# SELF-ORGANISATION OF INTESTINAL ORGANOIDS IS DRIVEN BY A YAP1-DEPENDENT REGENERATION PROCESS

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Friedrich Miescher Institute for Biomedical Research, Basel,

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Denise Serra

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# 1. ABBREVIATIONS

| | |
|---|---|
| 14.3.3 | 14-3-3 proteins |
| 3D | Three-dimensional |
| AJ | Adherens junction |
| AKT | Protein kinase B |
| ALPI | Intestinal alkaline phosphatase |
| AMOT | Angiomotin |
| AMPK | AMP-activated protein kinase |
| AP1 | Activator protein 1 |
| APC | Adenomatous polyposis coli |
| Ascl2 | Achaete scute-like 2 |
| Axin | Axis inhibition protein |
| Bmi1 | Polycomb ring finger BMI proto-oncogene |
| BMP | Bone morphogenetic protein |
| C-Abl | Yes/Src and Abelson tyrosine-protein kinase 1 |
| CBC | Crypt base columnar cell |
| CDK1 | Cyclin-dependent kinase 1 |
| CDK6 | Cyclin dependent kinase 6 |
| CDT1 | Chromatin Licensing And DNA Replication Factor 1 |
| CIP | Contact inhibition of proliferation |
| CK1$\alpha$ | Casein kinase alpha 1 |
| CRB | Crumb |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CTGF | Connective tissue growth factor |
| DDIT4 | DNA-damage-inducible transcript 4 |
| Dll1 | Delta like canonical Notch ligand 1 |
| Dll4 | Delta like canonical Notch ligand 4 |
| DNA | Deoxyribonucleic acid |
| DSS | Dextran Sodium Sulfate |
| DVL | Dishevelled |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| Ephb3 | Ephrin type-B receptor 3 |
| ErbB | ErbB protein family |
| FA | Focal adhesion |
| FAK | Focal adhesion kinase |
| FEnS | Foetal enterospheres |
| Fzd | Frizzled |
| GSK3 | Glycogen synthase kinase 3 |
| Hes1 | Hairy and enhancer of split-1 |
| Hopx | HOP homeobox |
| ICM | Inner cell mass |
| IL | Interleukin |
| ISC | Intestinal stem cell |
| Jag1 | Jagged 1 |
| Jag2 | Jagged 2 |
| KLF4 | Krupper-like factor 4 |
| KO | Knock-out |
| LATS1 | Large tumour suppressor kinase 1 |
| LATS2 | Large tumour suppressor kinase 2 |
| Lgr5 | Leucine-rich-repeat-containing G-protein-coupled receptor 5 |
| LINC | Linker of nucleoskeleton and cytoskeleton |
| Lrig1 | Leucine-rich repeats and immunoglobulin-like domains protein 1 |
| LRP5/6 | Low-Density Lipoprotein Receptor-Related Protein 5/6 |
| MMP7 | Matrix Metalloproteinase 7 |

| | |
|---|---|
| MOB1A/B | Mps one binder kinase activator like 1B |
| mRNA | Messenger ribonucleic acid |
| MST1/2 | Hepatocyte growth factor-like protein precursor 1/2 |
| mTOR | Mammalian target of rapamycin |
| NDR1/2 | Nuclear dbf2-related kinase 1/2 |
| NEUROG3 | Neurogenin 3 |
| NF2 | Neurofibromin 2 |
| NICD | Notch intracellular domain |
| NLK | Nemo Like Kinase |
| NuRD | Nucleosome remodelling deacetylase |
| OCT4 | Octamer-binding transcription factor 3 |
| OGT | O-GlcNAc transferase |
| Olfm4 | Olfactomedin 4 |
| P2 | Postnatal day 2 |
| P63/73 | Tumour protein 63/73 |
| PCP | Planar cell polarity |
| PolII | Polymerase II |
| POU2F3 | POU class 2 homeobox |
| PP1 | Protein phosphatase 1 |
| PP2A | Protein phosphatase 2A |
| PTEN | Phosphatase and tensin homolog |
| PTPN14 | Protein Tyrosine phosphatase non-receptor type 14 |
| RBP-J | Recombinant signal binding protein for immunoglobulin Kappa J region |
| revSC | Revival stem cell |
| Rho-A | Ras homolog family member A |
| RNA | Ribonucleic acid |
| Rock | Rho associated coiled-coil containing protein kinase 1 |
| Rspo | R-spondin |
| RUNX1/2 | Runt-related transcription factor 1 |
| SAV1 | Salvador homolog 1 |
| SCF | Skp1-Cul1-F-box |
| SET7 | SET-domain-containing lysine methyltransferase |
| SH3 | SRC Homology 3 |
| SMAD | Small mother against decapentaplegic |
| SPDEF | SAM pointed domain containing ETS transcription factor |
| TA | Transient amplifying |
| TAD | Carboxy-terminal transcription activation domain |
| TAO1/2/3 | Thousand and one amino acid protein kinase 1/2/3 |
| TCF | Lymphoid enhancer-binding factor/T-cell factor |
| TE | Trophectoderm |
| TEAD | TEA domain transcription factor |
| Tert | Telomerase reverse transcriptase |
| TF | Transcription factor |
| TGF | Transforming growth factor |
| TJ | Tight junction |
| TLE | Transducin-like enhancer protein |
| Trail | TNF-related apoptosis-inducing ligand |
| TSSs | Transcription start sites |
| VGLL4 | Vestigial like family member 4 |
| WNT3 | Wingless-Type MMTV Integration Site Family, Member 3 |
| YAP | Yes-Associated Protein |
| YAP1 | YAP1-1 |
| YAP2 | YAP1-2 |
| YES1 | Yes proto-oncogene 1 |
| β-cat | β-catenin |
| β-TrCP | Beta-Transducin Repeat Containing E3 Ubiquitin |

Abbreviation of amino acids are found in appendix section

# 2. SUMMARY

Self-organisation is a phenomenon in which ordered structures arise from local interactions of disordered elements. The collective behaviour of the elements confers properties to the system that are more than the sum of the properties of the elements. Indeed, the spontaneous formation of ordered structures can be explained by the dynamic and non-linear interactions of the distinct elements.

In multicellular organisms, cells constantly adjust their state according to the signals received by the neighbouring cells and the environment, leading to events of self-organisation that are observed e.g. during embryogenesis, tissue development and regeneration.

Cellular diversification, or symmetry breaking, appears within uniform starting condition through stochastic fluctuations and cell interactions that are sustained and amplified in feedback loops. Symmetry breaking brings the system from a homogeneous and disordered state to a state that is stable and well-defined. Exactly how the cells make distinct choices and influence the collective behaviour is still an open question. During my PhD I have investigated this pivotal point, fundamentally defining higher organisms, and thereby contributed to the understanding of how multicellular asymmetric structures arise from the self-organising behaviour of single stem cells.

Cellular self-organisation can be modelled *in vitro* through organoid culture. In the main study presented in this thesis "Self-organization and symmetry breaking in intestinal organoid development" (Serra et al., 2019) we characterize the formation of intestinal organoids from single cells. Intestinal organoids resemble the structure and function of the intestinal epithelium by containing all the tissue characteristic cell types which are similarly spatially distributed in crypts and villi. Intestinal organoids develop from a single proliferating cell that gives rise to an initially round and symmetric sphere; the sphere then break the symmetry by the differentiation of a first Paneth cell, one of the cell types of the intestinal epithelium. This event allows the succeeding formation of a stem cell niche and specification of crypt and villus domains.

The ability of a single cell to spatially and temporally organise into a functional organoid under homogeneous culture conditions was poorly elucidated, as were the driving transcriptional programs and the mechanisms leading to symmetry breaking. The absence of studies to describe this initial phase of organoid development has in part been due to the lack of technologies with adequate spatial and temporal resolution. In this manuscript, we progress and integrate several technologies for the use in organoid cultures, ranging from automated high-content imaging, multiplexed imaging and light sheet microscopy. These methods are combined with mRNA sequencing in order to allow us to characterize morphologically and molecularly the steps of organoid formation. Previous studies have revealed that several cell types of the intestinal epithelium dedifferentiate *in vivo* following injury. In this pluripotent state they are able to restore tissue homeostasis and these cells are also able to form into intestinal organoids. In our work we show that the different populations of stem cells and non-stem cells share a stereotypic pattern of organoid growth. The process of organoid

formation is driven by the transient activation of the mechanosensor and transcriptional regulator YAP1 in single cells. YAP1 initiates a regenerative response by reprogramming cells with diverse levels of differentiation into fast proliferating cells, and exploiting the plasticity of the intestinal epithelium. These cells, cultured in homogeneous growth-promoting conditions, divide and generate a round cyst that lacks expression of known marker genes of intestinal cell types. The emergence of intercellular variable activity of YAP1 in the cyst, probably due to local changes in cell crowding, enables the establishment of Notch-Delta lateral inhibition, which is responsible for cell fate specification in the intestinal epithelium. Cells with YAP1 activity express the Notch ligand DLL1 and differentiate into Paneth cells. This is the event that denote the break of the symmetry. Paneth cells release Wnt molecules that sustain cell proliferation and self-maintenance of the organoids. The formation of a Wnt gradient around Paneth cells allows the diversification of two functional regions in the organoid: 1) the highly proliferative crypt — containing transient amplifying cells and stem cells intermingled with Paneth cells. 2) the villus — populated by the differentiated enterocytes, Goblet and enteroendocrine cells. Failure of symmetry breaking prevents the formation of the stem cell niche and causes the differentiation of the cyst into a short-living enterocyst composed uniquely of enterocytes.

In conclusion, we describe the molecular mechanisms underlying the capacity of single cells to generate multicellular ordered and functional systems, such as the intestinal organoid. These findings can help shedding light into the principles governing formation, tissue organisation, as well as regeneration of multicellular organisms. Our findings reveal that the process of organoid formation recapitulates the regeneration of the intestinal epithelium YAP1-mediated following injury. This new knowledge will influence the future applications of intestinal organoids in research, and the interpretation of the experiments done with this model system.

# 3. INTRODUCTION

This thesis studies the mechanisms of self-organisation and symmetry breaking in intestinal organoid development. In the following introduction, I describe the current understanding and definition of self-organisation and symmetry breaking, and then proceed with a brief overview of the intestinal epithelium and of the intestinal organoid as a model system. Finally, I focus on the YAP1 protein as key player of self-organisation, in general, as well as in formation of intestinal organoids.

## 3.1. SELF-ORGANISATION

Self-organisation is a process in which global order arises from local interactions of individual agents. Several examples of self-organising systems can be find in nature. For instance, snowflakes that exhibit complex structures without a central organisation, and schools of fish or flocks of birds that create larger scale patterns following simple rules (stay close to the neighbour (positive feedback); and avoid collisions (negative feedback )). Similarly, tissues and organs arise when populations of cells coordinate their behaviour in space and time through the process of self-organisation. Cells give rise to a functional system that has new properties by interacting with each other and integrating signalling pathways, chemical and mechanical stimuli (Bryant and Mostov, 2008; Gilmour et al., 2017; O'Brien et al., 2002). At the microscale level, this happens when single cells sense neighbouring cells, and their local environment, and accordingly regulate numerous biological processes such as cell cycle, shape, differentiation, and polarization (Bryant and Mostov, 2008; Kim et al., 2018; Snijder and Pelkmans, 2011). These cellular behaviours are not determined genetically but depend on the establishment of non-linear interactions and feedback-loops that confer stability to the system and allow, at the multicellular level, the occurrence of tissue morphogenesis, homeostasis, and regeneration (Bryant and Mostov, 2008; Chau et al., 2012; Kim et al., 2018; Saetzler et al., 2011; Sasai, 2013; Wennekamp et al., 2013; Xavier da Silveira Dos Santos and Liberali, 2018).

