbit anti-Daughterless (Y.-N. Jan; 1:1000), rabbit anti-Asense (Y.-N. Jan; 1:5000) and goat anti-β-gal (1:1000, Biogenesis).

For the *scute* in situ hybridization, the protocol developed by the Bier laboratory was used (O'Neill and Bier, 1994), followed by anti-DIG-POD (1:2000, Roche, 11207733910). In situs were developed using the TS Plus Cyanine 3 System following the supplier's instructions (Perkin-Elmer, NEL741001KT).

Quantification and statistical analysis

Only clones located in the posterior midgut were considered for analysis and numerous clones of representative midguts were analyzed. In Fig. 1M and Fig. 5E, the number of cells per clone was counted. In Fig. 3E and Fig. 4H, $Delta^{-}$ GFP^{+}, $Delta^{+}$ GFP^{+}, as well as $Pros^{-}$ GFP^{+} and $Pros^{+}$ GFP^{+} cells were counted on images taken using a CoolSNAP Camera (Ropers Scientific) on a Leica DMRXE epifluorescence microscope. Two planes of focus, apical and basal, were analyzed for each field. Differences in total cell number (Fig. 1M, Fig. 5E) or in the proportion of $Delta^{+}$ and $Pros^{+}$ cells relative to GFP^{+} cells (Fig. 3E, Fig. 4H) between genotypes were tested for statistical significance using a standard normal distribution where $\varepsilon \geq 1.96$ indicates significance with 95% confidence. In Fig. 1M: wild type, n=215 clones; H^{E31}, n=334. Differences in the proportions of wild-type versus H^{E31} clones are significant for: 1-cell clones, ε=3.1; 2- to 5-cell clones, ε=3.2; ≥6-cell clones, ε=8.7. In Fig. 5E: wild type, n=139 clones; da^{10}, n=62. Differences in the proportions of wild-type versus da^{10} clones are significant for: 1-cell clones, ε=5.78; 2- to 5-cell clones, ε=5.62. Since there were no da^{10} clones with greater than 5 cells, the difference in proportions could not be tested.

For counting $Delta^{+}$ cells in *Hairless* mutants and in Fig. 3E at the 10 day time point: wild type, n=422 GFP^{+} cells; H^{E31}, n=202 GFP^{+} cells; $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$, n=983 GFP^{+} cells; $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ H^{E31}, n=331 GFP^{+} cells. The following differences in proportions were statistically significant: wild type versus H^{E31}, ε=3.7; H^{E31} versus $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ H^{E31}, ε=17.1. The difference in proportions between $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ and $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ H^{E31} is not significant (ε=1.7). For counting $Pros^{+}$ cells in Fig. 4H, we used a 6 day time point: wild type, n=294 GFP^{+} cells; H^{E31}, n=197 GFP^{+} cells; $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$, n=622 GFP^{+} cells; $neur^{IF65}$, n=327 GFP^{+} cells. Wild type is significantly different from $neur^{IF65}$ (ε=17.5). This statistical test was not used for cases in which the number of incidents was less than five.

EC density in Fig. 5D was determined on confocal stacks in which nuclear Pdm1 expression and nuclear size were measured and divided by the total area of the mutant or wild-type tissue. We found that $Delta^{+}$ cells and $Pros^{+}$ cells have nuclei ranging between 3 and 5 μm in diameter; we therefore use a cut-off of 7 μm to define the EC. Several representative stacks were analyzed. Error bars represent s.d. from the mean. In Fig. 6J, the number of clones at 10 days AHS that contained at least one $Pros^{+}$ cell was assessed in wild-type versus *Df(1)scB57* clones from Leica SPE confocal stacks. Twelve out of 32 wild-type clones contained at least one $Pros^{+}$ cell, compared with none out of 37 *Df(1)scB57* clones. This difference is statistically significant (P=0.0000256, Fisher's exact test).

For Fig. S2 in the supplementary material, confocal sections were used to count the total number of cells and the $Delta^{+}$, $Pros^{+}$ and phosphorylated Histone H3 $(PH3)^{+}$ cells per clone. Error bars represent s.d. from the mean. In Fig. S2A,B in the supplementary material: for the overexpression of Hairless, n=20 clones; wild type, n=25 clones. In Fig. S2D in the supplementary material: for the overexpression of Hairless, n=25 clones; wild-type, n=28 clones. Thirteen out of 25 clones overexpressing *Hairless* have at least one $PH3^{+}$ cell, compared with 3/28 in wild type (P=0.0008, two-tailed Fisher's exact test). Additionally, 4/25 clones overexpressing *Hairless* contained more than one dividing cell, compared with 0/28 for wild type (P=0.043, two-tailed Fisher's exact test).

RESULTS

Hairless is required for self-renewal of ISCs

Upon division of the ISC, Delta is inherited by both daughter cells and Notch is similarly present in both daughter cells (Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). This raises the possibility that Notch receptors are activated in both daughter cells. Since Notch activity in ISCs can cause terminal differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007), we hypothesized that expression of Notch target genes might be repressed in ISCs. The Hairless-Su(H) co-repressor complex acts in a limited number of developmental contexts to repress Notch target gene expression (Furriols and Bray, 2000; Koelzer and Klein, 2003; Koelzer and Klein, 2006; Morel and Schweisguth, 2000). The adaptor protein Hairless binds to Su(H) and is required for the repressor activity of Su(H) by recruiting the co-repressors CtBP and Groucho, as well as the Histone H3/H4 chaperone Asf1, thereby promoting efficient silencing through repressive chromatin (Bang and Posakony, 1992; Barolo et al., 2002; Brou et al., 1994; Castro et al., 2005; Goodfellow et al., 2007; Morel et al., 2001; Nagel et al., 2005). Upon Notch receptor activation, Notch is cleaved and the active form of Notch, NICD (Notch intracellular domain),

translocates to the nucleus where it interacts with Su(H) and replaces Hairless (reviewed by Kopan and Ilagan, 2009; Bray, 2006; Schweisguth, 2004). First, we examined the expression of Hairless in the intestine of adult flies. Hairless was detected in the nuclei of ISCs and EBs, as well as in a small number of differentiated progeny (see Fig. S1 in the supplementary material). By contrast, lower or undetectable levels of Hairless were present in more mature ECs (see Fig. S1 in the supplementary material). This expression is consistent with a role of Hairless in ISCs and/or ISC daughter cells.

