



Unravelling the identity and fate of Notch1-expressing cells within intestinal tumours

Larissa Mourão

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UNRAVELLING THE IDENTITY AND FATE OF NOTCH1- EXPRESSING CELLS WITHIN INTESTINAL TUMOURS

PhD Thesis to obtain the degree of Doctor from the Pierre and Marie Curie University (UPMC, Paris VI) and in accordance with the decision of the Doctorate Board

Prepared in the Laboratory of *Notch Signalling in Stem Cells and Tumours*, UMR3215/U934, Institut Curie, Paris

Doctoral school: Life Science Complexity, ED515

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**The public defence shall be held on
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DEDICATION

À minha Mãe e ao meu Pai

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ABSTRACT

Stem cells and cancer are inextricably linked and many tumours, including colorectal cancers, contain a small population of self-renewing cells, referred to as cancer stem cells (CSCs), able to give rise to proliferating but progressively differentiating cells that contribute to the cellular heterogeneity typical of solid tumours. Thus, the identification of CSCs and the factors that regulate their behaviour should have a profound impact on cancer treatment.

Notch signalling controls the maintenance and differentiation of stem cells in several tissues, including the intestine, where it is essential for stem cells maintenance. Based on these premises, my work was aimed at identifying and characterising the cells that express the Notch1 receptor in intestinal tumours *in vivo*, with the objective of getting insights into the cellular hierarchy of colon cancer cells.

We found that the Notch1 receptor is expressed in rare undifferentiated tumour cells that present self-renewal and multipotency *in vivo*, as they indefinitely give rise to marked differentiated tumour cells and fuel tumour growth. Our analysis on the transcriptomic profile of these cells confirmed our *in vivo* observations that Notch1+ tumour cells represent a specific population of highly proliferative tumour cells, expressing several, but not all, known markers of normal intestinal stem cells (ISCs). Indeed, their transcriptional signature highly correlates with normal ISCs. Given that the tumour cells we characterised appear not to carry *Apc* mutations, we hypothesise that during the earliest steps of tumorigenesis, normal Notch1+ ISCs are engulfed within the nascent tumour (in aberrant hyperproliferative crypts) and are able to grow and expand within this new ecosystem, as they are supported by extrinsic secreted growth factors from the neighbouring mutant cells. The concept that normal ISCs might contribute to tumour expansion highlights the complications that patients can encounter during treatment, since these cells share many features with their wild-type counterparts, making therapy deleterious to normal ISCs.

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GENERAL OUTLINE

The *Introduction* summarises **the past main works in the cancer field** and extends into the current literature on stem cells and their mirrored cells in cancer, the cancer stem cells. The present hypothesis of how cancer stem cells arise and may evolve with their tumour ecosystem are explained, their clinical relevance is highlighted, as well as the technical assays currently used to promote their identification and isolation are also developed.

The *second part of the Introduction* includes an **overview on the basic anatomy of the gastrointestinal tract**, with a special emphasis in the small intestine regions and its surrounding layers, continuing with the cellular composition of the small intestine and colon epithelium in homeostasis and disease, most focusing in colorectal cancer.

To conclude the *Introduction*, two major pathways controlling gut homeostasis, **Notch and Wnt signalling**, are molecularly and functionally described. This section characterizes the core signalling players, intestinal phenotypes, and their main target genes in both pathways, that are believe to cooperate to maintain intestinal homeostasis.

The *Objectives chapter* lists the **specific aims** of the project, followed by the *Materials and Methods* section.

Within the *Results part*, the **molecular and functional characterisation** of Notch1-expressing cells within genetically-induced intestinal adenomas and AOM/DSS-induced colon tumours are described. The **final conclusions** and **future directions** of this work are included in the *Discussion and Perspectives* chapter.

INTRODUCTION

HISTORICAL VIEW: THE FUTURE IS IN THE PAST, FROM DEVELOPMENT TO CANCER

In the middle of the 19th century, Rudolf Virchow proposed a theory about the origin of cancer by observing that solid tumours, highly proliferative, appeared to share histological similarities with foetal tissues, hypothesising that the studied malignancies were derived from latent embryonic-tissue remnants that persist in the developing organs following embryogenesis (Virchow, 1855). Rather than hypothesizing that cancer arises through activation of embryonic pathways within any cell of the body, Virchow argued that cancerous cells, like their non-cancerous counterparts, must originate from other living cells that most likely carry what we presently term as “stem cell properties”. Virchow’s concept was later developed by his student, Julius Cohnheim, along with Franco Durante. Both Cohnheim and Durante developed the “embryonal rest theory”, which assumes that cancer arises when *dormant* cells, that retained pluripotent capacity since early developmental stages, change later in life from their embryonic rest to an actively proliferative state.

In the late 19th century this theory was progressively replaced by the “dedifferentiation theory of carcinogenesis”, that postulates that adult differentiated cells undergo dedifferentiation upon oncogenic stimuli, later acquiring stem and proliferative properties and thus, tumour initiation capacity (Sell, 1993).

When the stem cell field started to broaden in the mid-20th century, the concept bringing together cancer and stem cells was resurrected. One of the most important studies offering experimental evidence about the link between tumour and stem cells was directed by John Dick and colleagues in end of the 20th century. In this study (further discussed in detail in the *Cancer Stem Cells chapter*), Dick and his group demonstrated, for the first time, that only a specific leukemic cell population, expressing a similar transcriptional profile of those expressed by normal hematopoietic stem cells, retained clonogenic capacity when transplanted in immunocompromised mice (i.e. were able to initiate tumorigenesis). These observations suggested that this particular leukemic population are governed by similar molecular mechanisms to those controlling normal hematopoietic stem cells. Importantly, this study led to the concept that not all cancer cells have the same potential of originating

secondary tumours, and that cancer development is sustained by a minor population of tumour cells, often referred to as Cancer Stem Cells.

Besides Virchow's attempts to understand the cells at the origin of cancer, he was the pioneer in describing tumour cellular heterogeneity; a phenomenon defined by the presence of different morphological cell types within a tumour that has been now widely accepted for many years. The Cancer Stem Cell theory has been assigned as one of the possible means by which heterogeneity arises within tumours. Such heterogeneity (that will be discussed in more details in the *Cancer Stem Cells chapter*) likely represents a major therapeutic hurdle, but its investigation is still challenging and the mechanisms generating it remain poorly understood.

The proposed theories of the last two centuries, along with Dick's previous observations, have risen countless difficult questions to address in the field of oncology. Two of the most pertinent questions, up to now unsolved or still object of debate, concern **1) the identity of cells capable to initiate tumourigenesis** and **2) which cancer cells drive intratumoural heterogeneity**.

STEM CELLS AND CANCER STEM CELLS: SO NEAR, YET SO FAR

GENERAL OVERVIEW ABOUT STEM CELLS

Stem cell

(noun)

Originally firstly derived from the German's word "Stammbäume" (family tree) and later adapted to the cell as "Stammzelle" (stem cell) in 1868 by Haeckel to describe the unicellular organism ancestor that gave rise to all multicellular organisms (Ramalho-Santos and Willenbring, 2007).

Virtually any tissue in the body is continuously enduring external aggressions. To counteract the loss of cells that die due to these environmental wear, epithelial tissues heavily rely on stem cells to replenish, in homeostatic conditions, and repair tissues.

Adult tissue-specific stem cells (SCs) comprise a population of long-lived cells defined on the basis of two essential features: unlimited **self-renewal** (implicating proliferative capacity), the key process where a stem cell produces daughter cells identical to itself, and their capacity of giving rise to at least one (unipotency) or multiple (multipotency) differentiated cell types in a given tissue (Leblond et al., 1967; Morrison and Spradling, 2008; Nassar and Blanpain, 2016). Notably, while all tissue SCs were initially considered multipotent, hence capable of giving rise to many distinct cell types, recent studies have shown that unipotent SCs can sustain the development, homeostasis, and repair in several tissues, namely the skin (Clayton et al., 2007), muscle (Starkey et al., 2011), the mammary gland (Van Keymeulen et al., 2011; Wuidart et al., 2016), the testis (Nakagawa et al., 2010), the prostate (Ousset et al., 2012) and very recently, in blood (Velten et al., 2017).

Found in all somatic tissues, adult SCs are responsible for maintaining long-term tissue turnover and also to respond to injury, ensuring the efficient replacement of damaged cells, while dodging inappropriate over-proliferation to limit cancer risk (Blanpain and Fuchs, 2014). Following this broad definition, adult SCs from several organs, including hair follicle (Jaks et al., 2008), neural (Morshead et al., 1994), intestinal, (Potten, 1998; Potten et al., 1974), mammary (Welm et al., 2002) and hematopoietic stem cells (Wilson et al., 2008) were originally proposed to be of slow-cycling nature (quiescent or dormant) due to their ability to retain DNA labels over time. Nowadays, quiescence is rather seen as a **reversible cell cycle state** defined by the absence of cell division (G0 arrest) from which cells may escape to re-enter the cell cycle upon physiological cell stimuli (Cheung and Rando, 2013) and,

contrary to what believed before, might not be a defining hallmark of stem cells. The state of dormancy (G0) likely evolved as a DNA integrity conservation mechanism and/or a system to preserve self-renewal ability to avoid premature stem cell exhaustion.

The observation that skeletal muscle has the ability to regenerate after injury was well reported in the mid-19th century (Scharner and Zammit, 2011). However, the cellular basis for this regenerative potential remained unknown for a century, until almost 60 years ago when Mauro, using electron micrographs, identified mononucleated cells, which he named “satellite cells” (Mauro, 1961). Without any functional data, Mauro hypothesized that this muscle cell could represent a muscle progenitor cell, similarly to those found in the developing embryo, able to regenerate muscle tissue upon injury. Mauro’s prediction turned out to be very accurate, when in the late 70’s, tracing experiments using [³H]thymidine labelling in adult muscles demonstrated that satellite cells have the ability to undergo activation and to rapidly enter the cell cycle in response to extrinsic signals, such as the ones produced by muscle damage, but are kept in a quiescent state during homeostatic condition (Snow, 1977).

In the hematopoietic system, the existence of quiescent HSC was formally demonstrated by two independent groups. Their results characterised a specific type of dormant Hematopoietic Stem Cells (HSCs) that divide about five times along the entire life of a mouse. These quiescent SCs are kept in the body as a cell reservoir to rapidly and efficiently activate self-renewal mechanisms in order to respond to bone marrow injury (Foudi et al., 2009; Wilson et al., 2008).

The coexistence of both actively dividing and quiescent adult SCs in some tissues, such as the intestinal epithelium, skin and blood (Li and Clevers, 2010), does not necessarily imply the existence of a hierarchy between these two cell types, but it can rather highlight their ability to transit (or interconvert) between the two states depending on tissue requirements, such as the hair follicle SCs that are mainly quiescent, but are activated to proliferate in periodic cycles to produce new hair cells.

The SCs present in the small intestine have been extensively used to explore this phenomenon of interconversion. Tian and colleagues took advantage of cell ablation experiments to show that +4 intestinal stem cells (considered as quiescent) can counteract for the loss of actively dividing stem cells (Tian et al., 2011). Another study conducted by Takeda, Jain and colleagues followed Tian’s work and completed the puzzle by demonstrating that both types of stem cells, actively dividing and quiescent, can interconvert

bidirectionally, i.e. both can give rise to either themselves or to the other type of cells upon division (Takeda et al., 2011).

More and more research studies in the stem cell biology field, mainly emerged from *in vivo* lineage tracing analyses, suggest that rather than relying on a single, hard-wired SC controlling homeostasis and regeneration upon injury, tissues contain **plastic** SCs and progenitors, characterised by differences in the molecular markers they express and their context-dependent functions.

The small intestinal epithelium, owing to its remarkably ability to respond to acute injury, has been extensively studied for the presence of plastic progenitor cells within the crypt compartment that defines the anatomical residence for SCs and progenitor cells. Two independent *in vivo* lineage tracing studies were used to understand degrees of plasticity of small intestinal progenitor cells. The first report, published by van Es and colleagues, analysed early stem cell progeny expressing the Notch ligand Delta-like1 (Dll1) at the +5 to +8 crypt positions, which are assumed to be the location within the crypt compartment that places immediate SC-derived daughters. Dll1+ cells were defined as secretory progenitors that give rise to small, short-lived clones of four different intestinal secretory lineages. Notably, long-chase experiments demonstrated that Dll1+ cells are not capable of self-renewal, as marked cells disappeared after 10 days following initial labelling, with the exception of Paneth cells that are known to hold greater life span, ranging from 6-8 weeks. Subsequently, upon irradiation-induced deletion of actively dividing SCs expressing the Lgr5 Wnt target gene, within the crypt compartment, Dll1+ cells undergo conversion into multipotent Lgr5+ SCs endowed with stem cell activity, as they give rise to labelled clones (ribbons in the villi) originated from cells located at the crypt bottom compartment, that extend all along the villi (van Es et al., 2012).

The second study, experimentally very similar to the study described above, was recently published by Tetteh and colleagues. Aware that secretory progenitor cells (Dll1+) had the potential to behave as multipotent and self-renewing SCs upon loss of Lgr5+ SCs, the authors hypothesised that absorptive progenitors could also present a similar extent of cell plasticity. To test this hypothesis, they performed lineage tracing analysis *in vivo* using the Alkaline phosphatase intestinal (Alpi) gene as a marker of enterocyte progenitors. Alpi-marked cells were shown to behave very similarly as Dll1+ cells in homeostatic conditions (i.e. originating small, short-lived clones of enterocytes). Following Lgr5+ SC targeted ablation, Alpi+ cells were shown to behave analogously to Dll1+ cells, as they convert to SCs and are able to give rise to the five intestinal cell types (Tetteh et al., 2016).

These research advances aided to refine the classical view of stem cell hierarchy, which assumes a unidirectional pyramid where only SCs are capable of tissue replenishment (as the one established for the hematopoietic tissue), by a novel model in which adult SCs are also heterogeneous, comprising several subpopulations of self-renewing cells with different regenerative capacity and moreover, that their lineage-committed progeny also retain substantial plasticity that can be awakened by a broad variety of non-homeostatic stimuli.

The molecular mechanisms allowing cell plasticity remain poorly characterised. So far, few studies have thoroughly addressed this cell property. In the mouse intestine, the mechanisms that allow interconversion between SCs and progenitors have been attributed to the interaction of a lineage-restricted transcription factor (namely the secretory determinant Atoh1) with a broadly permissive chromatin state present throughout the intestinal crypt compartment including both SCs and progenitors (Kim et al., 2014). This cellular plasticity and fate reversibility may represent evolving mechanisms for the organism self-preservation following injuries. However, the same molecular mechanisms underlying plasticity of a determined pool of cells, when gone skewed, may contribute in major ways to tumour initiation and also to its heterogeneity, if a tumour is already established. Recently, cell plasticity acquisition mediated by tissue repair has been hypothesised to contribute to the clonal expansion and heterogeneity observed in cancer evolution dynamics (Figure 1) (Donati and Watt, 2015).

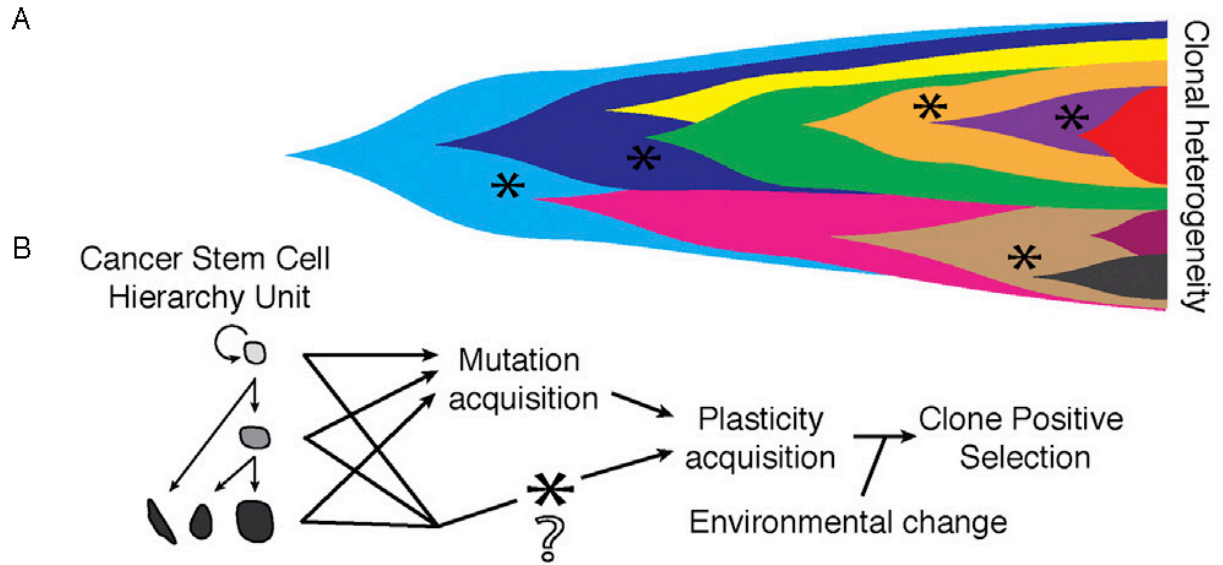


Figure 1. Clonal heterogeneity observed in cancer might be driven by cellular plasticity.

Concept that the mechanisms of stem cell plasticity during tissue repair might contribute to the evolutionary dynamics of cancer stem cell clones. In A) clonal evolution resulting in clonal heterogeneity over time (distinct clones are represented by different colours). In B) in tumours, clones of cancer stem cells with advantageous mutations will expand, while changes within the niche (asterisk) could also promote clonal evolution without the need of genetic mutations by conferring cell plasticity, as occurs during repair of healthy tissues (Donati and Watt, 2015).

For more than a century (and as pointed out in the *Historical view chapter*), the resemblance between the cell hierarchy of normal developing tissues and the cellular composition of tumours has intrigued many researchers (Müller, 1838). This observation led to the complementary ideas that common developmental pathways could be reactivated, since their activity is often involved in stemness maintenance and implicated in cancer initiation. Therefore, intratumoural heterogeneity could be hence generated through divisions and differentiation of “immature” tumour cells, localized in a virtual apex of a cellular hierarchy and carrying stem cell properties (Takebe et al., 2015). These stem cell-like populations were later termed as Cancer Stem Cells (CSCs) (Bonnet and Dick, 1997; Lapidot et al., 1994) and similarly to normal SCs, CSCs should be defined by their functional properties, such as self-renewal capacity for tumour propagation during an extended period and to be able to recapitulate the different cell lineages found in the primary tumours, hence promoting the heterogeneity that is found among different parts composing a tumour (Nassar and Blanpain, 2016). Intratumoural heterogeneity is defined at the histological level by the presence of different tumour cells within the same tumour and, consequently, at the transcriptional level by the heterogeneous expression of distinct markers.

To explain how tumours arise and why they are composed of different cell types, two models of tumour growth have been proposed; the “**Stochastic model**” and the “**Hierarchical model**”, also called “**CSC model**”.

Traditionally, cancer treatment has been designed to generically kill tumour cells based on the idea that *all* tumour cells have the same potential to proliferate, self-renew and fuel tumour growth. This notion follows the “**Stochastic model of tumour initiation**” (Figure 2). The stochastic model assumes that all tumour cells are equivalent/equipotent and, in a stochastic fashion, a proportion of tumour cells is endorsed with an advantageous trait, likely a mutation. These “upgraded” tumour cells (meaning virtually **any** cell present in a tumour), often referred as **TICs** (Tumour-Initiating Cells), will further expand and will generate larger clones at the expense of the differentiation of their counterparts. During the process of expansion, TICs can acquire additional mutations that are propagated within the tumour bulk, fuelling the heterogeneity observed. Importantly, over the lifetime of the tumour, any cancer cell can become therapy-resistant and cause relapse. Consequently, all tumour cells have to be eliminated for successful treatment; yet, in most cancers, complete remission is hardly

achieved and cancer-related death numbers have barely decreased over the past half-century.

The “*Stochastic model of tumour initiation*” has been challenged by a second model designated as “*Hierarchical*” or “*CSC model*” (Figure 2). This second model supports the idea that tumours are hierarchically organised in a unidirectional fashion, similar to normal tissues, and only **some** tumour cells endowed with stem cell properties are responsible for driving tumour development and hence, to generate the intratumoural heterogeneity observed. According to this model, the CSC-derived progeny has limited or no growth potential and carry, initially, the same genetic aberrations present in the CSCs, but they can later acquire new somatic mutations depending on their lifespan within the tumour. Plausibly, new somatic mutations occur within the CSCs located at the hierarchical apex, and these are believed to generate the clonal diversity observed that increases tumour heterogeneity. By definition, CSCs are undifferentiated tumour cells with unlimited self-renewal capacity and are believed to be present at low frequency (Beck and Blanpain, 2013).

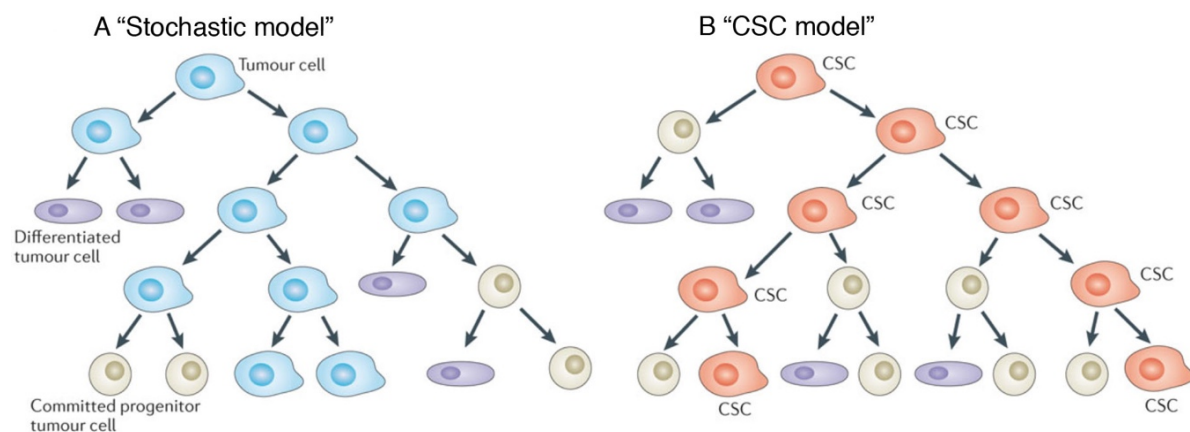


Figure 2. "Stochastic model" versus "CSC model".

In the stochastic model, all tumour cells (blue cells) have the same probability of expanding the tumour growth by generating terminally differentiated or progenitor cells. In the CSC model, only a small population at the apex of a hierarchy (orange cells) are able to self-renew and give rise to differentiated and progenitor cells. Adapted from Beck & Blanpain, 2013.

From another perspective, one could also envision a third model, somehow reconciling both models. In this third model (Figure 3), different mechanisms account for intratumoural heterogeneity and the tumour is viewed as a **dynamic ecosystem** where the combination of different CSCs and/or TICs displaying distinct panels of advantageous mutations give rise to more or less fit clones that outcompete among themselves – a phenomenon known as

clonal interference (Greaves and Maley, 2012). This relentless battle among different clones hinders complexity to the tumour phenotype and thus complicates patient's prognosis. Another layer of complexity to be highlighted in this model is the inherent presence of **cell plasticity**.

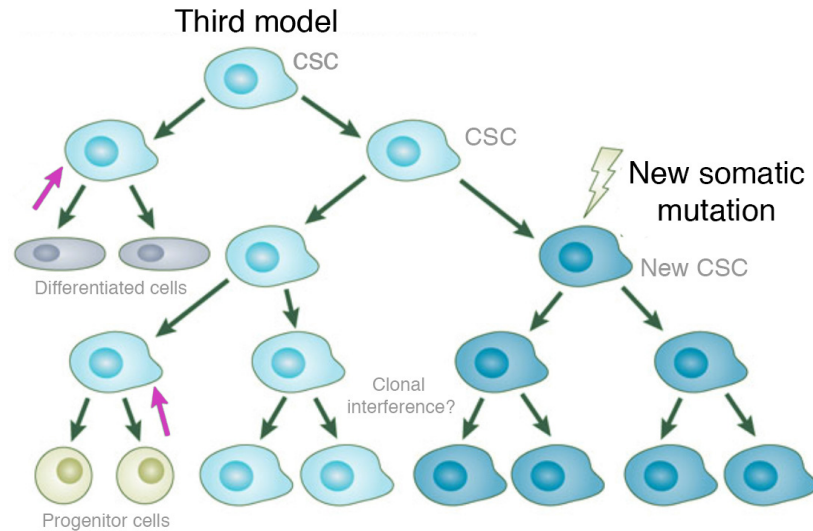


Figure 3. Third model.

The third model assumes that the tumour works as a constantly evolving ecosystem, in which CSCs or TICs (light blue cells) can acquire new mutations, generating other types of CSC (darker blue cells) that might outcompete for space (niche) and external signals (clonal interference), continuously driving intratumoural heterogeneity. Besides the acquisition of new mutations within the CSCs, the other cells comprising the tumour can also rewire their fate and dedifferentiate (pink arrows). Adapted from Beck & Blanpain, 2013.

Cellular plasticity is revealed in normal tissues upon injury, as some progenitors or even terminally differentiated cells may present a certain degree of flexibility and are able to revert to a SC-like state to heal tissue damage (Blanpain and Fuchs, 2014).

Besides the presence of tumour populations with different tumour-propagating abilities, constantly evolving and/or competing over time and space, some tumour cells have been characterised as adaptable to their environment and as such to respond to external signals, acquiring divergent functional fates. This tumour cell reprogramming initiates through a combination of competent signals that confer stem cell properties to non-CSCs, such as progenitors or any other tumour cell, previously recognised with a reduced or inexistent capacity of self-renewal (Schwitalla et al., 2013). Besides the important observation that any cell within the intestinal epithelia may give rise to a tumour (i.e. any cell can be a potential

cell-of-origin for tumour initiation), Schwitalla and colleagues demonstrated that stemness is a dynamic state, associated with different propensities for proliferation, differentiation, and apoptosis, thus producing functional variability within stem cell populations that results in high adaptability to environmental conditions.

Modelling a theory to explain what is observed in tumours, in particular tumour heterogeneity, is a challenging task. The first two models proposed represent very stiff scenarios, sidestepping cellular plasticity and also the **tumour microenvironment**; two key players in heterogeneity development (Junttila and de Sauvage, 2013). The third model takes into account these two features, rendering the main differences originally proposed in the stochastic and hierarchical models uncertain.

It is currently proposed that intratumoural heterogeneity arises through time as TICs or CSCs acquire genetic and epigenetic modifications that allow them to differentiate into several cancer cell types (Kreso et al, Science 2013).

Apart from which models are used to explain intratumoural heterogeneity and the identity of TICs, the concept of CSC is of considerable clinical relevance because it stresses the pivotal need for development of targeted therapies to improve the efficiency of existing oncologic treatments, and also confers a justification to explain patients relapse upon therapy.

Another feature pointed out in the CSC theory relies on clinical observations that CSCs may be intrinsically **less sensitive to conventional therapy**, such as ionizing radiation and chemotherapy, than the bulk of tumour cells (Dallas et al., 2009). As consequence, the model predicts that even if the tumour regresses initially and is macroscopically eliminated, the remaining resistant-CSCs can reinitiate cancer progression. As these CSCs survivors are now part of a highly-selected drug resistant population, this vestigial subset of tumour cells might undergo continuous genomic and epigenomic evolution over time and also have the possibility to disseminate to secondary organs, providing a second level of difficulty in treatment decisions after relapse.

Besides their inherited capacity of evolution within the tumour, CSCs are believed to coexist in a dynamic equilibrium with non-CSCs and to communicate with the tumour microenvironment. Any shift in this balanced system, through therapy selection for example, may enrich the CSCs and, subsequently, potentiate the negative effect of cancer, further reflected on the patient's outcome (reviewed in (Lee et al., 2016)).

The clinical relevance of CSCs has prompted intense research in experimental approaches for their identification, isolation and thorough characterisation.

In the late twentieth century, Dick and Bonnet offered one of the first experimental evidences for the presence of CSCs in acute myeloid leukaemia (AML) (Bonnet and Dick, 1997). Using a specific panel of hematopoietic stem cell (HSC) markers, they identified different subsets of leukemic cells derived from AML patients and showed that not all leukemic tumour cells had the potential to reform tumours upon transplantation in immunodeficient mice at limiting dilutions, but only a specific population that expressed HSC markers (CD34+ and CD38-).

After Dick and colleagues' work, several other studies verified the presence of human solid tumour cell populations able to recreate the original parental tumour by the use of **transplantation assays**, such as in colorectal cancer (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), in specific types of brain tumours (Singh et al., 2004), in breast cancer (Al-Hajj et al., 2003), pancreatic cancer (Li et al., 2007), lung cancer (Eramo et al., 2008), head and neck cancer (Prince et al., 2007), ovarian cancer (Zhang et al., 2008) and in skin cancer (Malanchi et al., 2008).

Overall, these studies demonstrate the feasibility of CSCs isolation by tumour dissociation and sorting (usually by flow cytometry), according to the presence of carefully **selected cell surface markers**. The next step in this process is to assess whether and which specific isolated populations are able to re-form **secondary tumours**, indefinitely, by serial xenotransplantation at limiting dilutions in immunocompromised mice.

A considerable drawback, often ignored, in this type of assays is that transplantations of tumour pieces, organoids derived from tumours and even cancer cell suspensions might represent a trauma to the recipient animals and can initiate a wound healing response, even in immunodeficient models. Consequently, host-cell contribution may confound the interpretations of stem cell-initiated tissue regeneration (Tang, 2012).

It is also very important to keep in mind that transplantation assays (Figure 4) are critical assays to investigate the potential of the population in question, in a specific experimental setting, although they do not entirely reflect what these populations can accomplish within their natural environment (Blanpain and Simons, 2013). Indeed, these assays have revealed a broader plasticity of epithelial stem cells than they exhibit under their *in vivo* physiological environment (Van Keymeulen et al., 2011). Another disadvantage imposed by

transplantations assays, except in rare experimental design settings, includes the lack of possibility in tracing the fate of the transplanted cells within their original niche.

Lineage tracing analysis, also called **fate mapping** (Figure 4), consists in the permanent genetic labelling of defined cells using reporter genes to identify all progeny derived from the originally marked cells. As the offspring of these cells receive the genetic label upon cell division, it is possible to follow the fate and lineages of defined cell populations in time *in vivo* and in their physiological environment. If only one differentiated cell lineage can be traced as being derived from a single cell, this cell is considered a unipotent SC. Instead, if many different lineages can be traced back to the same mother cell, this cell is a multipotent SC. Lineage tracing also allows to assess whether a SC can achieve long-term self-renewal by tracking down over a long period the production of marked cell lineages. Any candidate cell demonstrating either unipotency or multipotency and self-renewal capacity meets the minimal criteria used to define a stem cell (Barker, 2014).

Nowadays, lineage tracing has become the gold-standard assay to study stem cell behaviour *in vivo* and *in situ* and it has demonstrated that there is a significant difference between the potential of a given cell (i.e. testing its capacities upon transplantations) and its physiological behaviour in its natural environment or niche (Blanpain and Simons, 2013).

Besides the advantages mentioned before over transplantations assays, clonal analysis can provide evidence of hierarchical organizations in solid tumours *in vivo* (Driessens et al., 2012).

A limitation that has becoming more and more apparent within this method is that some mouse models do not faithfully report endogenous expression patterns of candidate stem cell markers.

Another caveat inherent to the lineage-tracing approach is that it can only be used, so far, in lab animals and therefore, the translation of these results to humans still requires many other validations (Tang, 2012). However, the CRISPR-Cas9 technology has been recently applied to allow the introduction of transgenes for *in vitro* human lineage tracing studies using patient-derived organoids (Shimokawa et al., 2017).

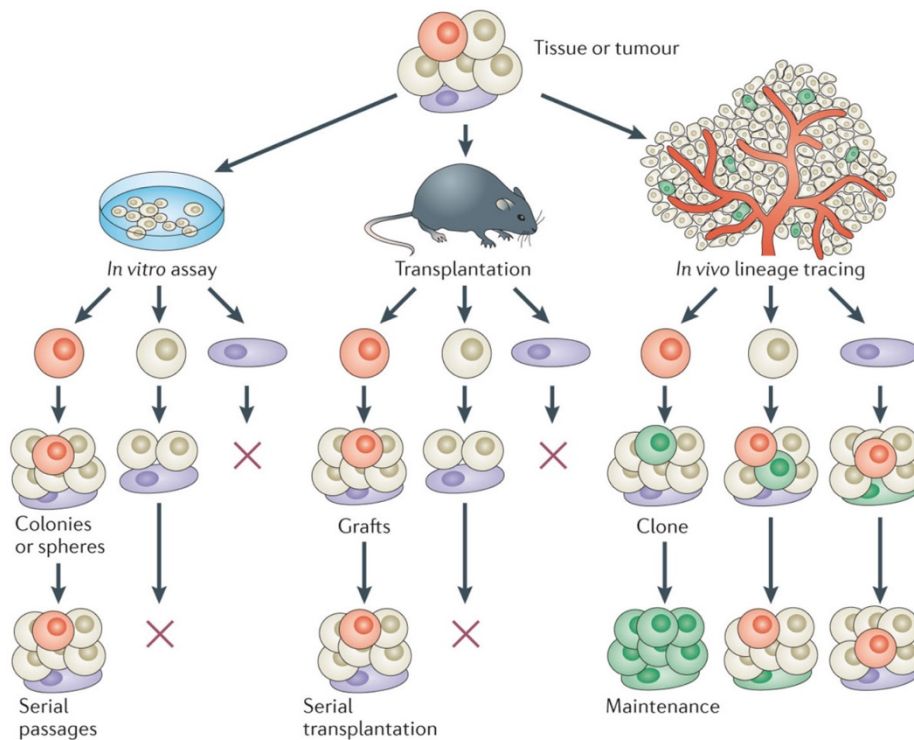


Figure 4. Commonly used methods to functionally test stem potential and to isolate CSCs.

Ex vivo assays, such as clonogenic assays, predicts self-renewal and differentiation capacity of a given cell population. Only the tumour cells that can be passaged for many cycles present self-renewal capacities. Progenitor cells cannot be serially transplanted and terminally differentiated tumour cells cannot form spheres *in vitro*, nor survive upon transplantation. Transplantations in recipient mice are often used to verify the presence of CSCs and to proceed to their evaluation. Lineage tracing is the gold-standard method that allows to assess both self-renewal and multipotent capacities of tumour cells *in vivo*. Transiently present clones are thought to be derived from progenitors, while persistent clones over long time are probably maintained by CSCs endowed with self-renewal potential. Analysis of the tissue-specific differentiation markers within clones can assess the degree of multipotency of a CSC population (Beck and Blanpain, 2013).