A key step in self-organisation is the breaking of symmetry, that arises when identical cells in an identical environment activate different genetic pathways which result in different cellular fates. (Xavier da Silveira Dos Santos and Liberali, 2018). Interestingly, symmetry breaking appears to be the result of the collective behaviour of the system and of the amplification of stochastic fluctuations in protein abundance and signalling at the single cell level (Mateus et al., 2009). Specifically, once this amplification reaches a certain threshold, it triggers a cascade of events within the single cell that modifies the differentiation potential, resulting in discontinuity of cellular homogeneity. This event introduces higher complexity to the system and initiates functional specification and diversification. This happens, for example, during blastocyst development where initially

equipotent cells start to segregate into inner cell mass (ICM) and trophectoderm (TE) cells (Anderson, 1972; Zhang and Hiiragi, 2018).

Cell-to-cell variability is a key property of multicellular systems. Each cell within a population of genetically identical cells is subjected, spatially and temporally, to a combination of intracellular and extracellular factors that can stochastically vary and influence cell state and behaviour differently in each cell, see results on page 34 (Mayr et al., 2019). Cell-to-cell variability creates the conditions for a seemingly homogenous population of cells to give rise to distinct cell types and reach an ordered structure. Without variability, all cells would have an all-or-none response to a stimulus and would all adapt their behaviour accordingly, preventing symmetry breaking events and the emergence of biological complexity (Xavier da Silveira Dos Santos and Liberali, 2018; Zhang and Hiiragi, 2018).

The study of environmental cues, intercellular dynamic interactions, and sources of cell-to-cell variability is important to understand the processes of self-organisation. To achieve this an approach integrating spatial and temporal analysis at the single cell level, and considering tissue functionality is needed. The intestinal epithelium has a high self-renewal rate and a simple structure, but still maintains a level of complexity through the diverse cell types populating it; it thus represents an interesting system for investigation of the phenomenon of self-organisation.

## 3.2. INTESTINAL EPITHELIUM

The intestinal epithelium is a highly specialised monolayer of cells responsible for nutrient uptake. To efficiently perform this task with increased absorptive surface, the tissue is folded in villi that projects into the lumen of the intestine. Villi are mostly populated by absorptive enterocytes which also expand their surface through a brushed apical membrane (microvilli). Villi are interspaced by crypts of Lieberkühn, which are highly proliferative epithelial invaginations, responsible for tissue homeostasis. At the bottom of the crypt is a stem cell niche, where crypt base columnar stem cells (CBCs) and the secretory Paneth cells reside, and in the upper part the transient amplifying (TA) compartment. Apart from absorptive enterocytes, villi are composed of two secretory cell types: Mucus-secreting goblet cells that form a protective barrier for the epithelium, and hormone-secreting enteroendocrine cells acting both locally and systemically. Less characterized are the rare differentiated Tuft cells that reside both in the crypt and villus domains. Recently, it has been shown that they play a role in sensing the lumen content and protecting the intestine from helminth infections (von Moltke et al., 2016). Of note, another sporadic cell type of the villi, the microfold cell or M-cell, controls immune responses by transporting antigens from the lumen to the intestine-associated lymphoid cells (Mabbott et al., 2013).

A constant flow of newly generated cells migrates upwards from the stem cell niche towards the villus compartment, while cells slowly acquire different lineages, where they are eventually shed in the lumen after few (4-5) days. Paneth cell progenitors, however, move back to the crypt where they differentiate and intermingle with stem cells and

supply essential niche factors such as WNT3[1], EGF[2], TGF[3], lactate, and Notch-ligand Delta. Indeed, deletion of Paneth cells leads to stem cells loss, highlighting their role in supporting and maintaining the stem cell niche (Sato et al., 2011). Furthermore, Paneth cells have a protective function for the intestinal epithelium by secreting antimicrobial enzymes and peptides such as Lysozyme or defensins.

## 3.3. NICHE SIGNALLING PATHWAYS

A fine balance of different signalling pathways plays an important part in sustaining the niche and the equilibrium in the tissue. Wnt, EGF, BMP[4] and Notch are among the key cascades and their activity has been well characterised in the crypt.

[1] WNT3 = Wingless-type MMTV integration site family, member 3
[2] EGF = Epidermal growth factor
[3] TGF = Transforming growth factor
[4] BMP = Bone morphogenetic protein

### 3.3.1. Wnt pathway

Wnt signalling is an important regulator of intestinal homeostasis; it stimulates secretory cells differentiation, stem cell maintenance, and proliferation. This was shown, for example, through *in vivo* experiments where Wnt inhibition leads to the disruption of intestinal crypts and the absence of stem and secretory cells (Korinek et al., 1998; Kuhnert et al., 2004; Pinto et al., 2003; van Es et al., 2012a).

The Wnt cascade can be further amplified by the Wnt agonist R-spondin (RSPO). In fact, transgenic *Rspo* expression causes crypt hyperproliferation (Kim et al., 2005). Wnt ligands are produced and released both from the underlying mesenchymal cells (WNT2B, WNT4, WNT5A, WNT5B) and from Paneth cells (WNT3), while the source of RSPO is still not determined (Farin et al., 2012; Gregorieff et al., 2005; Kabiri et al., 2014).

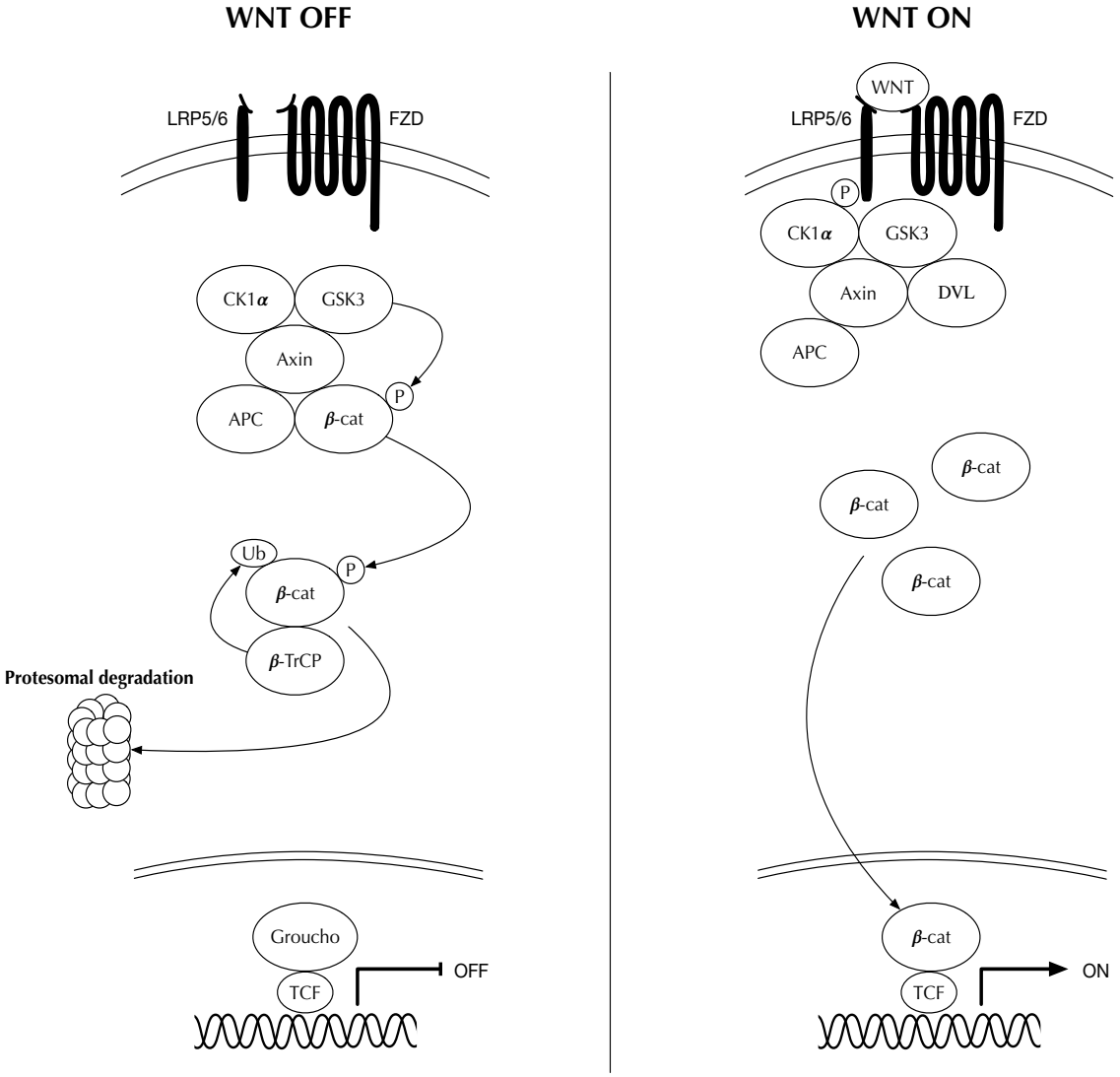


*Figure 1 Wnt/β-catenin pathway. In the absence of Wnt ligand, β-cat is trapped in the destruction complex (composed of Axin, GSK3, CK1α and APC), and is marked for proteasomal degradation. The binding of Wnt ligand to the receptor complex FZD-LRP5/6 induces the inhibition of the destruction complex and allows cytoplasmic accumulation of β-cat. β-cat enters the nucleus and drives transcription together with TCF after displacing the TCF repressor Groucho.*

Wnt ligands can induce three different cellular responses: The canonical Wnt/β-catenin cascade, the noncanonical planar cell polarity (PCP) pathway, and the Wnt/$Ca2^{+}$ pathway. The most studied and understood in the intestinal epithelium is the canonical Wnt/β-catenin pathway and therefore, it will be the only one in focus in this introduction. Pathway components and regulation of the cascade are now presented in brief (Figure 1).

The main effector of the canonical Wnt signalling is the transcriptional co-activator β-catenin (β-cat) that, together with TCF[1] transcription factors (TFs), drives the expression of context-specific target genes. When the Wnt pathway is inactive, cytosolic β-cat interacts with the β-cat disruption complex, which is composed of Axin[2], APC[3], GSK3[4] and CK1α[5]. The disruption complex phosphorylates β-cat and marks it for ubiquitination by ubiquitin ligase β-TrCP[6], and subsequent proteasome degradation (Ikeda et al., 1998; Krausova and Korinek, 2014). The binding of Wnt ligand to the transmembrane receptor complex Frizzled (FZD) - LRP5/6[7] promotes β-cat stabilization. In particular, the active receptors recruit Dishevelled (DVL) protein that provides a platform for the relocation of the disruption complex to the membrane preventing its activity (Gao and Chen, 2010; Kim et al., 2013; Li et al., 2012). This event allows β-cat accumulation and translocation to the nucleus where, after removing the transcriptional repressor groucho/TLE[8] from TCF, it drives Wnt response. β-cat induces for example the expression of Cyclin D and *c-Myc*, well known positive regulators of cell division. To restrain excessive proliferation, it promotes the expression of Axin2, thereby establishing a negative feedback loop on itself (Nusse and Clevers, 2017). Wnt-mediated expression includes also defensins/cryptidins and Matrix metalloproteinase 7 (MMP7), which are the proteins that mediate Paneth cell functions (Andreu et al., 2008; Farin et al., 2012; van Es et al., 2005a).

Along the crypt-villus axis, a Wnt signalling gradient is established through Wnt transfer between adjacent cells. Indeed, Wnt released by Paneth cells does not diffuse freely but binds to the relative FZD receptor expressed on neighbouring cells, e.g. CBCs. FZD-Wnt complexes are then diluted during cell divisions (Farin et al., 2016).