Next, we examined the role of *Hairless* in ISC maintenance. Growth of clones that were homozygous for the null allele *Hairless*^{E31} (H^{E31}) was examined using the mosaic analysis with repressible cell marker (MARCM) technique (Lee and Luo, 2001), in which GFP was heritably expressed in either the ISC and its progeny cells, forming a growing stem cell clone, or in the EB, forming a transient single-cell clone with a ~1-week turnover rate (Ohlstein and Spradling, 2006). Whereas wild-type clones proliferated over time and formed large clones by 14 days after heat shock (AHS) (Fig. 1A-D), H^{E31} mutant clones failed to grow, forming either very small groups of cells or remaining as single cells at 14 days AHS (Fig. 1E-H). To quantify the self-renewal potential of *Hairless* mutant clones, we counted the number of large stem cell clones (6 cells or more), small stem cell clones (2-5 cells), and single-cell clones (transient clones and stem cell clones that are non-proliferating) at 6 days AHS (Fig. 1M). This analysis revealed that only 4% of the H^{E31} mutant clones contained 6 cells or more, compared with 31% for wild-type clones. Furthermore, 55% of the H^{E31} mutant clones were single-cell clones (n=215), compared with only 42% of wild-type clones (n=334). These differences are statistically significant (see Materials and methods). H^{1} and H^{2} clones similarly lost self-renewing capacity over time and failed to produce large clones at 14 days AHS (data not shown), indicating that this phenotype is unlikely to result from unrelated mutations on this chromosomal arm. These results clearly show that *Hairless* mutant clones have a considerably diminished capacity to grow.

We next examined the fate of ISCs in *Hairless* mutants using Delta as a marker. At 10 days AHS, only 7% of cells in H^{E31} mutant clones were Delta^{+} (n=202; Fig. 1I-J‴ and Fig. 3E), whereas 18% of cells in wild-type clones were Delta^{+} (n=422), indicating that the ISC fate is not properly maintained upon loss of *Hairless* activity. We further examined differentiation in *Hairless* mutant clones. At 6 days AHS, 40% (n=158) of the H^{E31} mutant cells expressed the EC marker Pdm1 (Nubbin – FlyBase) (Lee et al., 2009) and 59% (n=675) had a large polyploid nucleus, a characteristic of ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), whereas only 0.5% (n=202) of the H^{E31} mutant cells expressed the ee marker Prospero (Pros) (Fig. 3E). This indicates that *Hairless* mutant cells can differentiate and, when they do so, they become ECs and not ees. This fate choice is consistent with the known role of Hairless in antagonizing Notch, as strong Notch signaling favors adoption of the EC fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Nevertheless, 27% (n=675) of the H^{E31} mutant cells had a small diploid nucleus and 14% had an intermediate size nucleus. Thus, many *Hairless* mutant cells appear to have lost ISC characteristics (i.e. the expression of Delta and the ability to form large clones over time) without properly differentiating into ECs.

The effect of *Hairless* overexpression on clone growth and cell fate was tested. *Hairless* overexpression for 6 days caused a large increase in average clone size (85±57 cells, n=20 clones; see Fig. S2A in the supplementary material), as compared with wild-type clones (14±10 cells, n=25 clones). Additionally, *Hairless* overexpression resulted in many clones containing at least one

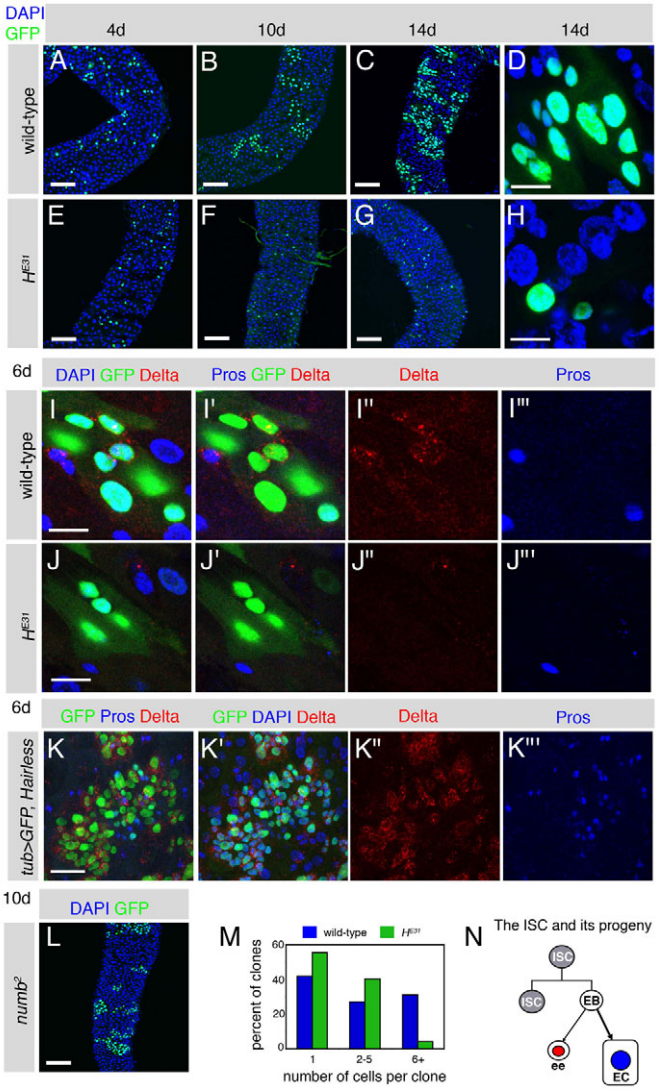

Fig. 1. *Hairless* is required for ISC self-renewal. The growth of positively marked clones (GFP, green) as well as the expression of Delta (red) and Pros (blue) were monitored over time in the posterior midgut (nuclear DAPI, blue). (**A-H**) The growth of positively marked wild-type (A-D) and H^{E31} mutant (E-H) clones was monitored over a 2-week period at 4, 10 and 14 days (d) after heat shock (AHS). (D,H) High-magnification views at 14 days. (**I-J‴**) Whereas wild-type control clones (I-I‴) usually contained 1-2 Delta^{+} small cells and 0-2 Pros^{+} cells, H^{E31} clones (J-J‴) lacked Delta^{+} small cells and Pros^{+} cells. Note that Delta expression was detected in some Pros^{+} cells in wild-type midguts (see I′). (**K-K‴**) Overexpression of *Hairless* in MARCM clones under the control of *tub-GAL4* over a 6-day period resulted in ectopic Delta^{+} ISC-like cells and ectopic Pros^{+} cells. (**L**) *numb*^{2} mutant ISCs proliferated into large clones by 10 days AHS. (**M**) Analysis of the size of wild-type and H^{E31} clones at 6 days AHS. For each category of clone size, the number of clones is given as the percentage of the total number of clones (wild-type, n=215 clones; H^{E31}, n=334 clones). The frequency of single-cell clones, corresponding to transient clones and non-proliferative ISC clones, was significantly increased upon loss of *Hairless* activity, whereas large H^{E31} mutant clones (6 cells or more) were rare compared with wild-type clones (see Materials and methods for statistics). (**N**) The ISC and its progeny. The ISC produces an ISC and an enteroblast (EB) upon division. The EB further differentiates into either an enteroendocrine cell (ee) expressing the transcription factor Pros or a large polyploid enterocyte (EC). Scale bars: 100 μm in A-C,E-G,L; 10 μm, in D,H,I-K.