Another important method to identify CSCs is to determine their functional importance, for example, in tumour growth. **CSC ablation techniques** have been now widely employed to functionally characterise important CSCs populations in many different tumours (Blanpain and Fuchs, 2014). Several approaches have been used to study the importance of a particular population in cancer, including monoclonal antibodies targeting specific cell surface markers (Jin et al., 2006), shRNA approaches (Zhou et al., 2014), engineered oncolytic viruses (Chen et al., 2012), activated immune system cells, CSC-directed differentiation therapy, laser-induced CSCs ablation or genetic ablation (Nakanishi et al., 2013). The

disadvantage of this system is the requirement of finding new specific cell surface markers for CSCs, in order to avoid ablation of SCs in healthy organs.

As an alternative to *in vivo* approaches, CSCs can be tested in **colony formation assays** (Shaheen et al., 2016) (Figure 4). CSCs isolated from solid tumours, such as colon (Lee et al., 2015), liver (Tomuleasa et al., 2010), ovary (Zhang et al., 2008) and skin (Fang et al., 2005) can form spheres in serum-free medium supplemented with tissue specific growth factors, such as epithelial grow factor (EGF), basic fibroblast growth factor (bFGF), b27 supplement, among others, in non-adherent conditions (Fatehullah et al., 2016). Clonogenicity, long-term renewal capacities and multilineage differentiation are features that can be assessed by spheroid colony formation assays, however it is important to keep in mind that not only SCs but also progenitors are capable to form spheres.

So far, the existence of CSCs in murine adenomas, with particular importance for this work, was neither properly, nor exhaustively investigated. Previous works reported by several groups have been challenging this question over the last decade, yet the experimental designs tailored to obtain an answer are essentially attempting to solve two distinct questions; which are the cells of origin that generate a tumour and/or which are the key oncogenic pathways driving tumorigenesis. These two questions are undoubtedly important in the cancer field, but they concern a sublayer from the original fundamental ones; **1) do CSCs really exist in tumours? and if so, 2) can somatic SC markers be used to track CSCs?**

A recent report presented by Yanai and collaborators challenged these two questions by using a multicolour lineage-tracing method to track the contribution of Bmi1+ and Lgr5+ tumorigenic cells (representing two well-known CSCs markers in the literature), within distinct mouse models to generate intestinal adenomas, adenocarcinomas and also colon tumours (Yanai et al., 2017). In this detailed descriptive study, despite the detected clonal expansion of Bmi1+ or Lgr5+ cells within proliferative tumours (i.e. an indication that the cells labelled by these markers can potentially be CSCs), their observations indicate that not all Bmi1+ or Lgr5+ tumour cells can act as CSCs, stressing the need of finding new additional markers to explore all distinct tumour populations endowed with stem cell activity.

NORMAL INTESTINAL PHYSIOLOGY AND COLORECTAL CANCER: A *TOUR INTO THE GASTROINTESTINAL TRACT IN HEALTH & DISEASE*

THE DIGESTIVE SYSTEM, FROM MACRO TO MICRO

Gastroenterology

(noun)

Combination of three Ancient Greek words; *gastér* (genitive *gastros*, stomach), *énteron* (intestine, piece of gut), and *logos* (reason).

The digestive system includes the gastrointestinal tract (GIT) and the accessory organs required for food digestion; the salivary glands, liver, gallbladder and pancreas. Its functions consist in the ingestion and digestion of food, where essential nutrients are absorbed to be distributed throughout the body and waste is eliminated. The intestine functions as a selective barrier, while digesting and absorbing essential nutrients, it prevents the entry of microorganisms and toxins into the body (Noah and Shroyer, 2013).

The GIT (Figure 5) consists of a hollow tube starting from the oral cavity (the mouth), where food is first processed, continuing through the pharynx, oesophagus, stomach and small and large intestines to the rectum and anus, where food is excreted.

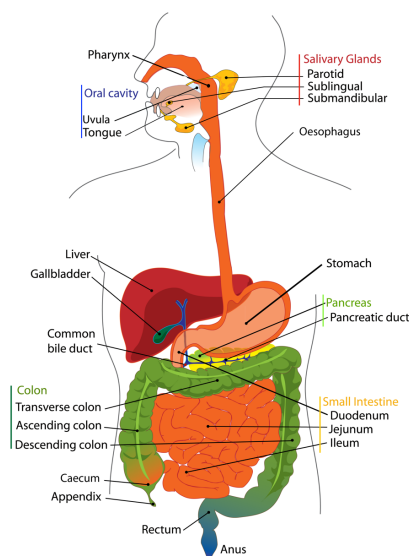


Figure 5. The location of organs composing the human digestive system.

The digestive system includes the GI tract (also termed the alimentary canal) and its accessory organs of digestion, such as the salivary glands, liver, gallbladder and exocrine pancreas. Nutrient uptake occurs in the small intestine. Adapted from Ruiz, M. and Gaspar, A. J, 2006.

Being partially in contact with the external environment and saturated with external stimuli, the GIT also plays an essential role in immune system homeostasis and surveillance.

The **small intestine** is subdivided in three regions, from the proximal to the distal axis; duodenum, jejunum and ileum.

The **duodenum** receives the combined pancreatic and hepatic digestive secretions to promote digestion of the food expelled from the stomach. The **jejunum** is where the majority of digestion and absorption take place. The last portion of the small intestine, the **ileum**, has the task of absorbing the soluble molecules that are then released to the bloodstream.

The **large intestine** starts in the caecum, continues in the **colon** (ascending, transverse, descending and sigmoid colon) and ends in the rectum.

The caecum is an intraperitoneal pouch receiving material from the ileum and starting its compression into faecal product into the colon.

The colon accumulates the remaining unabsorbed material to form faeces, and while the terminal stage of digestion occurs, it reabsorbs water, salts, sugars and vitamins.

The GIT is composed of four distinct concentric layers surrounding the lumen; the **mucosa**, the **submucosa**, the **muscularis externa**, and the **adventitia** (or **serosa**). Each one of these tissue layers presents a **specific** histology that reflects its function (Figure 6).

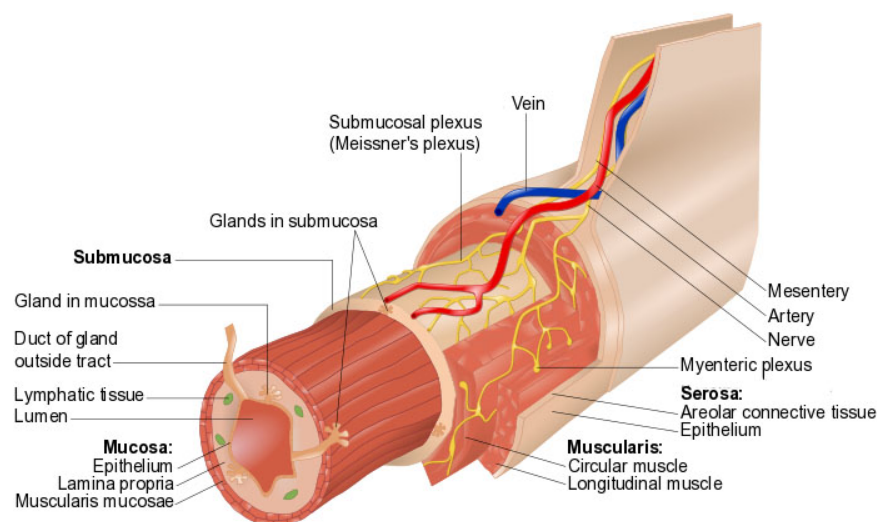


Figure 6. The gastrointestinal wall is build-up of four layers.

The gastrointestinal wall is build-up of four layers; the mucosa is the most internal layer and contains glands that secrete their content to the intestinal epithelium, the submucosa harbours blood and lymphatic vessels, the

muscularis externa promotes peristaltic movements and the serosa supports all the layers mentioned above. Image from OpenStax College, Layers of Gastrointestinal Tract, 2013.

The **mucosa** is the first layer found in contact with the digestive luminal environment, lining the entire GIT and being responsible for food nutrient absorption and secretion, while rigorously excluding passage of harmful molecules or hostile microorganisms to the body. It consists of epithelium, lamina propria (connective tissue), and muscularis mucosa (thin layer of smooth muscles). The mucosa is highly specialized in each organ of the GIT, and its structure depicts the varied needs of different regions of the gut. In the stomach, small intestine and colon, it is mainly composed of simple columnar or glandular epithelial cells that are specialized in nutrient and water absorption or secretion to either help in the digestive process or to protect the mucosa. Differently from the mid-lower ends of the GIT, the mouth, oesophagus, and anal canal harbour a protective mucosa composed of stratified squamous epithelial cells.

The next layer in contact with and structurally supporting the mucosa is the **submucosa**. This second layer consists of a dense irregular layer of connective tissue and contains blood vessels, lymphatic vessels, submucosal glands, and the Meissner's plexus (a nerve network that influences the adjacent smooth muscle of the muscularis externa).

The **muscularis externa** surrounds the submucosa and is composed of two smooth muscle layers, an inner circular layer, that prevents the food from travelling backwards, and outer longitudinal layer, that constricts the tract. These two layers move perpendicularly to one another to promote the peristaltic movements that drive the expulsion of the chyme.

The outermost layer of the gut wall is the **adventitia** or **serosa**. This layer is in fact composed of several sublayers and if the outermost layer is attached to surrounding tissue (the mesentery or peritoneal fold), it is then adventitia containing the connective tissue that supports the organ it surrounds. On the other hand, when the outermost layer lies adjacent to the peritoneal cavity, it is termed serosa.

The layers composing the gut wall and their structural organisations are of high relevance not only in health but in determining the extent of cancer progression and patient's prognosis. As a cancer evolves from the mucosa into lower layers, the stage is characterized as more advanced. Consequently, besides prognosis correlations, therapeutic treatments are also

prescribed according to the invasion into specific layers, reflecting the stages of colorectal cancer (see page 25 for detailed description).

THE GUT EPITHELIUM AND ITS ORGANISATION

Epithelium

(noun)

Combination of two Ancient Greek words; epí (upon) and thēlē (teat, nipple). Originally, this word referred to the skin on the breast, however in its literal translation, it is considered as misnomer.

While the small intestine plays a major role in absorbing nutrients from food and producing antimicrobial proteins, the large intestine mainly absorbs water and secretes high amounts of mucus to facilitate evacuation of the gut contents. These two different functions are reflected at the epithelial scale by a different architectural and cellular organisation. To maximise the capacity of absorption, the epithelial monolayer of the small intestine is composed of millions of finger-like projections, name villi, and by adjacent invaginations into the submucosa, called crypts of Lieberkühn (Figure 7A). The intestine is the fastest renewing tissue in the whole body (Figure 7B) and the entire epithelial population has a turnover rate of approximately 3-5 days. These specialised cell types derive from Intestinal Stem Cells (ISCs) present at the bottom of the crypts.

ISCs are highly proliferative cells (approximately one division every 22 hours) and are responsible for the production of rapidly cycling **transit-amplifying (TA)** daughter cells by symmetric cell division (Lopez-Garcia et al., 2010) to ensure the tremendous cell turnover required to maintain intestinal homeostasis. While proliferating, TA cells also actively migrate upwards, undergoing four to five cell divisions until reaching the crypt-villus junction, after which they undergo differentiation into one of the two major functional lineages of the gut, either absorptive (Enterocytes) or secretory (Paneth, Goblet, Enteroendocrine and Tuft cells), which further move upwards into the villus to accomplish their respective functions. Only one cell type, the **Paneth cells**, escapes this unidirectional upward route migrating downwards to the crypt base to intermingle between the ISCs to supply their neighbours with niche signals and produce antimicrobial proteins, such as defensins or cryptdins and the hydrolytic enzyme lysozyme, maintaining the small intestine free of bacteria (Sato et al., 2011b). To counteract the continuous production of progenitor and differentiated cells along the crypt-villus axis, within approximately four to five days, cells that reach the villus tip undergo anoikis, a programmed cell death induced upon cell detachment from extracellular matrix, and are extruded into the gut lumen. **Paneth cells** not only escape from the apical migration along the crypt-villus axis, but also escape the rapid cell turnover, having an average life span of six to eight weeks (Ireland et al., 2005).

The absorptive lineage (Figure 7C) constitutes the majority of cells found in the villus epithelium and is composed of **Enterocytes** (EC) that are in charge of nutrient uptake, from the intestinal lumen, and its transport across the epithelial layer. ECs are polarized cells displaying apical brush border (microvilli) that secrete hydrolases into the gut lumen to complete the digestion process and to increase the intestinal absorptive area.

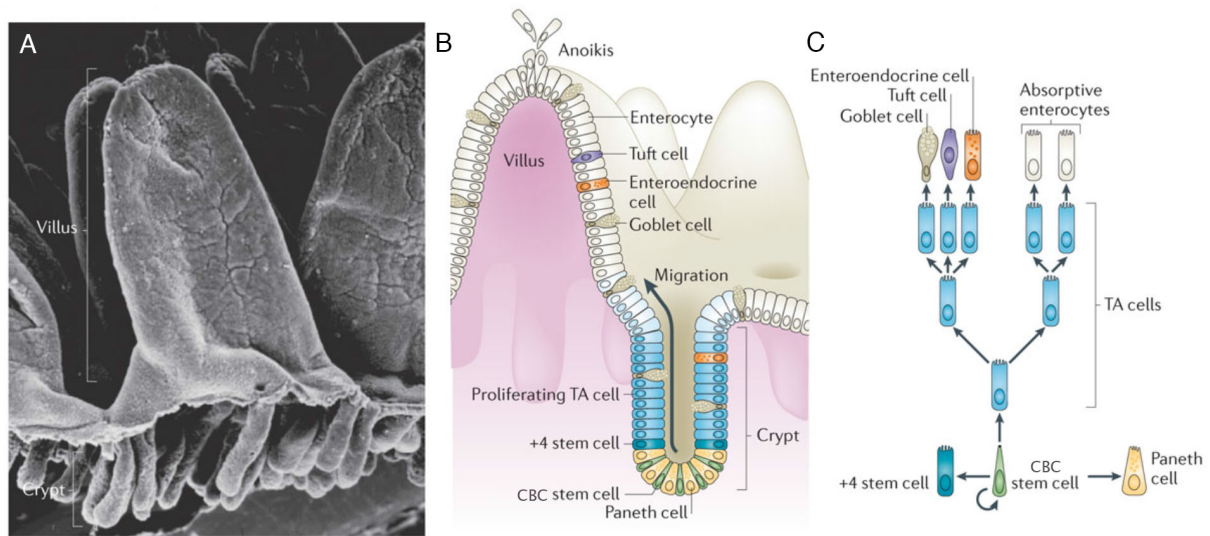


Figure 7. The small intestine architecture and its cellular composition.

In 7A Electronic microscopy scan of the small intestine to show its structural organisation. The diagram in 7B depicts the epithelial monolayer of the small intestine and the its residing different cell types. The crypt compartment harbours the stem cells (CBCs and +4 SCs) interspersed between paneth cells, and transit-amplifying (TA) progenitor cells. The villus domain contains exclusively in its entire length terminally differentiated cells, such as goblet, enteroendocrine, tuft and enterocyte cells, each carrying a defined function. The hierarchical cellular organisation in 7C shows the two options of lineages available for progenitor cell differentiation; secretory or absorptive. Adapted from Barker, 2014.

The secretory lineage (Figure 7C) is composed of four distinct cell types: the mucus-producing **Goblet cells**, the hormone-producing **Enteroendocrine** (EE) cells, the opioid-secreting **Tuft cells** and the lysozyme-producing **Paneth cells**. **Goblet cells** reside scattered throughout the length of the small intestine and are responsible for the production and maintenance of the protective mucous blanket necessary for the movement and the optimal dissemination of intestinal contents. **Enteroendocrine cells** (EE) or **Neuroendocrine cells** are also found dispersed throughout the epithelium of the GIT and

similarly to EC, these cells present microvilli-covered apical surfaces. In response to food intake stimuli and their spatial localization in the GIT, EE release digestive enzymes and also distinct peptide hormones to coordinate the function of the gut, liver and pancreas.

Tuft cells, also known as **brush cells**, carry an apical pack of microfilaments connected to a tuft of long and thick microvilli facing the lumen. These cells were recently functionally characterised as major regulators of type 2 immune responses during parasite infections in the intestine (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016).

Over the last decade, specific markers of the ISCs have been identified as defining **two distinct states** of activity that are characterized by separated anatomical and gene expression patterns. The rapidly dividing ISCs that are found mainly at the crypt base are called **Crypt base columnar cells (CBCs)**, originally discovered by Cheng and Leblond (Cheng and Leblond, 1974), are identified based on the expression of the following genes: **Notch1** (Fre et al., 2011), **Ascl2** (van der Flier et al., 2009b), **CD133** (or Prom1) (Zhu et al., 2009), **Lgr5** (Barker et al., 2007), **Smoc2** (Munoz et al., 2012), **Olfm4** (van der Flier et al., 2009a), **Ephb2** (Merlos-Suarez et al., 2011), **Lrig1** (Powell et al., 2012), **Msi1** (Potten et al., 2003) and **Sox9** (Formeister et al., 2009).

The second type of ISCs residing approximately four cell positions above the crypt bottom are termed **Label retaining stem cells (LRCs)** or **+4 cells**. Contrary to the CBCs, label-retaining experiments using tritiated thymidine (3H-TdR) or bromodeoxyuridine (BrdU) DNA incorporation, revealed that LRCs divide at a presumably slow rate (Potten et al., 2002). LRCs are described in the literature as cells expressing the **Notch1** receptor (Fre et al., 2011), **Bmi1** (Sangiorgi and Capecchi, 2008), **Hopx** (Takeda et al., 2011), and **mTert** (Montgomery et al., 2011) and may also be labelled by **Lrig1** (Powell et al., 2012), and **Musashi1** (Potten et al., 2003). LRCs are believed to serve as a **reserve stem cell population** that does not actively supply new epithelial cells but rather functions promptly following injury to promote epithelial repair when CBCs are depleted (Yan et al., 2012).

The ISCs markers identified, so far, derive from works mainly developed using lineage tracing analysis, in which the expression of a reporter gene reflects the expression of a **single** specific ISC marker within a specific isolated snapshot in time. Consequently, these studies require careful interpretation due to the fact that the use of a single ISC marker might not be enough to distinguish and define an ISC population. In this line of thought, one can say that 1) ISCs cannot be defined by the expression of a single gene and 2) ISC

populations are indeed heterogeneous and probably inter-related. Powell and colleagues suggested that an individual colonic SC population, labelled by a specific colonic SC marker, may carry a leading program (i.e. active cell cycle status and oxidative stress response), but may be capable of transitioning to or from another state (Powell et al., 2012). This ability of transitioning from different states, and so adopting the expression of other markers, highlights that the expression of individual SC markers might very well reflect cell state, making each population distinct from others at a given point in time, rather than specific fixed SCs (i.e. SCs that do not transit between one state to the other) populations within the same tissue. What governs these states and regulates SCs transitions, during homeostasis and upon injury, are still challenging and unsolved questions in the stem cell biology field.

The structural architecture of the colon, except for the lack of villi, closely resembles the one of the small intestine. However, compared to the small intestine, the colon contains in its cellular composition an outnumbered amount of Goblet cells and high occupancy by differentiated cells in a large part of the colon crypt. **Goblet-like cells** that co-express cKit and CD117 markers (Rothenberg et al., 2012), were identified as probable niche components for the colon SCs (similarly to Paneth cells in the small intestine).

COLORECTAL CANCER, A HETEROGENEOUS DISEASE

Cancer

(noun)

Approximatively 400 B.C., Hippocrates, the Father of Medicine, named masses of cancerous cells as “karkinos” (Greek word for crab). The word cancer was most likely applied because the finger-like invading cellular projections found within disease would call to mind the shape of a crab. Celsus (28-50 BC), a Roman physician, later translated the Greek term into cancer, the Latin word for crab.

Colorectal cancer (CRC) represents the third leading cause of cancer-related death worldwide, both in men and women. At early stages of the disease, the 5-year survival rate is over 90%, stressing the fact that early diagnosis is pivotal for the patient outcome (IARC, 2017). A well-defined sequence of genetic events occurs during CRC development, leading initially to aberrant crypt proliferation or aberrant crypt foci (ACF) formation, more diffused hyperplasia, adenomas or polyps (benign tumour nodules that arise from the glandular epithelium and project into the gut lumen), and further progression to adenocarcinomas *in situ* that might, eventually, lead to metastatic carcinomas if they acquire further mutations (Fearon and Vogelstein, 1990).

In clinical terms, CRC has been characterized with four stages, where the earliest stage is called stage 0 (or carcinoma *in situ*), and then ranging from stage I (1) through IV (4), with minor subdivisions among these. Roughly, in stage I, the cancer cells have not grown beyond the mucosa of the colon. The progression of these cancer cells through the muscularis mucosa into the submucosa (and possibly into the muscularis propria) with no dissemination to the lymph nodes nor distant sites comprises the stage II. When the cancer cells spread exclusively to one or three nearby lymph nodes regions (and not the nodes themselves), the tumour is staged as stage III. The stage IV englobes the presence of cancer cells within lymph nodes, to distant parts of the peritoneum and nearby or distant organs (Steele et al., 2013).

While some environmental factors like diet, lifestyle and others have been shown to have an impact on CRC risk (Potter, 1999), genetic germline and more commonly somatic mutations, principally in the Adenomatous polyposis coli (*APC*) tumour suppressor gene, invariably lead to CRC, and define its onset and progression (Fearon and Vogelstein, 1990; Kinzler et al., 1991). Most of CRCs are sporadic, driven by somatic mutations, whereas familial or inherited cases due to germline mutations have a lower weight and represent 10 to 15% of all cases.

The most prevalent CRC inherited syndrome, carrying an 80% lifetime risk for cancer development, is defined by **Lynch syndrome**, characterised as a highly penetrant dominant hereditary condition. Other rare genetic diseases, causing the emergence of multiple tumours at a young age, include **familial adenomatous polyposis (FAP)**, **Peutz-Jeghers syndrome (PJS)** and **juvenile polyposis syndrome (JPS)**.

Sporadic CRC is consequently more frequent to occur and its origin may be assigned to a combination of common and/or rare genetic variants that are believed to act cumulatively to increase the chances of disease development (Gryfe, 2009).

In both cases, mutations in the *Apc* gene that typically delete the central domain of β -catenin binding sites, were identified as the first and main genetic aberration to drive CRC tumorigenesis (Morin et al., 1997). The *Apc* gene encodes for a roughly 300-kDa protein and its best-established role is to negatively regulate the Wnt signalling pathway. In the absence of Wnt ligands, APC is part of a destruction complex, together with AXIN2, Glycogen synthase kinase-3 β (GSK3 β) and Casein kinase 1 (CK1), promoting β -catenin proteasomal degradation, thus preventing Wnt signal activation (Schneikert and Behrens, 2007). When both *Apc* alleles are inactivated, β -catenin phosphorylation and degradation are abolished, mirroring a constitutive Wnt signalling activation status at the cellular level. As a consequence, β -catenin translocates to the nucleus functioning as a transcriptional coactivator of, for example, proto-oncogenes, such as *c-Myc* and *Cyclin D1* (van de Wetering et al., 2002), and also Wnt pathway feedback regulators such as AXIN2 (Jho et al., 2002), NAKED1/2 (Rousset et al., 2001) and DICKKOPF1 (Niida et al., 2004).

In sporadic CRC, the vast majority of colorectal adenomas and carcinomas carry Wnt activating mutations. Notably, up to 80% of these somatic mutations occur in the *APC* tumour suppressor gene (Kinzler and Vogelstein, 1996), leading to **premature truncation** of the APC protein. However, accumulation of other specific mutations in a sequential-fashion underlies the progression of CRC, as proposed by Fearon and Vogelstein in the 1990's. In their original model for CRC tumorigenesis, a series of genetic alterations involving oncogenes and tumour suppressor genes are affected in a multistep process (Fearon and Vogelstein, 1990) (Figure 8).

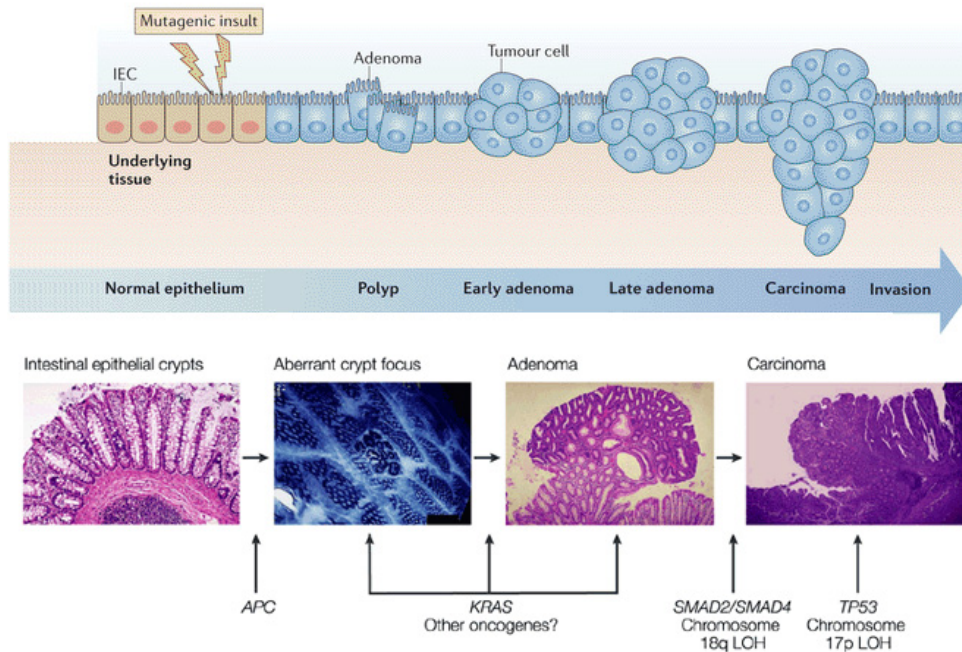


Figure 8. From polyp to cancer: adenoma to carcinoma sequential progression model and histology.

The model depicted is a simplified overview of the clinic-pathological modifications and genetic abnormalities in the progression of CRC. The initial step is driven by a genetic insult within the intestinal epithelium leading to adenoma formation associated with loss of the *Apc* gene. Adenoma cells start to rapidly proliferate and accumulate *in situ*, giving rise to ACFs (Aberrant crypt focus) that can further acquire mutations in the small GTPases *KRAS*, followed by loss of chromosome 18q along with *SMAD4*; a downstream effector of transforming growth factor- β (TGF β), and mutations in *TP53* in an established carcinoma. Images reproduced from (Fodde et al., 2001; West et al., 2015).

To build this model, Fearon and Vogelstein relied on the observations of a previous study by Lipkin and colleagues (Lipkin, 1988), in which biopsies derived from patients with polyposis displayed a widespread cellular hyperproliferation, suggesting that proliferation could be induced by loss or inactivation of the *Apc* gene, hence resulting in benign polyp formation and further assisting as a precursor to colorectal adenocarcinomas. Concomitantly with these observations, the inactivation of the *Apc* tumour suppressor gene represents one of the **firsts events** in both hereditary and non-hereditary CRC and it can be histologically traced by the presence of ACFs. The second step required for tumour progression is activating mutations in the oncogene *Kras* in one or some adenoma cells, that will then evolve into larger clones producing a dysplastic tumour. Intriguingly, gain-of-function mutations in the *Kras* gene are present in 50% of human CRCs, but mutations in *Kras* gene alone fail to drive tumour formation in mice. Subsequent additional mutations are required for adenoma to carcinoma progression, in particular the loss of the 18q chromosome that houses the

Smad2/4 genes, among others. At later stages, malignant transformation is driven by additional mutations in the TGF- β , PIK3CA, and TP53 pathways (Fearon, 2011). This model provides a list of the key mutations driving CRC and predicts that at least 7 distinct mutations are required. Genome-wide sequencing has predicted that approximately 80 mutated genes were present per colorectal tumour, but a reduced number of mutations (roughly less than fifteen) were found to be authentic driver mutations (Leary et al., 2008; Wood et al., 2007).

CURRENT OPINIONS ABOUT THE CELLULAR ORIGIN AND MAINTENANCE OF COLORECTAL CANCER

Whereas the mutations characterising the sequential steps of CRC formation are now well defined, it is still unclear which cells are the initial targets of oncogenic mutations and lay at the origin of tumour development. Several studies tried to uncover the cell-of-origin of CRC and results are still a matter of debate. Because the intestinal epithelium represents one of the most rapidly renewing tissues in our body, it was hypothesised that only the SCs present in this tissue could persist overtime to propagate their genetic alterations within their progeny. Hence, these cells were considered for a long time as the most suitable candidates to initiate cancer (Visvader, 2011).

Many studies attempted to tackle this hypothesis by introducing specific mutations targeting specific cell types (Barker et al., 2009; Maynard et al., 2014; Sangiorgi and Capecchi, 2008; Schwitalla et al., 2013; Zhu et al., 2009). All these studies claim to have found a specific population of cells that can contribute to tumour initiation, although the solely reliable message is that those tested oncogenic inputs are indeed capable of transforming the specific targeted SCs into tumorigenic cells. The relationship between normal SCs and CSCs is still a challenge to be obtained and more studies aimed at the expression analysis of the above assessed markers within spontaneously-derived tumours are required.

Furthermore, the hypothesis that only SCs are able to propagate a set of mutations through their daughters and generate a tumour was contradicted by a recent study (Schwitalla et al., 2013). In this work, Schwitalla and colleagues induced Wnt activating and Nuclear Factor kappa-B (NF- κ B) gain-of-function mutations specifically in terminally differentiated cells found in the gut epithelium. The authors found that the differentiated cell types affected by this combination of mutations could proliferate (generating ACFs) and they also started to express specific ISC markers and crypt markers. They concluded that **dedifferentiation of non-stem** cells that acquire tumour-initiating capacity is a phenomenon that might occur - implying that the ISCs are not the only cells capable of tumour initiation.

Until quite recently, direct evidence for the existence of CSCs within CRC had not been properly demonstrated as many studies using transplantations assays (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) have tested the stem cell **potential** of specific tumour cell populations, whereas stem cell **activity** assessment, that can be shown using clonal lineage

tracing experiments, is the ultimate analysis to decisively determine whether a tumour cell behaves as a CSC (Vermeulen and Snippert, 2014).

The latest report (detailed described in *Methods to identify and isolate cancer stem cells*) attempted to shed light to this question by performing lineage tracing analysis in murine adenomas using two well-known somatic ISC markers; Lgr5 and Bmi1. Their observations indicate that both markers can label tumour cells endowed with stem activity and potential, but not all cells carrying the expression of these could perform as a CSC. Their final conclusive remark is the requirement of further investigation using other somatic ISCs markers that can potentially undercover a tumour cell population that entirely behaves as CSCs (Yanai et al., 2017).

NOTCH SIGNALLING PATHWAY

INTRODUCTION TO NOTCH

Notch

(noun)

The origin of the word “Notch” dates from the mid-16th century, probably from Anglo-Norman French word “noche”, variant of Old French “osche”, of unknown origin. It describes an indentation or incision on an edge or surface.

The Notch signalling pathway is an evolutionary conserved mechanism used by metazoans to control cell fate decisions in multiple tissues, both during development and adult homeostasis. Its pleiotropic functions include control of differentiation, proliferation, and stem cell maintenance, normally triggered via cell-to-cell contact and involving long-range paracrine effects (Artavanis-Tsakonas et al., 1999; Cohen et al., 2010; De Jussineau et al., 2003; Sheldon et al., 2010).

Over 100 years ago now, the “Notch” phenotype was initially described by John S. Dexter in heterozygous mutant female *Drosophila melanogaster* as the appearance of indents (or notches) at the tips of their wings (Figure 9).

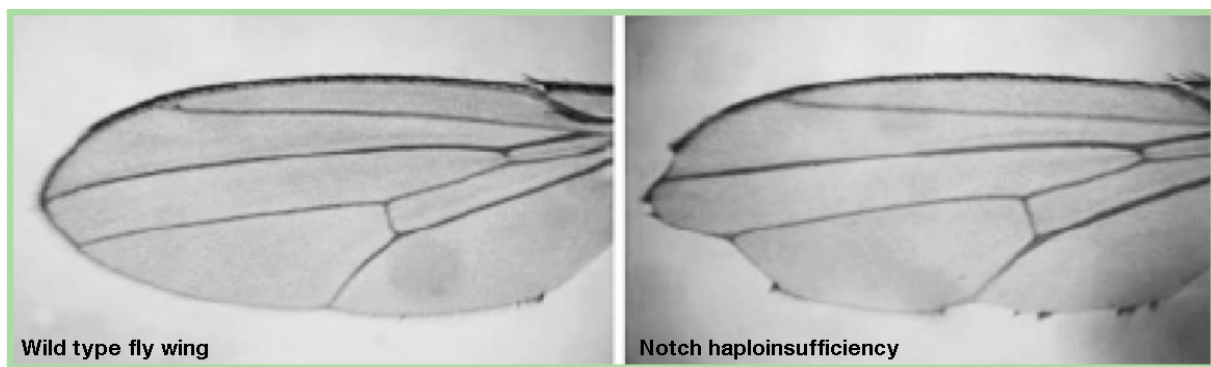


Figure 9. Notch haploinsufficiency phenotype in the *Drosophila* fly wing.

Image of a wild-type fly wing (left panel). Notch^{+/-} haploinsufficient fly wing containing notches at the border (right panel).

Rapidly after, Thomas H. Morgan identified the mutant allele responsible for this phenotype and in the beginning of the 1980's, Artavanis-Tsakonas and his colleagues cloned the Notch locus and unveiled the Notch protein primary structure as being a receptor (Wharton et al., 1985) composed of a large single-pass transmembrane protein, that is cleaved in the trans-

Golgi network and is presented on the cell surface in a heterodimeric form, consisting of a 110-kDa (Notch extracellular (NEC) domain) and a 180-kDa Notch transmembrane (NTM) domain, tethered together on the plasma membrane (Blaumueller et al., 1997).

Following the receptor's discovery, two fly Notch ligands with similar structures were discovered; **Delta** and **Serrate**, named after their effects on *Drosophila* wing development (reviewed in (Kopan, 1999)). The **Notch Intracellular domain (NICD)** requires two additional key components, namely **Suppressor of Hairless [Su(H)]** and the coactivator protein called **Mastermind (Mam)**, to form a nuclear complex and to promote Notch transcriptional activity (reviewed in (Bray, 2016)).