Interestingly, the Wnt target gene *Lgr5*[9] has been identified as CBCs marker gene, because it is expressed in a population of proliferating cells located between Paneth cells, and because it enables maintenance of long-term self-renewal of the intestinal epithelium (Barker et al., 2007). Of note, *Lgr5* marks also stem cells of colon, stomach and hair follicle (Barker et al., 2010; Barker et al., 2007; Jaks et al., 2008). LGR5 transmembrane receptor binds to RSPO and induces a Wnt signalling response through associated FZD–LRP5/6 complexes (de Lau et al., 2011). Furthermore, the interaction with RSPO prevents degradation of FZD receptors, adding a second positive layer of

[1] TCF = Lymphoid enhancer-binding factor/T-cell factor
[2] Axin = Axis inhibition protein
[3] APC = Adenomatous polyposis coli
[4] GSK3 = Glycogen synthase kinase 3
[5] CK1α = Casein kinase alpha 1
[6] β-TrCP = Beta-Transducin Repeat Containing E3 Ubiquitin
[7] LRP5/6 = Low-Density Lipoprotein Receptor-Related Protein 5/6
[8] TLE = Transducin-like enhancer protein
[9] Lgr5 = Leucine-rich-repeat-containing G-protein-coupled receptor 5

regulation on Wnt signalling (Farin et al., 2016). RNA[1] sequencing analysis of LGR5 positive and negative cells allowed for identification of new stem cell markers such as *Ascl2*[2], another Wnt target gene, and *Olfm4*[3], which is a secreted peptide with anti-inflammatory properties. Gain and loss of function experiments on *Ascl2* revealed its important role in regulating stemness in the intestinal epithelium (van der Flier et al., 2009).

### 3.3.2. EGF pathway

EGF signalling is a second key regulator of the stem cell population in the intestine. This has been shown by exogenous administration of EGF in rats which leads to increased proliferation in the intestinal epithelium (Marchbank et al., 1995). Like Wnt ligands, EGF is produced by Paneth cells and subepithelial mesenchyme (Farin et al., 2012; Sato et al., 2011). EGF ligand binds to the receptor EGFR[4], which is member of the ErbB[5] family of receptor tyrosine kinases highly expressed by stem cells and TA cells. EGF induces receptor dimerization and autophosphorylation, which allows for recruitment and activation of downstream effectors that initiate proliferation signals (Jorissen et al., 2003) (Figure 2). A negative feedback loop mediated by LRIG1[6] restricts the action of EGF and maintain tissue homeostasis. In particular, LRIG1, expressed by proliferating cells of the intestinal crypts, downregulates ErbB receptor expression and activity, while LRIG1 knock out (KO) increases the amount of stem and TA cells (Wong et al., 2012).

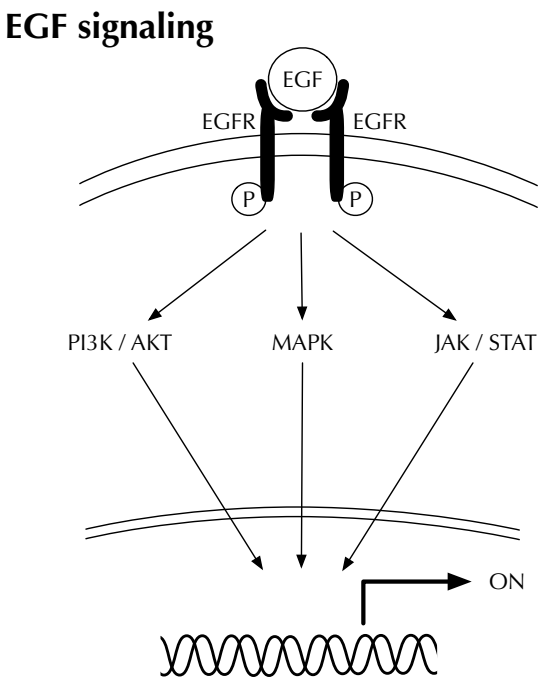


*Figure 2. EGF pathway. Activation of EGFR by the growth factor EGF induces receptor dimerization and auto-phosphorylation. This results in the recruitment and activation of down-stream signalling cascades such as PI3K/AKT, MAPK, JAK/STAT.*

[1] RNA = Ribonucleic acid
[2] Ascl2 = Achaete scute-like 2
[3] Olfm4 = Olfactomedin 4
[4] EGFR = Epidermal growth factor receptor
[5] ErbB = ErbB protein family
[6] Lrig1 = Leucine-rich repeats and immunoglobulin-like domains protein 1

### 3.3.3. BMP pathway

BMP signalling restrains cell proliferation in the intestine, although the exact mode of action remains unclear (Auclair et al., 2007; Haramis et al., 2004; He et al., 2004). The gradient of BMP and Wnt signalling cascades along the crypt-villus axis is opposite, with high expression of BMP ligands in the mesenchymal and villus cells, and low in the crypt compartment. BMP binding to the respective receptor activates SMAD[1] proteins that promote cell differentiation by gene expression regulation in the nucleus (Figure 3). BMP inhibitors, such as Noggin, are produced by the mesenchyme around the crypts and bind antagonistically to BMP ligands to prevent interaction with BMP receptors. Accordingly, BMP receptor KO or Noggin transgenic expression lead to hyperproliferative crypts (Haramis et al., 2004; He et al., 2004).

**BMP signalling**

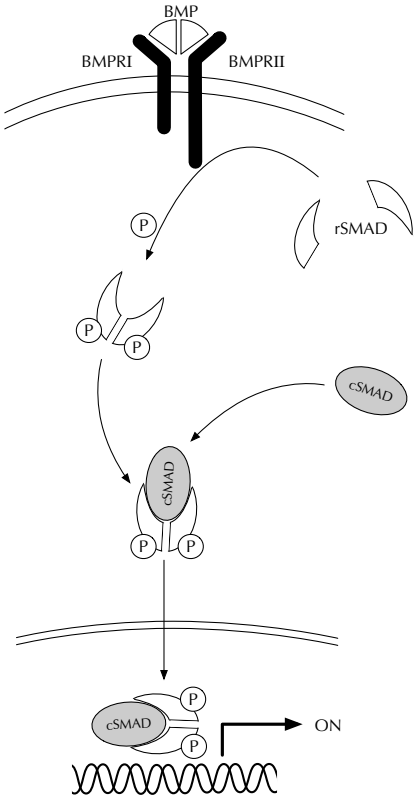


*Figure 3. BMP pathway. BMP ligands induce dimerization and activation of the receptors BMPRI and BMPRII. This event leads to phosphorylation and dimerization of two receptor-regulated SMAD (rSMAD) that are now able to bind common SMAD (cSMAD). The protein complex can translocate to the nucleus to regulate target gene expression.*

### 3.3.4. Notch pathway

Another signalling cascade active in stem cells and TA cells of the intestinal epithelium is the Notch pathway. Notch maintains proliferative cells and inhibits cell differentiation; moreover, Notch is an important regulator of cell fate through the mechanism of lateral inhibition (Fre et al., 2005; Pellegrinet et al., 2011; VanDussen et al., 2012). Notch signalling is initiated by receptor-ligand interaction between neighbouring cells — TA cells that express Notch receptors are prompted to acquire an absorptive fate while TA cells expressing Notch ligand are prompted to a secretory fate. In brief, binding of Notch ligand to the receptor triggers a series of proteolytic events on the receptor mediated by gamma secretase proteases that lead to the release of the Notch intracellular domain (NICD). NICD is then able to translocate into the nucleus and induce the expression of target genes through the association with the RBP-J[2] transcription factor (Figure 4).

[1] SMAD = Small mother against decapentaplegic
[2] RBP-J = Recombinant signal binding protein for immunoglobulin Kappa J region

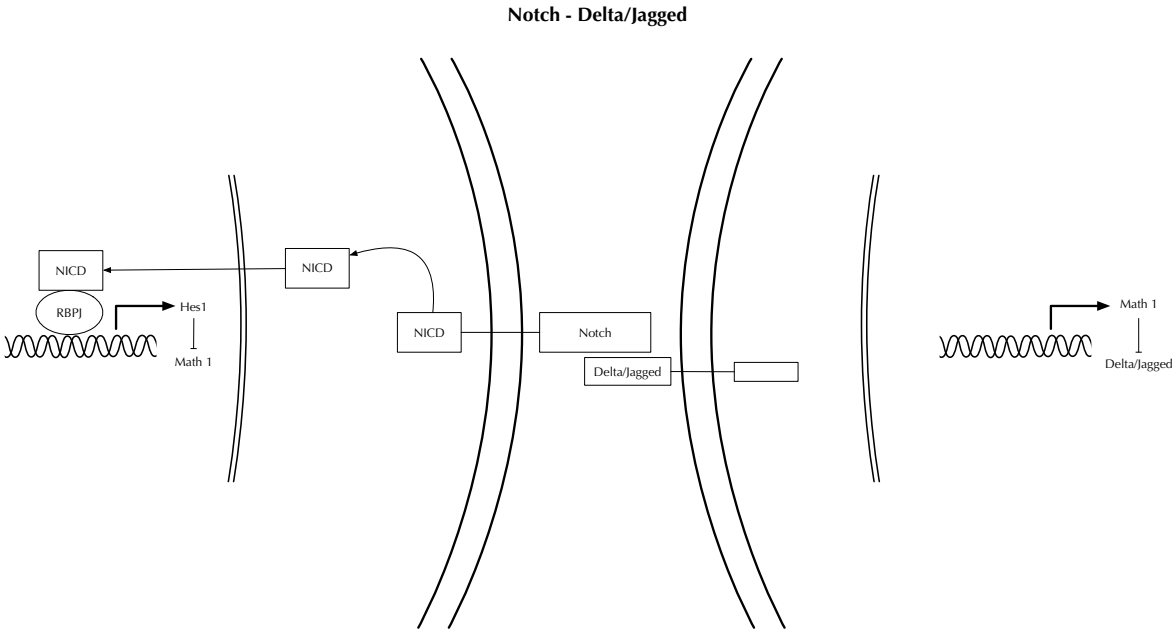


*Figure 4. Notch pathway. The interaction of Notch receptor with the ligand (Delta-like or Jagged) exposed on the membrane of an adjacent cell induces two subsequent cleavage events on the receptor and release of the Notch intracellular domain (NICD). NICD translocates to the nucleus and activate transcription of target genes, such as Hes1. Hes1 is a TF and represses the expression of the Math1 which normally promotes Notch ligand expression.*

Among the target genes, *Hes1*[1] is a TF that negatively regulates the expression of *Math1*[2], the inducer of Notch ligand expression and of secretory lineage (Sancho et al., 2015). In this way, Notch signalling restrains differentiation into the secretory fate. *Hes1* inhibition causes enrichment of secretory cells, reduction of enterocytes, and proliferation, while *Math1* deletion, in contrast, leads to depletion of secretory cells (Jensen et al., 2000; Shroyer et al., 2007; Yang et al., 2001). Similar results have been obtained with gamma secretase inhibitors that impede cleavage of NICD, and constitutively active Notch receptor (Fre et al., 2005; Milano et al., 2004; van Es et al., 2005b).

Notch1 and Notch2 receptors are expressed in proliferating cells in the crypt, while the Notch ligands *Dll1*[3] and *Dll4*[4] are expressed by Paneth cells and early secretory progenitors (Fre et al., 2011). Additionally, *Dll4*, along with Notch ligands *Jag1*[5] and *Jag2*[6], is expressed by secretory progenitors in the upper part of the crypt and by secretory cells in the villi (Schroder and Gossler, 2002; Shimizu et al., 2014). Due to this organisation, Paneth cells are able to activate the Notch signalling cascade in stem cells, preventing differentiation, while cells that exit the stem cell niche and are no longer in contact with Paneth cells stochastically downregulate Notch receptor and commit to secretory lineage. These latter cells that present Notch ligands on their membrane trigger NICD maturation in neighbouring cells, which causes them to eventually differentiate into enterocytes (Clevers, 2013).

[1]Hes1 = Hairy and enhancer of split-1
[2] *Math1* also known as *Atoh1* (atonal homolog 1)
[3] Dll1 = Delta like canonical Notch ligand 1
[4] Dll4 = Delta like canonical Notch ligand 4
[5] Jag1 = Jagged 1
[6] Jag2 = Jagged 2

Beyond the absence of Notch receptors, specification into one of three secretory cell types, Paneth, goblet and enteroendocrine cells, is dependent on the presence of several other signals. Paneth cell differentiation requires for example high Wnt and absence of Notch, and depends on TF SPDEF[1], without which both Paneth and goblet cell formation is impaired (Gregorieff et al., 2009). Low Wnt and an absence of Notch drive goblet and enteroendocrine fate. Goblet cells are also positively affected by Interleukins (ILs), while enteroendocrine cells are specified by the TF NEUROG3[2] (Gehart et al., 2019; Gerbe et al., 2016; Jenny et al., 2002; Lopez-Diaz et al., 2007; von Moltke et al., 2016). Tuft cells originate from DLL1 positive cells but are independent of MATH1 and are not considered secretory cells. Instead, Tuft specification is mediated by the TF POU2F3[3]. Moreover, they are also sensitive to and produce interleukins under helminths infection. ILs release increases goblet cell differentiation and mucus secretion, helping the elimination of the parasites (Gerbe et al., 2016; von Moltke et al., 2016).