mitotic cell (52%, *n*=25 clones; see Fig. S2C,D in the supplementary material), with one clone containing five cells undergoing division at the same time, including two that were directly adjacent, strongly suggesting that these two cells had acquired ISC fate. In addition, *Hairless* overexpression was sufficient to promote the formation of many ectopic small cells that expressed Delta and/or Pros (Fig. 1K-K″ and see Fig. S2 in the supplementary material). The increased number of both Delta$^+$ and mitotic cells indicates that the overexpression of *Hairless* induces ectopic ISCs. We conclude that Hairless is both necessary and sufficient to produce ISC-like cells, indicating that Hairless regulates the maintenance of ISCs.

By contrast, Numb, another negative regulator of Notch, plays no essential role in ISC self-renewal: both *numb*^{2} (Fig. 1L) and *numb*^{15} (data not shown) mutant cells produced large clones over time. We therefore conclude that *Hairless*, but not *numb*, is required for ISC self-renewal and maintenance.

Hairless acts in an *Su(H)*-dependent manner to maintain the ISC fate

Repression of Notch target genes by Hairless requires the sequence-specific DNA-binding protein Su(H), which recruits the repression complex onto DNA (Barolo et al., 2002; Furriols and Bray, 2001; Morel et al., 2001; Morel and Schweisguth, 2000). To test whether Hairless acts via Su(H) to regulate ISC maintenance, we generated clones lacking both *Hairless* and *Su(H)* activities (see Materials and methods). The loss of *Hairless* activity resulted in defective self-renewal (Fig. 2C-D′; see also Fig. 1), whereas loss of *Su(H)* activity led to an overspecification of small Delta$^+$ ISC-like cells (Fig. 2A-B′) (see Ohlstein and Spradling, 2007). Positively marked *Su(H)*$^{\Delta 47}$ *H*^{E31} double-mutant clones generated within *Su(H)*$^{\Delta 47}$ single-mutant unmarked clones proliferated and expressed the ISC marker Delta (Fig. 2E-H′). Therefore, the activity of *Su(H)* is required for the ISC loss seen in *Hairless* mutant clones and we conclude that *Su(H)* is epistatic to *Hairless* with respect to Delta expression and ISC self-renewal. This suggests that Hairless acts via Su(H) to repress Notch target genes in ISCs in order to promote stem cell maintenance. Interestingly, the loss of *Su(H)* led to an increased number of ISC-like cells, similar to Notch loss-of-function, whereas loss of *Hairless* activity led to a loss of ISC self-renewal, similar to the overexpression of an activated Notch receptor. This suggests that loss of Hairless/Su(H)-mediated repression, on its own, does not lead to ISC loss and that Su(H)-mediated target gene activation is also required for ISC loss in this context.

Deletion of the *E(spl)-C* suppresses the *Hairless* loss-of-ISC phenotype

We next sought to identify the Notch target genes that need to be repressed by Hairless in ISCs. The *E(spl)-C* genes are well characterized targets of the Notch pathway (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). We found that an *E(spl)mβ-lacZ* construct (Cooper et al., 2000) was highly upregulated in EB cells, whereas it was expressed at only low levels in ISCs (Fig. 3F-F″). Moreover, loss of *E(spl)-C* genes, using either *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ or the *Df(3R) gro*$^{32.2}$ combined with the *groucho* rescue construct, *P[gro+]*, resulted in an increased number of small Delta$^+$ ISC-like cells (Fig. 3A-A″,E; see Fig. S3A-C‴ in the supplementary material). This indicates that *E(spl)-C* genes are required in ISC daughter cells to promote EB differentiation and/or block self-renewal in presumptive EB cells. To test whether this phenotype was due to the loss of the *E(spl)-C* bHLH transcriptional repressors, we took advantage of an ‘activator’ version of m7 (HLHm7 – FlyBase), m7-VP16, in which fusion of the transcriptional activator VP16 to the bHLH m7 converts m7 from a transcriptional repressor into an activator (Jimenez and Ish-Horowicz, 1997). Expression of m7-VP16 similarly resulted in an increase in small Delta$^+$ ISC-like cells and a loss of Pros$^+$ cells (see Fig. S3F-F″ in the supplementary material). We therefore conclude that EB differentiation and/or inhibition of ISC self-renewal are likely to result from the loss of one or more *E(spl)-C* bHLH genes.

To test whether the loss of ISCs seen in the absence of *Hairless* activity was due to derepression of *E(spl)-C* genes, we examined the phenotype of *E(spl)-C Hairless* double-mutant clones. We found that deletion of the *E(spl)-C*, using either *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ or the *Df(3R) gro*$^{32.2}$ *P[gro+]*, rescued the growth defect of *Hairless* mutant clones at 6 and 10 days AHS (Fig. 3B-B‴,D; see Fig. S3D-E‴ in the supplementary material; compare with *Hairless* single-mutant clones in Fig. 1E-H,J-J‴). The observation of large clones at 10 days AHS clearly indicated that *Hairless E(spl)-C* double-mutant ISCs retained their ability to self-renew over time (Fig. 3D and see Fig. S3D-D″ in the supplementary material). Consistent with this, ISC-like Delta$^+$ cells were seen in *Hairless E(spl)-C*