Despite its pleiotropy in controlling distinct cellular processes, the core-signal transduction machinery of the Notch pathway is relatively "simple" - including a limited number of essential components, and conserved across species (Artavanis-Tsakonas et al., 1999; Bray, 2006; Kopan and Ilagan, 2009).

NOTCH RECEPTORS

Whereas only one Notch receptor is found in the fly system, mammals have **four Notch paralogs** (Notch1-4) that present both redundant and unique functions. Notch receptors (Figure 10) are large Type I transmembrane glycoproteins that are normally cleaved, at a site termed Site 1 (S1), during maturation in the trans-Golgi network. This proteolytic cleavage is mediated by a furin-like pro-protein convertase that yields a heterodimer that retains the N-terminal end of the protein (that forms the NEC domain), bound to the C-terminus end (that contains the NTM and intracellular (NICD) domains) and held together by non-covalent interactions between the N- and C-terminal halves of the Heterodimerization domain (HD). These heterodimers are then anchored in the cellular membrane and form the transmembrane protein complex (Blaumueller et al., 1997).

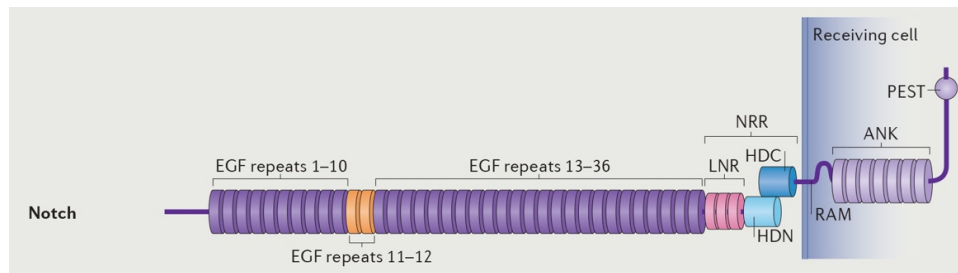


Figure 10. Molecular structure of the Notch receptor.

The EC domain of the Notch receptor is characterised by the presence of 36 EGF repeats, of which two are required for proper ligand-receptor binding. Following these EGF repeats, two heterodimeric portions (HDN at the N-term and HDC at the C-term) interact and, together with the cysteine-rich Lin12/Notch repeats (LNRs), form the negative regulatory region (NRR), which under absence of ligand occludes the cleavage site for ADAM proteases. The C-terminus of the Notch receptor includes the Notch intracellular domain (NICD) that harbours an RBPJ κ -associated module (RAM) domain, ankyrin (ANK) repeats, and ultimately a PEST domain, which regulates NICD degradation. Between the ANK repeats and PEST, NICD also contains several nuclear localization signals and a region that confer transactivation. Modified from Bray, 2016.

The EC domain harbours roughly 36 tandem EGF repeats, of which two motifs (11-12) mediate interactions with Notch ligands (Rebay et al., 1991; Shimizu et al., 1999), and others bind to Calcium to determine the structure and affinity of the receptor to its ligands. Following the EGF repeats, a single region termed (Notch) Negative Regulatory Region (NRR), composed of three cysteine-rich Lin12-Notch repeats (LNR) and one HD that harbours two sites for cleavage (S1 mentioned above and S2) and prevents receptor activation in the absence of ligand (Kopan et al., 1996).

The NTM domain is a single-pass transmembrane, followed by the RAM (RBP-j κ association module) domain that is found within the intracellular domain (juxtaposed to the NTM). The RAM domain forms a high affinity binding module with the RBP-j κ DNA-binding protein. C-terminally to the RAM domain, seven ankyrin repeats (ANK domain) flanked by two nuclear localization signals (NLS) required for nuclear translocation of NICD are found and a transactivation domain (TAD). Lastly, in the most C-terminal part of the receptor, a PEST [proline (P), glutamic acid (E), serine (S) and threonine (T) rich] motif regulates the stability of NICD (Blaumueller et al., 1997).

NOTCH LIGANDS

Five canonical Notch ligands, Delta-like1, 3 and 4 (Dll1, 3, 4), and Jagged1 and 2 (Jag1-2) have been identified in mammals. These ligands are integral cell surface proteins (Figure 11) characterized by the presence of a DSL domain (Delta, Serrate, and Lag2). DSL ligands are Type 1 transmembrane proteins containing multiple tandemly arrayed EGF repeats, some Calcium binding and some non-Calcium binding, in the extracellular domain. Jag1 and Jag2 contain a cysteine-rich domain (CRD) and are enriched in EGF repeats, whereas Dll1, Dll3 and Dll4 are devoid of CRD domain and are composed of less EGF repeats. Besides the DSL motif, the Notch binding domain contains a conserved motif termed Delta-OSM-11-like proteins (DOS) within the first pair of EGF repeats. The DOS domain is proposed to cooperate with the DSL domain for Notch binding and signalling, differently from the remaining EGF repeats. Following the DOS domain, all ligands display a variable number of EGF repeats prior to the transmembrane domain (D'Souza et al., 2008).

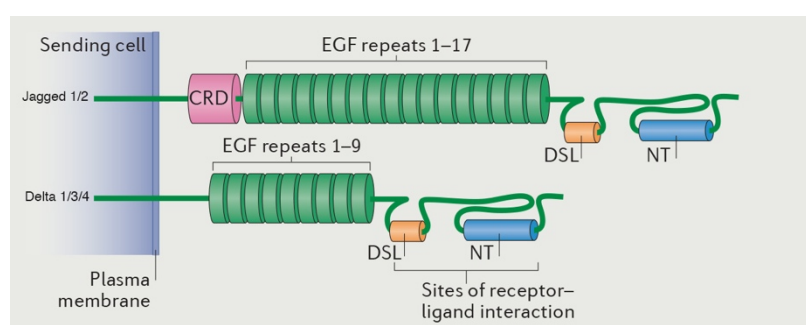


Figure 11. Schematic diagram of Notch ligands.

Notch ligands are characterised by an N-terminal DSL domain necessary for receptor interaction. The EC domains contain a variable number of EGF repeats and the ligands are divided in two categories depending on the presence/absence of a cysteine rich domain (CRD). Modified from Bray, 2016.

NOTCH ACTIVATION CASCADE AND REGULATION

Notch signal activation is triggered by the binding of DSL Notch ligands expressed in the signal-sending cell to Notch receptors that are present on adjacent cells (signal-receiving cell). Upon ligand-receptor binding, forces derived from the ligand present in the signal-sending cell will exert on the bound Notch receptor, promoting the displacement of the LNR domain, which will in turn generate a conformational change in the NRR domain and,

ultimately, will expose Notch S2 for cleavage by **A-Disintegrin-And-Metalloprotease (ADAM)** (van Tetering et al., 2009). ADAM cleavage gives rise to the **membrane-anchored Notch extracellular truncation (NEXT)** fragment that serves as a substrate for the next cleavage mediated by the γ -secretase complex at the membrane or in endosomal compartments. This multi-complex includes four components; **Presenilin, Nicastrin, APH-1** and **PEN-2** proteins (De Strooper et al., 1999). Proteolytic cleavage allows the release of the cytoplasmic domain of Notch receptors (NICD), which shuttles into the nucleus to associate with the **RBP-Jk** (recombination signal sequence-binding protein Jk or CSL), a DNA-binding transcription factor, and its transcriptional coactivators, acting directly to orchestrate transcription (Jarriault et al., 1995) (Figure 12).

More specifically, the interaction between NICD and RBP-Jk (via NICD RAM and ankryin domains) promotes the displacement of DNA bound corepressors complexes (eg. MTG8 (Salat et al., 2008), MTG16 (Engel et al., 2010), and SPEN, SHARP, CtBP (Kuroda et al., 2003; Oswald et al., 2005)) and the recruitment of **Mastermind-like (MAML)** proteins, ultimately generating a transcriptional activator complex that drives the expression of Notch target genes containing RBP-Jk binding sites, such as the bHLH transcription factors belonging to the **HES (hairy and enhancer of split) family**. The level and extent of transcriptional activity will depend on the amount and stability of NICD molecules, which is also regulated by post-translational modifications, such as ubiquitylation, that can promote NICD degradation.

In the absence of ligand, Notch NRR maintains the Notch receptor resistant to ligand-independent metalloprotease cleavages, disabling NICD pathway activation (Lai, 2004).

In mammals, the best-characterised Notch target genes belong to the Hes (Hairy Enhancer of Split) and Herp/Hey (Hes-related repressor proteins with Y-box) family of basic helix-loop-helix (bHLH) transcription factors. *Hes* and *Hey* genes encode nuclear proteins that usually repress transcription both actively, by interacting with co-repressors (such as Groucho homologs), and passively, forming non-DNA-binding heterodimers with bHLH activators (like the E47 transcriptional factor) inhibiting transcriptional activation. These proteins repress the activity of many other transcription factors, that are mainly repressors as well, such as Math and Mash (Iso et al., 2003).

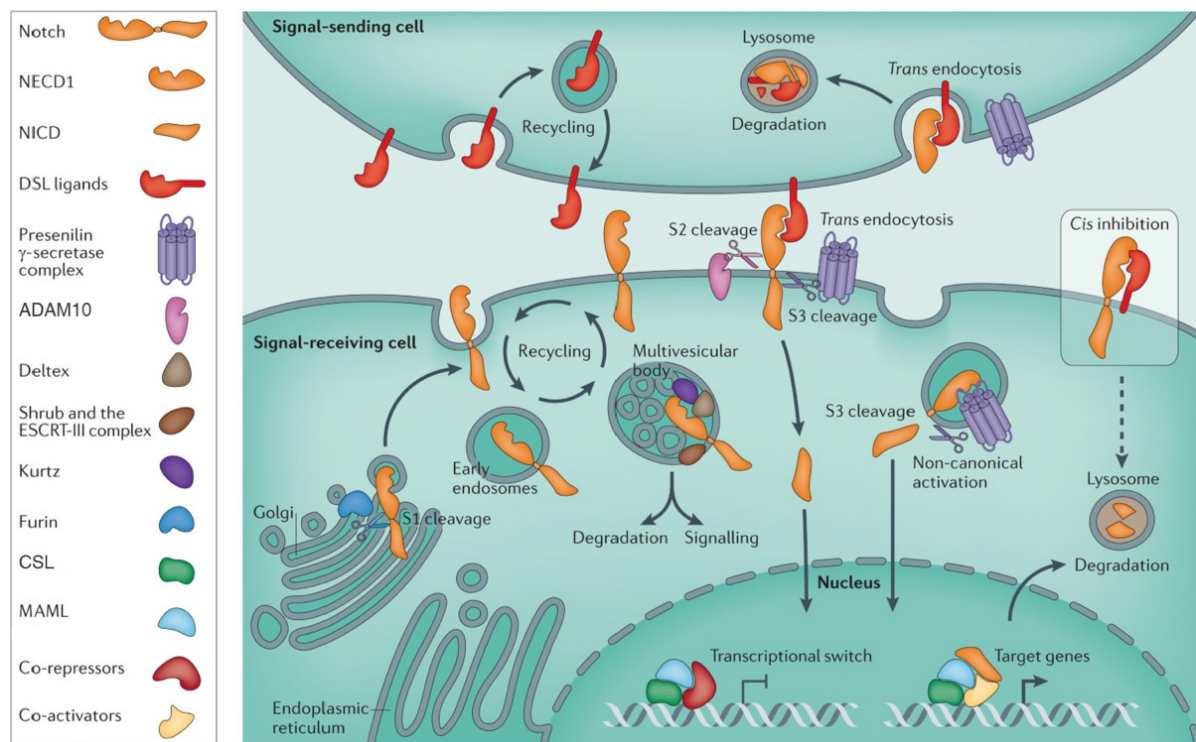


Figure 12. Overview of the Notch signalling cascade.

Three proteolytic cleavage steps are required for canonical Notch receptor signalling. The first (S1 cleavage) is mediated by the Furin protease, which produces a heterodimer composed of the Notch extracellular domain (NECD1) and the Notch intracellular domain (NICD). When a ligand binds a Notch receptor on the adjacent cell (termed *trans* interaction), two additional proteolytic cleavage events occur on the Notch receptor; the first promoted by TACE metalloproteinases (S2 cleavage), such as ADAM10, and the second by γ -secretase (S3 cleavage). The S3 cleavages produces the transcriptionally active NICD that translocates to the nucleus to subsequently interact with the DNA-binding protein CSL/RBP-J κ and the co-activator Mastermind-like (MAML) to promote transcription of its target genes. *Cis*-inhibition occurs when the Notch receptor binds to an adjacent ligand present on the same cell. This interaction in *cis* causes the internalization of the receptor, which is further transported to lysosomes to undergo degradation. Modified from (Guruharsha et al., 2012).

To provide an additional step of signal regulation, the availability of Notch receptors and ligands at the cell membrane is tightly controlled (for maintaining the correct levels of Notch expression and availability at the membrane). Given that each molecule of Notch receptor can only signal once and constitutive Notch signalling activation can potentially be harmful for the cell, the simplest way to fine-tune signals that are further translated into important cellular messages is to limit receptor and/or ligand expression at the spatial and temporal level. Within this step of spatiotemporal receptor/ligand regulation, post-translational modifications and trafficking have been assigned as important mechanisms that control

receptor/ligand availability and productive interactions (reviewed in (Kopan and Ilagan, 2009)). Among these mechanisms, **receptor** and **ligand glycosylation** of the EGF repeats, respectively, in the extracellular motif by the O-fucosyltransferase POFUT1 has been shown to be required for proper signalling establishment, relying on **Fringe** glycosyltransferase activity. Members of the Fringe family recognise O-Fucose residues and extend the glycosaminoglycan chain by the addition of N-acetylglucosamines (Bruckner et al., 2000; Moloney et al., 2000). In mammals, the presence of distinct Notch receptors/ligands and three Fringe proteins (Lunatic (Lfng), Manic (Mfng), Radical (Rfng) fringe) renders the full understanding of how Fringe regulates Notch signalling a complex ground (Johnston et al., 1997). Depending on which Fringe proteins and Notch ligand/receptor interact and which cell types express them, Fringe glycosylation can either enhance or reduce Notch activation.

Cell signalling and **endocytosis** are intimately and bi-directionally linked processes. Several developmental signalling pathways, such as RTK (receptor tyrosine kinase), TGF- β /Decapentaplegic, Hedgehog, Wingless, and, with no exception, the Notch signalling pathway, are regulated by endocytosis through the internalisation of plasma membrane receptors and/or ligands (Seto et al., 2002).

Many studies in zebrafish and flies have shown a different mechanism by demonstrating that **endocytosis and recycling of Notch ligands** to the plasma membrane are necessary to increase the affinity of ligand/receptor interactions and thus to trigger signalling (Becam et al., 2010; Matsuda and Chitnis, 2009). Two structurally unrelated RING-type E3 ubiquitin ligases, **Neuralized (Neurl)** and **Mindbomb-1 (Mib-1)** promote Jag1 and Dll1 ubiquitylation, respectively, further stimulating their endocytosis and signalling activities (Koo et al., 2005; Lai et al., 2001; Lai et al., 2005). In mice, Neurl genes are dispensable for normal development (Ruan et al., 2001), whereas Mib-1 has an embryonic lethal phenotype linked to Notch signalling disruption (Koo et al., 2007). It is proposed that Neurl acts downstream of Mib-1 controlling lysosomal degradation of internalized ligands and thereby regulating the level of ligand availability for Notch signals, while Mib-1 is seen as a mediator of Notch activation by releasing NICD, upon receptor-ligand endocytosis, thus promoting Notch activity (Song et al., 2006).

Differently from ubiquitination-mediated endocytosis of Notch ligands, Notch **receptor endocytosis** mechanism are mainly designed to regulate Notch protein levels at the cell membrane in order to regulate receptor availability for ligand binding. In mammals, another E3 ubiquitin ligase called **Itch** (AIP4 in humans) was identified in a natural mutant mouse line

(Perry et al., 1998) and was associated with Notch receptors as its substrate (Qiu et al., 2000). Murine Itch controls the degradation of non-activated Notch receptors by ubiquitination and consequently, its targeting to lysosomal degradation. Itch interactions with Notch seem to occur after the early steps of endocytosis implicating the mediation of other E3 ubiquitin ligases in this process. As a matter of fact, Notch receptors can also adopt two distinct fates: recycling in endosomes for later anchorage at the cell membrane or activation in a Deltex, ligand-independent manner (i.e. Deltex functionally antagonizing Suppressor of Deltex and its fly homolog NEDD4 that are proteins proposed to inactivate Notch molecules), after its partial cleavage into NICD form (Sakata et al., 2004).

Ligand-receptor interactions within the same cell (called in *cis*) control Notch receptor availability. At the end of the 20th century, two different labs carefully described the relationships between Notch and its ligand Delta using the fly wing margin as a model. The authors showed that ligands could exert a *cis*-inhibitory effect on Notch receptors, present on the same cells, to correctly specify the wing margin. This process driving these inhibitory interactions is called ***lateral inhibition*** and comprises the central mechanism for Notch action. By definition, lateral inhibition is a process by which a cell that acquired a particular fate interacts with its immediate neighbours to prevent them from adopting the same fate. It describes the inability or reduced ability of a cell to receive signals from neighbouring cells due to *cis* interactions between Notch receptors and ligands. Both studies in the fly wing mentioned above uncovered these ligand-receptor interactions and reached the analogous conclusion that the ratio between the ligand and the receptor expressed in the same cell can influence the signal activity result (de Celis and Bray, 1997; Micchelli et al., 1997). Molecularly, when a sending cell expresses higher numbers of Notch ligands than Notch receptors, all Notch molecules are *cis*-inhibited by Notch ligands (present within the same cell), although sufficient ligands remain accessible to interact with receptors on adjacent cells, making the cell capable of sending a signal. Conversely, when a receiving cell expresses higher numbers of Notch receptors than Notch ligands, some receptors are *cis*-inhibited by ligands, but sufficient Notch is available to bind with ligands from neighbouring cells, and thus to receive signals. For situations in which classical lateral inhibition occurs, Notch activation frequently inhibits ligand expression to polarize the signalling promoting thus a negative feedback. The exact mechanism to explain *cis*-inhibition remains elusive, but some reports suggest that Notch receptors are degraded after they have undergone ligand interaction (Becam et al., 2010; Glittenberg et al., 2006).

NOTCH SIGNALLING IN THE INTESTINE: FOR BETTER, FOR WORSE

The Notch signalling pathway controls the balance between proliferation and differentiation in the intestinal epithelium and thus, dictating specific cell fates. The expression of Notch receptors, DSL ligands, and Notch target genes was broadly analysed in the embryonic and mouse adult intestinal epithelium. From the four Notch receptors, Notch1 and Notch2 gain the podium for the most expressed genes within the ISCs and crypt progenitors. Delta1 and Delta4 ligands are mainly expressed in secretory lineages, such as Paneth (that confer the ISC niche) and Goblet cells, while Jagged1 and Jagged2 mirror the expression pattern of Notch1 and Notch2 expression within epithelial crypt cells (Sander and Powell, 2004). Notably, Notch3 and Notch4 are mainly expressed in the vasculature endothelium and the mesenchyme, respectively.

One of the first studies aimed at the characterisation of **Notch function** in the small intestine of rats was performed by Milano and colleagues in the mid-2000s. By *in vivo* administration of γ -secretase inhibitors (GSIs), the authors could observe a massive expansion of Goblet, Paneth and Enteroendocrine cells, as well as apoptosis of small intestinal epithelial crypt cells, and increased gene expression of the transcriptional activator Rath1 (the rat homolog of mouse Math1) known to specify secretory lineages (Milano et al., 2004).

The **direct role of Notch signalling** in controlling the segregation of each mature lineage from undifferentiated progenitor cells, as well as in the maintenance of the proliferative intestinal compartment was formerly demonstrated through mice carrying either a loss (van Es et al., 2005) or gain of function Notch mutation (Fre et al., 2005). Gut-specific inactivation of the major Notch effector RBP-J κ in adult mice results in the complete loss of proliferating cells and in their conversion into post-mitotic secretory cells. In a reciprocal study, Fre et al reported that the expression of a constitutively active form of the Notch receptor (NICD) in the developing intestine blocked secretory cell differentiation and increased the proportion of undifferentiated dividing cells, which extended outside of the crypt proliferative compartment, and were found all along the vertical axis of the villi, where normally no cell divisions ever occur. Conditional deletion of both Notch1 and Notch2 using tamoxifen inducible Villin-Cre^{ERT2} mice forced all ISCs to terminally differentiate into post-mitotic goblet cells. Conversely, conditional removal of Notch1 or Notch2 allows normal crypt development, demonstrating the extent of redundancy between Notch1 and Notch2 in the intestine (Riccio et al., 2008).

Collectively, these original studies demonstrated that Notch signals are essential to maintain proliferation of undifferentiated crypt progenitors (and SCs), as well as to control the binary cell fate decision of progenitors that will differentiate into either an absorptive or a secretory lineage. In addition, these reports have shown that affecting Notch signals in the gut originates severe phenotypes, validating its essential role in maintaining intestinal homeostasis, therefore, proposing that Notch signals may be mechanistically involved in carcinogenesis. Indeed, many studies have shown that Notch signalling is overexpressed or constitutively activated in many cancers including CRC (Fernandez-Majada et al., 2007; Reedijk et al., 2008; Rodilla et al., 2009). In fact, increased Notch signalling may be linked to the increased susceptibility of CRC development in some precancerous conditions.

To better understand and to explore different therapeutic alternatives for CRC, murine intestinal/colon tumours generated through aberrant Wnt signalling, as well as tumour cell lines, have been tested for pharmacological inhibition of Notch signalling.

Rodilla and colleagues found that Jagged1 was expressed at a significantly higher level in the normal colonic mucosa and adenomas of patients with FAP, compared to the normal intestine of healthy subjects. They also found that Notch signalling inhibition by deletion of its ligand Jag1 in *Apc^{Min}* mutant mice decreased adenoma formation, again supporting the notion that Notch inhibition could be a therapeutic avenue to explore (Rodilla et al., 2009). Likewise, *in vitro* pharmacological and siRNA-mediated Notch signalling inhibition in colon cancer cell lines seems to sensitize cancer cells to chemotherapy (Meng et al., 2009).

Work performed in Louvard and Robine group has shown that mice carrying constitutive Notch activation in an *Apc* mutant background rapidly develop benign adenomas, that are prevented from reaching more advanced stages (i.e. adenocarcinoma) (Fre et al., 2009). To investigate the functional role of Notch signals in CRC, they used a combined gain-of-function Notch mutant and the *Apc¹⁶³⁸* mouse model, previously shown to be genetically predisposed to intestinal tumour development, due to the stochastic loss of heterozygosity (or LOH) at the *Apc* locus (Fodde et al., 1994). When Notch signals were constitutively activated in the intestine of *Apc* heterozygous mice (using a Villin-Cre^{ERT2} mouse), the number of adenomas developed increased by 20-fold, compared with *Apc* control mice. Importantly, even if the number of adenomas was highly increased upon Notch activation, these tumours failed to progress into adenocarcinoma, stressing that Notch activation has a strong impact on the initial stages of tumour development, although it may not have a role in the progression to malignant carcinomas. Consistent with these observations, biopsies from human patients show that roughly 80% of both sporadic and hereditary (FAP) low-grade

adenomas present strong nuclear expression of the Notch target Hes1, whereas Hes1 is either not detectable or expressed at very low levels in human adenocarcinomas (Fre et al., 2009). Indeed, in early stages of human CRC, Notch is frequently activated, but the molecular mechanisms underpinning Notch activation are not understood. In this line of thought and contrary to the previous reports that propose targeting Notch as a therapeutic approach in CRC, this observation implies that blockage of Notch signals within early stage adenomas could have instead a potential deleterious effect, in which benign tumours could progress into more aggressive tumours.

Altogether, these findings reveal that Notch signals are highly implicated in CRC initiation, although the mechanisms underlying their contribution to potentially impair cancer progression are not fully understood.

WNT SIGNALLING PATHWAY

INTRODUCTION TO WNT

Wnt

(noun)

The term “Wnt” originates from the merge of the fly Wingless (Wg) and mouse homolog Int1 genes. The Wingless (Wg) gene was identified in the 1970’s and named after the resulting Drosophila melanogaster mutant phenotype that causes a drastic transformation of the wings up to the thoracic notum.

The Wnt signalling pathway is an evolutionary conserved pathway, which controls panoply of vital biological processes during early development and adulthood.

Wnt signalling was unveiled in the late 1970s thanks to the genetic screen of *Drosophila melanogaster* developmental mutants. Nüsslein-Volhard and Wieschaus showed that the **Wg** gene was driving key events for segmental and spatial organization of the fly body plan and, for this reason, they called it a “segment polarity gene” (Nusslein-Volhard and Wieschaus, 1980).

Few years after the discovery of Wg in the fly, a retroviral insertion mutagenesis screen uncovered the **Int-1** gene (later called **Wnt1** gene) as its mouse homolog (Rijsewijk et al., 1987). Int-1 was originally characterized as a proto-oncogene that is activated upon MMTV (mouse mammary tumour virus) retrovirus integration of viral DNA in virally induced mammary tumours (Nusse and Varmus, 1982).

Comparable to the Notch signalling pathway, Wnt signals regulate a vast range of cellular processes and, as these important signals (i.e. expression of Wnt proteins and antagonists) play crucial roles during embryogenesis, they require thus tight regulation, both temporally and spatially coordinated during development (Yamaguchi, 2001). Proper orchestration of Wnt signalling is necessary for embryonic development, from gastrulation and early pattern formation to organogenesis. In the adult life, Wnt signalling pathway plays a crucial role in tissue homeostasis maintenance and stem cell regulation, controlling several processes, such as cell proliferation, self-renewal, cell polarity, cell death and cell fate specification. Deregulation of this signalling pathway leads inevitably to disease, such as cancer (namely breast, colon and skin), skeletal defects and human birth defect disorders including the most common neural tube closure defect; spina bifida (Komiya and Habas, 2008; van Amerongen and Nusse, 2009).

WNT RECEPTORS

Frizzled (Fz) proteins are the main receptors for the secreted Wnt ligand family, characterized as seven-pass transmembrane receptors that share a common structure with their ligands: a conserved extracellular N-terminal cysteine-rich domain (CRD) (Bhanot et al., 1996) that provides a primary platform for ligand-receptor bound. In the mammalian genome, there are ten distinct **Fz** receptors that cooperate with low-density lipoproteins receptor-related proteins 5 or 6 (**LRP5 and LRP6** in vertebrates, or **Arrow** in the fly) to form a heterodimeric complex (Pinson et al., 2000; Tamai et al., 2000). LRP5 and LRP6 act as crucial co-receptors in the canonical Wnt signal transduction and represent key regulatory molecules. The importance of LRP co-receptors was unravelled by the finding that potent Wnt inhibitors, such as Dickkopf (Dkk, (Glinka et al., 1998)) and Wise (Itasaki et al., 2003), form a complex with LRP and promote its internalisation, making it unavailable for Wnt signal reception (Mao and Niehrs, 2003) .

WNT LIGANDS AND THEIR REGULATION

In mammals, Wnt signal transduction occurs through 19 distinct **secreted Cysteine-rich Wnt ligands**, which can be divided into 12 groups. The defining structure of Wnt proteins comprise an almost invariant positioning of 24 to 22 cysteine residues, extending across the whole molecule, most of which are thought to form disulfide bridges to hold a secondary structure. These secreted glycoproteins are highly hydrophobic and mostly found associated with cell membranes and the extracellular matrix (ECM) (Reichsman et al., 1996). The purification of Wnt proteins not only confirmed their hydrophobic properties, but also unravelled that Wnt proteins undergo several post-translational modifications, such as lipid modifications in the ER (Endoplasmic Reticulum) via the attachment of a palmitate moiety – a process mediated by the acyltransferase Porcupine (Por) (Zhai et al., 2004), N-glycosylation and the coordination of a series of intra-molecular disulphide bonds. After palmitoylation and proper folding in the ER, Wnts are primarily directed to the Golgi apparatus, where they encounter the transmembrane protein **Wntless (Wls)** that further ensures their transport to the plasma membrane and thus their secretion (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). After anchorage at the plasma membrane, Wls undergo clathrin-mediated endocytosis, to be later directed by the retromer complex (an evolutionarily conserved multiprotein complex) back to the trans-Golgi Network

where they are recycled and trafficked back (from the Golgi) to the plasma membrane. This loop mediated by the retromer complex enables WIs to proper direct Wnt secretion (Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008).

WNT CASCADE AND TARGET GENES

The Wnt signalling pathway has been divided into either canonical (β -catenin-dependent) or non-canonical (β -catenin-independent). Non-canonical Wnt pathway includes the Planar Cell Polarity (PCP) pathway that leads to actin cytoskeleton modifications mediating cell polarity, cell movements during gastrulation, and other processes (Veeman et al., 2003), and the Wnt/ Ca^{2+} pathway that acts through Ca^{2+} intra-cellular release and is thought to impact both canonical and PCP pathways (Miller et al., 1999), being important for cell adhesion and cell movements during gastrulation (Kuhl, 2002).

Initially discovered as part of the adherens junction complex with α -catenin (Ozawa et al., 1989), **β -catenin** also serves as a transcriptional coactivator of Wnt target gene expression. β -catenin levels are often low in unstimulated cells, but both soluble and membrane-bound protein accumulates in the cytoplasm and nucleus in response to Wnt signalling (Polakis, 2000).

In the absence of an extracellular Wnt stimulus (or in the presence of the Wnt antagonist, such as Dkk), free cytoplasmic β -catenin is actively directed by the **GSK-3 β /Axin/APC/CK1** multiprotein “**destruction complex**”, composed of **Glycogen synthase kinase-3 β (GSK-3)**, **Axin**, **Adenomatous Polyposis Coli (APC)** protein and **CK1 kinase**, for phosphorylation and ubiquitination, following its proteasomal degradation. In particular, GSK3- β and CK1 kinases promote sequential phosphorylation of highly conserved Ser and Thr residues of β -catenin (Liu et al., 2002).

Mutations in destruction complex members associated with various cancers result in inappropriate stabilisation of β -catenin and Wnt target gene expression when Wnt signals are off (Heisenberg et al., 2001; Satoh et al., 2000). For example, in the absence of a Wnt stimulus, the half-life of β -catenin was found to be 50 min in AtT20 cells, but 3 hours in the colon cancer cell line SW480, which carries a mutated APC protein (Munemitsu et al., 1996). The Wnt signalling pathway is active when Wnt proteins (released from or displayed on the surface of signalling cells) act on target cells by binding to the Fz/LRP complex at the cell surface. Wnt receptors transduce a signal to several intracellular proteins, including

Dishevelled (Dsh), a Wnt agonist), β -catenin, and the components of the destruction complex. Molecularly, the transduction of Wnt signals entails a ligand-induced conformational modification of the Fz/LRP co-receptor complex that in turn allows the activation of Dsh via its phosphorylation, resulting in the recruitment of the GSK-3 β /Axin/APC/CK1 complex from the cytosol to membrane, to promote LRP phosphorylation. Dsh is also recruited to the membrane and binds to Fz and Axin. The resulting complex formed at the cell membrane at Fz/LRP level induces the stabilization of β -catenin via either sequestration and/or degradation of Axin. Newly translated β -catenin protein is then allowed to accumulate in the cytosolic compartment, and can be further translocated to the nucleus, where it binds to the nuclear **TCF/LEF (T-cell specific factor/Lymphoid enhancer factor) transcription factors**, displacing co-repressors to promote activation of its target genes (Li et al., 2012) (Figure 13).

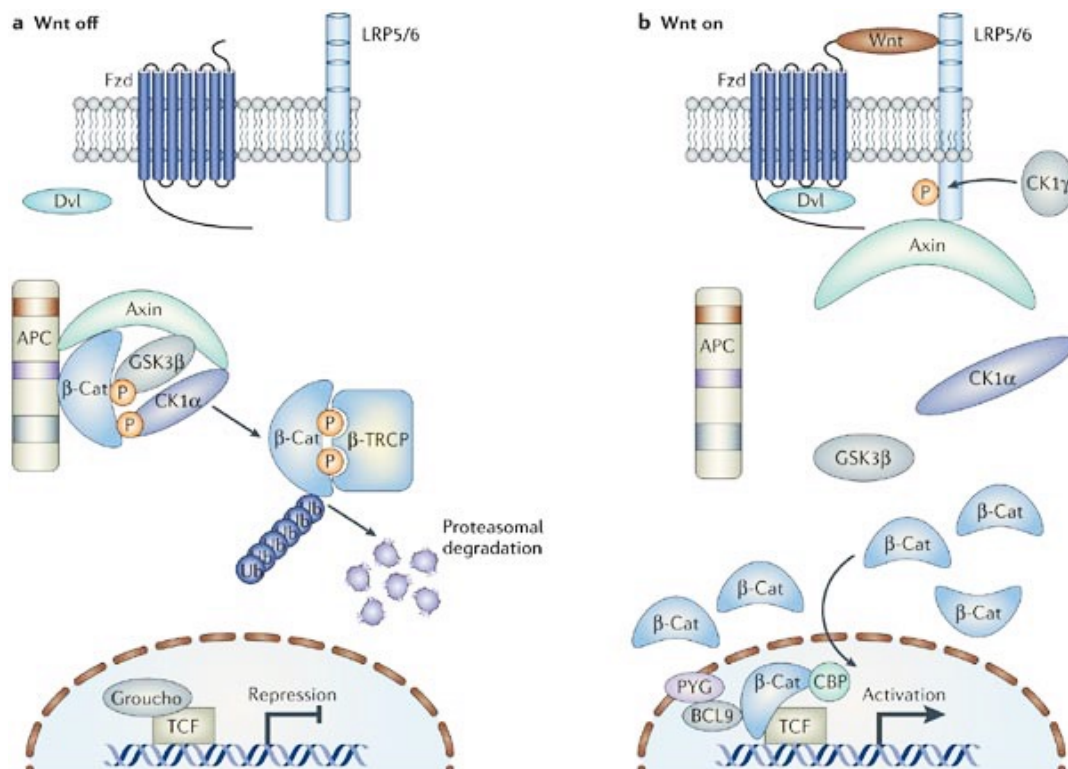


Figure 13. Simplified overview of the canonical Wnt signalling pathway.