## 3.4. INTESTINAL EPITHELIUM HOMEOSTASIS AND REGENERATION

The intestinal epithelium has great self-renewal ability, both in steady state condition and in regeneration. 14-16 LGR5 positive cells reside in the crypts of the mouse intestinal epithelium and divide with a frequency of around 24 hours. Crypt homeostasis is obtained at population level through LGR5 positive cell divisions and generation of new stem cells and/or TA. Stem cell loss by differentiation is balanced by symmetric division of other stem cells. This process of stochastic loss and replacement of stem cells is defined as neutral drift dynamics and leads to crypt monoclonality within 1-6 months, as shown by lineage-tracing experiments (Lopez-Garcia et al., 2010; Snippert et al., 2010). The stemness potential is also determined by the spatial position within the crypt where equipotent cells compete for limited space. Dividing stem cells that remain in the stem cell niche keep stem features, while cells that are displaced from this area become TA cells (Ritsma et al., 2014).

Stemness is no longer believed to be an inherent property of a cell, but is defined by the integration of intracellular and extracellular signals sent by the environment and neighbouring cells, see results on page 34 (Chacon-Martinez et al., 2018; Mayr et al., 2019)**.** Indeed, several studies report how progenitor or more committed cells re-acquire stemness functionality and restore tissue homeostasis after extensive crypt damage and stem cells loss. An example of this dynamics is exposure to radiation, following by DLL1 high secretory progenitor cells regaining contact with Paneth cells and niche factors causing them to dedifferentiate and maintain long-term absorptive and secretory lineages (van Es et al., 2012b). Similarly, Paneth cells can re-activate Notch signalling, re-acquire CBCs signature, and become multipotent (Yu et al., 2018). Absorptive progenitors,

[1] SPDEF = SAM pointed domain containing ETS transcription factor
[2] NEUROG3 = neurogenin 3
[3] POU2F3 = POU class 2 homeobox

marked by ALPI[1], are also able to reconstitute the LGR5 positive population of cells and the epithelial homeostasis following damage (Tetteh et al., 2016).

The existence of a second pool of quiescent, reserve stem cells that re-activates upon tissue damage is still under debate. They are believed to be slowly cycling cells located between the uppermost Paneth cell and the TA cells (position +4 from the bottom of the crypt, hence their name "+4 cells") and marked by expression of CBCs genes *Bmi1*[2], *Tert*[3], *Hopx*[4], and *Lrig1* (Grun et al., 2015; Montgomery et al., 2011; Munoz et al., 2012; Powell et al., 2012; Sangiorgi and Capecchi, 2008; Takeda et al., 2011; Wong et al., 2012). The exact nature of these "reserve stem cells" is still not clear, but some lineage tracing experiments have shown that +4 cells contribute to crypt regeneration after injury and that they are not responsible for tissue turnover in homeostatic condition (Buczacki et al., 2013; Tian et al., 2011).

In summary, studies up to date present remarkable plasticity in the intestinal epithelium that ensures tissue integrity. Both putative reserve stem cells and cells with varying level of differentiation are able to reacquire stem cell status when subjected to specific environmental cues. A possible explanation to cell plasticity comes from the observation that many intergenic regulatory regions are equally accessible to TFs in LGR5 positive cells and progenitor cells (both secretory and absorptive lineages), suggesting that cellular fate eventually remains to be determined by the totality of signals coming from neighbouring cells and the environment. (Kim et al., 2014).

## 3.5. ORGANOIDS AS A MODEL SYSTEM

Self-organisation can be modelled *in vitro* using organoid, embryoid, or gastruloid cultures. Organoids are three-dimensional (3D) structures of cells that mimic the spatial and temporal organisation and formation of organs and tissues and depict their functionality. The starting material can be a population of pluripotent stem cells or isolated organ progenitors cultured in a 3D extracellular environment. From these cultures, in the presence of specific niche and growth factors, it is possible to observe spontaneous growth into ordered structures.

In a similar way embryoids and gastruloids, both derived from aggregates of differentiating embryonic stem cells, can recapitulate initial phases of embryonic development, cell type specification, and body axes formation (Beccari et al., 2018; Harrison et al., 2017; Rossi et al., 2018; Simunovic and Brivanlou, 2017; van den Brink et al., 2014).

Many protocols have been developed in the past years to derive various organoid systems, such as intestinal, pancreatic, lung, brain and kidney, among others

(Barker et al., 2010; Dahl-Jensen and Grapin-Botton, 2017; Eiraku et al., 2011; Greggio et al., 2013; Huch et al., 2013a; Huch et al., 2013b; Lancaster and Knoblich, 2014; Lancaster et al., 2013; Rookmaaker et al., 2015; Rossi et al., 2018; Sasai, 2013; Sato and

[1] ALPI = Intestinal alkaline phosphatase
[2] Bmi1 = Polycomb ring finger BMI proto-oncogene
[3] Tert = Telomerase reverse transcriptase
[4] Hopx = HOP homeobox

Clevers, 2013; Sato et al., 2011; Sato et al., 2009; Simunovic and Brivanlou, 2017; Werner et al., 2017).

A common aspect across organoid cultures is the presence of multiple cell types. These cell types and their organisation depend on the tissue or organ of origin. Spatial organisation that arises in organoids results in the establishment of a stem cell niche, the expansion of progenitor cells, and the maintenance of differentiated cells. This makes organoids a good model system for the study of tissue development, homeostasis, disease progression, regeneration, and orchestration and organisation in multicellular organisms in general. The accessibility of these multicellular 3D-structures facilitates the study of many biological processes that are difficult to study *in vivo*. Indeed, organoids have most advantages of the *in vitro* culture systems, while maintaining the cellular complexity of the *in vivo* counterpart. Many organoid cultures can be easily expanded to reach an optimal sample size (hundreds or thousands) that makes large-scale studies of chemical and genetic perturbations feasible. Such models allow for compound and CRISPR[1] screens in a situation with preserved physiological cell types and interactions (Rookmaaker et al., 2015; Rossi et al., 2018; Xavier da Silveira Dos Santos and Liberali, 2018). Moreover, with the combination of mRNA[2] sequencing and high-content imaging technologies it is now possible to follow the development of an organoid at the single cell level, resulting in unprecedented morphological and molecular description of such process (Mayr et al., 2019). This allows for a simultaneously detailed and holistic understanding of the processes governing for example self-renewal, cell differentiation, and de-differentiation, as well as the dynamics in cell-cell and cell-environment interactions both in physiological conditions and disease. Indeed, organoids both derived from biopsy samples or genetic manipulations, are used to study the molecular pathogenesis of several illnesses, perform drug screenings to discover new possible efficient treatments, or identify targets for personalised medicine. Furthermore, they have been tested for cell therapy and engraftment into mouse models to regenerate injured tissues (Rookmaaker et al., 2015; Rossi et al., 2018; Schweiger and Jensen, 2016).

In the work reviewed in this thesis, small intestine mouse organoids have been employed to study the process of self-organisation and symmetry breaking and will be introduced further in the following subchapter.

### 3.5.1. Mouse intestinal organoids

The first intestinal organoid culture was established shortly after the discovery of the LGR5 receptor as a marker of crypt base columnar stem cells in the intestine (Barker et al., 2007; Sato et al., 2009). Both single LGR5 positive cells and whole crypts extracted from the mouse gut give rise to 3D crypt-villus structures that resemble the intestinal epithelium both morphologically and functionally, when grown in appropriate conditions that prevent apoptosis and sustain proliferation. Intestinal organoids are

[1] CRISPR = Clustered regularly interspaced short palindromic repeats
[2] mRNA = messenger ribonucleic acid

monolayers of cells spatially organized around a central lumen. Crypt regions budding outwards contain LGR5 positive stem cells, Paneth cells and TA cells. Villus regions are populated by enterocytes, goblet and enteroendocrine cells. In brief, intestinal organoids can be derived from a single LGR5 positive stem cell plated in the extracellular matrix (ECM) Matrigel with a uniform distribution of growth factors. The cell starts dividing and gives rise to a round symmetric sphere in which only few cells start differentiating into Paneth cells. Paneth cells are able to produce and secrete WNT3A to neighbouring cells, which is important for the formation and maintenance of a stem cell niche. The establishment of a WNT3A gradient around Paneth cells induces local hyperproliferation and the formation of a bud that will further develop into the counterpart of the crypt compartment of the intestinal epithelium. Cells that are not under Wnt stimulation will differentiate and form the villus compartment, see results page 32 (Serra et al., 2019). One of the advantages of using intestinal organoids is the possibility of long-term culture without affecting their properties. Intestinal organoids can be dissociated mechanically into small fragments or single cells and re-plated to generate new organoids that will develop within a week (Sato et al., 2009).

Culture conditions have been optimised to promote cell survival and organoid growth. Isolated intestinal cells undergo anoikis when detached from the ECM. This is a form of cell death characteristic of anchorage-dependent cells (Gilmore, 2005). Therefore, single cells are plated in Matrigel, a laminin rich supportive stroma produced by Engelbreth-Holm-Swarm (EHS) tumour cells that recreates the *in vivo* environment and allows cell survival through integrin signalling (Hughes et al., 2010).

Recently, synthetic ECMs have been applied to intestinal stem cell (ISC) culture in order to identify the minimal set of factors required for organoid formation. Polyethylene glycol hydrogels reproduce Matrigel physical properties, while fibronectin, laminin and collagen provide biochemical signals that sustain organoid development (Gjorevski et al., 2016). Cell proliferation is promoted in the intestinal crypts by Wnt signalling, therefore an exogenous source of WNT3A ligand is required until Paneth cells differentiate in the round spheroid. The addition in the culture medium of the Wnt agonist RSPO which binds to the LGR5 receptor, prevents the degradation of Wnt receptors and thereby increases Wnt signalling. Another growth factor supporting cell survival and proliferation is EGF. Noggin, a BMP inhibitor, is essential to maintain *Lgr5* expression and, therefore, stem cells properties (Wallach and Bayrer, 2017).

Foetal enterospheres (FEnS) are highly proliferative intestinal organoids derived from foetal murine intestine before postnatal day 2 (P2). They maintain a spherical structure and do not have differentiated cells. Importantly, FEnS grow independently of Wnt and RSPO and are composed of progenitor cells expressing low levels of adult stem cell markers. The maturation of the intestinal epithelium associates with presence of Paneth cell and *Lgr5* expression. Therefore, Wnt signalling seems to be involved in the transition from foetal to adult tissue. In support of this assumption, addition of exogenous Wnt molecules to single cells extracted before P2 allows the formation of mature budding organoids (Fordham et al., 2013). FEnS have different proliferative capacity and cell composition compared to adult organoids, and represent a useful tool to study processes of intestinal epithelium development *in vitro*.

Even though intestinal organoids are extensively used in research, a molecular understanding of how this model system develops and recapitulates self-organisation has been lacking. During my PhD studies, I have investigated the mechanisms and signalling pathways that drive organoid formation and identified the TF YAP1[1] as master regulator of this process.