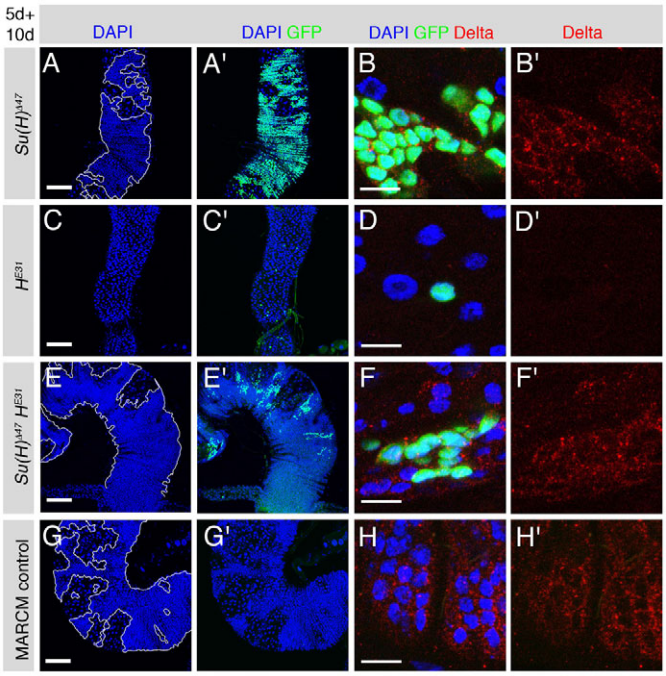

Fig. 2. *Su(H)* is required for the *Hairless* ISC maintenance defect. Clone growth (GFP, green; DAPI, blue) and Delta expression (red) were examined in clones produced using a two heat-shock protocol. Low (A,A′,C,C′,E,E′,G,G′) and high (B,B′,D,D′,F,F′,H,H′) magnification views of representative midguts are shown. (**A-B′**) *Su(H)*$^{\Delta 47}$ clones (outlined in A) contained ISC-like cells expressing Delta. (**C-D′**) *H*^{E31} clones failed to proliferate and did not express Delta. (**E-F′**) *H*^{E31} positively marked clones within *Su(H)*$^{\Delta 47}$ unmarked clones (outlined in E) proliferated and contained Delta-expressing ISC-like cells. (**G-H′**) Negative control clones. The potential loss of GAL80 expression independent of a recombination event was monitored in *Su(H)*$^{\Delta 47}$ single-mutant clones (outlined in G) in *y w P[hs-FLP] P[pTub-GAL4] P[UAS-nlsGFP]; FRT40A P[l(2)35Bg] Su(H)*$^{\Delta 47}$*/ FRT40A P[pTub-GAL80]; FRT82B P[pTub-GAL80] / MKRS* flies. This genotype produced virtually no GFP$^+$ cells, indicating that the spontaneous loss of GAL80 expression cannot account for the phenotype seen in E-F′. Scale bars: 100 μm in A,A′,C,C′,E,E′,G,G′; 10 μm in B,B′,D,D′,F,F′,H,H′.

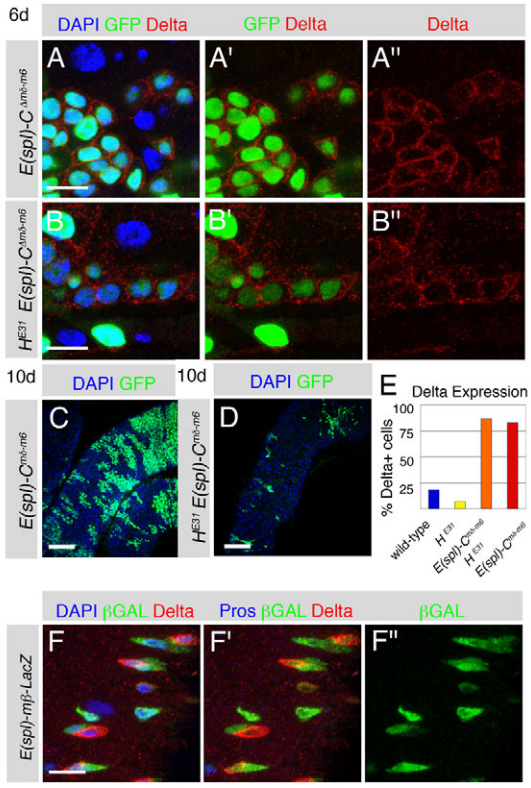

Fig. 3. Deletion of the *E(spl)-C* suppresses the *Hairless* ISC maintenance defect. (**A-B″**) Both *E(spl)-C*$^{mδ\text{-}m6}$ (A-A″) and *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} (B-B″) positively marked mutant clones (GFP, green; nuclear DAPI, blue) grew and contained many small Delta^{+} (red) ISC-like cells at 6 days AHS. (**C**,**D**) *E(spl)-C*$^{mδ\text{-}m6}$ clones (C) were larger than *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} clones (D) at 10 days AHS. (**E**) Analysis of the percentage of Delta^{+} cells in wild-type, *H*^{E31}, *E(spl)-C*$^{mδ\text{-}m6}$ and *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} clones at 10 days AHS. The difference between *H*^{E31} and either *E(spl)-C*$^{mδ\text{-}m6}$ or *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} was statistically significant, whereas the difference between *E(spl)-C*$^{mδ\text{-}m6}$ and *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} was not (see Materials and methods for statistics). (**F-F″**) *E(spl)-mβ1.5-lacZ* expression (β-galactosidase, green) was high in presumptive EBs adjacent to ISCs (Delta, red) that exhibited low *E(spl)-mβ1.5-lacZ* expression. Only basal nuclei are shown in this confocal section (Pros and DAPI, blue). Scale bars: 10 μm in A-B″, F-F″; 100 μm in C,D.

double-mutant clones (Fig. 3B-B″,E and see Fig. S3E-E‴ in the supplementary material). Quantification of Delta^{+} cells showed that *H*^{E31} *E(spl)-C*$^{Δmδ\text{-}m6}$ mutant clones contain a similar proportion of Delta^{+} cells (83%, *n*=331 cells) as *E(spl)-C*$^{Δmδ\text{-}m6}$ mutant clones (87%, *n*=983 cells) at 10 days AHS, a significant rescue of the 7% of Delta^{+} cells in *Hairless* mutants clones (*n*=202 cells; Fig. 3E). We note, however, that *E(spl)-C* single-mutant clones were larger than *Hairless E(spl)-C* double-mutant clones (Fig. 3C,D). We interpret this difference in clone growth to suggest the existence of additional Notch targets that act to limit self-renewal and are subject to derepression in the *Hairless E(spl)-C* mutant cells. Expression of these targets would therefore limit the growth of *Hairless E(spl)-C* double-mutant clones. Taken together, our data indicate that *E(spl)-C* genes promote EB differentiation and/or block ISC self-renewal in presumptive EB cells and that their inhibition in the ISC by Hairless-mediated repression prevents ISC loss.