In the absence of a Wnt signal (A), β -catenin is sequestered by the destruction complex, which mediates its phosphorylation and proteasomal degradation. Interaction of a Wnt ligand with its specific receptor complex that contains a Frizzled family member and LRP5 or LRP6 (B) triggers the formation of Dsh–Fz complexes and the phosphorylation of LRP by CK1 gamma, facilitating the relocation of Axin to the membrane and the inactivation of the destruction complex, allowing β -catenin to accumulate and enter the nucleus, where it interacts with members of the TCF/LEF family. In the nucleus, β -catenin transforms the TCF proteins into effective transcriptional

activators by displacing Groucho/TLE proteins and recruiting several coactivator proteins. After dissipation of the Wnt signal, β -catenin is exported from the nucleus by the APC protein and TCF proteins revert to actively repressing the target gene program. From (Barker and Clevers, 2006).

In the absence of Wnt signals, TCF acts as a transcriptional repressor by forming a complex with Groucho/TLE repressors (Cavallo et al., 1998).

The binding of β -catenin displaces Groucho/TLE and converts TCFs into transcriptional activators, translating the Wnt signal into transcription of specific target genes (Daniels and Weis, 2005).

The mechanism that allows β -catenin nuclear import is still unclear. β -catenin contains no recognizable NLS (Nuclear Localisation Signal) and because its nuclear localisation is stimulated by overexpression of TCF/LEF (Behrens et al., 1996), it has been hypothesized that β -catenin is imported by a piggy-back mechanism. However, LEF is not essential for β -catenin import, as mutated β -catenin that does not bind LEF can enter the nucleus (Prieve and Waterman, 1999).

A large number of Wnt targets, such as Axin2, LEF-1, Dkk, Fz (among many others), have been identified that include members of the Wnt signal transduction pathway itself, which promote feedback control during Wnt signal transduction (Logan and Nusse, 2004).

PREVIOUS WORK

NOTCH1+ ISCs CONTRIBUTE TO TUMOUR FORMATION

ISCs have been hypothesised as the cells of origin of intestinal cancer (Barker et al., 2009). To investigate whether Notch1+ ISCs could contribute to tumour initiation and growth, a Notch1 knock-in inducible Cre line that was generated in our laboratory (Fre et al., 2011) was used to perform lineage tracing analysis prior tumour formation. To this aim, a transgenic mouse model, in which a fluorescent reporter would be expressed according to Notch1 expression, in an inducible manner, was also generated by crossing the Notch1-Cre^{ERT2} mice (Fre et al., 2011) (referred to as N1) to a Cre-sensitive bi-fluorescent reporter line Rosa26^{mT/mG} (referred to as mTmG) (Muzumdar et al., 2007). In this reporter mouse, membrane-associated tomato (mT) fluorescent protein is expressed in all cells until tamoxifen injection, after which membrane-associated GFP (mG) marks the cells targeted by Cre recombination and its progeny. The compound mice thus generated (termed N1/mTmG mice) were then crossed to *Apc*^{+/-1638N} mice (Fodde et al., 1994) (henceforth named *Apc*^{+/-}), carrying a heterozygous germline mutation in the *Apc* tumour suppressor gene (Figure 56), which causes the spontaneous development of few intestinal adenomas (5 to 6 tumours maximum), initially detectable at around six months of age, upon somatic LOH of the wild-type *Apc* allele. The triple transgenic mice obtained from these crosses are hereafter referred to as N1/mTmG/*Apc*^{+/-}.

Previous observations in our lab had shown that early stage adenomas from N1/mTmG/*Apc*^{+/-} mice were composed of a large proportion of cells derived from Notch1-expressing cells, in a clonal distribution, when Notch1-expressing cells were traced prior to tumour formation (Figure 14). This not only reflects that intestinal adenomas present a polyclonal origin, corroborating Novelli and Yanai's findings (Novelli et al., 1996; Yanai et al., 2017), but also stresses the contribution of Notch1+ ISCs to tumour initiation and/or tumour growth.

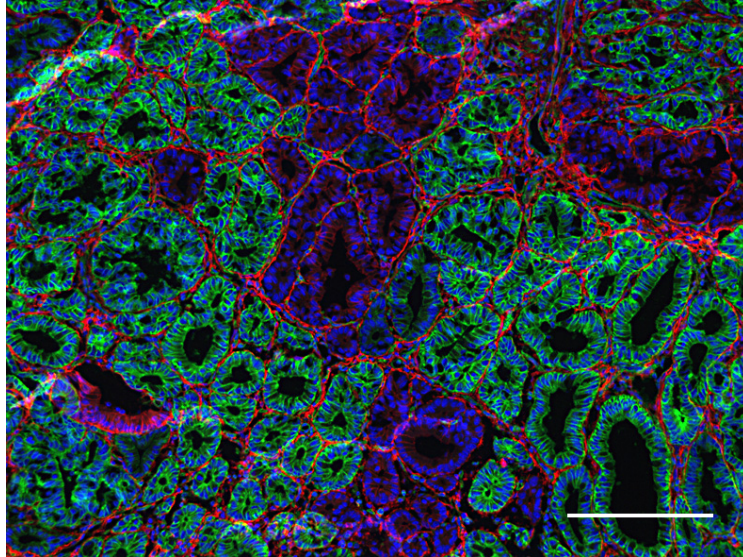


Figure 14. Notch1-expressing cell fate mapping before tumour development.

N1/mTmG/Apc^{+/-} mice were induced at 4 weeks of age (by a single pulse of tamoxifen) before tumour emergence. Notch1+ tumour cells (in green) clonally expanded during tumour development, as observed by the presence of large GFP marked clones. DNA is stained by DAPI in blue and the Tomato protein is in red. Scale bar represents 100 μ m.

NOTCH SIGNALS ARE ACTIVE IN EARLY STAGES ADENOMAS

Using another mouse line characterised in our lab that expresses GFP under the control of the Hes1 promoter, a direct transcriptional target of Notch signal activation (Fre et al., 2011), we detected a high proportion of Hes1+ cells in early stage adenomas generated from Apc^{+/-} mice, implying that Notch1 is not only expressed but that the pathway is also potentially active in these tumours (Figure 15).

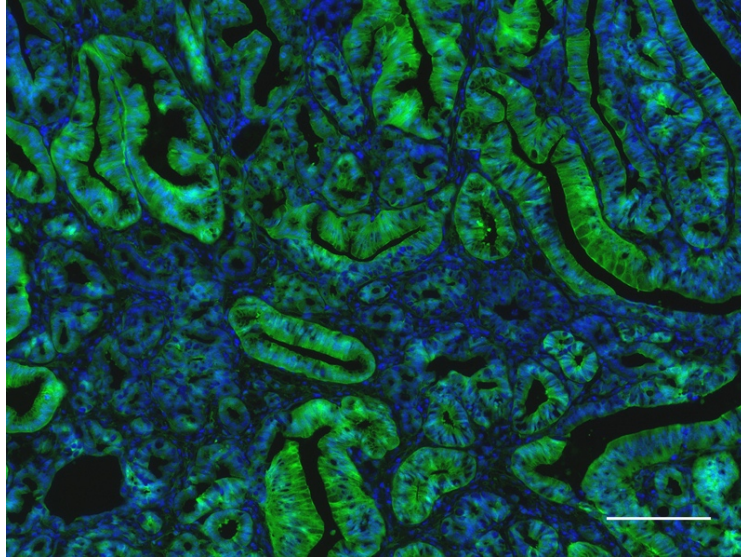


Figure 15. *Hes1* is expressed in *Apc* mutant adenomas.

Hes1/Apc^{+/-} mice display, at the same age as *N1/mTmG/Apc^{+/-}*, intestinal adenomas containing large number of cells expressing *Hes1* (reflected by the cytoplasmic GFP reporter gene in green). DNA is detected by DAPI in blue. Scale bar represents 100 μ m.

Based on these initial results, we decided to explore whether the Notch1 receptor was expressed in already formed tumours, using lineage tracing analysis *in vivo*, in *Apc^{+/-}* mice that spontaneously generate early stage intestinal adenomas.

OBJECTIVES

Colorectal tumours present a high cellular heterogeneity, and they consequently show varied responses to therapy. Therefore, it is important to define and molecularly characterise CSCs in these types of tumours, in order to improve targeted therapies. Nevertheless, the identification and isolation of CSCs is still challenging due to the lack of reliable CSCs markers.

In an attempt to isolate stem cell-like populations in intestinal tumours, we decided to assess if a marker of normal ISCs, the Notch1 receptor that labels both CBCs and LRCs, could be used to identify CSCs in tumours. The work of several labs has established that the Notch signalling pathway is an essential regulator of stem cell survival in the normal gut, and it plays a crucial role in promoting colon cancer in cooperation with Wnt signal activation (Fre et al., 2005; Fre et al., 2009; Pellegrinet et al., 2011; Riccio et al., 2008; Rodilla et al., 2009; van Es et al., 2005).

The extremely rapid cell renewal rate in the intestine suggests that only intestinal stem cells or very early progenitors persist long enough in this tissue to develop cancer. For this reason, long-lived crypt stem cells are considered as the best targets for oncogenic mutations.

Previous work conducted by my supervisor in the laboratory of Prof. Louvard showed that Notch1, consistently with its essential role in intestinal homeostasis and in cell fate determination (Fre et al., 2005), is expressed in multipotent crypt stem cells (Fre et al., 2011). Based on these results, we decided to use the expression of Notch1 as an experimental tool to mark putative “stem cell-like” populations within mouse intestinal tumours and follow their fate *in vivo* by lineage tracing analysis.

Our experimental design offered the possibility to characterise CSCs in tumours already formed, representing a novel and different setup from previous studies, in which tumours are generated upon genetic targeting of key oncogenic signalling pathways in distinct intestinal cell types, a system that tests the cell of origin but does not assess the expression of SC markers within intestinal tumours.

The **main objective** of this study was to **identify potential CSCs in spontaneously generated tumours, in order to pinpoint candidate therapeutic targets.**

The major aims of my PhD project were:

- 1) Identification and characterisation of Notch1-expressing cells and derived lineages in intestinal tumours developed upon loss of the tumour suppressor *Apc*.
- 2) Isolation and molecular characterization of Notch1+ tumour cells and Notch1+ normal ISCs. Upon cell dissociation and sorting by flow cytometry, these cells were characterized at several levels:
 - 2.1 Defining their transcriptional signature (by analysing the expression of known SC markers, degree of differentiation and signalling pathways).
 - 2.2 Exploring the proliferation potential of Notch1+ tumour cells *in vivo*. Determining the degree of heterogeneity within the Notch1+ tumour cell population by performing single cell transcriptomic analyses and single-molecule FISH.
- 3) Assessment of the tumourigenic capacity of Notch1+ tumour cells and their response to chemotherapeutic drugs *ex vivo*.
- 4) Investigation of the contribution of Notch1-expressing cells to tumour growth in AOM/DSS-induced colon tumours.

RESULTS

1. CHARACTERISATION OF NOTCH1-EXPRESSING CELLS WITHIN INTESTINAL TUMOURS

NOTCH1 IS EXPRESSED IN RARE CELLS IN BOTH THE EPITHELIAL AND THE STROMAL COMPONENTS OF INTESTINAL TUMOURS

The Apc^{+/-} mouse line used is known to display tumours, almost exclusively in the small intestine, at around 6 months of age (Fodde et al., 1994). To analyse the cells expressing Notch1⁺ within these tumours, N1/mTmG/Apc^{+/-} tumour bearing mice (i.e. older than 6 months) received a single dose of tamoxifen and were analysed 24 hours later. In this reporter mouse, after tamoxifen injection, the switch from Tomato to GFP protein expression at the cell membrane allow to us to mark individual cells that can be efficiently isolated and sorted by flow cytometry. The presence of rare cells expressing GFP (Notch1⁺ cells) scattered throughout the tumour, both of epithelial and stromal origin, was readily detected by GFP fluorescence. Notably, the overall distribution of GFP⁺ cells in different tumour regions was heterogeneous and we hypothesise that this could be due to tamoxifen accessibility that might not be homogeneous within the tumour.

Immunostaining for the epithelial cell marker EpCAM (Epithelial cell adhesion molecule) confirmed the presence of tumour epithelial cells labelled by Notch1 (Figure 16).

Some stromal tumour cells (the majority displaying a classical spindle-shaped morphology) were also marked by Notch1 (Figure 16, highlighted by asterisk).

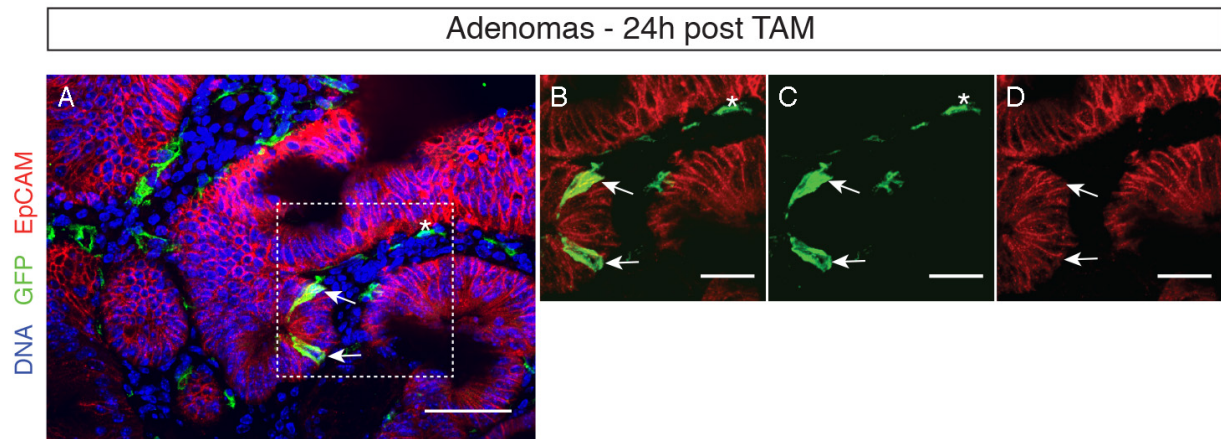


Figure 16. Immunostaining anti-EpCAM in adenoma sections 24 hours post tamoxifen induction.

EpCAM is labelled in red, Notch1 expression is reflected by GFP fluorescence in green and DNA was marked by DAPI in blue (A). Magnification panels display the merge of both red (anti-EpCAM) and green (Notch1+ tumour cells) channels (B), following split channels (GFP in C and anti-EpCAM in D). Arrows show two epithelial cells marked by Notch1 and asterisk highlights a Notch1-expressing stromal cell. Scale bars represent 20µm in A and 10µm in B, C and D.

Their identity was explored by immunostaining using an anti-alpha Smooth Muscle Actin (α SMA) antibody (Figure 17), that can mark myofibroblasts, pericytes and smooth muscle cells in vessels, and an anti-CD31 antibody (not shown) that labels endothelial cells and platelets, monocytes, neutrophils, and some types of T-cells. Notch1+ stromal cells were often marked by either of these two markers, and validation of their identity as fibroblasts or endothelial cells was confirmed in these cells.

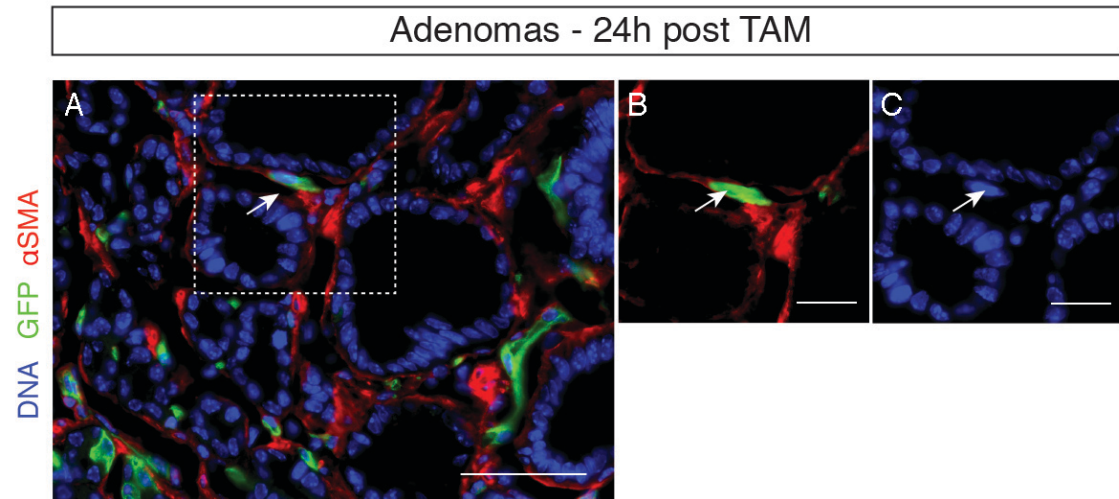


Figure 17. Immunostaining against α SMA performed in adenoma sections after 24 hours post tamoxifen induction.

α SMA is labelled in red, Notch1 expression is reflected by GFP fluorescence in green and DNA was marked by DAPI in blue (A). Magnification panels show the merge of both red (anti-EpCAM) and green (Notch1+ tumour cells) channels (B), following DAPI channel (C). Arrows show one stromal cell marked by GFP in between two tumour glands. Scale bars represent 50 μ m in A and 20 μ in B and C.

Despite the observation that Notch1 is also marking a heterogeneous stromal population, we pursued our study focusing exclusively on the tumour epithelial cells.

The quantification by FACS analysis of tumour cells marked by Notch1 revealed (in agreement with immunostaining observations) that Notch1-expressing epithelial cells represent a rare tumour cell population comprising $1,2\% \pm 0,3\%$ of Tumour Epithelial Cells (TEC, Figure 18).

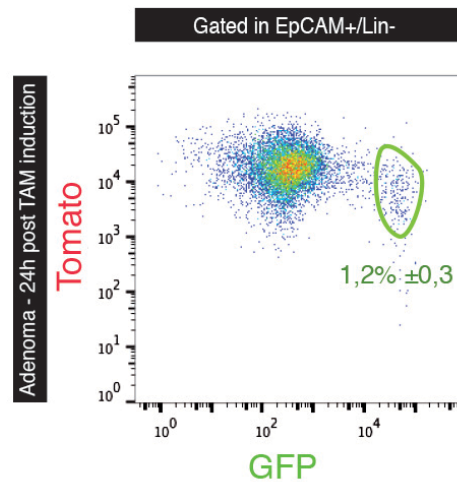


Figure 18. Gate strategy to quantify, analyse and sort Notch1+ tumour cells by FACS.

Tumour dissociated cells were gated within the DAPI- population (live cells), and doublets were excluded within the SSC-W/FSC-A (SSC-W^{high}) gate, following exclusion of Lin+ tumour cells and selection of EpCAM+ tumour cells that represent the TEC, in which the GFP+ percentage was quantified (within the Tomato/GFP channels here depicted).

NOTCH1-EXPRESSING TUMOUR CELLS ARE UNDIFFERENTIATED AND PROLIFERATIVE

To evaluate the differentiation state of Notch1-expressing tumour cells, the expression of distinct differentiation markers commonly used in the normal gut epithelium, such as anti-Lysozyme (marker for Paneth cells), Agglutinin (Ulex Europaeus Agglutinin, labelling both Paneth and Goblet cells), Chromogranin A (for Enteroendocrine cells) and anti-Mucin2 (labelling Goblet cells) was assessed by immunofluorescence (Figure 19). We observed the complete lack of co-expression of GFP and any of these markers, indicating that Notch1+ tumour cells are undifferentiated.

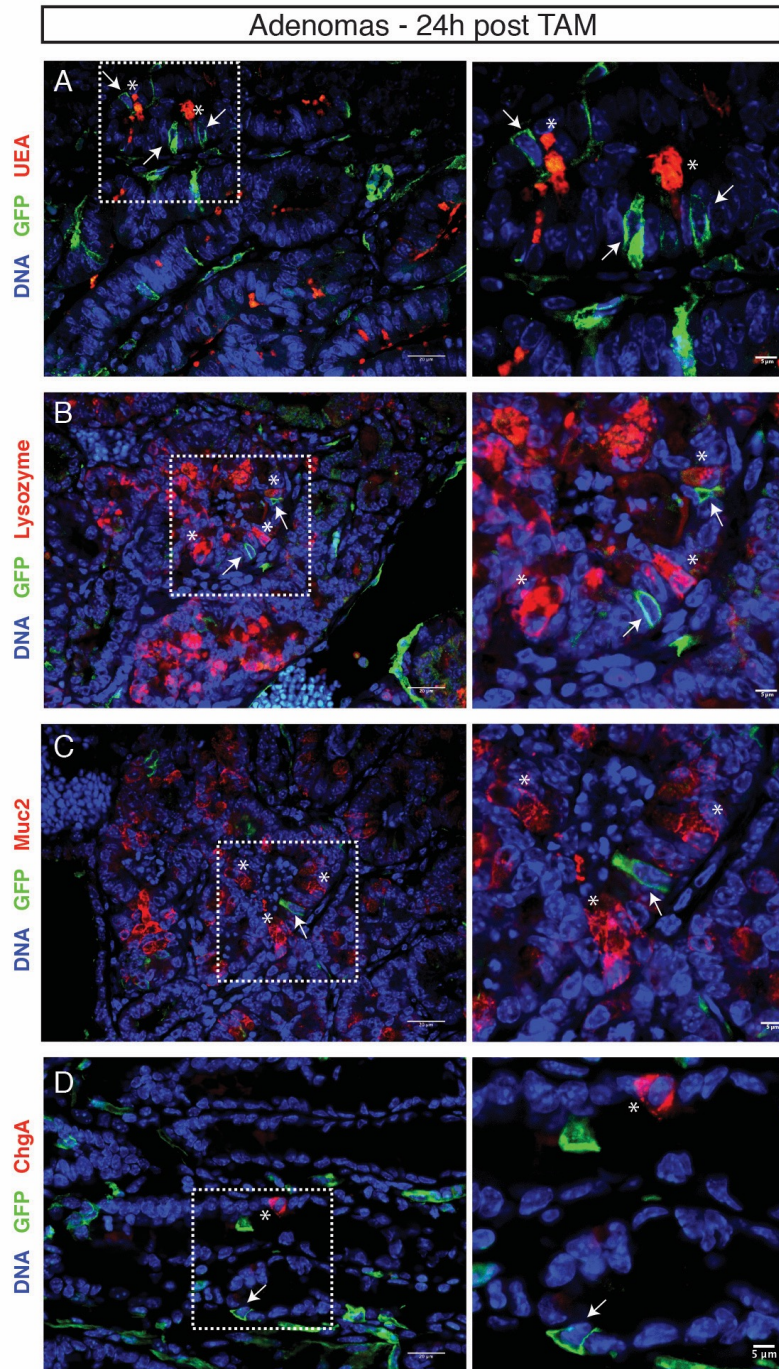


Figure 19. Notch1+ tumour cells comprise an undifferentiated cell population.

From top to bottom: immunofluorescent stainings of tumour sections using Agglutinin (UEA, labelling mainly Goblet cells but also Paneth cells, panel A), anti-Lysozyme (marker for Paneth cells, panel B), anti-Mucin2 (marker for Goblet cells, panel C) and anti-Chromogranin A (for Enteroendocrine cells, panel D), all in red. Notch1+ tumour cells are reflected by GFP expression in green and DNA was marked using DAPI, in blue. Arrows highlight GFP+ tumour cells and asterisks indicate tumour cells expressing the differentiation markers tested. Scale bars represent 20 μ m. Magnifications of selected areas of each section are shown in the right panels (scale bars represent 5 μ m).

We confirmed these results by assessing relative mRNA expression levels of intestinal secretory lineage genes, including Lysozyme, Mucin2 and Gob5 by qRT-PCR in cDNA from sorted Notch1-expressing and non-labelled tumour cells (Figure 20). We extended the differentiation status analysis of Notch1+ tumour cells by selecting one enterocyte marker, Alpi (Alkaline Phosphatase Intestinal) (Figure 20). This preliminary transcriptional analysis confirmed the undifferentiated state of Notch1+ tumour cells detected by immunofluorescence, as GFP+ cells display a significant reduction in mRNA levels of several differentiation markers for both secretory and absorptive lineages compared to non-labelled cells.

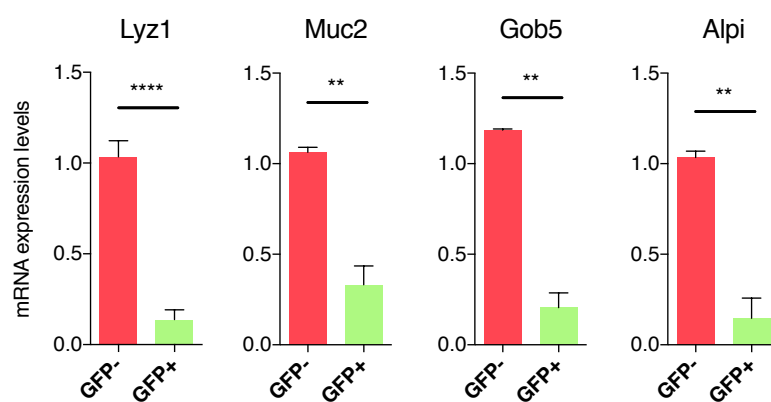


Figure 20. Differentiation status of Notch1-expressing tumour cells by qRT-PCR analysis.

Non-marked tumour cells (red bars) and Notch1+ tumour cells (GFP+ cells, green bars) were FAC-sorted for RNA extraction and cDNA synthesis. Expression of Lysozyme1 (Lyz1), Mucin2 (Muc2), CLCA1 (Gob5) and Alkaline phosphatase (Alpi) was quantified relatively to the expression of housekeeping genes (18S and β -actin) and normalized to 1 in the GFP- control for each gene. Three representative experiments are shown. Error bars represent the standard deviation. * represents a p-value <0.05, ** p-value <0.01 as calculated by Welch's t-test.

To determine the proliferation status of Notch1+ tumour cells (GFP+), two different proliferation markers were used; immunofluorescence using the anti-Ki-67 antibody (marking cells in G1, S and G2/M phases) and BrdU incorporation assay after a 2 hours *in vivo* pulse (incorporated during S phase). We found that approximately 50% of GFP-marked cells (Notch1+) were positive for Ki-67 and 20% for BrdU (Figure 21). This initial proliferative analysis showed that a large proportion of Notch1+ tumour cells are actively cycling, although the presence of quiescent or slow cycling cells within the tumour marked by Notch1 could not be excluded. As CSCs responsible for tumour relapse and dissemination were theorized to be retained in a dormant state for long time intervals, and thus to escape

conventional chemotherapeutical drugs that mainly target actively dividing cells (Sosa et al., 2014), we sought to better define whether a proportion of Notch1-expressing tumour cells could be maintained in a non-proliferative state by performing continuous BrdU incorporation experiments (schematic representation of BrdU incorporation experiment in Figure 57; *Material & Methods section*). After 5 days of continuous BrdU feeding (prior tamoxifen recombination), the percentage of Notch1+ tumour cells (induced by one pulse of tamoxifen at day 4) that had incorporated BrdU increased from 20% (at 24 hours + 2 hours BrdU pulse, Figure 21A) to 60% (Figure 21B – 5 days of continuous BrdU feeding + 24 hours TAM pulse prior culling), indicating that these cells, at a population level, do not seem to be quiescent and that they, most probably, simply divide asynchronously (Figure 21).

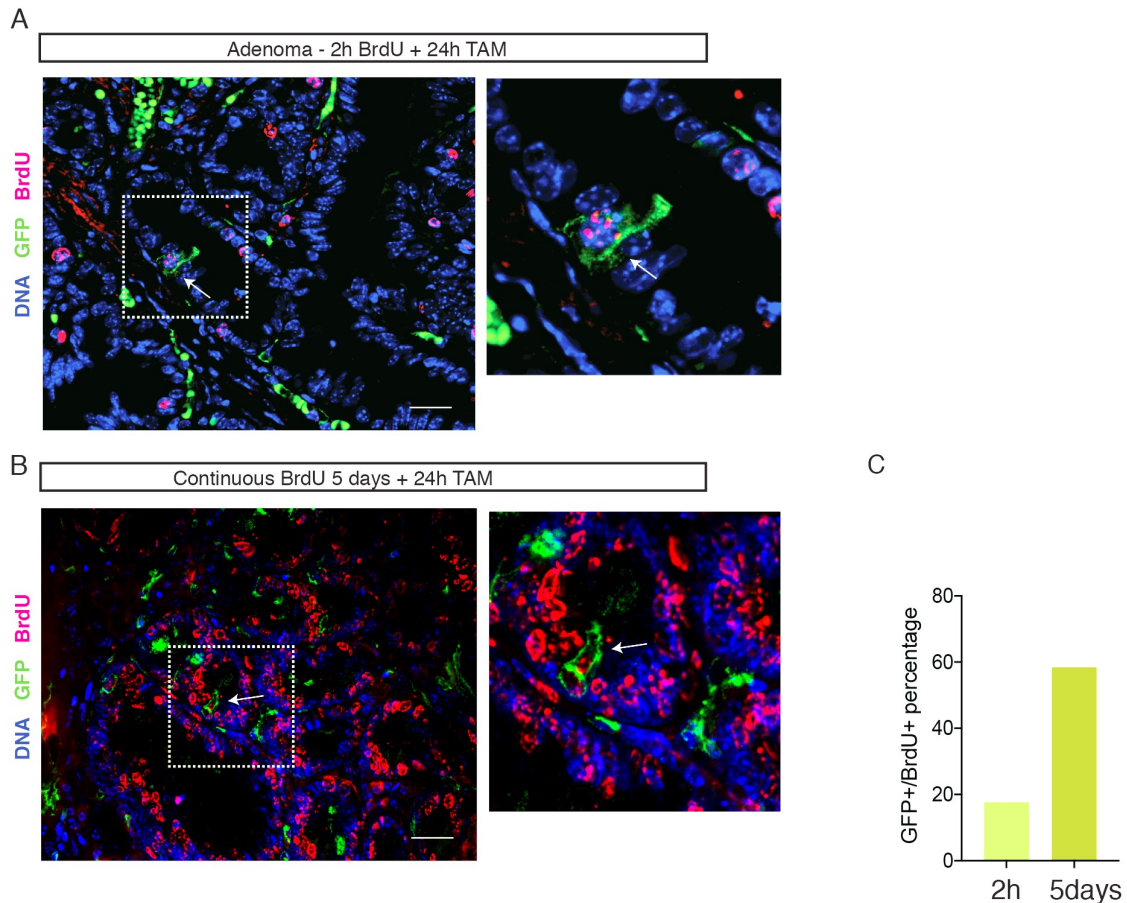


Figure 21. Continuous BrdU incorporation experiments reveals that the majority of Notch1+ tumour cells are actively proliferative.

In A) immunostaining performed with anti-BrdU antibody in adenoma section shows a Notch1-expressing tumour cell (marked by GFP expression, in green), positive for BrdU (in purple) after 2 hours BrdU injection. In B) the same immunostaining was performed as in A) in adenoma sections of mice kept for 5 days in continuous BrdU feeding and a 24 hours tamoxifen injection at day 4 (24 hours prior culling). In both images, arrows highlight GFP+/BrdU+ tumour cells. Scale bars represent 20 μ m. In C) Quantification of GFP+/BrdU+ tumour cells percentage after 24 hours post tamoxifen induction + 2 hours BrdU incorporation (light green bar, 2h) and GFP+/BrdU+ tumour cells percentage after 5 days in continuous BrdU feeding and a 24 hours tamoxifen injection before culling (darker green bar, 5 days).

We also analysed adenoma sections from 1 to 2 months chase for the presence of GFP+ singlets (a potential indication that Notch1+ tumour cells and/progeny did not divide, data not shown). We did not observe isolated GFP+ tumour cells within these animals, reinforcing our observations that the great majority of Notch1+ tumour cells are actively cycling.

Based on these observations, we were prompted to ask whether the Notch1+ tumour cells had a different proliferative capacity than non-labelled tumour cells. To study the cell cycle

dynamics of these two cell populations (GFP+ and GFP-), we analysed the distribution of the different cell cycle phases by FACS using Hoechst 33342 (a fluorescent double-stranded DNA dye). Notch1-expressing tumour cells showed a significant increase in the percentage of cells in S and G2/M phases compared to non-marked cells (Figure 22). These results demonstrate that the Notch1 receptor is expressed in a population of undifferentiated cells endowed with high proliferative capacity within intestinal adenomas.

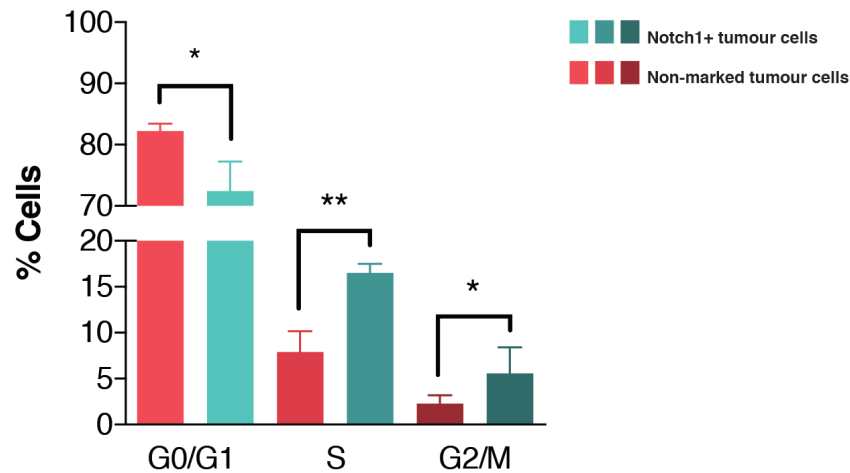


Figure 22. Cell cycle distribution analysis by FACS using Hoechst incorporation assay.

Notch1+ (turquoise bars) and non-marked tumour (red bars) cells were analysed by FACS to probe their cell cycle distribution. Mean percentage of cells in the different phases of the cell cycle (G0/G1, S, G2/M) was quantified based on their DNA content after Hoechst 33342 incorporation. Results represent the average of 4 independent replicates. Error bars represent the standard deviation. * represents a p-value <0.05, ** p-value <0.01 as calculated by Welch's t-test.

NOTCH1+ AND LGR5+ TUMOUR CELLS POORLY OVERLAP WITHIN ADENOMAS

To molecularly characterise the tumour cells that express the Notch1+ receptor, RNA from sorted Notch1-expressing and non-labelled tumour cells was obtained and a panel of specific genes containing proliferation and ISC markers (see list in Table 2) was selected to assess their expression levels within both tumour cell populations by qRT-PCR. First, as our control, we confirmed that sorted GFP+ cells indeed express higher levels of GFP and Notch1, but also of the two direct Notch target genes Hes1 and Nrarp (Figure 23), indicating that the pathway is active in Notch1+ tumour cells. Consistently with the cell cycle analysis

performed by FACS, Notch1+ tumour cells present higher expression levels of Cyclin D1 (CCND1) compared to GFP- cells and they show enriched expression of some ISC markers, such as Olfm4, Ascl2, Hopx and Musashi1 (Msi1) (Figure 23).