## 3.6. YAP AND THE HIPPO PATHWAY

The Hippo pathway is a highly conserved cascade of kinases that controls tissue growth, regeneration, cell survival, and proliferation by activating Yes-Associated Protein (YAP) through phosphorylation events. YAP is a transcriptional co-regulator initially identified as an interacting protein of YES1[2]. In particular, a short proline-rich region of YAP was shown to bind the SH3[3] domain of YES1, as well as of other proteins (Sudol, 1994). In addition, the WW domain, another important region in YAP structure that mediates protein interaction, was described shortly after. The WW domain is characterized by the presence of two tryptophan (W) and recognises a PPxY motif (where P is proline, x any amino acid and Y tyrosine) (Chen and Sudol, 1995; Sudol et al., 1995). The PPxY motif is found in the activation domain of several TFs (Komuro et al., 2003; Mosser et al., 1998; Strano et al., 2001; Yagi et al., 1999), highlighting the co-transcriptional activity of YAP. YAP does not itself contain a DNA[4]-binding domain, but exerts transcriptional modulation through the carboxy-terminal transcription activation domain (TAD), when brought into proximity of promoters by interaction with TFs (Yagi et al., 1999). Several YAP splicing isoforms exist and have been grouped into YAP1-1 (YAP1) isoforms when carrying one WW domain and YAP1-2 (YAP2) when carrying two WW domains (Sudol et al., 1995). Further YAP isoform variability is mediated by differences in length and subdomains of TAD (Gaffney et al., 2012).

### 3.6.1. Hippo pathway dependent modulation of YAP activity

YAP is a direct downstream effector of the Hippo pathway (Figure 5). YAP is active and able to translocate into the nucleus when dephosphorylated (Hippo signalling cascade off), while inactive when phosphorylated on specific residues (Hippo signalling cascade on). The signalling cascade of the Hippo pathway starts with TAO1/2/3[5]-mediated phosphorylation of the activation loop of the serine/threonine kinase MST1/2[6] (also known as STK4/3) (Boggiano et al., 2011; Poon et al., 2011). Then, active MST1/2 dimerises with the protein SAV1[7] at the plasma membrane and phosphorylates both SAV1 and MOB1A/B[8] (Callus et al., 2006; Praskova et al., 2008). This in turn creates a

[1] YAP1 = Yes-associated protein 1
[2] YES1 = Yes proto-oncogene 1
[3] SH3 = SRC Homology 3
[4] DNA = Deoxyribonucleic acid
[5] TAO1/2/3 = Thousand and one amino acid protein kinase 1/2/3
[6] MST1/2 = Hepatocyte growth factor-like protein precursor 1/2
[7] SAV1 = Salvador homolog 1
[8] MOB1A/B = Mps one binder kinase activator like 1B

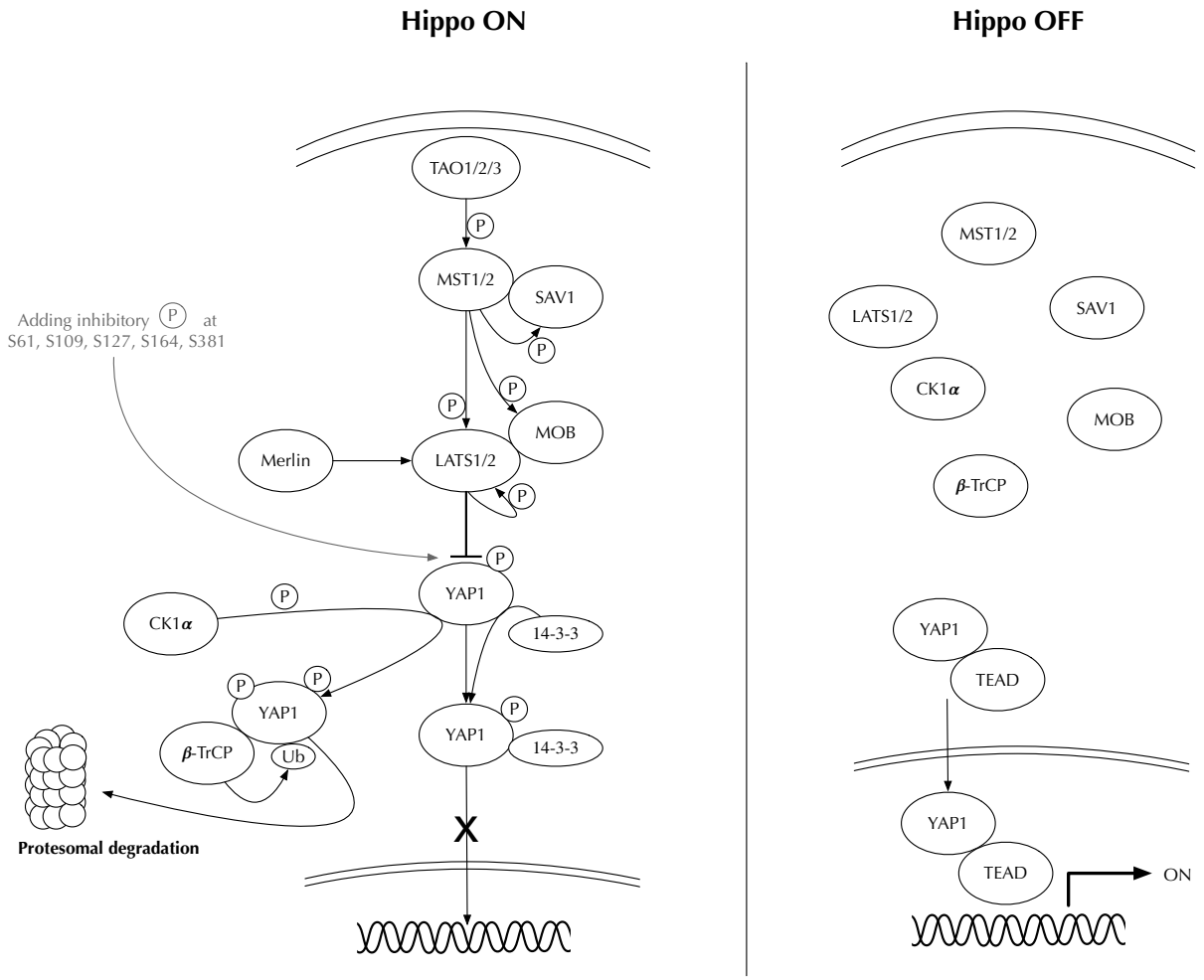


*Figure 5. Hippo pathway. The Hippo signalling cascade is mediated by a series of phosphorylation events that end with the recruitment and inhibition of YAP1 protein. Therefore YAP1 is inactive when the Hippo pathway is ON, and active when the Hippo pathway is OFF. Active YAP1 is able to interact with TF partners, such as TEAD, and regulate target gene expression in the nucleus.*

protein scaffold complex able to recruit and activate LATS1[1] and LATS2[2] (Hergovich et al., 2005; Hergovich et al., 2006). Of note, LATS1/2 translocation to the cellular membrane is also facilitated by the interaction with the protein Merlin[3] (Yin et al., 2013). Finally, LATS1/2 phosphorylation by MST1/2 (Chan et al., 2005; Tamaskovic et al., 2003) primes LATS1/2 autophosphorylation in the activation loop to be fully functional.

Active LATS1 and LATS2 can directly phosphorylate YAP on five serine amino acids found within the consensus motif HxRxxS (where H is histidine, x any amino acid, R arginine and S serine) and inhibit it (Hao et al., 2008; Zhao et al., 2007; Zhu et al., 2015). The five serine amino acids are S61, S109, S127, S164 and S381. Therefore, a mutated form of YAP with substitution of all the five serine amino acids with alanine (YAP5SA) can be used to study the effect of YAP activity. Phosphorylation of S381 and S127 have the highest impact on YAP functionality. Phosphorylation of S127 leads to YAP inhibition through several means, as it allows the interaction between YAP and 14-3-3 proteins that

[1] LATS1 = Large tumour suppressor kinase 1
[2] LATS2 = Large tumour suppressor kinase 2
[3] Merlin is the protein name while Neurofibromin 2 (NF2) is the gene name

maintain YAP in the cytoplasm (Basu et al., 2003; Dong et al., 2007; Muslin and Xing, 2000; Zhao et al., 2007), just like it may interfere with the transcriptional role of YAP by blocking part of the TAD or the transcription factor binding domains. Dephosphorylation of S127 by the protein phosphatase 1 (PP1) completely re-establishes YAP activity (Wang et al., 2011a). Phosphorylation of S381 promotes further phosphorylation by CK1. This phosphorylation is then recognised by β-TRCP, a subunit of the ubiquitin protein ligase complex SCF[1], that induces YAP polyubiquitination and degradation (Fuchs et al., 2004; Ma et al., 2018; Zhao et al., 2010).

At the transcriptional level, LATS2 expression is positively regulated by YAP (among other TFs), therefore creating a feedback loop that prevents YAP hyperactivation but maintains tissue homeostasis (Furth and Aylon, 2017; Moroishi et al., 2015).

### 3.6.2. Hippo pathway independent modulation of YAP activity

Several other proteins that are not core components of the Hippo pathway, such as AMOT[2], Claudin 18, CDK1[3], PTPN14[4], and α-catenin interact with YAP and prevent its nuclear localisation (Nakajima et al., 2017; Schlegelmilch et al., 2011; Wang et al., 2011b; Yu et al., 2015; Zhao et al., 2011). Moreover, additional kinases including NDR1/2[5] and AKT[6] have been reported to phosphorylate YAP on S127 and thereby inhibit its activity (Basu et al., 2003; Zhang et al., 2015). YAP cytosolic sequestration is also mediated by methylation of lysine 494 by SET-7[7], while its nuclear localisation is primarily dependent on the binding of the TF TEAD[8] (Oudhoff et al., 2013). Not all YAP phosphorylation events have an inactivating effect. Indeed, YAP transcriptional activity increases by tyrosine phosphorylation of Y357 by c-Abl[9], or S128 phosphorylation by NLK[10] (Hong et al., 2017; Levy et al., 2008; Li et al., 2016; Moon et al., 2017; Taniguchi et al., 2015). The latter prevents binding of YAP to 14-3-3[11] even when the Hippo pathway is on. YAP activity and localisation is regulated also by cellular metabolic conditions and sensors of energy stress. This modulation can be explained by the knowledge that YAP promotes cell proliferation, and cell proliferation requires adequate levels of cellular energy.

One of these sensors is AMPK[12]. Under glucose deprivation AMPK directly blocks YAP activity by a phosphorylation that impedes YAP-TEAD interaction, and indirectly through LATS1/2 activation (Mo et al., 2015). When glucose is abundant, another detector of metabolic status, OGT[13] (part of the Hexosamine biosynthesis pathway) interacts with

[1] SCF = Skp1-Cul1-F-box
[2] AMOT = Angiomotin
[3] CDK1 = Cyclin-dependent kinase 1
[4] PTPN14 = Protein Tyrosine phosphatase non-receptor type 14
[5] NDR1/2 = Nuclear dbf2-related kinase 1/2
[6] AKT = Protein kinase B
[7] SET7 = SET-domain-containing lysine methyltransferase
[8] TEAD = TEA domain transcription factor
[9] C-Abl = Yes/Src and Abelson tyrosine-protein kinase 1
[10] NLK = Nemo Like Kinase
[11] 14-3-3 = 14-3-3 proteins
[12] AMPK = AMP-activated protein kinase
[13] OGT = O-GlcNAc transferase

YAP and prevents LATS-mediated inhibition (Peng et al., 2017). Similarly, glucocorticoids and the mevalonate pathway are positive YAP stimulators (Sorrentino et al., 2014; Sorrentino et al., 2017). Furthermore, cellular stress, such as hypoxia, oxidative, or endoplasmic reticulum stress, regulates the Hippo pathway and YAP activity in order to maintain tissue homeostasis (Lin et al., 2017; Ma et al., 2015; Shao et al., 2014; Wu et al., 2015).

## 3.7. YAP IN MECHANOTRANDUCTION

YAP is a mechanosensor protein able to translate physical and mechanical cues into biochemical signals that regulate cell behaviour. YAP activity is therefore influenced by the cellular microenvironment and the physical and spatial conditions.