E(spl)-C genes play a non-essential role in enteroendocrine differentiation

E(spl)-C mutant cells do not express the ee markers Pros [Fig. 4A-B‴,H and see Fig. S3 in the supplementary material; only 0.5% of mutant cells are Pros^{+} (*n*=622 cells) compared with 6.1% of cells in wild-type clones (*n*=294 cells)] and Allatostatin (data not shown). Expression of m7-VP16 produced similar phenotypes to those when *E(spl)-C* is lost (see Fig. S3F-F″ in the supplementary material). By contrast, EC cells were properly specified, as shown by both large nuclear size (>7 μm) and expression of the EC marker Pdm1 (Fig. 4B-D and see Fig. S3C in the supplementary material; note that Pdm1 appeared to also mark early ECs with a nucleus smaller than 7 μm). The density of large ECs in *E(spl)-C* mutant tissue was similar to that of wild-type tissue, whereas the density of Pdm1^{+} cells was slightly increased in the *E(spl)-C* mutant tissue (Fig. 4B-D). Thus, loss of *E(spl)-C* genes led to a large excess of Delta^{+} ISC-like cells, a normal density of differentiated EC-like cells and a loss of Pros^{+} ee-like cells. Despite the large increase in small Delta^{+} cells, no general hyperplasia of the tissue was observed (Fig. 4B-D). We conclude that genes of the *E(spl)-C* are important to limit ISC fate specification and to promote ee differentiation, but are dispensable for the production of ECs. Since Notch signaling is required for EC fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), this implies that Notch targets other than those encoded by the *E(spl)*-C act to promote EC differentiation. Furthermore, whereas the *E(spl)-C* genes are important for ee differentiation, this role could be bypassed by reducing Delta signaling activity using a *neuralized* (*neur*) mutation. Indeed, expression of Pros was restored in *neur E(spl)-C* double-mutant clones (Fig. 4E-G‴), showing that *E(spl)-C* genes are not strictly required for ee specification.

Daughterless is required for ISC maintenance

A major function of the E(spl) bHLH repressors in both *Drosophila* and vertebrate development is to inhibit the activity of the bHLH Daughterless (Da)/E47-based dimeric transcriptional activators (reviewed by Kageyama et al., 2007; Alifragis et al., 1997; Gigliani et al., 1996; Heitzler et al., 1996; Oellers et al., 1994). Consistent with this, we found that Da is expressed in cells of the ISC lineage (see Fig. S4 in the supplementary material). To test the possible role of *da* in this tissue, we studied the growth of *da* mutant clones. We found that ISCs are lost in *da*^{10} (Fig. 5C-E) and *da*^{3} (data not shown) clones, as seen by loss of clone growth and loss of Delta^{+} ISC cells. *da* mutant clones consisted of single or pairs of polyploid EC-like cells, showing that *da* is important for ISC maintenance.

Da-dependent bHLH transcriptional activity is mediated through E-box motifs. To test whether Da regulates ISC-specific gene expression, we examined the expression of a known Da/proneural target (Reeves and Posakony, 2005), *miranda* (*mira*), using a reporter gene in which the *mira* coding sequence is replaced with nuclear GFP (*mira-promoter-GFP*; Materials and methods and see Fig. S6 in the supplementary material). We found that *mira-promoter-GFP* was specifically expressed in ISCs (Fig. 5F-G″). Moreover, mutation of the seven E-box motifs present in this construct largely eliminated ISC-specific gene expression (Fig. 5H-I″), indicating that E-box sites are necessary to drive such expression. In addition, multimerized E-boxes in front of a minimal promoter were sufficient to direct expression in ISCs, albeit weakly (see Fig. S5 in the supplementary material). We conclude that Da-binding motifs can mediate ISC-specific gene expression.

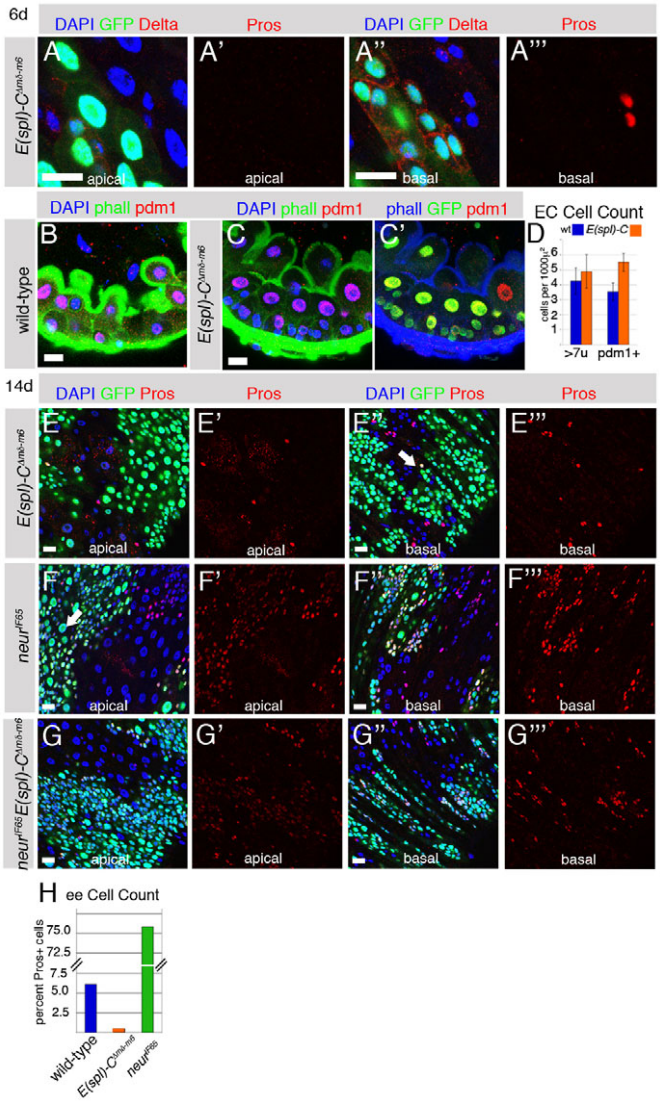

Fig. 4. *E(spl)-C* genes regulate enteroendocrine differentiation. (**A-A‴**) The expression of Pros as a marker for ee fate and polyploidy (DAPI, blue) as a marker for EC fate were assessed in *E(spl)-C* and *neur* mutant clones (GFP, green). EC nuclei were detected in apical planes (A). A schematic of cells in apical/basal planes is shown in Fig. S3B in the supplementary material. Apical (A,A′) and basal (A″,A‴) views are shown of a 6-day AHS *E(spl)-C*$^{mδ\text{-}m6}$ mutant clone. *E(spl)-C*$^{mδ\text{-}m6}$ mutant clones produced many ISC-like cells expressing Delta (red in A,A″) but not Pros (red in A′,A‴). (**B-C′**) At 6 days, *E(spl)-C*$^{mδ\text{-}m6}$ mutant clones (GFP, green in C′) had an increase in small nuclei (DAPI, blue) but overall tissue architecture (phalloidin, green in B,C and blue in C′; Pdm1, red) was similar to the wild type (B). (**D**) The density and specification of large (>7 μm) polyploid ECs was unaffected by the deletion of the *E(spl)-C*, although a slightly higher number of cells expressing Pdm1 was present. Error bars represent s.d. from the mean. (**E-E‴**) Apical and basal low-magnification views of a field of 14-day *E(spl)-C*$^{mδ\text{-}m6}$ mutant cells. Polyploid ECs were properly specified (E), whereas Pros was not expressed in *E(spl)-C*$^{mδ\text{-}m6}$ mutant cells (E-E‴; an exceptional Pros$^+$ cell is indicated in E″ by an arrow). (**F-F‴**) Apical and basal low-magnification views of a field with 14-day *neur*^{IF65} mutant cells. As previously noted (Ohlstein and Spradling, 2007), *neur*^{IF65} mutant clones contain few polyploid ECs (arrow in F) but many ectopic Pros$^+$ ee-like cells (F-F‴). (**G-G‴**) Apical and basal low-magnification views of a field with 14-day *neur*^{IF65} *E(spl)-C*$^{mδ\text{-}m6}$ mutant clones. Many Pros$^+$ ee-like cells were detected (G′,G‴). (**H**) Quantification of the number of Pros$^+$ ee cells. *E(spl)-C*$^{Δmδ\text{-}m6}$ mutant clones lack Pros$^+$ cells (0.5%, *n*=622 cells) compared with *neur*^{IF65} mutant (76%, *n*=327 cells) and wild-type (6%, *n*=294 cells) clones. See Materials and methods for statistics. Scale bars: 10 μm.