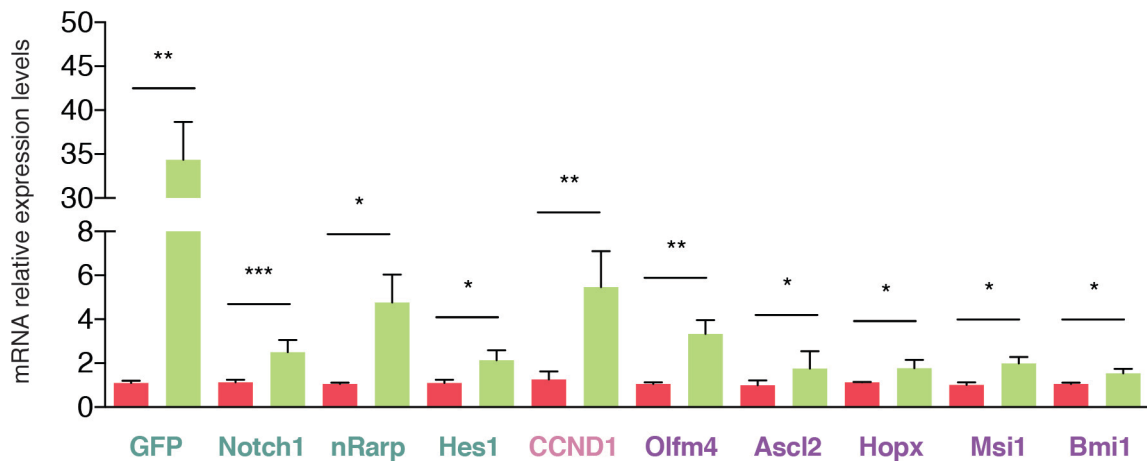


Figure 23. qRT-PCRs showing that Notch1+ tumour cells have active Notch signalling, are enriched in “stem” genes expression and are more proliferative than non-marked cells.

Non-marked (red bars) and GFP+ (Notch1+, green bars) tumour cells were FAC-sorted independently for RNA extraction and cDNA synthesis. Expression of GFP, Notch1, nRarp, Hes1, CCND1 (Cyclin D1), Olfm4, Ascl2, Hopx, Musashi1 (Msi1) and Bmi1 transcripts were quantified relatively to the expression of housekeeping genes (18S and β -actin) and normalized to 1 in the respective GFP- control for each gene. Three representative experiments are shown. Error bars represent the standard deviation. * represents a p-value <0.05, ** p-value <0.01 as calculated by Welch's t-test.

Surprisingly, while in normal crypts the Notch1 and Lgr5 transcripts are co-expressed in ISCs, Notch1+ tumour cells show a significant reduction in Lgr5 expression compared to non-labelled cells (Figure 24).

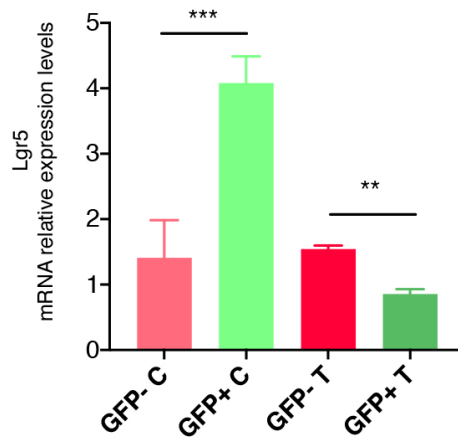


Figure 24. *Lgr5* expression is reduced in Notch1+ tumour cells compared to non-labelled tumour cells.

Non-marked crypt (GFP- C, pink bar), non-marked tumour cells (GFP- T, red bar) and Notch1+ expressing normal (GFP+ C, light green) and tumour (GFP+ T, dark green) cells were FAC-sorted from crypts from small intestines and adenomas from N1/mTmG/Apc^{+/-}, respectively. Expression of *Lgr5* was quantified relative to the expression of housekeeping genes (18S and β -actin) and normalized to 1 in the respective GFP- control for each gene. Three representative experiments are shown. Error bars represent the standard deviation. * represents a p-value <0.05, ** p-value <0.01 as calculated by Welch's t-test.

We next sought to explore the potential separation of these two tumour cell populations without losing their spatial localisation within tumours. For this purpose, we attempted to perform single-molecule RNA FISH for Notch1 and *Lgr5* transcripts. Tumour sections derived from N1/mTmG/Apc^{+/-} mice were co-labelled using Notch1-Cy5 and *Lgr5*-TMR probes. For membrane detection and cell number quantification, sections were also stained using Phalloidin (that binds to actin F) and DAPI (to mark DNA). Preliminary quantification of these sections revealed that Notch1+/Lgr5+ tumour cells (cells expressing both transcripts) seem to be reduced in comparison with Notch1+/Lgr5+ ISCs (in normal crypt), but these results are not statistically significant (Figure 25, quantification graph) and require a more extensive analysis to be conclusive.

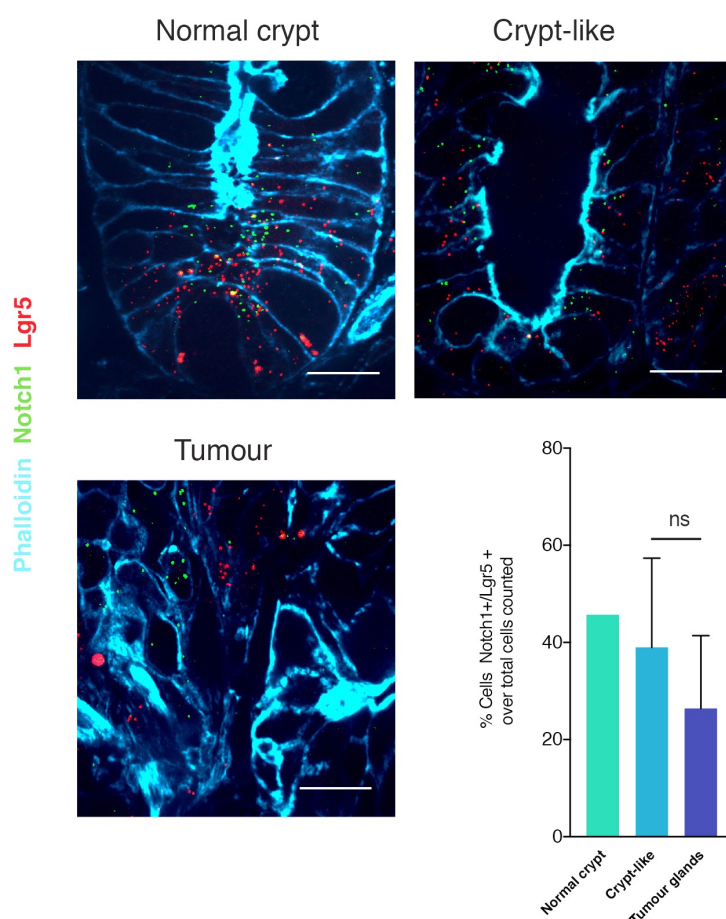


Figure 25. Single-molecule RNA FISH analysis.

Adenoma sections were co-labelled with Notch1 (green dots) and Lgr5 probes (red dots). Phalloidin (in cyan) was used to mark cell membranes and DAPI (not shown) to visualize DNA. Graph represents the number of cells that are co-labelled by Notch1 and Lgr5 probes (over the total cells, marked by Phalloidin, counted per each section) in normal crypt (green bar), in crypt-like structures (within the tumour, turquoise bar) and in tumour glands (dysplastic tumoural regions, dark blue bar). Error bars represent the standard deviation.

To better examine the relationship between Notch1+ and Lgr5+ tumour cells, we generated tumours using Lgr5/Apc^{+/-} mice and extracted RNA from sorted Lgr5-expressing (GFP+) and non-labelled tumour cells as well as from intestinal crypt cells (GFP+ and GFP-) to assess the levels of Notch1 expression in these four populations. Notably, in our Apc background, Lgr5+ tumour cells comprise 0,72% $\pm$ 0,2% of the TEC (Figure 26) as shown by FACS quantification, while in the crypts the Lg5+ population comprises approximately 10% of the total epithelial cells.

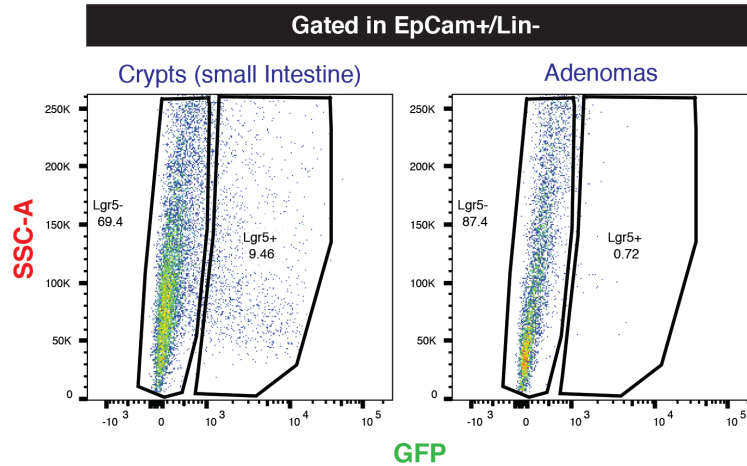


Figure 26. Gate strategies for sort and quantification of Lgr5+ crypt and tumour cells by FACS.

Small intestine crypts and tumours derived from Lgr5/Apc^{+/-} mice were isolated and dissociated into single cells. Crypt and tumour cells were gated within the DAPI- population (live cells), and doublets were excluded within the SSC-W/FSC-A (SSC-W^{high}) gate, following exclusion of Lin⁺ tumour cells and selection of both EpCAM⁺ crypt (Small Intestine) and tumour (Adenomas) cells, further gated in the SSC-A/GFP channels, here depicted.

Lgr5-expressing tumour cells display a decrease in Notch1 and nRarp expression compared to non-marked tumour cells, supporting our observation that these two tumour populations (Notch1⁺ and Lgr5⁺) do not completely coincide within adenomas (Figure 27). Consistent with our observations in Notch1⁺ ISCs, Lgr5⁺ cells isolated from normal crypts express higher levels of Notch1 than non-labelled crypt cells (Figure 27).

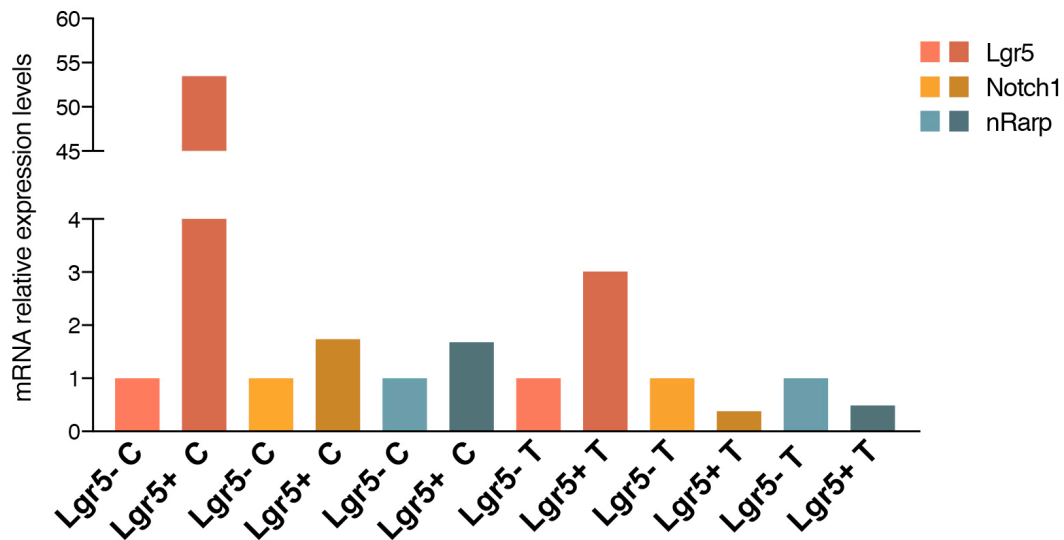


Figure 27. Lgr5+ tumour cells express lower levels of Notch1 and nRarp than non-labelled tumour cells.

Lgr5- and Lgr5+ crypt cells (C) and Lgr5- and Lgr5+ tumour cells (T) were FAC-sorted from crypts from small intestines (C) and adenomas (T) from Lgr5/Apc^{+/+}, respectively. Expression of Lgr5, Notch1 and nRarp was quantified relatively to the expression of housekeeping genes (18S and β -actin) and normalized to 1 in the respective Lgr5- control for each gene. One representative experiment is shown.

Altogether, the collected data show that Notch1-Cre^{ERT2} mice label a rare undifferentiated tumour cell population, highly proliferative, that expresses some ISC markers, but not Lgr5, suggesting that we might target a specific tumour cell population distinct from the one described by Yanai et al, using the Lgr5-Cre^{ERT2} mice, which was shown to contribute to tumour propagation (Yanai et al., 2017).

2. CHARACTERISATION OF NOTCH1-DERIVED TUMOUR PROGENY

NOTCH1+ TUMOUR CELLS CLONALLY EXPAND CONTRIBUTING TO TUMOUR GROWTH AND HAVE SELF-RENEWAL CAPACITY

According to our proliferation analysis, Notch1-expressing tumour cells are more proliferative than the non-labelled tumour bulk. Notably, Notch1-expressing tumours cells already present two (undifferentiated state and proliferation capacity), out of four features (multipotency and self-renewal capacity), required for their classification as CSCs. We then decided to track their fate by clonal analysis *in vivo*.

To verify whether Notch1-expressing cells were able to generate differentiated lineages within intestinal tumours, we further examined tumour-bearing N1/mTmG/Apc^{+/-} mice at different time points (from 4 days up to three months) after Cre^{ERT2} induction by tamoxifen. This fate mapping analysis revealed that Notch1-expressing tumour cells rapidly generate clones of marked tumour cells, visible already at 4 days post-tamoxifen, and that such clones expand (i.e. increase in cell number) over time (Figure 28).

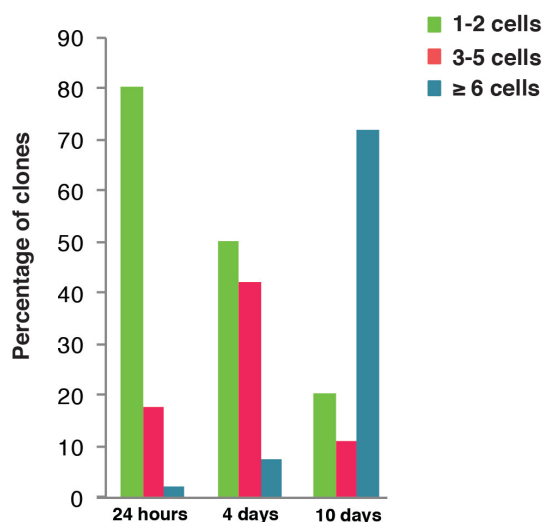


Figure 28. Clonal analysis of Notch1+ cells within adenomas.

GFP+ tumour cells were quantified at different time points (24 hours, 4 days and 10 days) after tamoxifen induction. Green bars represent single cells or doublets, red bars represent clones of 3-5 tumour cells and blue bars represent clones of 6 or more tumour cells.

We did not pursue the quantification of clones (clonal analysis, Figure 28) within adenomas because two major technical difficulties were encountered; 1) at 24 hours the number of singlets was indeed very high, as necessary for this kind of analysis, however, the number of neighbouring recombined cells (i.e. Notch1+ tumour cells that were adjacently positioned and stochastically marked by GFP expression) was not negligible (around 17%, counted as “3-5 cells”), which renders this analysis confusing, since the goal of clonal analysis is to start from single cells in order to follow their clonal fate over time, and 2) even after dilution of tamoxifen (aiming at reducing the number of recombined cells) we obtained different variable results; the same non-negligible number of 3-5 adjacent cells per clone or, on the contrary, no label detection, both resulting in the unfeasibility of scoring single clonal events.

Quantification of Notch1-expressing tumour cells was then assessed using two methods: by FACS analysis, we quantified the percentage of GFP+ cells over the TEC (Figure 29), and by immunofluorescence in tumour sections (Figure 31), in which the GFP+ area was quantified over the total surface of the tumour that was defined by the expression of Tomato fluorescent protein.

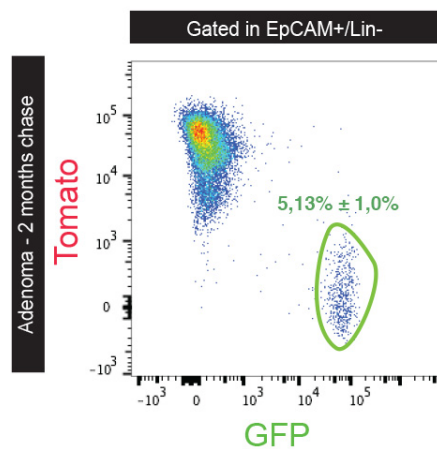


Figure 29. Gate strategy to analyse Notch1+ tumour cells clonal expansion (representation of 2 months chase).

Tumours derived from N1/mTmG/Apc^{+/-} mice injected for different chases were isolated and dissociated accordingly to their induction time-point into single cells. Tumour cells were gated within the DAPI- population (live cells), and doublets were excluded within the SSC-W/FSC-A (SSC-W^{high}) gate, following exclusion of Lin+ tumour cells and selection of EpCAM+ cells that were gated within the Tomato/GFP channels. Tom^{neg}/GFP^{pos} population (highlighted in green) represents the progeny of Notch1+ tumour cells.

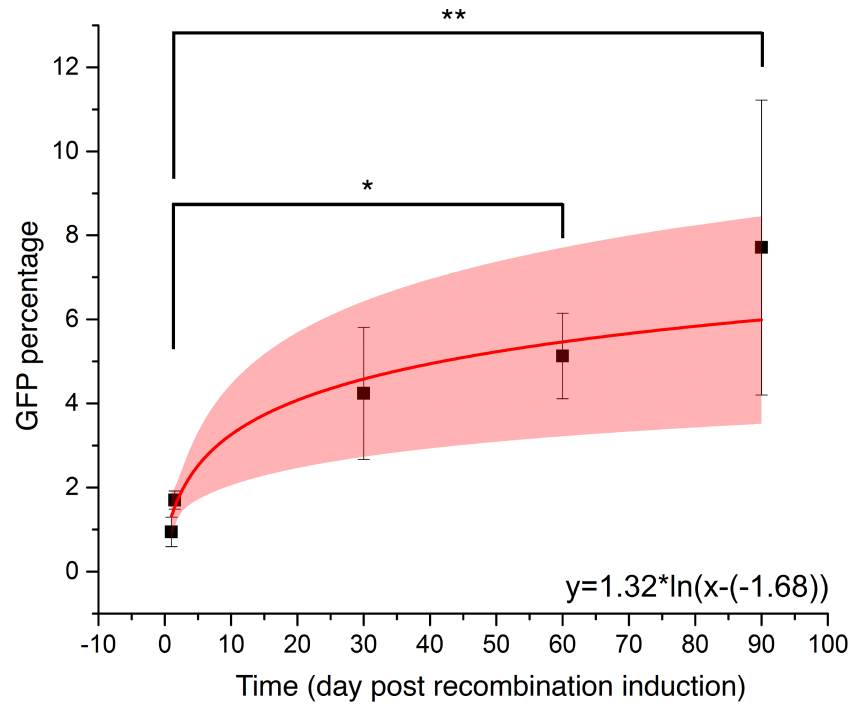


Figure 30. Notch1+ tumour cell expansion quantification by FACS and modelling of progression.

A set of N1/mTmG/Apc^{+/-} tumour bearing mice was injected with tamoxifen and culled at different time-points (24 and 36 hours for short chases and 30, 60 and 90 days for long chases). GFP+ tumour cells were quantified over the total TEC population by FACS. At least three representative experiments are shown. Error bars represent the standard deviation. * represents a p-value <0.05, ** p-value <0.01 as calculated by Mann-Whitney non-parametric t-test. A two-parameter logarithmic function, $y=b*\ln(x-a)$ trend line is given (in red). $R^2(\text{COD}) = 0,87$.

Our experimental approach requires the use of Apc^{+/-} tumour-bearing mice (i.e. around 6 months old, that tend to succumb around 2 to 3 months later due to tumour burden, anaemia, and other complications) and consequently, a 3-month chase is the longest period possible for analysis.

From 24 hours post tamoxifen up to three months, Notch1+ tumour cells increase from 1,2% $\pm$ 0,3% to 7,7% $\pm$ 3,5% within the TEC (validated by clonal expansion analysis by GFP area quantification, Figure 32). A two-parameter logarithmic function ($y=1.32*\ln(x-(-1.68))$) was used to model the rate of expansion of Notch1+ tumour cells quantified by FACS. This function predicts that the Notch1+ tumour cells will continue to slowly grow after 3 months of chase, although due to high variance in the last time-point (3 months chase) experimentally assessed, the model requires more experimental data about this specific time-point and ideally (experimentally unfeasible as mentioned above), the percentage of GFP+ in longer chases (> 3 months, also unfeasible), to be applicable with certainty.



Figure 31. Schematic representation of GFP+ area quantification using Tomato (Tom) and GFP fluorescence.

Adenomas were dissected and sectioned for imaging. The images reflecting the total surface of Tom and GFP fluorescence were adjusted for threshold, allowing the quantification (in pixel) used to obtain the total surface of GFP+ tumour cells within each section. Panels highlighted by dashed lines are representative treated images using threshold adjustment for area quantification (using the total number of dark pixels).

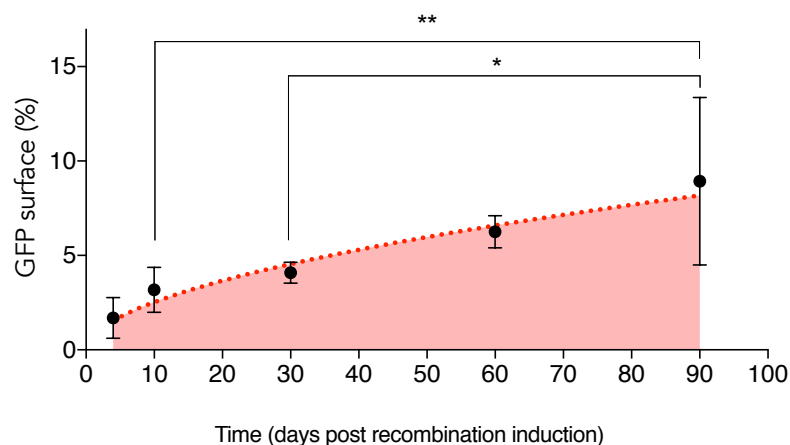


Figure 32. Clonal expansion based on Tomato and GFP fluorescence in adenoma sections.

A set of N1/mTmG/Apc^{+/+} tumour bearing mice was injected with tamoxifen and culled at different time-points (4 and 10 days for short chases and 30, 60 and 90 days for long chases). GFP+ surfaces were quantified over the total Tomato surface and displayed in percentage. Error bars represent the standard deviation. * represents a p-value <0.05, ** p-value <0.01 as calculated by Mann-Whitney non-parametric t-test. Red dashed line simply connects all points.

To verify that the two approaches used to quantify the rate of *in vivo* expansion of Notch1+ tumour cells were not biased by technical errors and biological material variability, we compared both results and we show that they nicely correlate (Figure 33).

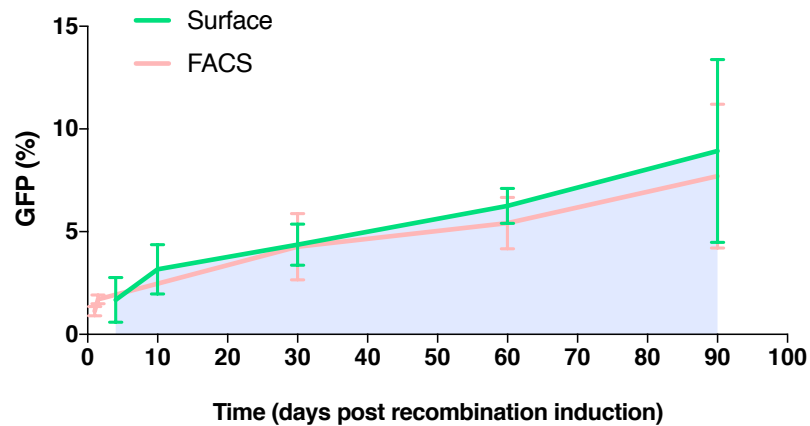


Figure 33. Correlation of FACS and Surface quantification methods.

Quantifications derived from FACS (Figure 30) and GFP (Figure 32) surface methods were merged to verify individual trends and their correlation. Notably, each individual time-point contains different animals analysed in both techniques. Pink trend line shows FACS quantifications and green trend line depicts surface calculations.

Altogether, the few recombined Notch1-expressing cells observed after 24 hours induction seem to rapidly expand within the first 1 month (interval of expansion in which is observed the highest slope), and are predicted to indefinitely outspread within adenomas, even though in a slow rate (as modelled by the logarithmic function), suggesting that these cells are endowed with self-renewal capacity.

NOTCH1-EXPRESSING TUMOUR CELLS ARE MULTIPOTENT

To address whether Notch1+ tumour cells were capable of generating different cell types that are found within adenomas, we assessed the expression of proliferation and differentiation markers in the progeny of these cells. Immunostaining analysis showed that Notch1-derived lineages contain tumour cells that are actively dividing (as shown by PCNA expression, a proliferation marker for G1/S phase) and express all the differentiation markers we assessed, including Lysozyme, Mucin2, and Chromogranin A (Figure 34), indicating that Notch1+ cancer cells, like normal ISCs, have multilineage potential (Fre et al., 2011). Whether these tumour cells expressing these differentiation genes are fully functional, like in the normal intestine, remains unclear.

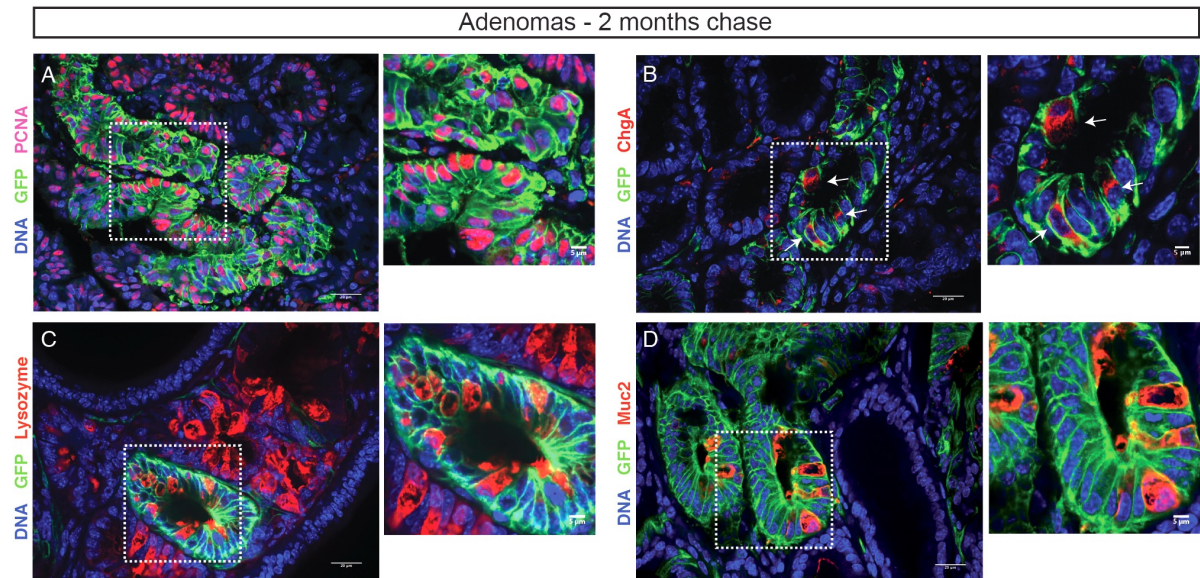


Figure 34. *Notch1*+ tumour cells are multipotent.

Immunostainings of tumour sections after 2 months chase using PCNA (proliferation marker, panel A), anti-Lysozyme (marker for Paneth cells, panel C), anti-Chromogranin A (for Enteroendocrine cells, panel B) and anti-Mucin2 (marker for Goblet cells, panel D), all in red. *Notch1*+ tumour cells are reflected by GFP expression in green and DNA was marked using DAPI, in blue. Arrows highlight Chromogranin A+ tumour cells within a GFP+ clone. Scale bars represent 20 μ m. Magnifications of selected areas of each section are shown in the right panels (scale bars represent 5 μ m).

NOTCH1-DERIVED LINEAGES COMPRISE A HETEROGENEOUS POPULATION

To molecularly characterise the *Notch1*-derived progeny, qRT-PCR was performed on three sorted populations; *Notch1*+ tumour cells, their progeny (a mixed population of daughter and *Notch1*-expressing tumour cells) and non-marked cells. To carry out this experiment, tumour-bearing mice was induced with a pulse of tamoxifen, following 1 to 2 months chase to allow labelling of *Notch1* progeny. One day prior tumours dissection, mice were re-injected with a single pulse of tamoxifen to re-label new *Notch1*-expressing cells. Thanks to this experimental trick, *Notch1*-expressing cells separation from their progeny was achievable by FACS due to the expression of the Tomato protein. In this particular setup, the Tomato protein persists in *Notch1*-expressing cells after a 24 hours pulse (hence these cells are GFP+ and Tom+) while it is absent in the progeny that is marked only by GFP, but Tom-, Figure 35).

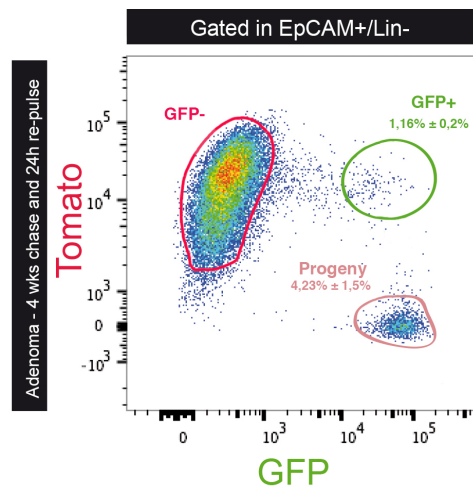


Figure 35. Representative FACS plot showing the gate strategy to separate GFP-, GFP+ (Notch1+ tumour cells) and the progeny derived from Notch1 tumour cells.

The progeny of Notch1+ tumour cells (highlighted in pink) is devoid of Tomato protein expression, while new Notch1+ tumour cells (green gate), induced 24 hours prior analysis, are GFP^{hi}/Tom^{hi}. The three populations (GFP-, GFP+ and progeny) were sorted after 1 and 2 months chases for RNA extraction.

Following sorting of these cell populations, qRT-PCR analysis for a specific set of genes (Figure 36) showed that Notch1-derived progeny (GFP+ and Tom-) express intermediate mRNA levels between non-labelled (GFP- and Tom+) and Notch1+ tumour cells (GFP+ and Tom+) for all tested genes; Notch1, nRarp, Olfm4, Lysozyme and Lgr5 (with the expected exception of GFP).

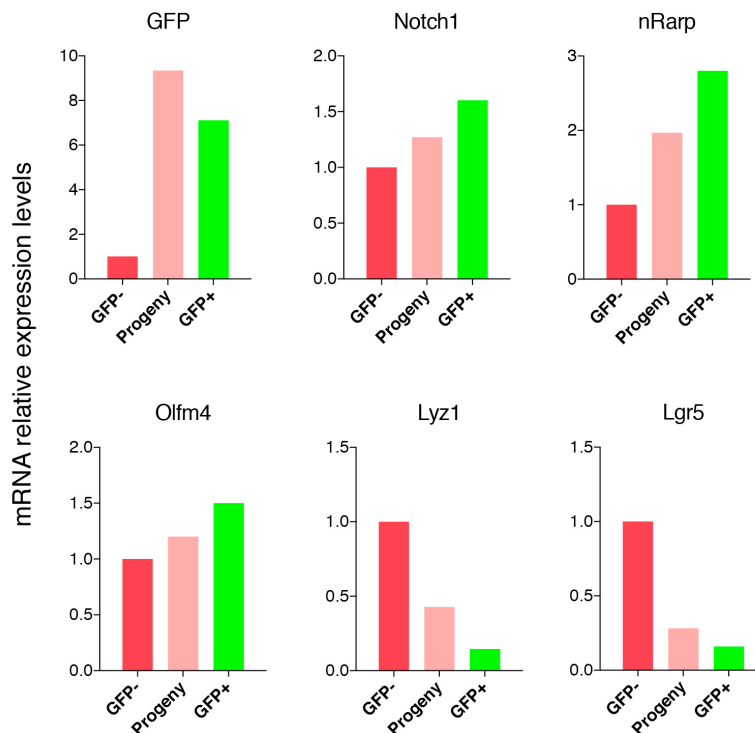


Figure 36. qRT-PCRs showing that Notch1-derived progeny have intermediated levels, in a “ladder profile” for all tested genes (with the exception of GFP).

GFP- tumour cells (red bars), Notch1-derived progeny (pink bars) and GFP+ (Notch1+, green bars) tumour cells were FAC-sorted for RNA extraction and cDNA synthesis. Expression of GFP, Notch1, nRarp, Olfm4, Lysozyme1 and Lgr5 transcripts were quantified relatively to the expression of housekeeping gene (18S) and normalized to 1 in the respective GFP- control for each gene. One representative experiment is shown.

Concerning the expression levels of the transcripts tested, these three cell populations display what we named a “ladder profile”, as the progeny of Notch1+ tumour cells present intermediate levels of all tested genes, potentially indicating that it is composed of a “mixed” cell population including both differentiated and Notch1+ tumour cells. This analysis not only supported our previous observations indicating that Notch1+ tumour cells have lower expression levels of Lgr5, but also demonstrated that the progeny derived from these tumour cells is enriched in Lgr5 expression when compared to the cell that gave rise to them (Notch1-expressing tumour cells).

These experiments confirmed, at the molecular level, that Notch1+ tumour cells are multipotent, contributing in parallel to tumour growth and intratumoural heterogeneity.