### 3.7.1. Cell-ECM interaction and cell spreading

Both high stiffness in the ECM and cell spreading induce increased formation of focal adhesions (FAs), which in turn activate YAP via multiple mechanisms (Yeh et al., 2017). One such mechanism involves focal adhesion kinases (FAKs) which regulate YAP subcellular localisation by modulating actin-myosin and stress fibre dynamics through RHO-A[1] and ROCK[2] signalling (Dupont et al., 2011; Zhao et al., 2012). Normally, YAP can move freely in and out of the nucleus but under tense cytoskeletal conditions the nuclear exit is prevented (Dupont et al., 2011; Sansores-Garcia et al., 2011). Several studies describe a correlation between actin cytoskeleton and YAP functionality, but the exact mechanism underlying this observation is still not clear (Aragona et al., 2013; Wada et al., 2011). For example, F-acting capping/severing proteins block YAP nuclear localisation by destabilisation of the cytoskeleton in low mechanical stress conditions. YAP inactivation is also observed in dense populations of cells. Dense cells have a small surface interacting with the ECM, establish less FA contacts, and experience weaker mechanical stress compared to sparse cells (Aragona et al., 2013). Conversely, the induction of mechanical stress or inhibition of F-actin capping/severing proteins on dense cells will rescue YAP activation. These observations highlight again the fundamental role of mechanical cues in modulating YAP functionality (Aragona et al., 2013). The mechanical regulation on YAP is permissive for additional modulation on YAP activity by the Hippo pathway (Zhao et al., 2012). Indeed, it has been shown that depletion of LATS1/2 does not induce YAP activity in the presence of a soft ECM (Aragona et al., 2013).

Alternative mechanisms have been proposed to link cytoskeleton and YAP status: 1) YAP interaction with the cytoskeleton exposes or blocks regulatory sites of the protein (Halder et al., 2012); 2) YAP regulators are either trapped or released by the cytoskeleton under defined mechanical cues. For instance, the interaction of actin with the aforementioned protein AMOT reduces the antagonistic binding of AMOT to YAP. However, phosphorylation of AMOT by LATS prevents the actin-AMOT contact (Chan et al., 2013; Dai et al., 2013; Mana-Capelli et al., 2014). AMOT, in turn, promotes activation of the

[1] RHO-A = Ras homolog family member A
[2] ROCK = Rho associated coiled-coil containing protein kinase 1

Hippo pathway component Merlin, and LATS phosphorylation in a positive feedback loop (Li et al., 2015).

The cytoskeleton and nucleoskeleton are connected through the protein complex LINC[1]. Therefore, the cytoskeleton transmits mechanosensing responses inside the nucleus that influence the nucleus architecture. Of note, the actin cytoskeleton can induce nucleus flattening and nuclear pore stretching allowing YAP nuclear transport (Elosegui-Artola et al., 2017).

The positive regulation on YAP by FAK and the additional FA components integrins is also mediated through activation of the SRC signalling cascade. In brief, SRC can phosphorylate both LATS1, preventing LATS1 activity (Elbediwy et al., 2016; Kim and Gumbiner, 2015; Si et al., 2017), and YAP-TAD, promoting YAP nuclear localisation and interaction with TEAD (Li et al., 2016). A positive feedback loop is then established by YAP which induces FA protein expression and cytoskeleton stability (Nardone et al., 2017).

### 3.7.2. Cell crowding and cell polarity

Cell crowding is another environmental signal that regulates YAP activity. YAP promotes cell proliferation but once the cell population has reached a defined size, the population inhibits YAP to maintain tissue homeostasis (Zhao et al., 2007). Cells regulate YAP status in response to sensing their spatial context through FA, desmosomes, adherens junctions (AJs), and tight junctions (TJs)(Ma et al., 2018). At AJs, for example, the proteins Kibra and Merlin initiate the Hippo pathway by interaction with LATS, and limit tissue growth (Xiao et al., 2011; Yin et al., 2013; Yu et al., 2010). E-cadherin homophilic binding and recruitment of α- and β-catenin, components of AJs, can induce density dependent YAP nuclear exit and contact inhibition of proliferation (CIP) (Kim et al., 2011). In particular, E-cadherin/catenin complexes respond to cell-cell contact by activating LATS and thereby reduce cell proliferation. Complete growth inhibition is observed only under high cell confluence. In this condition, cells adhere to a small ECM area and experience low mechanical cytoskeleton tension that modulate once again YAP subcellular localisation (Aragona et al., 2013). α-catenin can also bind directly to phosphorylated YAP S127 and 14-3-3 at AJs. Such connection prevents YAP de-phosphorylation by the protein phosphatase 2A (PP2A), and YAP nuclear localisation (Schlegelmilch et al., 2011).

In addition, cell density increases the expression of PTPN14 which mediates cytosolic transfer of YAP from the nucleus and retention at AJs (Liu et al., 2013; Wang et al., 2012).

Finally, cell polarity is another influencer of YAP activity. Crumb proteins (CRB), for example, localize at the apical domain of the cell membrane and facilitate the interaction of LATS and YAP at the apical junctions, therefore promoting YAP phosphorylation and cytoplasmic localisation (Szymaniak et al., 2015).

[1] LINC = Linker of nucleoskeleton and cytoskeleton

## 3.8. YAP TRANSCRIPTIONAL ACTIVITY

The transcriptional activity of YAP varies in different cell types and environmental conditions, and depends on the interacting TF. TEAD1-4 are among the best characterized partners of YAP but several others have been identified, such as AP1[1], SMAD, OCT4[2], KLF4[3], RUNX1/2[4], ErbB4 and p63/p73[5] (Alarcon et al., 2009; Galli et al., 2015; Komuro et al., 2003; Lapi et al., 2008; Okazaki et al., 2012; Stein et al., 2015; Yu et al., 2015; Zanconato et al., 2015). TEAD modulates YAP activity by enabling YAP interaction with DNA and also by controlling YAP subcellular localisation. In fact, TEAD deletion leads to YAP nuclear exclusion even in the absence of LATS inhibition (Lin et al., 2017). Several studies report how YAP-TEAD regulate gene expression by binding prevalently to distal enhancers and super enhancers located more than 10 kilobases away from the relative transcription start sites (TSSs) (Galli et al., 2015; Stein et al., 2015; Zanconato et al., 2015). These genes are involved in cell cycle progression and cell growth, and their expression is enhanced by AP1 interaction with the YAP-TEAD complex (Ota and Sasaki, 2008; Zanconato et al., 2015; Zhao et al., 2008). Among YAP-TEAD target genes, CTGF[6] is a well-known growth factor that sustains proliferation and anchorage-independent growth, while CDK6[7] blocks cellular senescence (Xie et al., 2013; Zhao et al., 2008). Gene expression regulation by YAP-TEAD does not always lead to increased expression of target genes. When YAP-TEAD interact with the nucleosome remodelling deacetylase (NuRD - works by inactivating promoter acetylation) it mediates transcriptional repression, as reported for example for the genes *Trail*[8] and *DDIT4*[9] (Kim et al., 2015).

YAP-TEAD transcriptional activity is regulated by VGLL4[10] competitive binding to TEAD. VGLL4 is another protein that lacks DNA binding capacity but carries a binding domain for TEAD and acts as a potential tumour suppressor by impeding the expression of pro-survival and growth promoting genes (Jiao et al., 2014; Zhang et al., 2014).

On the other hand, YAP can promote the major regulator of cell growth mTOR[11] by suppressing its negative regulator PTEN[12] (Tumaneng et al., 2012). Furthermore, YAP recruits the Mediator complex to the DNA target sites which induces RNA PolII[13] promoter release and high rate of transcription (Galli et al., 2015).

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[1] AP1 = Activator protein 1
[2] OCT4 = Octamer-binding transcription factor 3
[3] KLF4 = Krupper-like factor 4
[4] RUNX1/2 = Runt-related transcription factor 1
[5] P63/73 = Tumour protein 63/73
[6] CTGF = Connective tissue growth factor
[7] CDK6 = Cyclin dependent kinase 6
[8] Trail = TNF-related apoptosis-inducing ligand
[9] DDIT4 = DNA-damage-inducible transcript 4
[10] VGLL4 = Vestigial like family member 4
[11] mTOR = Mammalian target of rapamycin
[12] PTEN = Phosphatase and tensin homolog
[13] PolII = Polymerase II

# 3.9. HIPPO PATHWAY IN THE INTESTINAL EPITHELIUM

The Hippo pathway is an important signalling cascade that preserves tissue homeostasis in the intestinal epithelium and ensures regeneration after injury. YAP1 protein expression is active in stem cells and TA cells of the crypt. YAP1 promotes proliferation through TEAD interaction but drives Goblet cell specification by binding to the TF KLF4 (Camargo et al., 2007; Imajo et al., 2015). *In vivo* experiments highlight the role of the Hippo pathway in maintaining tissue balance: *Yap1* overexpression or ablation of the negative regulators *Mst1-2* cause expansion of the proliferative compartment, and decrease differentiation in the villi (Camargo et al., 2007; Zhou et al., 2011). At the same time, high YAP1 activity establishes a negative feedback loop onto itself by inducing LATS2 expression (Dai et al., 2015; Moroishi et al., 2015). YAP1 transcriptional regulation is dispensable during homeostasis — indeed *Yap1* depletion during embryogenesis does not prevent normal development of the intestine. However, inhibition of YAP1 is still required to confine crypt proliferation. This was shown by removing components of the Hippo pathway cascade which leads to increased YAP1 functionality, growth of the undifferentiated domain, and eventually tumorigenesis (Cai et al., 2015; Zhou et al., 2011). YAP1 deficiency, though, hinders crypt restoration after injury. This suggests that the constant expression of an inactive form of YAP1 allows the vulnerable intestinal epithelium to be poised for regeneration (Cai et al., 2015).

### 3.9.1. YAP1 and intestinal regeneration

The role of YAP1 during intestinal regeneration has been extensively studied. As an example, YAP1 activity is observed in the altered tissue architecture of the gut after mouse whole body irradiation. Here, YAP1 drives a regenerative program by suppressing Paneth cell differentiation, and reprogramming LGR5 positive cells into fast proliferating progenitor-like cells that are able to repopulate the damaged crypt. In particular, YAP1 downregulates the transcription of Wnt target genes, including the ISCs markers *Lgr5*, *Olfm4*, and *Ephb3*[1], and the Paneth cells markers *Wnt3* and *Spdef*, and simultaneously promotes proliferation through the EGF pathway (Gregorieff et al., 2015). YAP1-mediated cell reprogramming occurs also in the repairing epithelium of the colon after Dextran Sodium Sulfate (DSS[2]) treatment. Epithelial disruption causes changes in extracellular matrix and cytoskeleton architecture that in turn stimulates YAP1 transcriptional activity to drive tissue restoration. Cells of the regenerating colon are characterised by loss of secretory and adult stem cell markers, and have a genetic profile similar to the one of cells of an early developmental stage, i.e. the foetal epithelium (Yui et al., 2018). Interestingly, this highly proliferative foetal-like state is reached also in inflamed intestine after helminthes infection, irradiation, or LGR5 positive cell ablation (Nusse et al., 2018). This observation highlights the occurrence of a common mechanism of transient reprogramming into a primitive fate that is induced by different types of damage and driven by YAP1 to re-establish crypt integrity. More generally, YAP1 activity

[1] Ephb3 = Ephrin type-B receptor 3
[2] DSS is a chemical compound that induces colitis

facilitates the enrichment of a fast proliferating pool of cells with stem potential which are able to repopulate the injured tissue. Indeed, it has been proposed that a YAP1-dependent expansion of quiescent progenitors, named revival SCs (revSCs), can also reconstitute the stem cell niche following damage (Ayyaz et al., 2019).

### 3.9.2. YAP1-Wnt pathway crosstalk

The interplay between Hippo and Wnt signalling pathways during tissue homeostasis and regeneration is still under debate. Several studies report diverse mechanisms by which YAP1 can inhibit the Wnt cascade. For example, the binding of cytosolic YAP1 to DVL or to β-cat can prevent DVL or β-cat nuclear localisation and Wnt target genes expression. Alternatively, YAP1 association with the destruction complex can facilitate the interaction between β-cat and the ubiquitin ligase that induces β-cat degradation (Azzolin et al., 2014; Barry et al., 2013; Imajo et al., 2012). At the same time, Wnt signalling was shown to promote YAP1 activity by disassembling the destruction complex and allowing YAP1 nuclear translocation (Azzolin et al., 2014). Moreover, WNT3A ligand can also trigger non-canonical Wnt pathway, together with WNT5A, and stimulate YAP1 dephosphorylation and function. In turn, active YAP1 drives the expression of secreted factors that hinder β-cat protein stabilization, and inhibit Wnt pathway (Park et al., 2015). Conversely, other studies sustain a positive role of YAP1 in promoting Wnt signalling. They report a correlation between active YAP1 and nuclear β-cat in mouse intestine under YAP1 overexpression or MST1-2 depletion (Camargo et al., 2007; Zhou et al., 2011).