achaete-acute complex genes are required for ee, but not ISC, specification

Class I bHLH family members (Da/E47) can act as homodimers (Ellenberger et al., 1994; Murre et al., 1991; Oellers et al., 1994), as heterodimers with class II bHLH proteins (Powell and Jarman, 2008), and, at least in the *Drosophila* wing, evidence suggests that Da acts in concert with a Zn-finger transcription factor (Jafar-Nejad et al., 2006). Da/E47 members have a broad expression pattern, such that class II bHLH partners with a more restricted expression pattern often confer cell- or tissue-specificity to the heterodimeric complex. In the *Drosophila* embryo, Da is ubiquitous and acts with proneural bHLH factors of the *achaete-scute* complex (*AS-C*) that are expressed in adult midgut precursor cells to regulate their fate (Tepass and Hartenstein, 1995). Since Da expression was fairly general in the intestine, we similarly reasoned that a putative Da bHLH class II protein might be specifically expressed in the ISC to promote E-box-dependent ISC-specific expression, as seen for *mira*. To identify ISC-specific bHLH genes, we compared the transcriptional profile of wild-type posterior midguts with those mutant for *Su(H)* that contained a large excess of ISC-like cells and a mild increase in Pros$^+$ ee-like cells (data to be presented elsewhere). From this analysis, two class II bHLH family genes were highly upregulated: *scute* (60-fold) and *asense* (22-fold). The Asense protein was detected in a subset of Pros$^+$ ee cells (Fig. 6A-A″). Since Scute antibodies are no longer available, we used in situ hybridization to detect the *scute* RNA. *scute* transcripts were detected in *Su(H)* mutant clones that contain many ISC- and ee-like cells (Fig. 6B,B′), as well as in single basal cells with small nuclei in the heterozygous tissue outside of clones (Fig. 6C), suggesting that *scute* is expressed in the ISC, EB and/or ee.

We therefore investigated the role of *scute* and *asense* in ISC maintenance and ee differentiation using *Df(1)scB57*, which lacks all four *AS-C* genes (*achaete*, *scute*, *lethal of scute* and *asense*). No effect on clone growth or on Delta$^+$ expression was observed (Fig. 6D-I). We conclude that the *scute* and *asense* genes are not required for ISC maintenance. The bHLH genes *amos* and *atonal* were similarly not essential for ISC maintenance (data not shown). Whether proneural proteins act redundantly in the maintenance of ISCs or, alternatively, whether Da acts as a heterodimer with a non-proneural bHLH protein or as a homodimer, remains to be studied. We next studied ee differentiation and found that *Df(1)scB57* clones are devoid of Pros$^+$ cells (Fig. 6F,I,J). These expression and genetic data indicate that *asense* and/or *scute* are necessary for ee differentiation. Conversely, overexpression in ISCs of *asense* or *scute* using *esgGAL4 GAL80ts* increased the number of Pros$^+$ cells (Fig. 6J-O), indicating that *asense* and *scute* are sufficient to promote ee differentiation.

DISCUSSION

Adult stem cells self-renew and, at the same time, give rise to progeny that eventually differentiate. Our work provides evidence that one of the strategies used to maintain the identity of ISCs in *Drosophila* is to repress the expression of Notch target genes. Consistent with our finding, the loss of a general regulator of transcriptional repression, the Histone H2B ubiquitin protease