3. 3D *IN VITRO* STUDIES TO ASSESS CLONOGENIC CAPACITY AND DRUG RESISTANCE

NOTCH1+ TUMOUR CELLS HAVE A POOR CLONOGENIC CAPACITY *IN VITRO* AND RETAIN THE WT APC ALLELE

Clonogenic assays, such as 3D *in vitro* organoid cultures, can be used to address the self-renewal capacity of distinct cell populations. They can be considered as an indirect method to characterise CSCs populations based on their *in vitro* growth capacity (Beck and Blanpain, 2013). This system allows a functional readout of “stemness” (i.e. self-renewal and differentiation capacities) within a given cell population by testing its ability to form miniguts (in the case of a normal ISC population, (Sato et al., 2009)) or cystic organoids (in the case of intestinal tumour cells, (Sato et al., 2011a)) when supplemented with the appropriate growth factor cocktail and cultured in a 3D extracellular matrix.

To define the clonogenic potential of Notch1+ tumour cells and its progeny, tumours derived from mice injected with one single pulse of tamoxifen for 24 hours or after 1 month chase were collected and GFP+ and GFP- tumour cells were isolated by FACS and plated at low density (300-500 cells/per well) in Matrigel drops in the continuous presence of the following four factors: EGF, Noggin, B-27, N2, and with Y-27632 ROCK inhibitor (that helps to avoid anoikis, added for only 24 hours after plating).

Unexpectedly, when we compared the tumour organoids originated from Notch1+ tumour cells and from Notch1 progeny to the ones formed by GFP- tumour cells, we observed that GFP- tumour cells formed, on average, at least two times more cysts than the GFP+ tumour cells in both experiments (Figure 37A and B). Another interesting observation is that the GFP- clonogenic potential is, within different experiments, variable depending on the pool of tumours, reflecting the intrinsic heterogeneity of the tumour bulk. Conversely, GFP+ tumour cells showed consistency in their cyst forming efficiency, even in low clonogenic conditions (i.e. the number of cysts formed per 100 cells plated is stable within different experiments), reflecting their homogeneity at a population level.

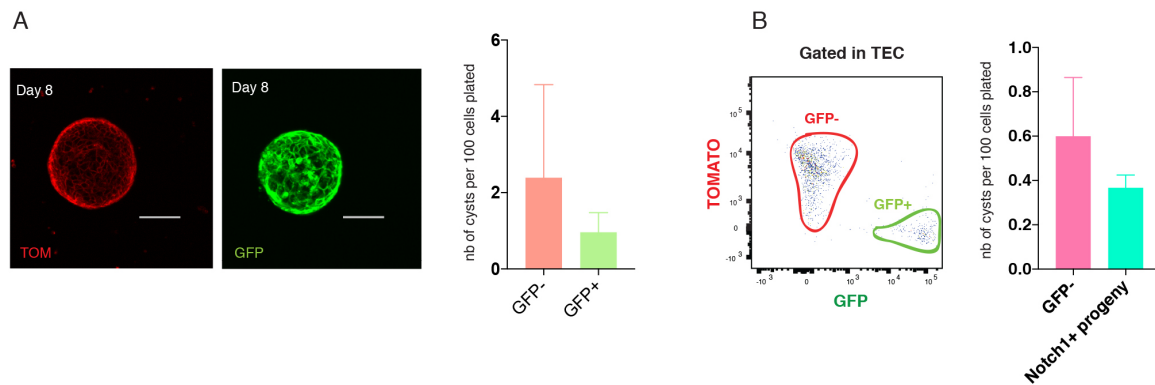


Figure 37. 3D Clonogenic assays to quantify Notch1-tumour cells cyst forming efficiency.

In A) Images represent GFP- (in red, Tomato) and GFP+ (in green) cysts that were quantified at day 8. Scale bars represent 100 μm. Graph shows quantification of cysts at day 8. Efficiency of cyst formation was calculated counting the number of cysts per 100 tumour cells plated. Orange bar represents GFP- tumour cells and green bar represent GFP+ tumour cells. More than three representative experiments are shown. Error bars represent the standard deviation. In B) FACS plot depicting gate strategy to sort the progeny derived from Notch1+ tumour cells (GFP^{hi}/Tom^{neg} tumour cells highlighted in green, gated in TEC; EpCAM+/Lin-) and the non-labelled tumour cells (GFP-, indicated in red). Graph shows quantification of day 8 and the efficiency was calculated as in Figure 37A). Pink bar represents GFP- tumour cells and turquoise bar represent Notch1-derived progeny within adenomas. Error bars represent the standard deviation.

These results are in contradiction with our *in vivo* observations, showing that Notch1-expressing tumour cells are highly proliferative and clonogenic.

Previous observations in our lab (data not published) have shown that some tumour organoids derived from *Apc*^{+/-} tumours carry the wild-type *Apc* allele, while some others have undergone LOH at the *Apc* locus. This observation not only corroborates the idea that not all adenomas are monoclonal, but also prompted us to investigate the *Apc* LOH in tumour organoids derived from single Notch1+ tumour cells. To assess the percentage of LOH, the same FAC-sorting and clonogenic protocols were employed. The tumour organoids originated from single Notch1+ and non-labelled tumour cells were picked after 2 weeks in culture and genotyped for the *Apc* mutant and wild-type alleles. Surprisingly, all GFP+ tumour cysts (derived from Notch1+ tumour cells) analysed carried the wt *Apc* allele, whereas non-GFP spheroids (derived from non-labelled tumour cells) were either heterozygous for *Apc* (*Apc*^{+/-}) or mutant (carrying only the mutant allele, *Apc*^{-/-}, Figure 38).

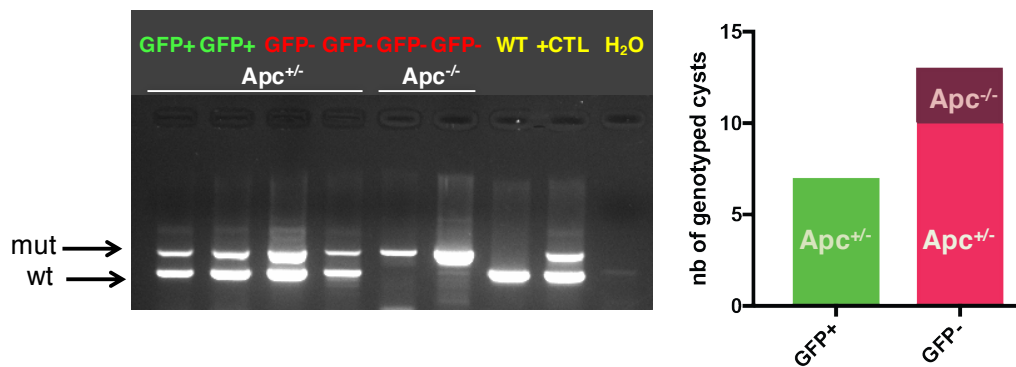


Figure 38. Representation of genotype of manually picked cysts for *Apc* mutant and wild-type alleles. Graph shows quantification.

The gel shows amplified products for *Apc* mutant (mut) and wild-type (wt) alleles from gDNA extracted from individually picked cysts derived from GFP+ and GFP- sorted tumour cells grown in culture for 20 days in 3D conditions. Graph shows the number of genotyped cysts categorised by genotype. Green bar represents cysts derived from GFP+ tumour cells (all Apc^{+/-}), pink bar represents cysts derived from GFP- tumour cells genotyped as Apc^{+/-} and dark red bar represents cysts derived from GFP- tumour cells genotyped as Apc^{-/-}.

Due to limitations in quantification by standard PCR analysis, the allelic ratio for the wild-type and mutant *Apc* alleles was quantified by quantitative Real-Time PCR (qRT-PCR) on genomic DNA (gDNA) extracted from FAC-sorted Notch1-expressing and non-marked tumour cells. To determine the *Apc* allelic ratio, we generated a standard curve using gDNA extracted from Apc^{-/-} and Apc^{+/-} at known concentrations that ranged from 50% Apc^{+/-} up to 100% Apc^{-/-} as initial gDNAs inputs. Based on fold change amplification levels of the *Apc* wt amplicon (relative to housekeeping genes), we estimated that Notch1+ tumour cells are approximately 55% ± 2.5% (n = 3) mutant for *Apc* (consequently 45% wt for *Apc* or lost 5% ± 2.5% of the wt allele at population level), whereas non-labelled tumour cells are estimated to be 65% ± 2.5% (n = 2) with the exception of one replicate that is predicted to be mainly mutant (due to its low relative fold change) (Figure 39).

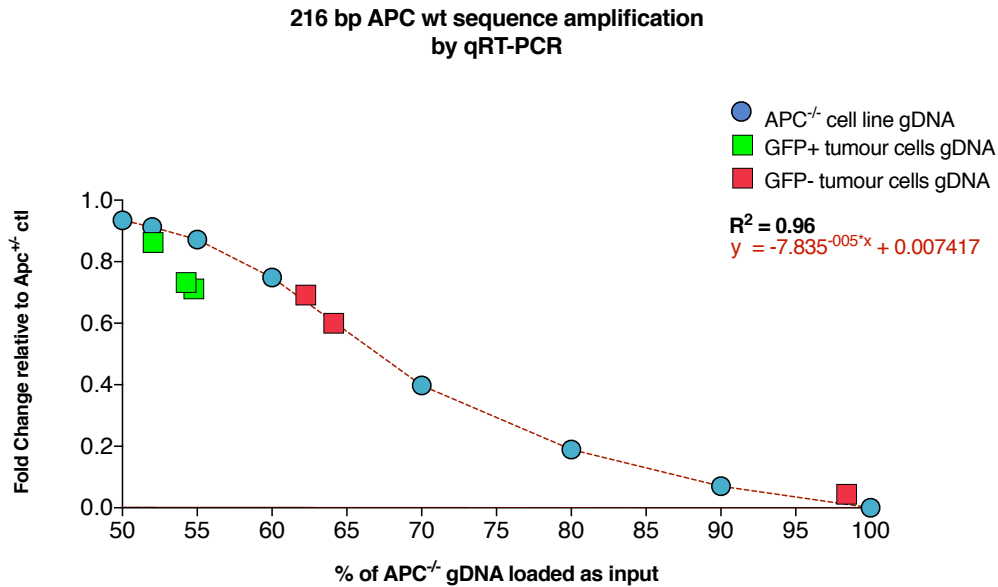


Figure 39. *Apc* allelic ratio quantification by qRT-PCR using standard curve generated with gDNA extracted from an *Apc*^{-/-} cell line and gDNA extracted from *Apc*^{+/+} from young mice.

Notch1+ and GFP- tumour cells were FAC-sorted for gDNA extraction. Using *Apc*^{-/-} and *Apc*^{+/+} gDNAs as reference (input loaded to generate a standard curve representing different percentages of *Apc* LOH) for wt sequence amplification, the percentage of LOH at the tumour population level was determined. Three replicates for both populations (Notch1+ in green squares and GFP- in red squares) are quantified.

Besides the validation of our initial results assessed by PCR (Figure 38), this quantitative analysis supports our initial hypothesis that the majority of Notch1+ tumour cells might retain the *Apc* allele, a plausible cause for their poor growth capacity *in vitro* after FAC-sorting.

To tackle this hypothesis, we first decided to test whether Notch1+ tumour cells would increase their cyst formation capacity upon co-culture with secretory tumour cells marked by UEA (that labels both Goblet and Paneth cells). Tumours derived from mice injected with one single pulse of tamoxifen for 24 hours were collected and GFP+ and GFP- tumour cells were isolated by FACS and plated at low density (300-500 cells/per well) in Matrigel drops in the continuous presence of the four factors mentioned before and in the presence UEA+ tumour cells (in a 3:1 ratio, i.e. 3 UEA+ tumour cells and 1 GFP+ tumour cell) that were also FAC-sorted. The cyst formation efficiency within the GFP+ population (or GFP-) did not increase as predicted (Figure 40), and we speculate that UEA+ secretory tumour cells might not offer the right signals (i.e. expression of Dll1 Notch ligand and the production of Wnt3a) required for *in vitro* growth.

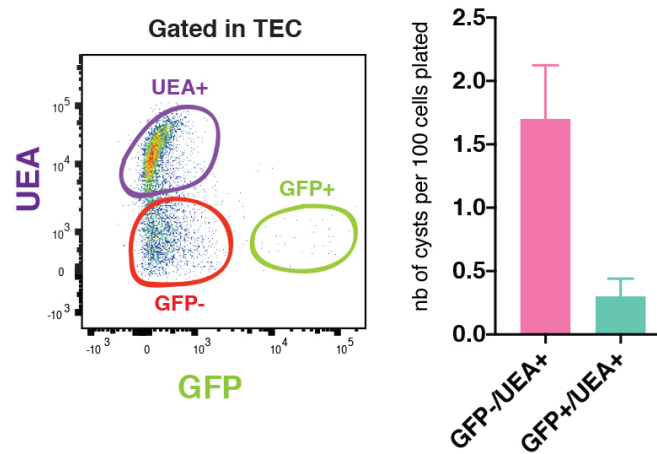


Figure 40. Gate strategy to FAC-sort Notch1+, GFP- and UEA+ tumour cells.

Tumours derived from N1/mTmG/Apc^{+/-} mice induced 24 hours before the experiment were dissociated into single cells. Tumour cells were gated within the DAPI- population (live cells), and doublets were excluded with the SSC-W/FSC-A (SSC-W^{high}) gate, following gate within the Lin-/EpCAM+ channel and further gate within the UEA/GFP channel. UEA^{neg}/GFP^{pos} population highlighted in green represents the progeny of Notch1+ tumour cells (GFP+). UEA^{pos}/GFP^{neg} (UEA+) population is indicated in purple and UEA^{neg}/GFP^{neg} (GFP-) in red.

The next step was to verify whether Notch1+ single tumour cells, derived from mice induced with tamoxifen 3 days before dissection (to increase the number of recombination events, i.e. Notch1+ tumour cells labelled by GFP expression), would show a better organoid forming efficiency *in vitro* without sorting, as this approach would be potentially less aggressive to the cells and could also provide factors secreted by neighbouring tumour cells, required for the growth of Notch1+ tumour cells, as we hypothesised that occurs *in vivo*.

In these conditions, the GFP+ cyst forming efficiency was still almost 40 times lower (at day 8) compared to GFP- tumour cells (Figure 41).

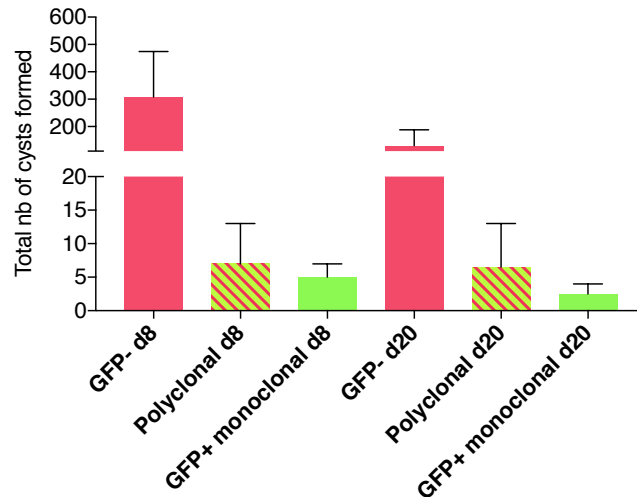


Figure 41. Averaged number of cysts formed in two independent co-culture experiments of Notch1+ and non-labelled tumour cells.

Adenomas from two animals were dissociated independently in single cells and plated individually in 3D conditions. Graph shows the average number of cysts present at day 8 and 20 after plating for these two pools of tumours. Red bars represent the total number of GFP- tumour cysts formed, striped bars represent the total number of polyclonal cysts (containing GFP+ and GFP- cells and probably derived from cell aggregation) and green bars represent GFP+ monoclonal cysts. Two replicates are shown and error bars represent the S.E.M.

Importantly, in this experiment the initial number of plated cells was not assessed because these cells were not FAC-sorted. Therefore, the number of GFP+ monoclonal cysts formed at day 8 was counted without taking in account the initial number of Notch1+ tumour cells plated at 3 days post tamoxifen induction. By lacking this information, the number of cysts formed by each tumour cell population (GFP+ and GFP-) is not very informative, as we cannot extract the cyst forming efficiency of each population.

At day 20, the total number of GFP- cysts was decreased by approximately 25% relative to the cysts counted at day 8 (Figure 41). The number of polyclonal cysts was stable from day 8 to day 20 (Figure 42). Although the number of GFP+ monoclonal cysts was slightly decreased from day 8 to 20, their size seems to be increased after passaging (Figure 43).

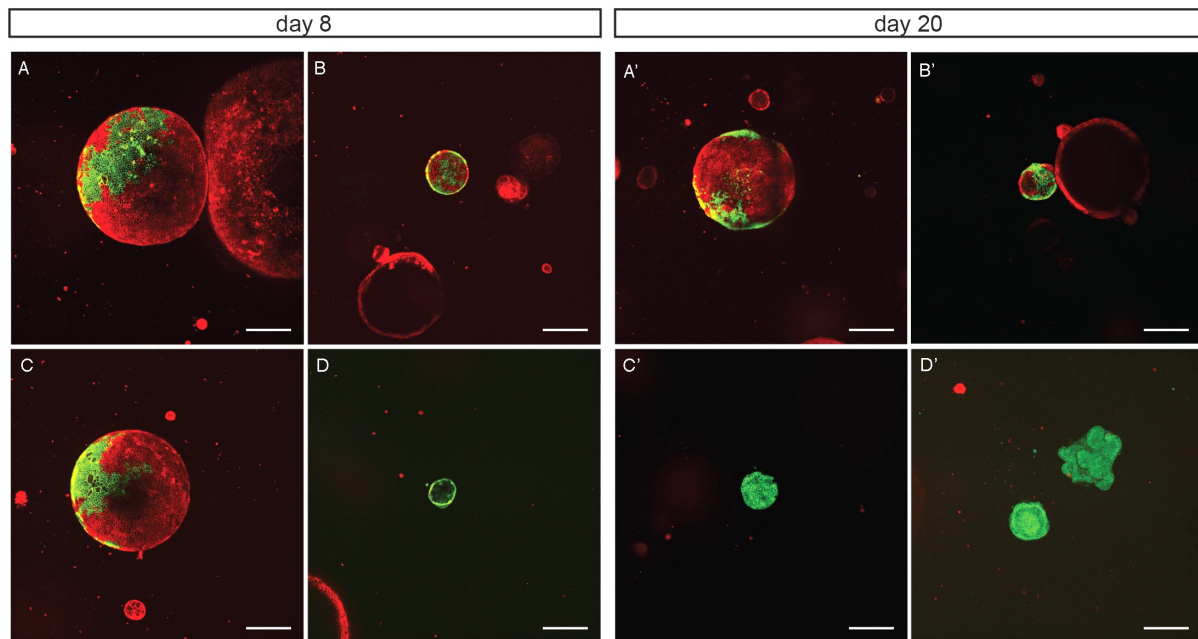


Figure 42. Representative images of cysts formed in co-culture of Notch1+ tumour cells with non-labelled tumour cells at day 8 and day 20 after plating.

At day 8 after plating, large polyclonal (due to cell aggregation) and GFP- monoclonal cysts are formed ($> 400\mu\text{m}$ diameter, panel A). GFP+ monoclonal cysts have a reduced diameter ($< 200\mu\text{m}$, panel D) compared to non-labelled and polyclonal cysts (panels A, B and C). After 20 days in culture, GFP+ monoclonal tumour organoids with reduced diameter (compared to polyclonal or GFP- cysts) persist in culture, some adopting a non-cystic morphology (panel D'). Scale bars represent $200\mu\text{m}$.

The majority of GFP+ cysts found in co-culture experiments, at day 23 (one passage at day 20), were of monoclonal origin (Figure 43) and their diameter was increased (as compared to day 20, no passaging), demonstrating that Notch1-derived tumour progeny is very efficient in re-forming cysts upon dissociation (due to the greater diameter observed) and recapitulating what we observed *in vivo*, upon long chases experiments.

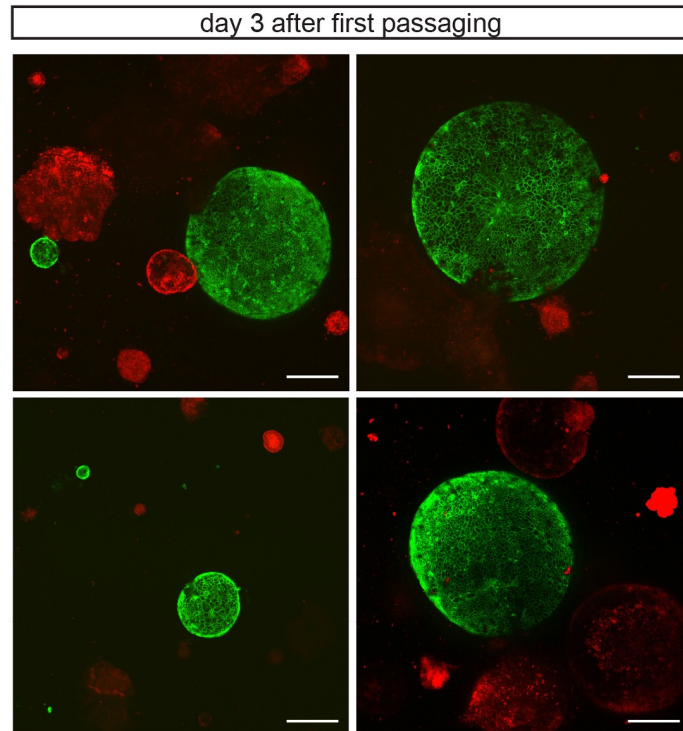


Figure 43. Representative images of cysts formed in co-culture of Notch1+ tumour cells with non-labelled tumour cells at day 23 (3 days after first passage).

At day 3 after passaging, large monoclonal GFP+ cysts are formed (> 200 μ m diameter). Scale bars represent 200 μ m.

Similarly to normal intestinal crypts, where ISCs require cell-to-cell contact with Paneth cells that serve as a niche producing signalling molecules (such as Wnt, EGF, and Notch ligands (Sato et al., 2011b)), this co-culture experiment demonstrates that Notch1+ tumour cells require other cancer cells for their growth *in vitro*, which might suggest that this population can be originated from normal ISCs, corroborating with our observations that these cells retain the wild-type *Apc* allele.

DRUG RESISTANCE REFLECTS APC LOH IN VITRO

Despite our observations that Notch1+ tumour cells have reduced clonogenic capacity (when grown in the absence of non-labelled tumour cells), we decided to investigate their sensitivity to 5-fluorouracyl (5-FU), a chemotherapeutic drug commonly used for patients with advanced CRC. To determine the best concentration to be used in our organoids, we assessed three different 5-FU concentrations; 150 μ M, 15 μ M and 1,5 μ M in tumour organoids derived from

Apc^{+/-} mice. We determined that the optimal final concentration was 15 μ M (32% of survival Figure 44A), as organoids in the presence of 1,5 μ M 5-FU had comparable survival rates to mock control (55% and 45% of survival, respectively) and, conversely, in the long-term presence of 150 μ M 5-FU, almost 80% of cysts died and the 20% that remained after treatment displayed a reduction in growth when compared to untreated cysts (Figure 44B).

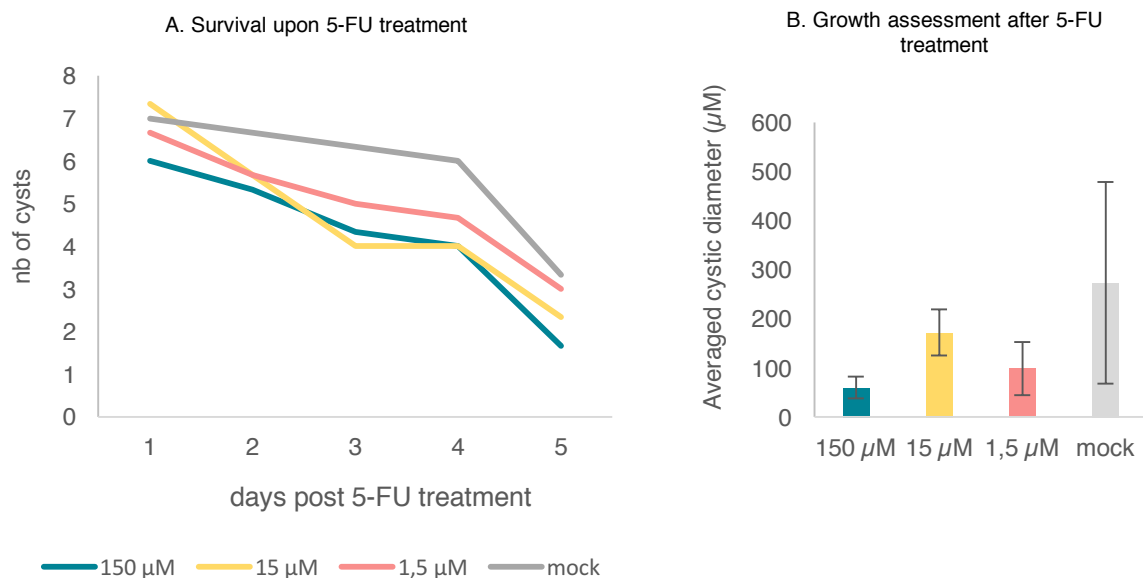


Figure 44. 5-FU drug titration for in vitro experiments.

In A) three concentrations were tested and the number of cysts was quantified from day 1 (first day of 5-FU treatment or mock, DMSO) up to day 5. Blue line indicates 150 μ M of final 5-FU, yellow line 15 μ M and pink line 1,5 μ M. DMSO was used as mock control. In B) the average diameter of cysts was measure to assess the impact of 5-FU on cyst growth. One representative experiment is shown.

5-FU works as an antimetabolite to prevent cell proliferation by inhibiting the enzyme thymidylate synthase, thus blocking the thymidine formation required for DNA synthesis (Wigmore et al., 2010). Tumour drug resistance has been attributed to many intrinsic or acquired properties displayed by CSCs, including DNA repair ability, overexpression of anti-apoptotic proteins, and drug efflux transporters and detoxifying enzymes (Vinogradov and Wei, 2012). We then sought to determine whether 5-FU treatment would select for *Apc*^{-/-} mutant cells. For this purpose, cysts derived from tumours of *Apc*^{+/-} mice were cultured in the presence of 5-FU (15 μ M) for 15 days and were individually picked and genotyped (at day 0 and day 15) for wild-type and mutant *Apc* alleles.

The majority of tumour organoids (86,6%) at day zero (starting day of 5-FU treatment) were genotyped as $Apc^{+/-}$. After 15 days in the presence of 5-FU, only 37,5% of all genotyped cysts were $Apc^{+/-}$, against 71,42% within the untreated cysts.

We did not pursue with our initial aim of testing 5-FU drug response of Notch1-derived cysts because 1) we show that the majority of Notch1-derived cysts are $Apc^{+/-}$ (Figure 38), as they derived from Notch1+ tumour cells that are also $Apc^{+/-}$ (Figure 39), and 2) based on these results of drug resistance, we hypothesised that 5-FU treatment selectively kills $Apc^{+/-}$ cysts *in vitro*, being plausibly to predict that Notch1-derived organoids would not survive upon 5-FU treatment.

4. TRANSCRIPTIONAL SIGNATURES OF NOTCH1-EXPRESSING TUMOUR CELLS AND NOTCH1+ ISCs

THE TRANSCRIPTIONAL SIGNATURE OF NOTCH1+ TUMOUR CELLS CLOSELY CORRELATES WITH NOTCH1+ ISCs SIGNATURE

In addition to the initial molecular characterisation of Notch1-expressing tumour cells, genome-wide transcriptional signatures of these cells were obtained by RNA-seq and Affymetrix analyses of FAC-sorted GFP+ and GFP- tumour cells. These two approaches confirmed that Notch1-expressing tumour cells are undifferentiated (downregulation of expression of differentiation genes, such as *Alpi*, *Apoa1/4*, *ChgA/B*, *DCLK1*, *Clca3*, *Defa21/21*, *Lyz1*, *Muc2/6*, among others) and also that the expression of some ISC markers, including *Ascl-2*, *Hopx*, *Lrig1*, *Smoc2* and *Olfm4*, is enriched in these cells, compared to non-labelled tumour cells. Notably, both analyses corroborated our qRT-PCR results that show decreased transcriptional levels of *Lgr5*, as well as upregulation of proliferation genes such as *Ki67* and *CCNB1* (Cyclin B1) expression within Notch1+ cells.

We also confirmed the Notch1 upregulation in Notch1+ expressing tumour cells (as shown by manual qRT-PCR), but members of Wnt signalling pathway (i.e. *Apc*, *Axin2*, *CTNNB1*, *Dsh*, *GSK3*) seem to be expressed at the same level in both Notch1+ and non-labelled tumour populations, with the exception of *Tcf4*, *Lef1* and *Dkk* genes, that are downregulated in Notch1+ tumour cells.

We took advantage of the available transcriptomic signatures (i.e. up-regulated gene sets in a given cell population) in order to correlate with our Notch1+ tumour signature. The GSEA computational tool allows determining whether a defined panel of genes (i.e. a signature) shows statistically significant, concordant differences between two biological samples (Subramanian et al., 2005). The first results obtained within this type of analysis are an enrichment score (ES) associated with its FDR q-value to verify statistical significance. The ES defines the extent of the positive or negative correlation of a given biological sample (in our case GFP+ and GFP-) with a specific set of genes.

We extracted a panel of genes defined as the “proliferation signature of ISCs” established by Merlos-Suarez et al. Confirming our previous results on the proliferative activity of Notch1+

cells, the Notch1+ tumour signature highly correlates with the ISC proliferation signature, but negatively correlates with a Wnt signature gene set (obtained from Nusse's Lab website, Figure 45).

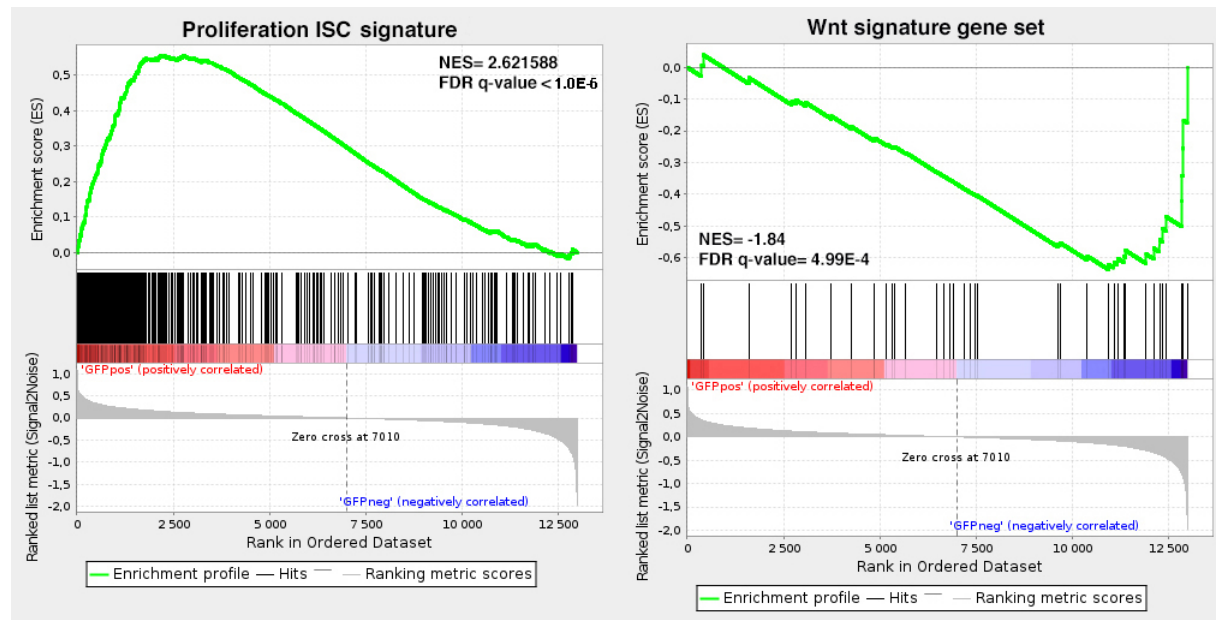


Figure 45. GSEA analyses of Notch1+ tumour cells against ISC Proliferation and Wnt signatures.

GSEA analyses performed in Notch1+ and non-labelled tumour cells whole transcriptomics to evaluate the enrichment of signatures associated with proliferation (Merlos-Suarez et al., 2011) and Wnt signalling genes (manually created list based on Nusse's lab database; <http://web.stanford.edu/group/nusselab/cgi-bin/wnt/>). NES represents the enrichment score corrected for multiple tests, and expresses the extent of the correlation with the transcriptomic signature associated with the GFP+ and GFP- transcriptional signatures with a large number of permutations. The false discovery rate (FDR) is the estimated probability that a gene set with a given NES represents a false positive finding.

To test our hypothesis that Notch1+ tumour cells might originate from Notch1+ ISCs, transcriptomic analysis by Affymetrix of Notch1+ ISCs sorted from normal intestinal crypts was performed. Indeed, GSEA analysis in which the comparison of the transcriptomes of normal Notch1+ ISCs with Notch1+ tumour cells was scored, showed that Notch1+ tumour cells (GFP+) positively correlate with our Notch1+ ISC signature with a Normalised Enrichment Score (NES) of 2.01 (Figure 46).

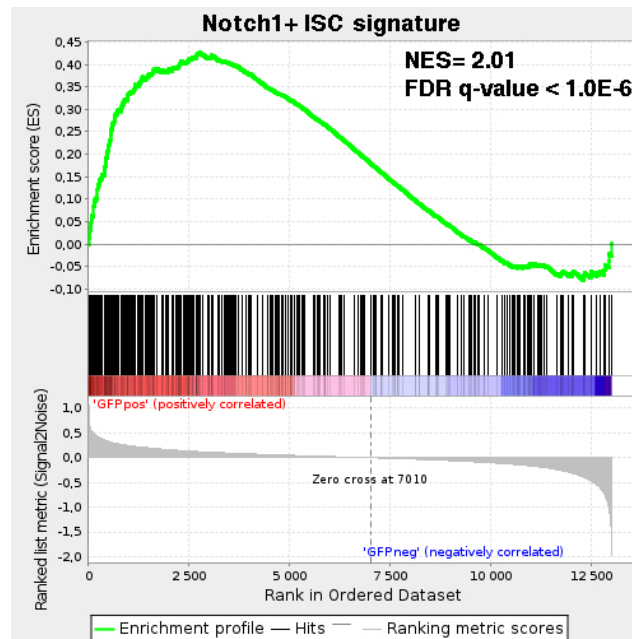


Figure 46. Correlation between *Notch1*+ tumour cells and *Notch1*+ ISC signatures.