The interplay between YAP1 and Wnt pathway is highly complex and discrepancies in results described above might arise from different approaches and mouse models used. YAP1 functionality is context dependent and influenced by interacting proteins, both in the nucleus and in the cytoplasm. Moreover, different levels of YAP1 protein abundance and associated TFs can lead to diverse response, such as suppression of differentiation or promotion of secretory lineage fate (Imajo et al., 2015).

## 3.10. AIM OF THE THESIS

Intestinal organoids represent a unique model system to study collective behaviour and self-organisation of single cells. Starting from a population of single LGR5 positive stem cells it is possible to culture 3D crypt-villus structures that mimic the intestinal epithelium both morphologically and functionally. Although signalling pathways and factors essential to support organoid growth and homeostasis have been extensively described, knowledge on the mechanisms underlying their development is still missing. In particular, how single cells have the capacity to collectively organise spatially and temporally in a functional organoid is poorly understood. The aim of this thesis is to characterise the mechanisms of organoid formation and to identify the transcriptional programs, intercellular communications and cell-environment interactions that lead to the gain of new properties and cellular specification.

# 4. RESULT I

ARTICLE

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# Self-organization and symmetry breaking in intestinal organoid development

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***The entire article can be found in the appendix section.***

## 4.1. SUMMARY

In this article, we describe the initial phases of organoid development that lead to the crucial event of symmetry breaking, represented by the differentiation of the first Paneth cell, allowing the maturation of a functional crypt-villus structure.

We follow the generation of organoids from single cells and identify molecular and morphological attributes by combining high resolution imaging of fixed samples at sequential time points, live imaging of reporter cell lines, and RNA-sequencing analysis.

We observe that not only LGR5 positive stem cells but also LGR5 negative cells are able to give rise to mature organoids, and that they follow the same stereotypic pattern of growth. However LGR5 negative cells do so at a lower efficiency. Upon tissue dissociation and single cell isolation, YAP1 initiates a regenerative program that induces cell de-differentiation, fast cell proliferation, and formation of round symmetric cysts. Once cell-to-cell variability in YAP1 localisation and activity arises in the cyst, probably do to local cell crowding, Notch-Delta lateral inhibition is established and this results in the differentiation of Paneth cells and stem cell niche formation. Consequently, villi composed mainly of enterocytes but also goblet and enteroendocrine cells emerge. If the symmetry breaking fails, due to absence of Notch-Delta lateral inhibition, the cyst is not be able to reconstitute a stem cell niche and will develop into a round enterocyst populated by enterocytes only.

In conclusion, our results uncover the molecular mechanisms by which single cells form ordered structures of higher level of complexity when exposed to growth-promoting conditions and via cell-cell interactions, recapitulating the process of self-organisation.

## 4.2. CONTRIBUTION

This work represents the main project of my PhD, thus, I designed, performed, and analysed many experiments. It is also the result of a fruitful collaboration with Urs Mayr and Andrea Boni within our laboratory.

As the one who initiated this project, I initially tested culture and immunostaining conditions of intestinal organoids to set up protocols for time course experiments, and started describing organoid growth phases.

All of us have been involved in the molecular and morphological characterisation of the process of organoid formation starting from a single cell through immunostaining and high throughput imaging of fixed samples at sequential time points. Moreover, our shared efforts using complementary methods allowed the identification of a common path of development for LGR5 positive stem cells and LGR5 negative cells. Andrea followed the organoid growth starting from a single cell by live imaging using a light sheet microscope. Urs performed multiplexed protein staining of the same biological sample (iterative indirect immunofluorescence imaging - 4i) and, from the data collected, inferred a pseudo-time trajectory of organoid development. I focused on bulk RNA-sequencing of samples of chronological progression and described organoid generation through RNA dynamics. Looking at transcripts and transcription factor binding motifs enrichment at initial phases of organoid development, I identified YAP1 protein as putative driver of organoid formation by inducing a regenerative response in single cells. To further validate these results, I chemically and genetically perturbed YAP1 and the Hippo pathway, and observed that YAP1 is necessary but not sufficient to drive organoid development as Wnt signalling is also required in the first 48 hours of growth. Moreover, I found that a transient and cell-to-cell variable activity of YAP1 is necessary to allow symmetry breaking. YAP1 homogeneous activation or YAP1 homogeneous inactivation leads to a failure of symmetry breaking and enterocyst formation. To better understand how YAP1 cell-to-cell variability promotes symmetry breaking, I looked at single cell RNA-sequencing at 72 and 120 hours of organoid development and combined the observations with the ones of Urs obtained by 4i imaging. Together, we found that YAP1 cell-to-cell variability enables the establishment of a Notch-Delta lateral inhibition and subsequent differentiation of a first Paneth cell. Finally, I treated single cells with several compounds inhibiting Notch pathway and perturbing Paneth cells formation to further study the correlation between Notch-Delta signalling and Paneth cells appearance.

# 5. RESULT II

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REVIEW

## Exploring single cells in space and time during tissue development, homeostasis and regeneration

**Urs Mayr[1,2,*], Denise Serra[1,2,*] and Prisca Liberali[1,2,‡]**

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*These authors contributed equally to this work

***The entire review can be found in the appendix* section.**

## 5.1. SUMMARY

In this review, we first discuss why we need to characterise both individual cells and population of cells both spatially and temporally to understand how self-organising systems arise. We then present how a combination of current technologies can allow us to quantitatively bridge spatial and temporal information at the single-cell level and generate a mechanistic understanding of tissue development, homeostasis, and regeneration.

## 5.2. CONTRIBUTION

This review is the final result of exciting discussions between Urs Mayr, Prisca Liberali and me. In the first part, I describe cell state dynamics during tissue development, homeostasis, and regeneration, and the importance of studying their regulation by temporal and spatial conditions, under the titles: Introduction, Cell state changes during development, homeostasis and regeneration: integrating signals in time and space, Spatial regulation of cell states, and Temporal regulation of cell states. In the second part, Urs proceeds to examine existing methods and technologies that together shed light on the rules of tissue organization.

# 6. RESULT III

## *Regenerative landscape of intestinal organoids*

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***The entire unpublished manuscript can be found in the appendix section.***

## 6.1. SUMMARY

Development of intestinal organoids from single intestinal stem cells recapitulates the regenerative capacity of the intestinal epithelium. To unravel molecular mechanisms orchestrating organoid formation and regeneration of intestinal tissue, we developed a high-content image-based screening assay for an annotated compound library. We generated multivariate feature profiles for hundreds of thousands of individual organoids to quantitatively describe the phenotypic landscape of organoid development. The resulting phenotypic fingerprints were then used to infer regulatory genetic interactions from a single screen, establishing a novel paradigm in genetic interaction screening applied to an emergent system. This allowed the identification of modules of genes that regulate cell identity transitions and maintain the balance between regeneration and homeostasis, unravelling novel roles for several pathways, among them retinoic acid signalling. We then characterized a crucial role for retinoic acid nuclear receptors in controlling the exit from the regenerative state and in driving enterocyte differentiation. By combining quantitative imaging with RNA sequencing we confirmed the role of endogenous retinoic acid signalling and metabolism for initiating transcriptional programs that guide intestinal epithelium cell fate transitions and identified a small molecule inhibitor of retinoid X receptor, RXR, that improved intestinal regeneration *in vivo*.

## 6.2. CONTRIBUTION

***This manuscript represents the main project of*** Ilya Lukonin.

I have contributed to this work by setting up and testing a protocol for RNA extraction from mouse intestinal organoids in our laboratory, providing RNA sample and helping with the analysis of the RNA-sequencing data.

# 7. DISCUSSION

In this thesis, I present the results of the study "Self-organization and symmetry breaking in intestinal organoid development". We address the fundamental question of how multicellular asymmetric structures arise as a result of the self-organising behaviour of single stem cells.

During development, homeostasis, and regeneration cells are constantly exposed to a changing environment and to dynamic interactions with neighbouring cells. Investigation of molecular mechanisms inducing distinct cell choices and collective behaviour can help us understand the biological processes and principles at the basis of cellular organization. Here, we characterize, from a single cell, the formation of a complex multicellular 3D-structure, such as the intestinal organoid. We find that not only LGR5 positive stem cells, but also cells of different degree of differentiation have the potential to generate intestinal organoids. This reflects the plasticity of the intestinal epithelium that allows the tissue to repair after injury. In particular, our results show that transient YAP1 activation initiates a regenerative program in isolated single cells that promotes fast proliferation and development of round symmetric cysts. The pivotal break of symmetry is indeed driven by intercellular variability in YAP1 activity. Symmetry breaking enables lineage segregation by establishment of Notch/Delta lateral inhibition and Paneth cell differentiation, and leads to the subsequent specification of crypt and villus domains.

In order to interrogate cells at the exact break of homogeneity, we make use of different quantitative technologies, ranging from automated high-content and 4i imaging, light sheet microscopy, and RNA sequencing. We generate a unique combination of data modalities that give us high spatial and temporal resolution, both at the single cell and organoid level. This allows us to describe the occurrence of self-organization and investigate the importance of cell-to-cell variability in this process. In conclusion, our finding elucidates the mechanisms by which single cells, exposed to homogeneous conditions, exploit their intrinsic potential to generate diverse functional multicellular structures.

## 7.1. YAP1 STIMULATES A REGENERATIVE PROGRAM DRIVING ORGANOID FORMATION

Multiple studies have reported YAP1-dependent reprogramming of several intestinal cell types *in vivo*, as a means to regenerate damaged epithelium (Ayyaz et al., 2019; Gregorieff et al., 2015; Nusse et al., 2018; Yui et al., 2018). In particular, Gregorieff and colleagues showed that YAP1 promotes tissue repair by enriching the pool of rapid cycling cells that can repopulate the epithelium. This is obtained reprogramming LGR5 positive stem cells in transient-amplifying-like cells through inhibition of the Wnt homeostatic program, and stimulating proliferation through the EGFR pathway (Gregorieff et al., 2015). Our *in vitro* data reveal that YAP1 activates a similar program in single cells at the initial phase of organoid formation. Genes of canonical Wnt

pathway and intestinal cell type markers are downregulated, while genes of the YAP1-induced regenerative program are upregulated, including some foetal genes (Fig. 3 and Extended Data Fig. 6e (Serra et al., 2019)). These results suggest that YAP1 induces cell dedifferentiation and transition to a highly proliferative undifferentiated state, and that the process of organoid formation resemble a regenerative response, exploiting the plasticity of the intestine. Previous works from other groups also show that the capacity of single cells to give rise to organoids is not restrained to stem cells (Tetteh et al., 2016; van Es et al., 2012b; Yan et al., 2017). Our time course imaging of sorted LGR5 positive stem cells and LGR5 negative cells shows that organoids formed from the two populations share the same stereotypic pattern of organoid growth. Stem cells, however, have a higher efficiency in generating organoids (Fig. 1, Fig. 2d, f, h, Extended Data Fig. 1d, e, Extended Data Fig. 2f, Extended Data Fig. 3 (Serra et al., 2019)). The difference in efficiency between LGR5 positive and negative cells could be due to the fact that some fraction of the negative cells have lost the reprogramming potential. For instance, terminally differentiated cells located in the distal part of the villi could require more complex mechanisms to dedifferentiate. It has been shown that enterocytes which exit the crypt are unable to revert their fate and restore the damaged epithelium *in vivo* (Tetteh et al., 2016). The ability of distinct intestinal cell types to dedifferentiate and self-organise into a functional organoid needs further investigation. To address this question, chemical or genetic tools could be applied to enrich and sort each subpopulation of cells in order to compare their efficiency in generating organoids while assessing changes in their transcriptional profiles.