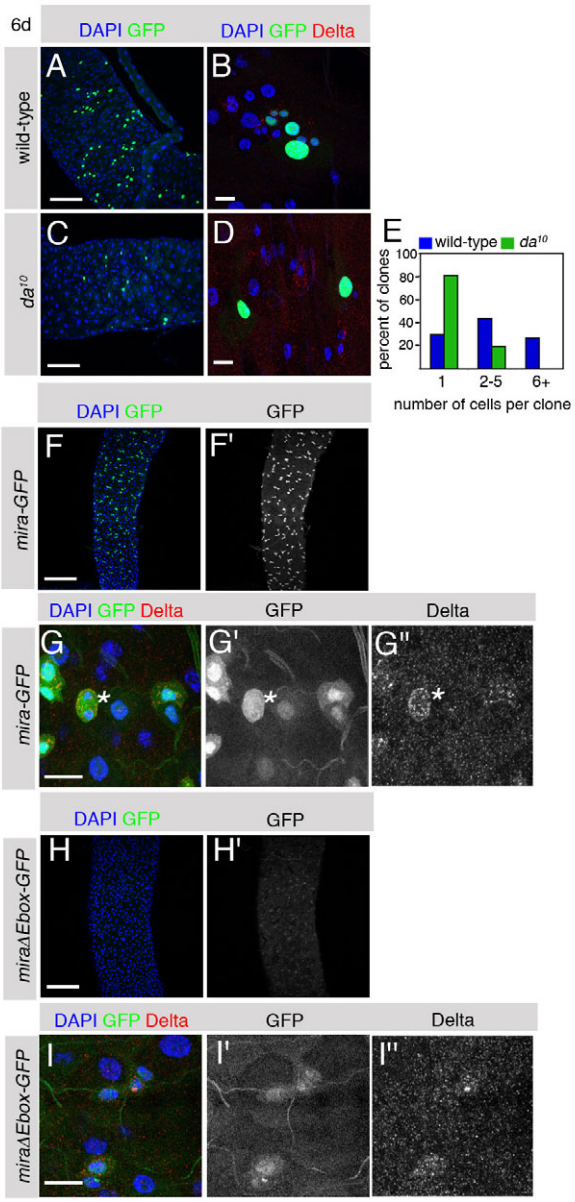

Fig. 5. *daughterless* is required for ISC identity. (**A-D**) Clone growth (nuclear GFP, green; DAPI, blue) and expression of Delta (red) were examined in wild-type (A,B) and da^{10} (C,D) clones at 6 days AHS. da^{10} mutant clones (C,D) did not contain Delta$^+$ cells, failed to grow and were composed of single or pairs of differentiated EC cells. (**E**) Quantification of clone size [number of cells in wild-type (blue, *n*=139) and da^{10} mutant (green, *n*=62) clones] at 6 days AHS. For each clone size category, the number of clones is given as the percentage of the total number of clones. The frequency of single-cell clones, corresponding to transient clones and non-proliferative ISC clones, was significantly increased upon loss of *da* activity, whereas large da^{10} mutant clones (6 cells or more) were not seen (see Materials and methods for statistics). (**F-G″**) The role of Da-binding motifs was assessed in the context of a *mira-promoter-GFP* transgene (*mira-prom-GFP*) that was specifically expressed in ISCs (nuclear GFP, green in G and white in G′; Delta, red in G and white in G″; DAPI, blue). A dividing ISC is marked by an asterisk in G. Pairs of GFP$^+$ cells were also seen, probably owing to inheritance of GFP by ISC progeny cells. (**H-I″**) Mutation of the seven E-box motifs in the *mira-promoter-GFP* transgene (*miraΔEbox-prom-GFP*) largely abolished nuclear GFP expression (compare nuclear signals in G′ and I′). Scale bars: 100 μm in A,C,F,F′,H,H′; 10 μm in B,D,G-G″,I-I″.

Scrawny, gives a similar phenotype to *Hairless* (Buszczak et al., 2009). Additionally, several recent studies indicate that transcriptional repression of differentiation genes may be a central hallmark of stem cells in general (Dejosez et al., 2008; Jepsen et al., 2007; Liang et al., 2008; Maines et al., 2007; Pietersen and van Lohuizen, 2008).

Two models have been proposed for Hairless activity. One proposes that Hairless competes with NICD for interaction with Su(H), thereby preventing transcriptional activation of Notch target genes by low-level Notch receptor activation (Bang et al., 1995; Morel et al., 2001). A second, non-exclusive, model proposes that Hairless antagonizes the transcriptional activation of Notch target genes by tissue-specific transcription factors other than Notch (Barolo et al., 2002; Barolo et al., 2000; Castro et al., 2005). Since the loss of Su(H) can suppress the phenotype of *Hairless* on ISC clone growth, we propose that Hairless promotes ISC maintenance by repressing the transcription of genes that would otherwise be activated by Notch signaling in ISCs (Fig. 7). Thus, Hairless appears to set a threshold level to buffer Notch signaling in ISCs. In the absence of this repression, the expression of *E(spl)-C* genes and other Notch targets would lead to loss of the ISC fate. Importantly, our findings suggest a mechanism for how the transcriptionally repressed state is turned off and activation of the differentiation program is initiated: high activation of Notch in EBs displaces Hairless from Su(H) and leads to expression of the *E(spl)-C* genes (Fig. 7).

E(spl)-C bHLH repressors act in part through their ability to inhibit bHLH activators (Kageyama et al., 2007). Our data demonstrate that Da is also essential to maintain ISC fate and that E-box Da-binding sites are required to promote ISC-specific enhancer activity. Thus, we propose that activation of *E(spl)-C* genes by Notch in EBs downregulates Da bHLH activity and thereby contributes to turning off ISC identity in the differentiating cell (Fig. 7). The specificity of ISC-specific E-box expression might be due to the ISC-specific expression of a bHLH family member. Although our array analysis raised the possibility that Scute may be specifically expressed in ISCs, our genetic analysis indicates that *scute* function is not essential for ISC maintenance. Alternatively, specificity of gene expression might result from inhibition of bHLH activity in the EB and differentiating daughters, possibly by E(spl)-bHLH factors, rather than by the ISC-specific expression of a Da partner. It is also possible that a non-bHLH, ISC-specific factor restricts the Da-dependent bHLH activity to ISCs in a manner similar to the synergism observed in wing margin sensory organ precursors (SOPs) between the Zn-finger transcription factor Senseless and Da (Acar et al., 2006; Jafar-Nejad et al., 2006).

Recently, a role for the Da homologs E2A (Tcf3) and HEB (Tcf12) has been found in mammalian ISCs marked by the expression of Lgr5 and, in this context, E2A and HEB are thought to heterodimerize with achaete-scute like 2 (Ascl2), which is essential for the maintenance and/or identity of Lgr5$^+$ ISCs (van der Flier et al., 2009). In *Drosophila*, however, *AS-C* genes are not essential for ISC maintenance, but appear to play a role in enteroendocrine fate specification. The observation that Da bHLH activity is required for the identity of both *Drosophila* ISCs and mammalian Lgr5$^+$ ISCs suggests that there might be conservation at the level of the gene expression program. Additionally, the bHLH genes *Atoh1* (*Math1*) and *Neurog3* are both important for differentiation of secretory cells in the mammalian intestine (Lee et al., 2002; Yang et al., 2001). Clearly, further analysis of the control of Da/E2A bHLH activity, as well as of the gene networks downstream of Da/E2A, will be of great interest.