GSEA analyses performed in *Notch1*+ (GFPpos) and non-labelled tumour (GFPneg) cells whole transcriptomics to evaluate the enrichment of *Notch1*+ ISC signature in this matrix. NES and FDR q-value are indicated.

Because our results demonstrate that *Notch1*+ tumour still retain, at a population scale, the wild-type *Apc* allele and that this tumour population also seems to be transcriptionally related to *Notch1*+ ISCs, with the intriguing difference of having lower levels of *Lgr5* expression (than non-labelled tumour cells), we decided to correlate the transcriptomic signature of *Lgr5* tumour cells, in which both *Apc* alleles were floxed (Schepers et al., 2012), with our *Notch1*+ tumour signature (Figure 47).

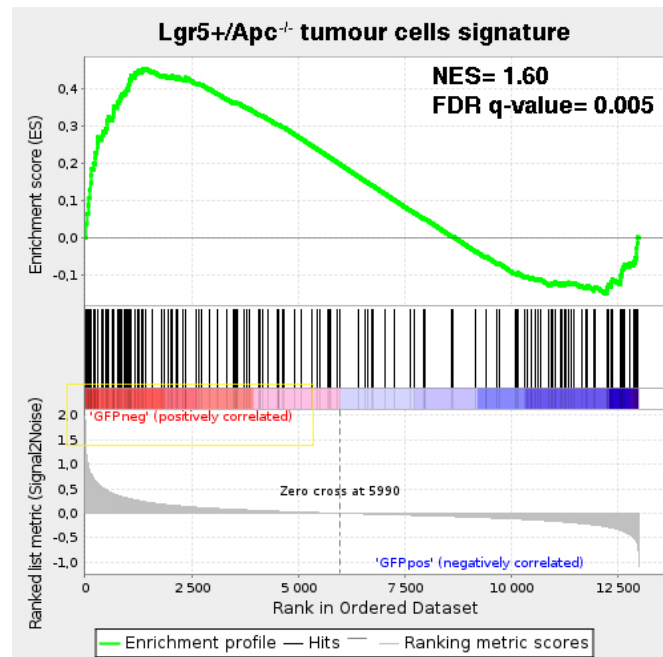


Figure 47. *Lgr5*⁺/*Apc*^{-/-} tumour cells signature positively correlates with non-labelled tumour cells signature.

GSEA analyses performed in Notch1+ and non-labelled tumour cells whole transcriptomics to evaluate the enrichment of *Lgr5*⁺/*Apc*^{-/-} tumour cells signature within the given matrix. NES and FDR q-value are indicated.

Once more, we confirmed that Notch1+ tumour do not correlate with *Lgr5* tumour cells. Instead, as depicted by the GSEA graph, non-labelled tumour cells (“GFPneg”, highlighted by the yellow box) are positively correlated to the gene set that characterises the *Lgr5*⁺ tumour population. Moreover, this results also indicate that Notch1+ tumour cells do not correlate with a signature derived from cells that lost both *Apc* alleles, reinforcing our previous results showing *Apc* wild-type allele retention in this tumour cell population.

We next determined the gene set that exclusively characterises Notch1+ tumour cells (i.e. a gene set that includes only tumour-specific genes) by eliminating genes that were co-expressed between Notch1+ ISCs and Notch1+ tumour cells and also upregulated genes in Notch1+ ISCs (relatively to the normal non-labelled crypt cells) to investigate whether these two populations present distinct biological features, with the ultimate goal of finding specific altered processes within the tumoural transcriptomic. GO (Gene Ontology) enrichment analyses of these two transcriptomic sets revealed that, in some extent, the same biological processes are found within both gene sets (Figure 48), including metabolism, cell cycle and DNA repair.

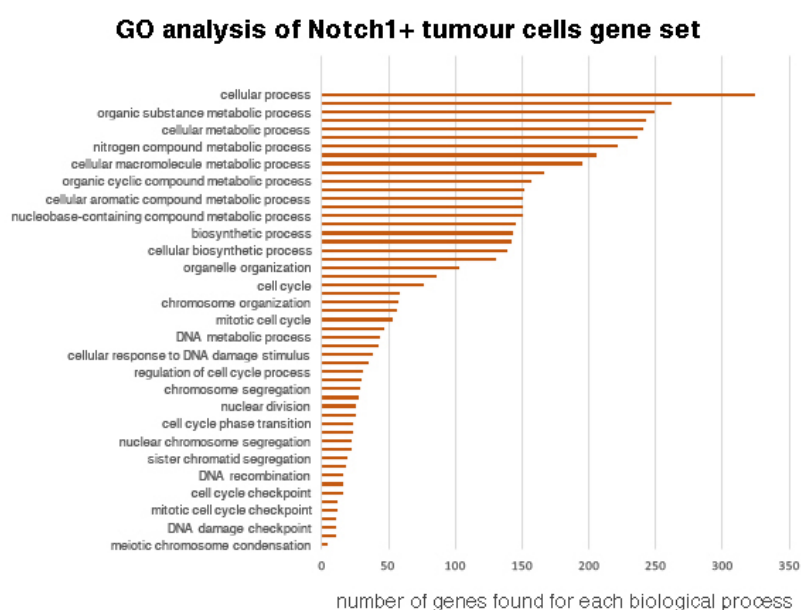
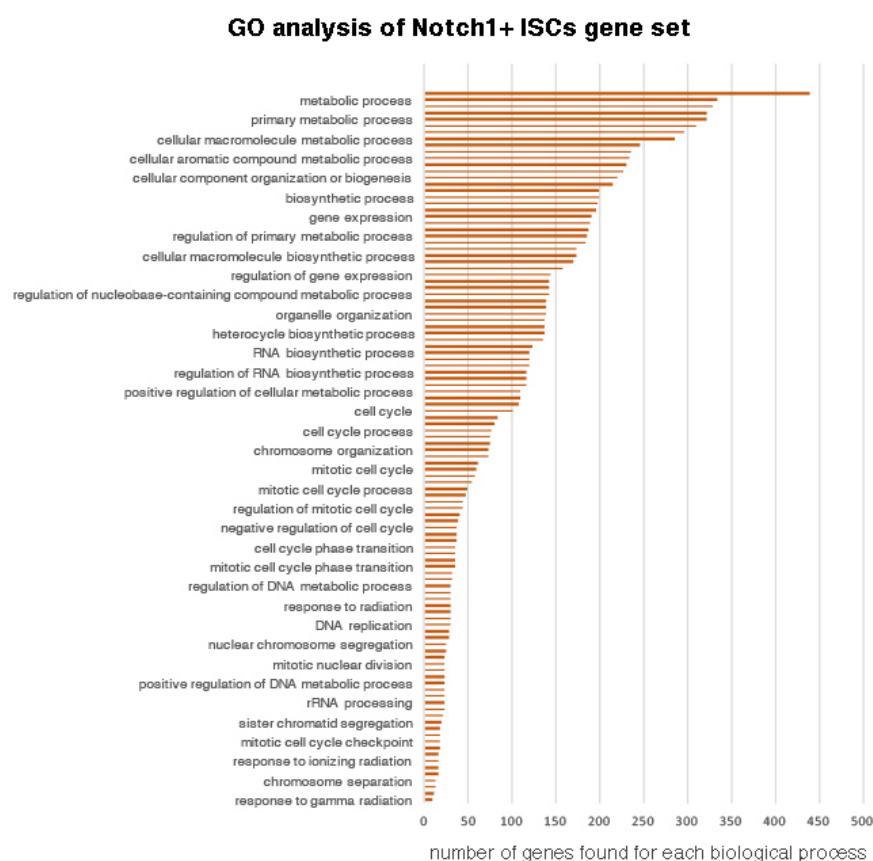


Figure 48. GO analyses representing the major biological processes found within the gene sets of Notch1+ ISCs and Notch1+ tumour cells.

GO analyses were performed using LAGO (Logically Accelerated GO Term Finder, an on-line available tool: <http://go.princeton.edu/cgi-bin/LAGO>). All results displayed are statistically significant ($p\text{-value} < 1 \times 10^{-6}$), Bonferroni correction was applied to correct the threshold of significance used for multiple comparisons.

Overall, the obtained GO results indicate that Notch1+ ISC and Notch1+ tumour cells are very similar transcriptionally, even when the gene sets determined here were built discarding common genes expressed among these two populations. This data shows that the gene sets used are composed of several genes that fit in the same biological processes, once again reinforcing our hypothesis that Notch1+ tumour cells might be Notch1+ ISCs.

SINGLE-CELL ANALYSIS OF NOTCH1+ TUMOUR CELLS

Single-cell RNA-seq (scRNA-seq) technology has emerged as a promising tool for detection of rare cells among complex cell populations. The development of distinct single-cell techniques has allowed comprehensive detailed studies of individual cells both in homeostatic conditions and disease, such as cancer, that are undetectable within bulk tissue analyses that naturally comprise averaged datasets, displaying low or inexistent cell-to-cell variation resolution (Wang and Navin, 2015).

The comparison between the transcriptomic signatures of Notch1+ and non-labelled tumour cells using STRING (a database that predicts protein-protein interactions), revealed that Notch1+ tumour cells seem to be expressing families of proteins that interact with each other at physical and functional levels, whereas the signature of non-labelled cells displays a panel of multiple protein families that are not all inter-connected and that often highlight families of proteins known to be involved in secretory and absorptive differentiation (Figure 49).

The ability to derive genome-wide mRNA expression data from a population of cells has indeed proven useful in many studies over the past two decades, although traditional expression experiments have limitations in providing measurements that are averaged over thousands of cells, which can cover or even misrepresent signals of interest (Bacher and Kendzierski, 2016). In attempt to dissect a possible heterogeneity within the Notch1+ tumour cell population (not detected by bulk transcriptomics), that seem to be defined by a panel of genes that are highly interconnected, we performed scRNA-seq using the C1™ system from Fluidigm. We obtained the transcriptomes of 28 Notch1-expressing tumour single-cells and 2 non-marked UEA+ single-cells as controls.

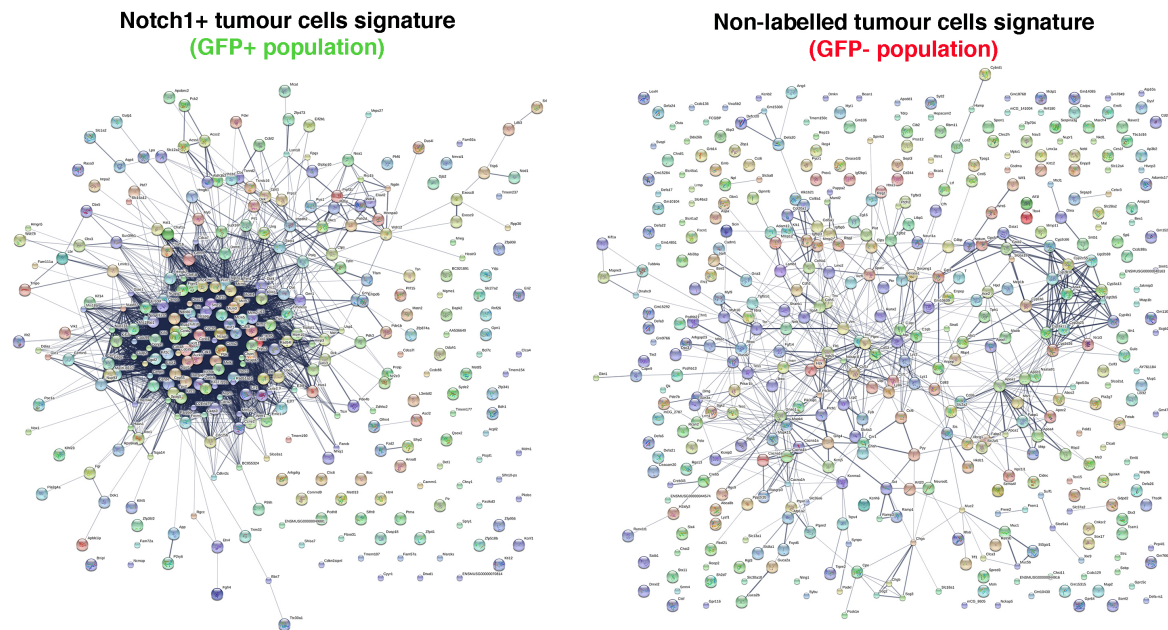


Figure 49. STRING analyses of Notch1+ tumour cells and non-labelled tumour cells signatures.

Comparison between Notch1+ tumour and non-labelled tumour cells signature using STRING; an on-line database of known and predicted protein-protein interactions, that include direct (physical) and indirect (functional) associations, based on computational prediction.

Our control to define whether the number of single cells sequenced was sufficient to obtain statistically significant results that are aimed at determining the level of heterogeneity within the Notch1+ tumour population, was to correlate the average number of reads (i.e. counts) per gene from the 28 Notch1+ expressing single-cells with the average number of reads per gene from RNA-seq bulk cells (Figure 50). The correlation between transcriptomic profiles of single-cells and bulk shows that the 28 single-cell transcriptomic profiles do not correlate with the bulk transcriptomic profile obtained by RNA-seq ($R^2=0.269$). This poor correlation suggests that the number of single cells sequenced is currently not sufficient to proceed with a statistical significant single-cell analysis and hence, information about the level of heterogeneity within the tumour population marked by Notch1 is still lacking.

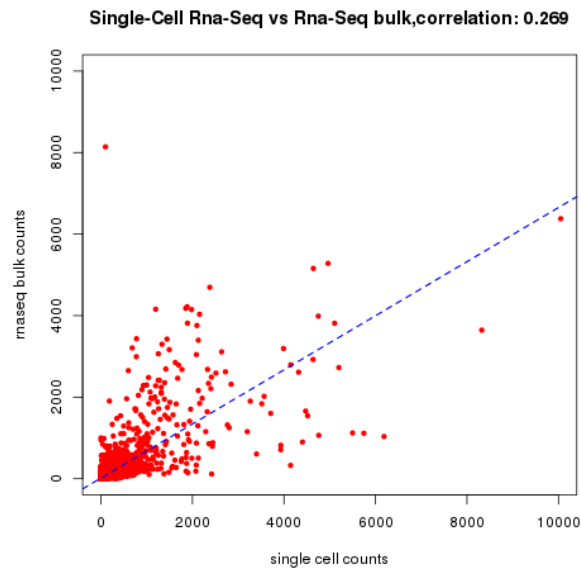


Figure 50. Comparisons of single cell and bulk population transcriptomic profiles.

The X-axis represents the expression level (as counts/gene) in individual Notch1+ tumour single-cells, while the Y-axis represents the gene expression level from the bulk population. The Correlation coefficient is indicated.

5. IDENTIFICATION AND CHARACTERISATION OF NOTCH1+ TUMOUR CELLS IN CHEMICALLY INDUCED COLON TUMOURS

NOTCH1-EXPRESSING TUMOUR CELLS CLONALLY EXPAND WITHIN COLON TUMOURS

To examine the presence and the contribution of Notch1+ tumour cells in the most predominant tumour location in patients, the colon, we chemically induced colon tumours in a set of N1/mTmG mice by administration of azoxymethane (AOM), followed by 2 cycles of exposure to the inflammatory agent dextran sodium sulphate (DSS, protocol presented in Figure 58, *Material and Methods section*). This method has been widely used to study colon carcinogenesis due to its reproducibility and potency (De Robertis et al., 2011).

We performed fate mapping analysis of Notch1+ cells within colon tumours (Figure 51) by administration of a single pulse of tamoxifen in AOM/DSS-treated N1/mTmG mice and analysis of the tumours after different chase periods (24h, 48h, 15 days and 2 months, Figure 52).

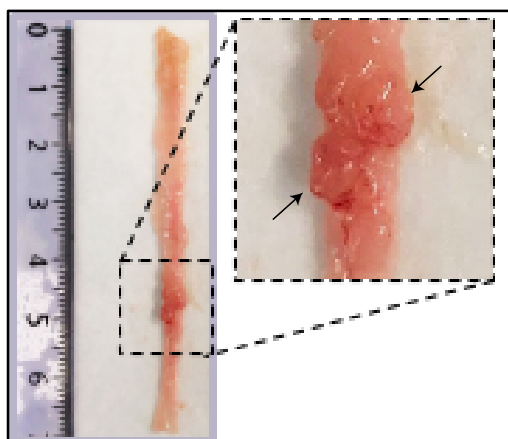


Figure 51. Colon of N1/mTmG mouse displaying AOM/DSS induced tumours.

Representative image of AOM/DSS induced colon tumours (indicated by black arrows) developed in N1/mTmG mice.

All colon tumours analysed after 24 hours post-tamoxifen induction (n = 8) contained scattered tumour cells expressing GFP (Notch1+ cells), both of epithelial and stromal origin (Figure 52A). At day 15 after tamoxifen induction, all tumours analysed (n = 5) contained Notch1+ cell derived clones (Figure 52C) and after 2 months chase (Figure 52D) the GFP+ clones appeared to be enlarged when compared to those from the previous time-point,

suggesting that Notch1+ colon tumour cells potentially continue to expand within time, similarly to the Notch1+ tumour cells found in small intestine adenomas.

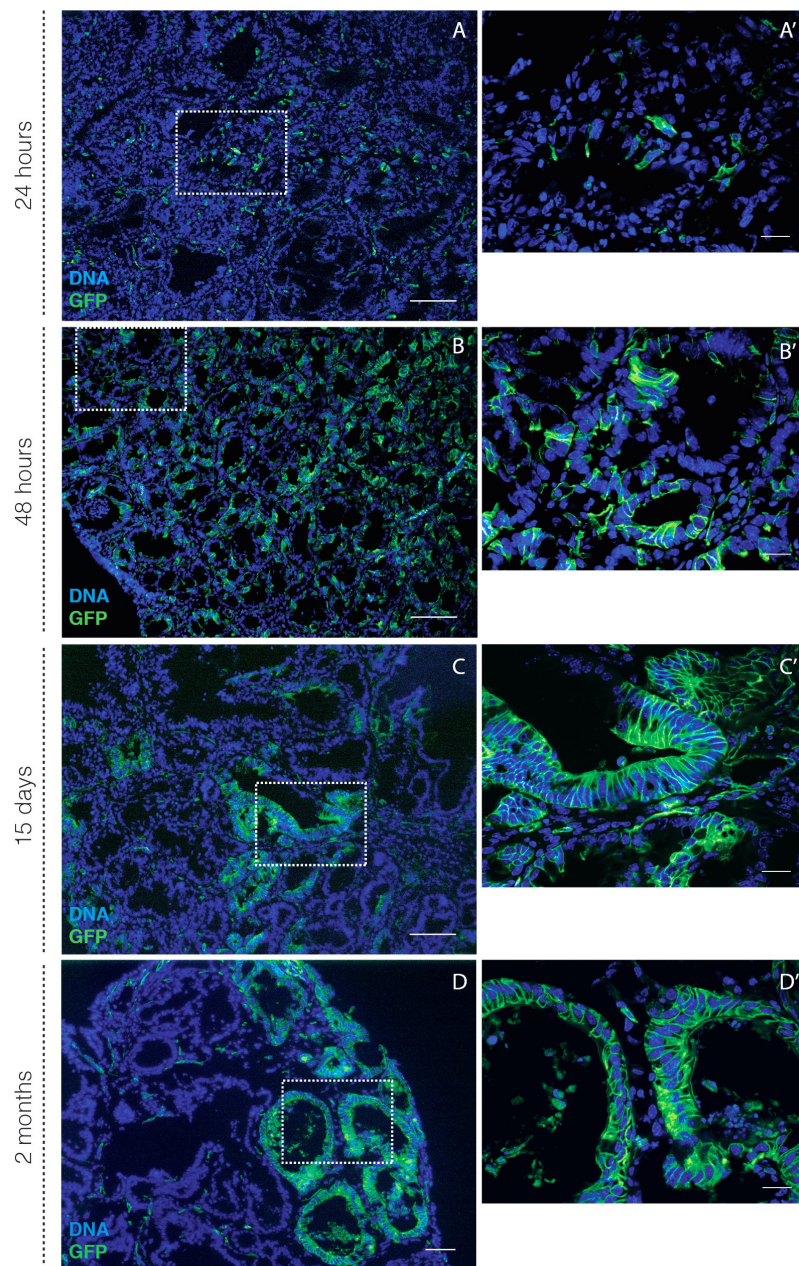


Figure 52. Representative sections of Notch1+ fate mapping analysis in AOM/DSS induced colon tumours.

Representative sections of colon tumours analysed at different time-points after tamoxifen injection (24h, 48h, 15 days and 2 months). Notch1+ colon tumour cells and Notch1-derived clonal progeny are reflected by GFP expression (in green) at the cell membrane. DNA is marked using DAPI in blue. Scale bars represent 50µm in A, B, C and D panels and 10µm in corresponding magnification panels.

NOTCH1+ COLON TUMOUR CELLS GIVE RISE TO BOTH PROLIFERATIVE AND MUCIN2+ COLON TUMOUR CELLS

To evaluate whether Notch1+ colon tumour cells were capable of giving rise to clones containing proliferative cells (as predicted since these cells expand over time), and also to differentiated tumour cells, such as Goblet cells, we assessed the expression of Ki67 (for proliferation) and Mucin2 (for Goblet cells) in the progeny of these cells. Immunostaining analysis showed that Notch1-derived lineages contain tumour cells that are actively dividing (as shown by Ki67 expression) and also Goblet cells (expressing Mucin2), suggesting that Notch1+ colon tumour cells might have the same multilineage potential (even though only one marker for one cell type was assessed in this preliminary analysis), as the one shown for Notch1+ adenoma cells, possibly producing distinct cell lineages within colon tumours and thus, potentially contributing to intratumoural heterogeneity. Whether Notch1+ colon tumour cells can give rise to other cell lineages is still to be determined.

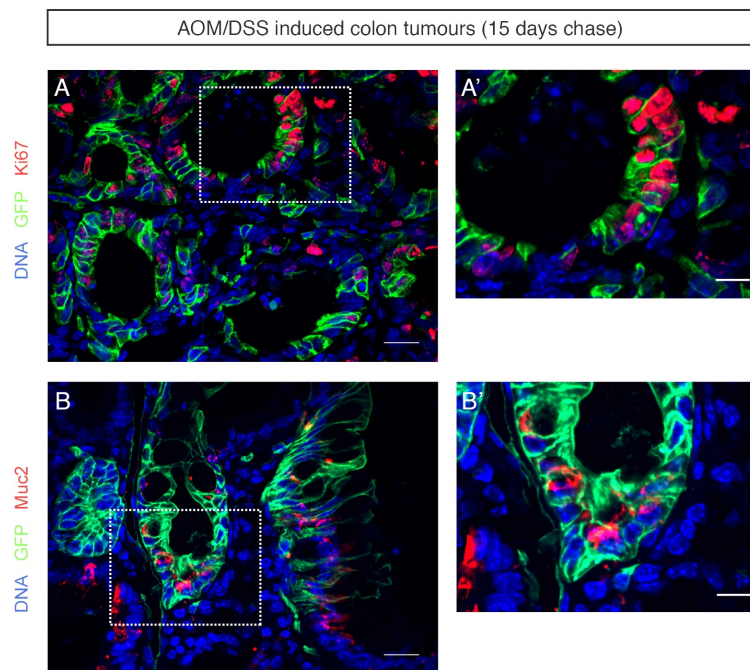


Figure 53. Notch1+ colon tumour cells generate clones that contain actively dividing tumour cells and differentiated Goblet cells.

Immunostainings of tumour sections 15 days after Cre induction using Ki67 (proliferation marker, panel A) and anti-Mucin2 (marker for Goblet cells, panel B) all in red. Notch1+ colon tumour cells are reflected by GFP expression in green and DNA was marked, using DAPI, in blue. Rectangles highlight areas of the tumour containing GFP+ tumour clones expressing Ki67 (A') or Mucin2 (B'). Scale bars represent 20 μ m in A and B and 10 μ m in A' and B'.

FINAL REMARKS, DISCUSSION & PERSPECTIVES

The main focus of my project was to investigate whether a gene (Notch1) expressed in normal stem cells and essential for their maintenance and proper differentiation could pinpoint putative CSC within tumours. We have chosen to test our hypothesis in a mouse model that spontaneously generates adenomas, by LOH at the *Apc* locus, instead of directly targeting specific mutations in ISCs, which would reflect the importance of the cell of origin and/or oncogenic hit, rather than exploring whether CSCs really exist in intestinal tumours.

We believe that this experimental design is the best to verify the presence and to characterise specific tumour cell populations with stem features, if the right marker(s) is/are used.

In this work, using the Notch1 receptor as a tool to potentially mark tumour cells with stem cell properties, we have identified and characterised a novel rare population of CSCs, that are undifferentiated, highly proliferative and consequently, are able to expand in significant proportions over time, giving rise to proliferative and differentiated tumour cells (an indication of multipotency) contributing to the heterogeneity observed within intestinal tumours.

Most importantly, our studies also suggest that the Notch1+ tumour cells we have found, might be originated from ISCs that find themselves in a new environment (i.e. the tumour niche), probably benefiting from the tumour stroma, surrounding tumour cells and secreted factors to survive and to further expand, promoting thus intratumoural heterogeneity, that is believed to be driven (in one of the models stressed in the *Cancer Stem Cells chapter*) by CSCs. We believe that our discoveries might have an impact in the way tumours can be seen and understood, as our work suggests that cells within the normal tissue, carrying specific primed functions, such as ISCs, can be found within tumours and hence adapt in this new niche (in this case the tumour), without necessarily carrying the driver mutation required for tumourigenesis (i.e. LOH of *Apc* gene in CRC).

1. CHARACTERISATION OF NOTCH1-EXPRESSING CELLS WITHIN INTESTINAL TUMOURS

Our fate mapping analysis, using Notch1 as a potential marker of CSCs, revealed that we label a very small population of epithelial tumour cells. So far, we have tested several antibodies against Notch1, but none is reliable for immunostaining of adult mouse tissues and we thus we cannot properly estimate the recombination rate (i.e. mosaicism) in our Notch1-Cre^{ERT2} mouse line. Therefore, we are, so far, not able to know with certainty the real percentage of cells expressing Notch1 within adenomas.

The characterisation of these tumour cells indicates that they are undifferentiated in terms of expression of known differentiation markers, both at the transcriptional and protein levels, like normal ISCs labelled by Notch1 in the crypt (Fre et al., 2011).

Our first attempt to characterise Notch1+ tumour cells was by investigating whether this population could be dichotomised in actively dividing and slow cycling tumour cells. BrdU incorporation assays demonstrated that the majority of these cells were cycling. We could not rule out that the few Notch1+ tumour cells we have scored as BrdU- might be of slow cycling nature. To verify this, we analysed tumour sections derived from 1-2 months chase and we did not detect any single GFP+ tumour cell, prompting us to conclude that a population of quiescent cells marked by Notch1 is likely inexistent.

We also found that Notch1+ tumour cells were more proliferative than non-marked cells, which suggests that these cells have a growth advantage within tumours.

Interestingly, we showed that Notch1+ tumour cells are enriched in the expression of known ISC markers, such as Ascl2, Hopx, Lrig1, Olfr4 and Smoc2, however they seemed to express lower levels of Lgr5 transcripts compared to non-labelled tumour cells. Corroborating this result, Lgr5+ tumour cells seem to express lower levels of Notch1 and the Notch1 target nRarp compared to Lgr5- tumour cells. Within the normal intestinal crypt, Notch1 predominantly marks +4 ISCs and other crypt progenitors, with only 30% of Notch1-labelled cells residing within the CBC compartment (Fre et al., 2011). These results prompt us to speculate that the majority of the tumour cell population labelled by Notch1 might originate from +4 ISCs that are known to express lower Lgr5 levels than CBCs.

+4 ISCs are believed to be of quiescent (or slow cycling) nature (Montgomery et al., 2011; Powell et al., 2012). If our hypothesis is correct, meaning that Notch1+ tumour cells might be originated from these cells, one would think that they could maintain their slow cell cycle

rates within the tumour, nonetheless what we show is exactly the opposite: Notch1+ tumour cells are more proliferative than non-labelled tumour cells. As pointed out in the Introduction (*Stem Cells and Cancer Stem Cells chapter*), ISCs can interconvert between cycling and resting states (Takeda et al., 2011). We hypothesise that, if Notch1+ tumour cells are indeed derived from +4 ISCs, these tumour cells might receive different/stronger signals (i.e. mitogenic signals sent by surrounding cells) from those that exist within the crypt and thus might be “forced” to interconvert from a slow cycling to an actively dividing state.

We’ve shown that Notch1+ cells within tumours poorly overlap with Lgr5+ tumour cells. Notably, Lgr5 expression has been reported in some studies to represent a good prognostic marker in CRC, as Lgr5 may counteract some features present in aggressive tumours, such as anchorage-independent growth, loss of cell-cell adhesion, enhanced migration and EMT (Walker et al., 2011; Ziskin et al., 2013).

Tian and colleagues have shown that targeted ablation of Lgr5+ ISCs (using a diphtheria toxin receptor gene knocked into the Lgr5 locus) does not perturb the homeostasis of the small intestine epithelium because other cell types, such as Bmi1+ ISCs, can compensate for deletion of this population (Tian et al., 2011). The work of Tian along with Yanai’s results (showing that Lgr5+ tumour cells clonally expand in adenomas and present (cancer) stem cell activity), suggest that Lgr5 could be thus considered as a prospective therapeutic target in CRC, as the homeostasis of the normal intestine would not be compromised by the lack of these cells. On the other hand, conditional gene inactivation (using the villin-Cre^{ERT2} promoter, that targets all the gut epithelium) of both Notch1 and Notch2 results in complete conversion of the crypt progenitors into post-mitotic Goblet cells (Riccio et al., 2008). Moreover, the same phenotype is observed upon disruption of Notch signals (by simultaneous inactivation of Dll1 and Dll4 genes (Pellegrinet et al., 2011)). Our work suggests that Notch1+ and Lgr5+ tumour cells might be independent populations in adenomas, however, the fact that our results may pinpoint a novel tumour cell population previously uncharacterised, that appears distinct from the one(s) expressing high levels of Lgr5, is rather informative in terms of tumour biology, than of possible medical relevance, as Notch1 is not envisaged as a good therapeutic candidate for CRC.

It is worth to highlight that the insertion of the Cre^{ERT2} cassette in the Notch1 locus generates a null allele. Even if Notch1 heterozygous mice do not display any appreciable differences from wild-type animals, the possibility that crypt stem cells carrying one functional Notch allele may behave slightly differently than wild-type cells cannot be rigorously ruled out on the basis of our analysis.

2. CHARACTERISATION OF NOTCH1-DERIVED TUMOUR PROGENY

As mentioned above, Notch1+ tumour cells are highly proliferative, which led us to analyse their clonal expansion *in vivo*. By performing lineage tracing analysis after a single pulse of tamoxifen, we showed that the rare tumour cells that we label using Notch1Cre^{ERT2} mice significantly expanded in time. Because the last time-point quantified (90 days) showed high variation in the percentage of GFP+ cells over total TEC, we are unable to conclude with certitude whether the expansion rate of Notch1+ tumour would continue to grow in time or if it would reach a plateau, where the number of newly formed tumour cells would be equal to the number of GFP+ that would undergo apoptosis. Although, the rate of GFP+ expansion in tumours was best fitted with a two-parameter logarithmic function, $y=b*\ln(x-a)$, that predicts that the GFP+ cells will continue to slowly expand within the tumour (as the function possesses no horizontal asymptote) over time. However, the large biological variance within the last time-point (90 days), results in a significant uncertainty of the fit and as evidenced by the 95% confidence interval, more experimental data is required to ascertain this prediction. Notably, the last time-point assessed requires wary interpretation. Since Apc^{+/-} mice die at around 8-9 months of age due to tumour burden, severe anaemia and other complications, the Cre^{ERT2} recombination has to be induced exactly at 6 months of age, implying a risk that tumours by this age might not yet be formed. We cannot rule out the possibility of having induced mice prior to tumour formation and thus including inaccurate expansion measurements in our 3 months quantifications.

Previous studies in our laboratory had shown that induction of Cre^{ERT2} recombination using the Notch1 promoter in 3-month old Apc^{+/-} mice (tumour-free) leads to the appearance of large clones occupying vast parts of total tumour area, a phenotype that we did not observe when inducing recombination at 6 months of age, suggesting that recombination probably occurred after tumour formation. Another factor of variability is the gender. Apc^{+/-} females tend to carry fewer tumours that appear with a longer latency than males. Interestingly, three out of the four replicates we analysed 90 days after tamoxifen administration were performed on females and their averaged GFP+ tumour cells percentage was of 6,1%, whereas the sole replicate containing mainly tumours derived from males had an average of 12,4% GFP+ tumour cells. Other replicates using only males have to be analysed, not only to determine whether gender has an impact in the expansion of Notch1+ tumour cells (since the number of recombined cells, at 24 hours post-induction, is the same between males and females), but also to provide robustness to the proposed model.

In addition to the expansion rate of Notch1-derived progeny, we analysed the identity of these tumour cells and we show that the GFP+ clones within adenomas are enriched in proliferative and differentiated cells, an indication that Notch1+ tumour cells indeed contribute to the intratumoural heterogeneity observed.

In attempt to decipher transcriptional changes (for a specific panel of genes) within the Notch1+ and Notch1-derived tumour cell populations (compared to non-labelled tumour cells), we separated by FAC-sorting these three populations within the same experiment and assessed their relative mRNA levels for the genes of interest, such as Notch1 targets and ISC and differentiation markers. With the exception of Hes1 gene (and GFP gene as internal control), the progeny of Notch1+ tumour cells display intermediate levels of gene expression for all genes tested. Interestingly, the levels of Lgr5 seem to be enriched in the daughter cells originated from Notch1+ tumour cells. Because we show intermediate levels of expression for the selected gene set (what we named “ladder profile”), we believe that this result might indicate that the progeny of Notch1+ tumour cells are a “mixed” population (which is also observed by immunostainings using differentiation markers), comprised of both proliferative and differentiated tumour cells.

We now hypothesise a scenario in which Notch1+ cells are able to fuel the tumour with the vast majority of cells that are in its composition. Besides the confirmation at the molecular level that Notch1+ tumour cells are multipotent, we can also appreciate that “the ladder profile” offers a representative view of the role of this particular population in tumour growth. Hypothetically, if this work was conducted in a non-mosaic system that could allow us the tracing for more than 3 months (such as what has been performed in the normal gut epithelium (Fre et al., 2011)), it would be possible to test if, at some point, all tumour cells would become GFP+. Due to our system limitations, these results prove that Notch1-derived cells represent both proliferative progenitors and differentiated tumour cells, and reinforce the notion (also supporting immunostaining analyses) that Notch1+ tumour cells indeed fuel tumour growth, by expanding and producing a diverse progeny composed of different heterogeneous cell types present in these tumours.

Strikingly, Notch1+ tumour cells seem to give rise to a population that contains intermediate levels of Lgr5, prompting us to question which is the genuine hierarchical position of Notch1+ cells within these adenomas relative to the Lgr5+ tumour cell population (Figure 54).

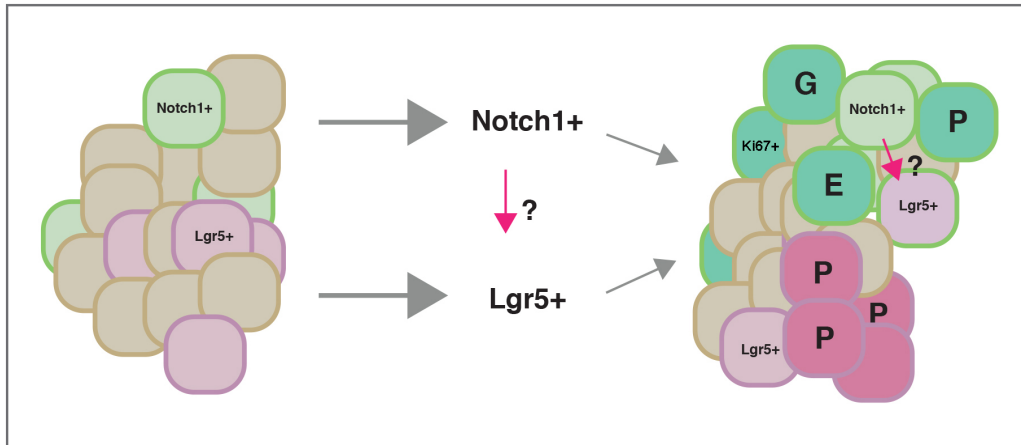


Figure 54. Model depicting the Notch1+ tumour cells hierarchical position relatively to Lgr5+ tumour cells.

Notch1+ tumour cells (light green cells) give rise to differentiated cells, including Paneth (P), Goblet (G), Enteroendocrine (E), proliferating tumour cells (Ki67+) and may as well generate Lgr5+ tumour cells (light pink/green border cell), as indicated by the pink arrows, since the levels of Lgr5 expression are increased within the progeny derived from Notch1 tumour cells (dark green cells, E, G, P and Ki67+).

3. 3D *IN VITRO* STUDIES TO ASSESS CLONOGENIC CAPACITY, *IN VITRO* BEHAVIOUR AND DRUG RESISTANCE

By probing the colony forming efficiency of Notch1+ tumour cells, we showed that Notch1+ tumour cells have a lower capacity of forming cysts than non-labelled tumour cells.

This observation, along with previous results obtained in our laboratory that demonstrate that a proportion of cysts derived from *Apc*^{+/-} adenoma cells, when plated without FAC-sorting, still retains the wild-type *Apc* allele (i.e. does not undergo LOH), led us to propose that the Notch1+ tumour cells might carry the wt *Apc* allele. If proven true, this would be a plausible explanation for their poor clonogenic efficiency when plated as individual tumour cells, since they could still require paracrine signals that are not available in 3D *in vitro* conditions.

When we individually picked cysts derived from Notch1+ tumour cells, we confirmed the presence of the wild-type *Apc* allele in all the GFP+ genotyped cysts, whereas a proportion of the cysts originated from non-labelled tumour cells have undergone complete LOH.

We next show that at a population scale, Notch1+ tumour cells seem to carry approximately 45% of wild-type *Apc* allele, whereas non-labelled tumour cells vary from 35% to 0%.

Altogether, these data might provide an elucidation for the decreased *in vitro* clonogenic capacity observed within the Notch1+ tumour cell population, when compared to non-marked tumour cells that might have an advantage in *in vitro* growth due to higher levels of *Apc* loss at a population scale. It is reported in the literature (Sato et al., 2011b) that normal ISCs have very inefficient colony forming efficiency (roughly 1%) when plated as isolated single cells after FAC-sorting, and that this capacity dramatically increases when they are co-cultured in the presence of Paneth cells that produce essential niche signals to promote ISC growth and survival. We can imagine that if the majority of Notch1+ tumour cells indeed retains the wild-type *Apc* allele, this could explain their poor colony forming efficiency when plated as single cells, as they would lack important paracrine signals for their growth and survival, and thus they might behave as normal ISCs in *in vitro* conditions.

According to this speculative model (Figure 55), Notch1+ cells within tumours might represent normal ISCs that were engulfed within the tumorous glands during the initial steps of tumour formation and, similarly to their crypt counterparts, they show reduced clonogenic capacity *in vitro* after dissociation and isolation by FACS.

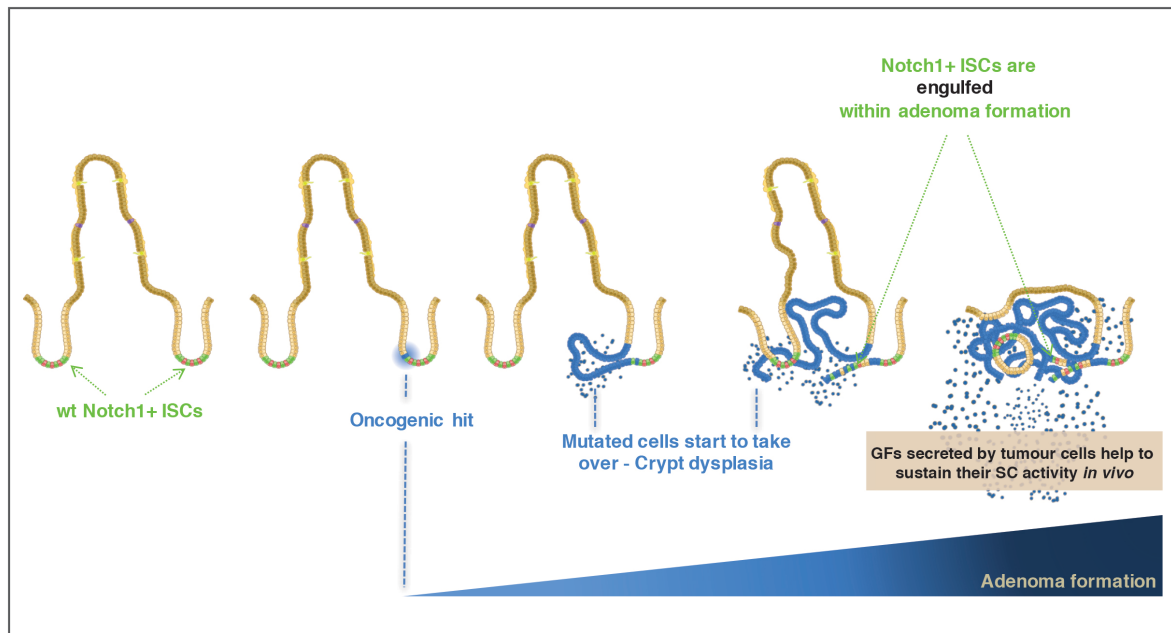


Figure 55. Hypothetical model to depict how Notch1+ ISCs are engulfed during tumour development.

An oncogenic hit, such as loss of the tumour suppressor *Apc*, initiates crypt hyperplasia (according to the bottom up model for tumourigenesis). Notch1+ ISCs (in green), located in the SC crypt compartment might get surrounded by mutant tumour-initiating cells. As mutant cells expand, they secrete growth factors (GFs), providing signals that will potentiate the growth of Notch1+ ISCs. The adenoma continues to develop over time, and as Notch1+ ISCs remain proliferative, they produce differentiated cells, contributing to intratumoural heterogeneity. Cartoon sketched by Jacquemin, G.

To test this model, we are currently investigating the growth capacity and morphology (that might reflect their tumourigenic transformation, if they grow as cysts instead of budding organoids) of FAC-sorted Notch1+ ISCs isolated from N1/mTmG/*Apc*^{+/-} mice and grown in co-culture with non-labelled adenoma cells. With this experiment, we intend to verify whether our hypothesis that proposes that Notch1+ tumour cells are normal ISCs that were engulfed within the tumours holds true.

Because it is now well accepted that clonogenic assays do not totally reflect the intrinsic potential of a particular cell population (Blanpain and Simons, 2013), the ultimate experiment to assess our hypothetical model would be to test whether Notch1+ ISCs could generate tumours *in vivo*, when co-transplanted with non-labelled tumour cells in orthotopic implants in the colon wall.

MATERIALS AND METHODS

TRANSGENIC MOUSE MODELS

The Notch1-Cre^{ERT2} was previously described by (Fre et al., 2011). This line was crossed to the Cre-sensitive bi-fluorescent reporter line Rosa26^{mT/mG} (referred to as mTmG) described in detail by (Muzumdar et al., 2007). The compound mice were then crossed to Apc^{+/-1638N} mice (Fodde et al., 1994) (henceforth named Apc^{+/-}), that contain a heterozygous germline mutation in the *Apc* tumour suppressor gene (Figure 56). The triple transgenic mice obtained from these crosses are hereafter referred to as N1/mTmG/Apc^{+/-}.

To identify Notch1-expressing cells in tumours, triple transgenic N1/mTmG/Apc^{+/-} mice were injected with a single dose of tamoxifen (0.1 mg of tamoxifen/g of mouse body weight), when they were between 6 and 9-month-old to ensure they already presented intestinal tumours. When induced mice were culled 24 hours after tamoxifen injection, only cells expressing the Notch1 receptor, but not their progeny, would be visualised, as the average division time of tumour cells is 24 hours. In this experimental setting, GFP-labelled cells represent the cells where Cre-mediated recombination has occurred. As controls, Apc^{+/-}/mTmG and Apc^{+/-} littermates were used. Of note, no recombination was observed in N1/mTmG/Apc^{+/-} mice without tamoxifen induction.

To investigate the profile of Lgr5+ expressing tumour cells within the same intestinal tumours described above, Apc^{+/-} mice were crossed with the Lgr5-EGFP-ires-Cre^{ERT2} (hereafter called Lgr5-Cre^{ERT2}) knock-in mouse line (Barker et al., 2007), generating the Lgr5-Cre^{ERT2}/Apc^{+/-} compound hereafter denoted as Lgr5/Apc^{+/-}. In the Lgr5-Cre^{ERT2} line, GFP expression is driven by the Lgr5 promoter (Figure 56). Notably, due to strong stability of the GFP protein, the immediate daughters of the Lgr5+ cells carry a residual signal of GFP.

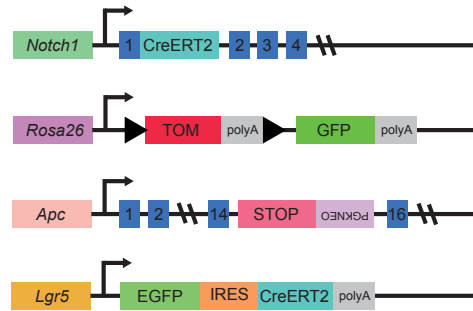


Figure 56. Schematic representation of the mouse models used for this project.

Notch1-Cre^{ERT2} mice are crossed to a double fluorescent reporter (*Rosa26*^{mT/mG} mice) and further crossed to *Apc*^{+/-} mice carrying a chain-termination mutation in the *Apc* gene. The *Lgr5*-Cre^{ERT2} mice are also crossed to the *Apc*^{+/-} mice and *Lgr5*⁺ tumour cells were visualized by EGFP expression.

To evaluate the contribution on tumour growth of Notch1⁺ tumour cells in murine colon tumours by lineage tracing analysis, N1/mTmG mice were generated and AOM/DSS treatment (see AOM/DSS treatment description below) was administrated in mice ranging from 5 to 7-months-old. As the introduced genetic modifications (Cre^{ERT2} in the one of the Notch locus and mT/mG in the *Rosa26* loci) do not generate any developmental defects nor other healthy issues later on in adulthood, these compound mice are characterized as phenotypically normal and thus the colon tumours generated are purely derived from the administered AOM/DSS treatment.

GENOTYPE OF TRANSGENIC MOUSE LINES

Genomic DNA was extracted from ear biopsies using the following lysis buffer 1 (25 mM NaOH/0,2 mM EDTA) for 1hour at 98°C. The resulting lysate was cooled and neutralization buffer 2 (40 mM Tris-HCl pH 5.5) was added in equal amount as buffer 1. Samples were centrifuged briefly, following PCR in the presence of 2.0 mM MgCl₂, 200 μM dNTPs, 100 nM of each primer, and 2 U of Taq DNA polymerase (Invitrogen) with the following primers:

Table 1. List of primers used for mouse genotyping.

Gene	Forward primers	Reverse primers
Notch1-CRE ^{ERT2}	5'-ATAGGAACCTCAAATGTCGCG-3'	5'-CACACTTCCAGCGTCTTTGG-3'
APC ^{T635/N}	5'-TGCCAGCACAGAATAGGCTG-3'	R1 5'-TGGAAGGATTGGAGCTACGG-3' R2 5'-GTTGTCATCCAGGTCTGGTG-3'
Rosa ^{mTmG}	5'-AAAGTCGCTCTGAGTTGTTAT-3'	R1 5'-TAAGCCTGCCGAGAAGACTC-3' R2 5'-GAAAGACCGCGAAGAGTTTG-3'
Lgr5-EGFP-ires-Cre ^{ERT2}	5'-GTAGTTATTCGGATCATCAGCT-3'	5'-GCTGCCACGACCAAGTGACAGC-3'

TAMOXIFEN TREATMENT

N1/mTmG/Apc^{+/-} triple transgenic mice and N1-Cre^{ERT2}/R26^{mTmG} compound mice ranging from 5 months to 9-months-old were induced with one single intra-peritoneal injection of tamoxifen (0.1 mg of tamoxifen/g of mouse body weight). Tamoxifen was dissolved in Ethanol and then diluted in sunflower seed oil at 10 mg/ml.

HISTOLOGY AND IMMUNOFLOUORESCENCE LABELLING

Freshly dissected intestines and tumours from N1/mTmG/Apc^{+/-} were washed in 1X PBS and fixed at room temperature with 4% PFA in PBS under agitation for 2 hours. The samples were then transferred to 30% Sucrose (24h at 4°C) or 70% Ethanol and embedded in OCT (VWR) or in paraffin, respectively. Samples were sectioned either in a cryostat or microtome at 5µm. For immunofluorescence staining, frozen sections were incubated with 0,3% Triton-blocking buffer (5% FBS and 2% BSA). Paraffin-embedded sections were rehydrated through a gradient of Ethanols. Subsequently, antigen retrieval was achieved by boiling in 10mM citrate buffer (20 minutes) for all antibodies. The following primary antibodies were used: chicken anti-GFP (1:800, ab13970), rabbit anti-Lysozyme (1:500, Dako A009902), rabbit anti-Chromogranin A (1:200, Immunostar 20086), rabbit anti-Mucin2 (1:100/200, Clone PH497), mouse anti-PCNA (1:1000, a29), rabbit anti-Ki67 (1:200, ab15580), mouse anti-BrdU (1:200, BD 347580), rabbit anti-EpCAM (Abcam, ab32392), rabbit anti-αSMA (1:400, ab5694). Secondary antibodies were incubated in PBS for one to two hours at room temperature. The following secondary antibodies were used: anti-chicken AlexaFluor488 (1:500, Invitrogen A-11039), anti-rabbit AlexaFluor633 (1:500, Invitrogen A-21071), anti-mouse AlexaFluor633 (1:500, Invitrogen A-21202), anti-mouse Cy3 (1:500, Jackson

laboratories 92557), anti-rabbit Cy3 (1:500, Jackson laboratories 91144). Identification of secretory cells on frozen sections was performed using Ulex Europaeus Agglutinin I (UEA) directly coupled to Cy3 (1:50, Sigma-Aldrich). Nuclei were stained with DAPI.

BRdU INCORPORATION IN VIVO

BrdU (5-Bromo-2'-deoxyuridine, Sigma-Aldrich) was dissolved in PBS at a concentration of 20mg/ml. To detect cells in S phase, BrdU was intraperitoneally injected (50mg BrdU/kg of mouse body) two hours before culling (Figure 57A). To assess putative quiescence (Figure 57B), mice were kept on continuous BrdU feeding (diluted in drinking water at a concentration of 0.8 mg/ml supplemented with 5%wt/vol sucrose) for 5 consecutive days. BrdU dilution in drinking water was renewed every 2 days and protected from light.

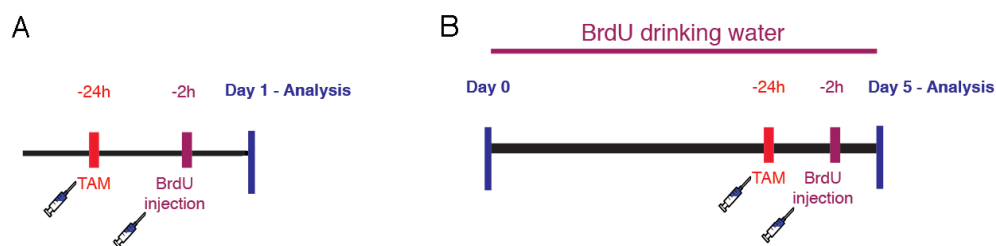


Figure 57. Scheme to illustrate 2 hours BrdU pulse to identify cells in S phase (A) and continuous BrdU incorporation assay to test putative quiescence (B).

In A) N1/mTmG/Apc^{+/-} tumour-bearing mice received a pulse of tamoxifen for 24 hours and one injection of BrdU 2 hours before death. In B) N1/mTmG/Apc^{+/-} tumour-bearing mice were kept in continuous BrdU feeding for 5 days (day 0 to day 5). To detect Notch1⁺ expressing tumour cells, all animals (n = 4) were injected with one single pulse of tamoxifen for 24 hours and one injection of BrdU by i.p. (50mg/kg of mice) 2 hours prior death (to ensure labelling).

SINGLE-MOLECULE RNA FISH

This experiment was performed in collaboration with the laboratory of Hans Clevers (Hubrecht Institute, The Netherlands). N1/mTmG/Apc^{+/-} tumours were dissected and processed accordingly to the protocol of Lyubimova et al. (2013). Single-molecule RNA FISH for Notch1 and Lgr5 probes were performed by M. van den Born and J. van Es at the Hubrecht Institute following the same protocol described by Lyubimova et al., 2013.

IMAGE ACQUISITION AND TREATMENT

Stained sections were analysed using an Upright Widefield Apotome Zeiss microscope equipped with CoolSNAP HQ2 camera (DAPI, GFP, CY3, CY5 and DIC filters) and Upright Confocal Spinning Disk Roper/Zeiss (405nm, 440nm, 491nm, 561nm and 634nm lasers). Raw images were acquired with the Axiovision (Apotome) and Metamorph (Confocal) softwares. Images were analysed using either ImageJ or Photoshop softwares.

TUMOUR CELL DISSOCIATION FOR FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

N1/mTmG/Apc^{+/-} and control Apc^{+/-}/mTmG mice ranging from 6 months to 9-months-old were induced with tamoxifen and sacrificed 24 hours later or later time-points (chase). In order to quantify Notch1-expressing cells (GFP+), tumours were dissected in DMEM/F12 (2% PS), following incubation in 5mM EGTA at 4°C, shaking gently, to remove potential contaminant cells from the normal tissue. Tumours were then minced in small pieces with razor blades and incubated in diluted TrypLE Express (66:100) in 1X PBS, shaking (180rpm) for 45 minutes at 37°C. Trypsin was inactivated with 10% of cold FBS. The cell suspension obtained was then filtered through a 40 µm cell strainer and cells were counted upon centrifugation for 5 min at 450g, following re-suspension in DMEM/F12 (2% PS). To stain cells, they were incubated in Flow buffer (DMEM/F12, 5mM EDTA, 1% BSA, 1% FBS and 10 U/ml DNase) during 25 minutes at 4°C with the following antibodies: EpCAM-PE (PE; R-Phycoerythrin) (1:100, Biolegend clone G8.8), CD45-APC (APC; Allophycocyanin) (1:100, Biolegend clone 30-F11), CD31-APC (1:100, Biolegend clone MEC13.3), Ter-119-APC (1:100, Biolegend clone TER-119). To exclude non-viable cells, DAPI (1:1000, Sigma-Aldrich) was added. Cells were then washed and filtered directly into FACS tubes (40 µm strainer). Analysis was carried out on a FACS-LSRII and sorting on a FACS-Aria III (Becton Dickinson). For cell sorting, three different collecting media were used: RTL lysis buffer supplemented with Beta-mercaptoethanol for RNA extraction (Qiagen), Phenol-chloroform-isoamyl alcohol mixture (Sigma-Aldrich) for genomic DNA extraction and cell culture medium for cell culture experiments (see composition below).

The FlowJo software was used for data analysis.

SMALL INTESTINE CRYPTS DISSOCIATION FOR FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

N1/mTmG/Apc^{+/-}, N1/mTmG and Lgr5/Apc^{+/-} small intestines were harvested and flushed with cold 1X PBS with a 5ml syringe. The intestines were then placed in a clean Petri dish with 10 ml of cold 1X PBS and opened longitudinally and washed once more to remove the remaining intestinal contents, following incubation in 10 ml 1PBS containing 2% Penicillin/Streptomycin and Gentamycin (1:500) for 20 minutes, room temperature. The intestines were then cut into small pieces of approx. 2 mm x 2 mm on a petri dish, with the help of scissors and the obtained small pieces in a 50 ml Falcon tube for further incubation in 20 ml of 2 mM EDTA in HBSS during 30 minutes at 4°C. PBS containing EDTA was removed and new 20 ml of fresh cold 1X PBS was added to the intestine pieces. Crypts were obtained by serial fractioning, following TrypLE Express (ThermoFisher Scientific, 33:100) in 1X PBS incubation for 5 minutes at 37°C to obtain single cells. TrypLE Express was inactivated with 10% of cold FBS (Sigma-Aldrich). To confirm crypt separation, 20 µl of supernatant sample was checked under the microscope. The cell suspension obtained was then filtered through a 40 µm cell strainer and cells were counted upon centrifugation for 5 min at 450g, following suspension in DMEM/F12 (2% PS). To stain cells, they were incubated in Flow buffer (DMEM/F12, 5mM EDTA, 1% BSA, 1% FBS and 10 U/ml DNase) during 25 minutes at 4°C with the following antibodies: rat anti-EpCAM(CD326)-PE-Cy7 (PE; R-Phycoerythrin) (1:100, Biolegend cat no. 118215/16), rat anti-CD45-APC (APC; Allophycocyanin) (1:100, Biolegend cat no. 103111/2), rat anti-CD31-APC (1:100, Biolegend cat no. 102509/10), rat anti-Ter119-APC (1:100, Biolegend cat no. 116211/12). After staining, cells were washed 3 times with Flow buffer. To mark non-viable cells, cells were stained with DAPI (1:1000, Sigma-Aldrich). Cells were then washed and filtered directly into FACS tubes (40 µm strainer). Analysis was carried out on a FACS-LSRII and sorting on a FACS-Aria III (Becton Dickinson). The FlowJo software was used for data analysis.

3D CELL CULTURE CONDITIONS FOR FAC-SORTED CELLS

Once isolated tumour cells were FAC-sorted, these were centrifuged at 450g during 5 minutes and the pellet was embedded in basement membrane matrix (Matrigel, Corning) mixed in a 1:1 proportion with DMEM/F12 (2% PS, Gibco) supplemented with the following growth factors; 50ng/ml EGF (Peprotech, cat no. 315-09), 100ng/ml Noggin (Peprotech, cat

no. 250-38), 1X B-27 (ThermoFisher Scientific, cat no. 17504044), 1x N-2 (Gibco, cat no. 17502001) and 10 μ M Y-27632 (ROCK inhibitor, Sigma-Aldrich), termed hereafter Complete tumour medium. After resuspension in Matrigel:Complete tumour medium, 300 tumour cells were seeded per 20-25 μ l of Matrigel drop, following 30 minutes of polymerization at 37°C. Complete tumour medium was added to the respective wells and later refreshed every 5 days.

For passaging, the drops of Matrigel were scratched with the help of a 1 ml tip and dissolved with cold 1X PBS. Tumour spheroids were then mechanically dissociated with the help of a 10 μ l tip fixed in a 1 ml tip or enzymatically detached using TrypLE Express (66% in DMEM F/12, 2% PS) for 5 minutes at 37°C. Dissociated spheroids were then centrifuged at 450g for 5 minutes and pellet resuspended in Matrigel:Complete tumour medium and plated as described above.

FLUORESCENCE-ACTIVATED CELL SORTING CELL CYCLE ANALYSIS BY HOECHST INCORPORATION

After intestinal tumour dissociation into single cells, cells were fixed in 2% PFA in 1X PBS for 20 minutes at 4°C without agitation. Cells were washed twice in 1X PBS upon centrifugation at 400g, 4 minutes at 4°C, and staining was proceed as described above. After staining, the cells were incubated in pre-warmed 1X PBS Hoechst 33342 (1:100, stock at 10mg/ml Sigma-Aldrich) solution for 20 minutes at 37°C, protected from any source of light. After this incubation, cells were filtered directly into FACS tubes (40 μ m strainer). The FlowJo software was used for data analysis.

RNA EXTRACTION FROM FAC-SORTED CELLS

Total RNA extraction was performed by using the miRNA universal kit (Qiagen) following the manufacturer instructions. The integrity of RNA samples used for RNA-sequencing and qPCR analyses presented in this work was evaluated with a Bioanalyzer using the RNA 600 Pico lab chip (Agilent) accordingly to company instructions. The amount and integrity of RNA samples selected for RNA-sequencing was assessed with a Bioanalyzer and 2200

Tapestation system (Agilent) and the RNA integrity number (RIN) was confirmed to be higher than 8 for all the twelve samples.

REVERSE TRANSCRIPTION AND QUANTITATIVE RT-PCR

Reverse transcription of RNA samples was performed using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific). Random hexamer and gene-specific primers (listed in Table 2) were used for reverse transcription. Real-time PCR quantification of gene expression was systematically performed in triplicate using SYBR Green I Master (Roche) on a ViiA 7 RT-PCR System (ThermoFisher Scientific). The efficiency of the primers used for real-time quantification was evaluated relatively to the slope obtained by the quantification of a standard curve, and the presence of a single amplicon at the expected size was checked on an 2% agarose gel. Results were normalized on the average of the expression of 18S, GAPDH and β -actin housekeeping genes and the relative quantification was obtained by applying the $-\Delta\Delta C_t$ method described by Pfaffl (Pfaffl, 2001):

$$\text{Relative expression} = \text{Efficiency}^{-\Delta\Delta C_p}$$

Table 2. List of primers (design against the mouse genome) used for qRT-PCR analysis.

Gene	Forward (5'-3')	Reverse (3'-5')	Product length (bp)
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	150
β -actin	GGTGACGAGGCCAGAGCAAGA	CGACCAGAGGCATACAGGGACA	278
GAPDH	GAGGCCGGTGCTGAGTATGTCG	GGCAGAAGGGGCGGAGATGAT	113
GFP	GCAAGGGCGAGGAGCTGTTCA	GTGTCGCCCTCGAACTTCAC	348
Notch1	TGTCAATGTTTCGAGGACCAG	TGACGTCAGCATGTGAGTTG	426
Hes1	ACACCGGACAAACCAAGAC	GTCACCTCGTTCATGCACTC	295
nRarp	TGGTGAAGCTGTTGGTCAAG	CTTGGCCTTGGTGATGAGAT	127
Olfm4	TGGCCCTTGGGAAGCTGTAGT	ACCTCCTTGGCCATAGCGAA	266
Ascl2	CTACTCGTCGGAGGAAAG	ACTAGACAGCATGGGTAAG	191
Hopx	TTCAACAAGGTCAACAAGCACCCG	CCAGGCGCTGCTTAAACCATTCT	105
mTert	AGCGGGATGGGTTGCTTTTAC	CACCCATACTCAGGAACGCC	115
Bmi1	GGAGAAGAAATGGCCCACTACC	TTGGCCTTGTCACCTCCAGA	133
Lgr5	ACCCGCCAGTCTCCTACATC	GCATCTAGGCGCAGGGATTG	198
Mucin2	AAACTGCTCTCTGGACTGCC	CAGGAACACGCACAGGTTTG	371
Gob5	CCCAAATGACCAAAACCAAC	CGCTTTGTACATAGGCAGCAGCA	296

Lysozyme1	CCCAGCACCCAGTTAGAACA	GGCACAGCTCACTAGTCCTC	251
Alpi	TCCTACACCTCCATTCTCTATGG	CCGCCTGCTGCTTGTAG	199
Aldh1b1	CTCCCGAACCCAATCCCAAA	ATGCCCAATGACCTCACCTG	129

TRANSCRIPTOME ANALYSES BY NEXT GENERATION SEQUENCING AND AFFYMETRIX

The transcriptomic-based characterisation of Notch1-tumour cells was performed by two different methods; Illumina RNA-sequencing and Affymetrix microarray. We carried out two different approaches in order to obtain a reliable signature (hereafter termed “Robust signature”) for this cell population due to inherent drawbacks of each individual tested method. The transcriptomic profiling of Notch1+ ISCs was obtained by Affymetrix microarray.

All transcriptomic experiments were performed using three biological replicates, consisting of cells pooled from at least 4 mice per replicate. Notably, to obtain the minimum amount of RNA input required for RNA-sequencing and Affymetrix microarray, three groups of 5-7 N1/mTmG/Apc^{+/-} mice were used and each biological replicate represents a pool of tumour cells derived from distinct tumours and mice. To establish the Notch1+ normal ISCs signature, a pair of female and male mice were used in each replicate.

Both transcriptome analyses were performed by N. Menssouri (Institut Curie, France).

GENOMIC DNA EXTRACTION FROM FAC-SORTED CELLS FOR QUANTITATIVE RT-PCR ANALYSIS

Genomic DNA extraction in sorted cells was conducted using Phenol-chloroform-isoamyl alcohol mixture (Sigma-Aldrich), following Ethanol precipitation. gDNA was quantified and qualified using the Nanodrop System (ThermoFisher Scientific). gDNA was diluted at a final concentration of 5ng/ μ l to perform qRT-PCR analysis using the protocol described above.

To perform an accurate standard curve to quantify the percentage of Apc wt and mutant within the Notch1+ and GFP- tumour populations, Apc^{-/-} gDNA from a cell line was used (a kind gift of Dr. Ron Smits). Table 3 includes the list of primers used.

Table 3. Gene specific primers used for qRT-PCR analysis for Apc LOH quantification.

Gene	Forward primers	Reverse primers & Product lengths
APC-A2	5'-TCAGCCATGCCAACAAAGTCA-3'	APC-A2/APC-C2 = wt amplicon 216bp APC-A2/Neo3 = mutant amplicon 227bp
APC-C2	5'-GGAAAAGTTTATAGGTGTCCCTTCT-3'	
Neo3	5'-CACTTCATTCTCAGTATTGTTTG-3'	
18S	5'-GTAACCCGTTGAACCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3' = 150bp
GAPDH	5'-GAGGCCGGTGCTGAGTATGTCG-3'	5'-GGCAGAAGGGGCGGAGATGAT-3' = 113bp

ISOLATION OF GENOMIC DNA EXTRACTION FROM ORGANOID IN 3D CULTURE

Cystic organoids were removed from matrigel drops by scratching with the tip of 1 ml pipette using cold 1X PBS and transferred to a clean Petri dish to facilitate individual picking and to avoid contamination from other surrounding organoids. Using a 10 μ l pipette, organoids and spheroids were harvested individually and transferred to an Eppendorf containing 10 μ l of lysis buffer (10 mM Tris pH 8.5, 50 mM KCl, 0.01% gelatin and 0.1 mg/ml Proteinase K). Lysis was achieved by placing the samples (in a thermoplate) at 50 °C during 50 minutes and rising to 90 °C for another 10 minutes to inactivate Proteinase K.

AOM/DSS COLON CARCINOGENESIS EXPERIMENTAL PROTOCOL

To induce colon carcinogenesis, we adjusted the protocol from De Robertis (De Robertis et al., 2011) and a group of 20 males N1/mTmG mice ranging from 5 to 7 months of age were administered a single intraperitoneal injection of Azoxymethane (AOM, Sigma #A5486) followed by Dextran Sulfate Sodium (DSS, MP Biomedicals #160110) treatment (3% in drinking water) the day after the AOM injection for 5 consecutive days (Figure 58). The health and body weight of mice were monitored daily during and after treatment. To verify the presence of colon tumours, 2 mice were killed 1 month after the first cycle of DSS treatment but these only showed signs of inflammation and no tumours were detected. For this reason, another cycle of DSS (3% in drinking water) was conducted for 3 days. This verification was repeated one month after the second cycle of DSS with 2 mice that harboured an important number of colon tumours.

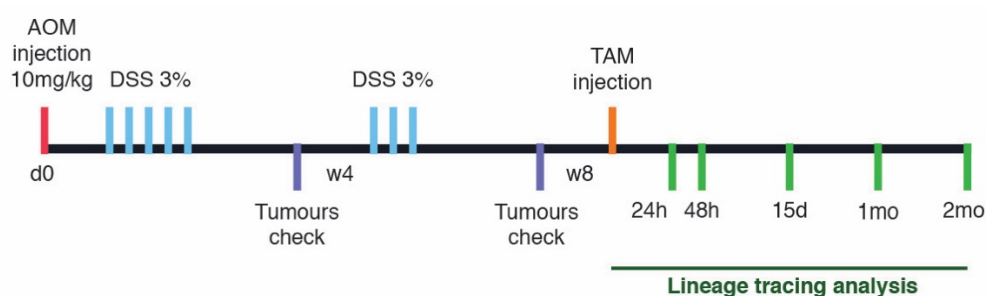


Figure 58. Schematic of the AOM/DSS protocol and lineage tracing analysis in chemically induced colon tumours.

Animals injected with 10 mg/kg AOM at day 0 (d0) followed 2 cycles of 3% DSS ad libitum (blue bars), separated by 3 weeks of recovery on standard drinking water. At weeks 4 (w4) and 8 (w8, purple bars) control animals were killed for evaluation of tumour occurrence. Lineage tracing analysis started after w8 upon tamoxifen (TAM) injection and animals were culled at different time-points (green bars).

The whole group of mice was then injected with one single intraperitoneal injection of tamoxifen and killed at different time-points (24 hours, 48 hours, 2 weeks, 4 weeks and 8 weeks) for lineage tracing analysis.

STATISTICAL ANALYSIS

The Prism software was used for descriptive statistical analyses. Results shown as histograms represent means, and error values are represented as standard deviation or S.E.M. as indicated in each figure.

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"Tell me with whom you walk and I will tell who you are"

- *Ancient Spanish proverb*