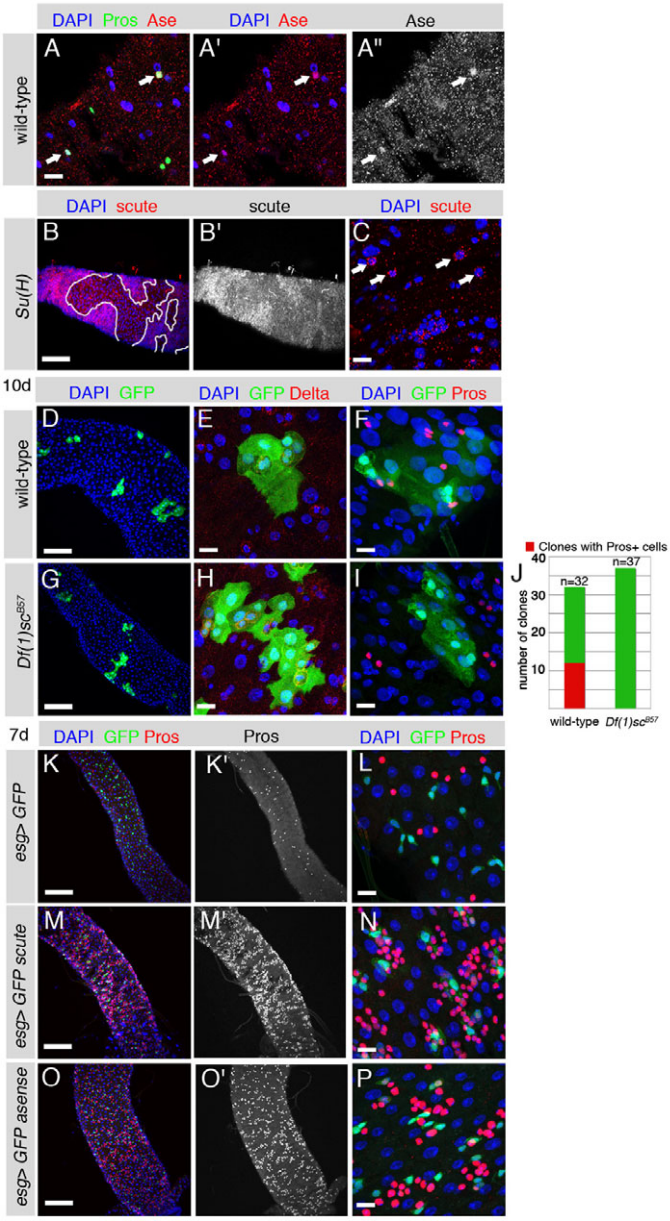

Fig. 6. *achaete-scute* complex genes are dispensable for ISC fate, but act in enteroendocrine fate. (**A-A″**) Expression of the bHLH protein Asense (Ase, red in A,A′, white in A″) was specifically detected in a subset of Pros$^+$ cells (arrows; DAPI, blue; Pros, green). (**B-C**) *scute* RNA (red) was detected by fluorescent in situ hybridization in small nuclei cells both within and outside (arrows in C) of the *Su(H)* mutant clone area (identified by DAPI staining and outlined in B). (**D-J**) *Df(1)sc^{B57}* mutant clones (G-I) grew similarly to wild-type control clones (D-F) and contained Delta$^+$ ISCs as well as polyploid ECs (Delta, red in E,H; DAPI, blue; GFP, green). However, *Df(1)sc^{B57}* mutant clones did not contain Pros$^+$ cells (red). (J) Quantification revealed that one-third of wild-type clones at 10 days AHS contained at least one Pros$^+$ cell (red bar, 12/32 clones), whereas *Df(1)sc^{B57}* (0/37) did not contain Pros$^+$ cells (P=0.00003, Fisher's exact test; see Materials and methods for statistics). (**K-P**) Expression of *scute* (M-N) and *asense* (O-P) in ISCs and EBs of adult flies using *esgGAL4Gal80ts* produced ectopic Pros$^+$ ee cells, as compared with control flies (K-L; GFP, green, Pros, white or red; DAPI, blue). Scale bars: 100 μm in B,B′,D,G,K,K′,M,M′,O,O′; 10 μm in A-A″,C,E,F,H,I,L,N,P.

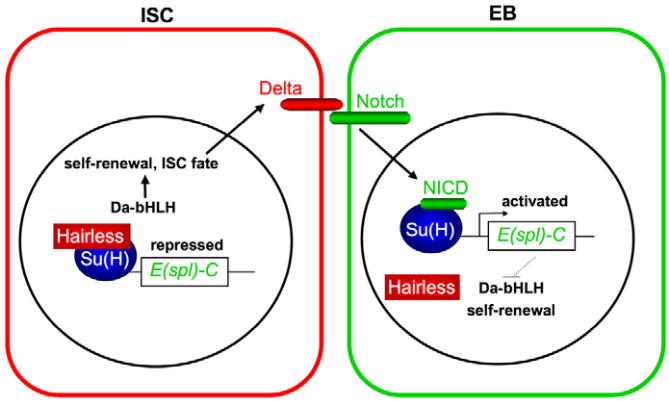

Fig. 7. Model for ISC maintenance. We propose that Hairless prevents ISC loss by repressing expression of Notch target genes, including the *E(spl)-C* genes. We further propose that Da-dependent bHLH activity promotes ISC identity, including the ability to self-renew and to express Delta. Delta, in turn, activates Notch in the adjacent EB (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007), releasing the intracellular domain of Notch (NICD). We speculate that, in response to Notch activation, the E(spl)-bHLH repressors downregulate Da-dependent bHLH activity in EBs as described in other systems (reviewed by Kageyama et al., 2007; Alifragis et al., 1997; Gigliani et al., 1996; Heitzler et al., 1996; Oellers et al., 1994), thereby shutting off ISC identity and promoting differentiation. The solid lines represent interactions for which we provide evidence, whereas the dashed line represents a proposed mechanism based on interaction data from other systems.

Our data suggest that ISC fate is promoted both by inhibition of Notch target genes through Hairless/Su(H) repression and by activation of ISC-specific genes through bHLH activity. How then is asymmetry in Notch activity eventually established between the two ISC daughters to allow one cell to remain an ISC and one cell to differentiate? We can envisage three types of mechanism that would allow for asymmetry of Notch signaling.

First, the binary decision between the ISC and EB fates might result from a competition process akin to lateral inhibition for the selection of SOPs (Heitzler and Simpson, 1991). In this process, feedback loops establish directionality by amplifying stochastic fluctuations in signaling between equivalent cells into a robust unidirectional signal. Our finding that the Da activator and E(spl)-bHLH repressors are important to properly resolve ISC/EB fate is consistent with this type of model. Activation of the Notch pathway in one of the daughter cells could then lead to the changes in nuclear position previously noted (Ohlstein and Spradling, 2007).

Second, the asymmetric segregation of determinants could bias Notch-mediated cell fate decisions. The cell fate determinants Numb and Neur are asymmetrically segregated in neural progenitor cells to control Notch signaling (Bardin et al., 2004; Knoblich, 2008). However, we find no evidence for the asymmetric segregation of these proteins in dividing ISCs (A.J.B., unpublished). Additionally, our data indicate that Numb is not important to maintain ISC fate. We cannot exclude, however, the possibility that another, unknown Notch regulator is asymmetrically segregated to regulate the fate of the two ISC daughters.

A third possibility is that after ISC division, one of the two daughter cells receives a signal that promotes differential regulation of Notch. Indeed, it has been noted that the axis of ISC division is tilted relative to the basement membrane, resulting in one of the

progeny maintaining greater basal contact than the other (Ohlstein and Spradling, 2007). An extracellular signal coming either basally or apically could bias the Notch-mediated ISC versus EB fate decision. For instance, Wg secreted by muscle cells could act as a basal signal to counteract Notch receptor signaling activity in presumptive ISCs (Lin et al., 2008). This could be accomplished by Wg promoting bHLH activity or gene expression. Indeed, Wg has been demonstrated to promote proneural bHLH activity in *Drosophila* (Couso et al., 1994; Phillips and Whittle, 1993; Tomoyasu et al., 1998).

These models are not mutually exclusive, however, and proper control of ISC and differentiated cell fates during tissue homeostasis might involve multiple mechanisms.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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