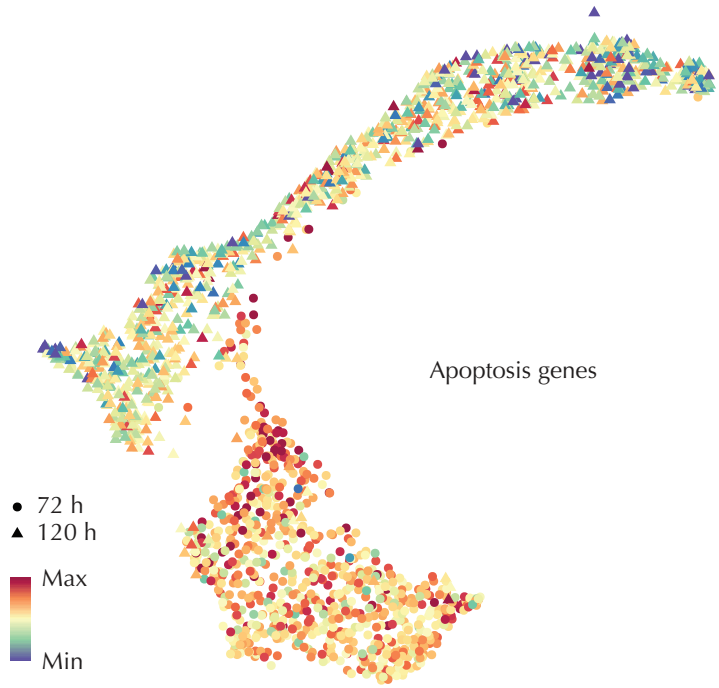


*Figure 6. scRNA-seq, apoptosis related genes. Visualisation of single cells at 72 and 120 hours of organoid growth. Colours are based on summed expression of apoptotic genes (Bad, Bak1, Bax, Bcl10, Bid, Bnip3, Bnip3l, Bok, Casp1, Casp12, Casp14, Casp2, Casp3, Casp4, Casp6, Casp8, Dffa, Dffb, Fas, Nod1, Pycard, Rnf7, Tnfsf10, Trp53bp2, Trp53inp1, Traf3).*

### 7.1.1. Highly proliferative and undifferentiated cells have a short $G_1$-phase

YAP1 induces a highly proliferative state in single cells and live imaging experiments show that these cells have a cell cycle rate of around 8 hours, while developing into a cyst. The dual-colour FUCCI organoid line allows the tracking of the cell cycle phase transition $G_1$ – $S/G_2/M$ by following the expression of antiphase oscillating fluorescently labelled proteins. In brief, the

protein CDT1[1], that has highest accumulation during $G_1$-phase, is fused to a red-emitting fluorescent protein. Conversely, the protein Geminin has highest accumulation during $S/G_2/M$ and is fused to a green-emitting protein (Sakaue-Sawano et al., 2008). Using this organoid line, we could observe a very short $G_1$ phase in the cells with fast cell cycle (data not shown). Such a short $G_1$-phase has, in several tissues, been linked to reduced propensity to differentiate and maintain a rich pool of cycling cells (Calegari and Huttner, 2003; Coronado et al., 2013; Lange and Calegari, 2010; Lange et al., 2009). At the single cell level, quick transitions between $G_1$ and S phase, and perhaps absence of checkpoints, might lead to the accumulation of DNA damages and make the cell more sensitive to perturbations. On the other hand, fast cell proliferation might provide an advantage at the tissue level by giving rise to an abundant population of cells that can rapidly organise into a functional 3D-structure. We see in our experiment that as soon as a cyst is developed, the division rate slows down and damaged cells are shed into the lumen. A challenge while preparing cells for RNA sequencing is isolating single cells from early time points of organoid differentiation. When extraction is successful, our sequencing data show high transcription of apoptotic genes at 72 hours (Figure 6). These observations could be further confirmed by staining for DNA damage and apoptosis markers and monitoring checkpoint proteins during the first days of organoid formation.

### 7.1.2. What are exactly cells in the undifferentiated foetal-like state?

Following several types of intestinal tissue damage YAP1 induces cell de-differentiation into a foetal-like state, to drive epithelial repair (Nusse et al., 2018; Yui et al., 2018). Interestingly, genes distinctive of this cellular state are also enriched in early phases of organoid formation. However, an in-depth characterisation of the immature population of cells and comparison with foetal cells is still missing. Adult organoids, even while transitioning through a phase of cellular de-differentiation, differ from FEnS (Foetal Enterospheres, see above Mouse intestinal organoids). This is observed both morphologically but also by the capacity of FEnS to grow independently of Wnt (Fordham et al., 2013). Whereas self-organization of adult intestinal organoids resembles the regeneration of the impaired epithelium, growth of FEnS represents the tissue maturation during development. A better understanding of the differences between these two systems, and what is their respective differentiation potential could help the design of prospective clinical applications. Additionally, FEnS and adult organoids could be used in a more fundamental research setting to identify molecular targets and compounds that could improve the development and healing of the intestinal tissue in disease.

### 7.1.3. YAP1 is necessary but not sufficient to drive organoid formation

The data presented in this thesis show that YAP1 activity is necessary but not sufficient to initiate a regenerative program, which also requires Wnt signalling. On the other hand, our bulk time-course RNA sequencing analysis reveals downregulation of canonical and upregulation of alternative Wnt target genes during the first days of

[1] CDT1 = Chromatin Licensing And DNA Replication Factor 1

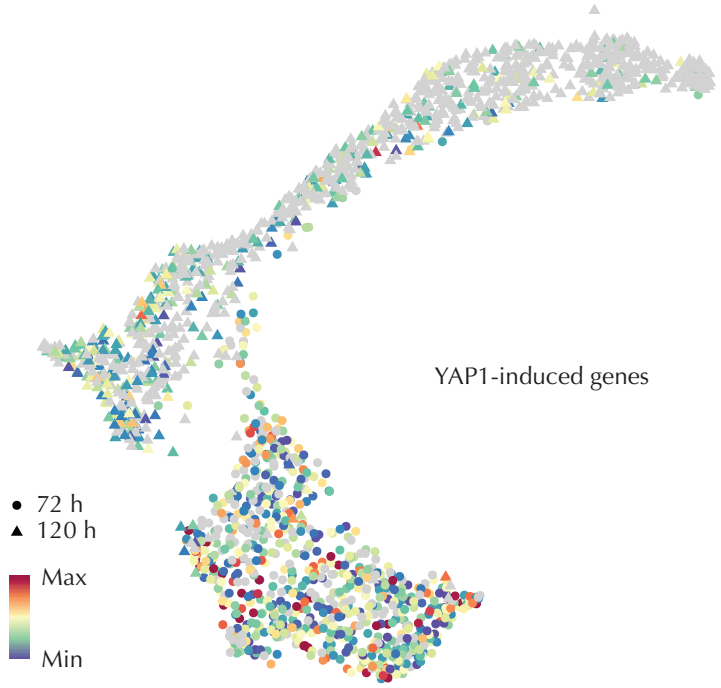


*Figure 7.scRNA-seq, YAP1-induced gene expression. Visualisation of single cells at 72 and 120 hours of organoid growth. Colours are based on summed expression of secreted factors genes YAP1-induced (Ctgf, Cyr61, Igfbp4, Bmp4, Wnt5b).*

organoid development (Extended Data Fig. 6e (Serra et al., 2019)). Using the reporter line TCF-GFP for the key TF that associates with β-cat, it seems that active canonical Wnt signalling reappears after Paneth cell differentiation (Extended Data Fig. 10h (Serra et al., 2019)). Therefore, the role of the canonical and alternative Wnt pathways in the induction of organoid formation and symmetry breaking, as well as downstream effectors of the YAP1 signalling pathway should be investigated further. The fact that YAP1 becomes cytosolic with Wnt removal 48 hours after plating single cells leads to the interesting hypothesis the Wnt cascade could potentiate a mechanically-induced YAP1 activation.

As mentioned in the introduction, WNT3A ligand triggers both the canonical and non-canonical Wnt cascades (PCP). The alternative pathway can activate YAP1 leading to the production of inhibitory secreted proteins targeting the canonical Wnt pathway. Whether such mechanisms are at play in intestinal organoids has still to be addressed. The data presented here support this hypothesis, as WNT3A is required to initiate the repair program, and upregulation of such secreted factors (*Ctgf, Igfbp4*) is detected by both bulk RNA sequencing within the first 24 hours of organoid culture and scRNA sequencing at 72 hours (Figure 7, Figure 8). More perturbation should be introduced to further characterise the YAP1-Wnt interplay.

To discriminate between the roles played by the Wnt canonical and non-canonical pathways, one could selectively inhibit β-cat signalling with the antagonists DKK1, tankyrase inhibitors, or *Lrp5/6* KO in single cells, and observe whether these cells are able to develop organoids. Substitution of WNT3A with ligands that are specific for the

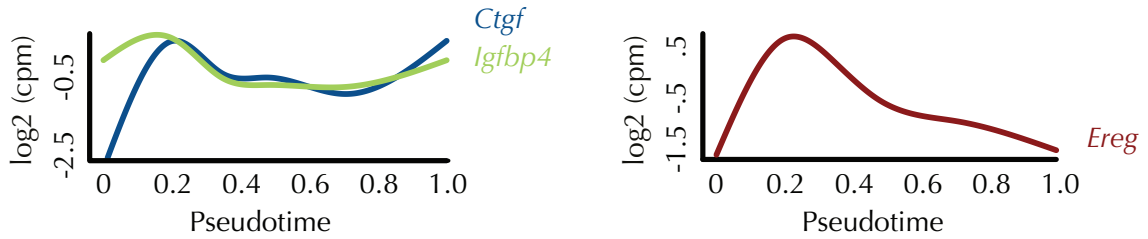


*Figure 8. Bulk-RNA Expression of YAP1-induced genes and Ereg. Expression profiles of genes encoding the secreted factor, as induced by YAP1(Ctgf, Igfbp4) and of the YAP1 downstream regulator Ereg mapped on pseudotime.*

alternative Wnt cascade could shed light on the role(s) of non-canonical Wnt in inducing YAP1. Looking at β-cat target genes expression in the context of YAP1 overactivation, (e.g. *Yap1* inducible-OE organoids or *Lats1/2*-DKO organoids), one could verify that YAP1 activity inhibits canonical Wnt pathway. Further insight could be gained by generating and imaging β-cat or TCF4 TF fluorescent reporter lines.

The study of Gregorieff and colleagues argued that YAP1 activates EGFR signalling by inducing increased transcription of the receptor ligand EREG (Gregorieff et al., 2015). We also observe a peak of expression of *Ereg* at 24 hours of organoid development by bulk RNA-sequencing, when there is a general enrichment of YAP1 target genes (Figure 8). In our work, the ligand EREG is considered to be the direct link between YAP1 and EGFR and we make use of exogenous EREG to mimic YAP1 activation. This treatment leads to more single cells that give rise to fully grown organoids, and to a reduced enterocyst phenotype compared with untreated control. However, we have not investigated further whether EREG is the main effector of YAP1 in our system. This could be tested by generating organoids lacking the *Ereg* gene and monitor efficiency of organoid formation and symmetry breaking. Finally, one could try to rescue *Yap1* inducible-KO or YAP1 inhibition by overexpressing *Ereg* or overstimulating EGFR.

### 7.1.4. What triggers YAP1 activity?

The current work suggests that YAP1 acts as a sensor of tissue integrity and is an inducer of a regenerative program that drives intestinal organoid formation. However, it does not address by which mechanisms and what pathway initiates the YAP1 response in single cells. Our working hypothesis is that when isolating single cells from the mouse intestine or from mature organoids at the initiation of the organoid culture we induce injury to the epithelium. Indeed, cells lose contact to each other and with the underlying mesenchyme, change their architecture, and start dying. Alteration in tissue compactness and cell spreading can activate YAP1 (Panciera et al., 2017). Furthermore, the Matrigel

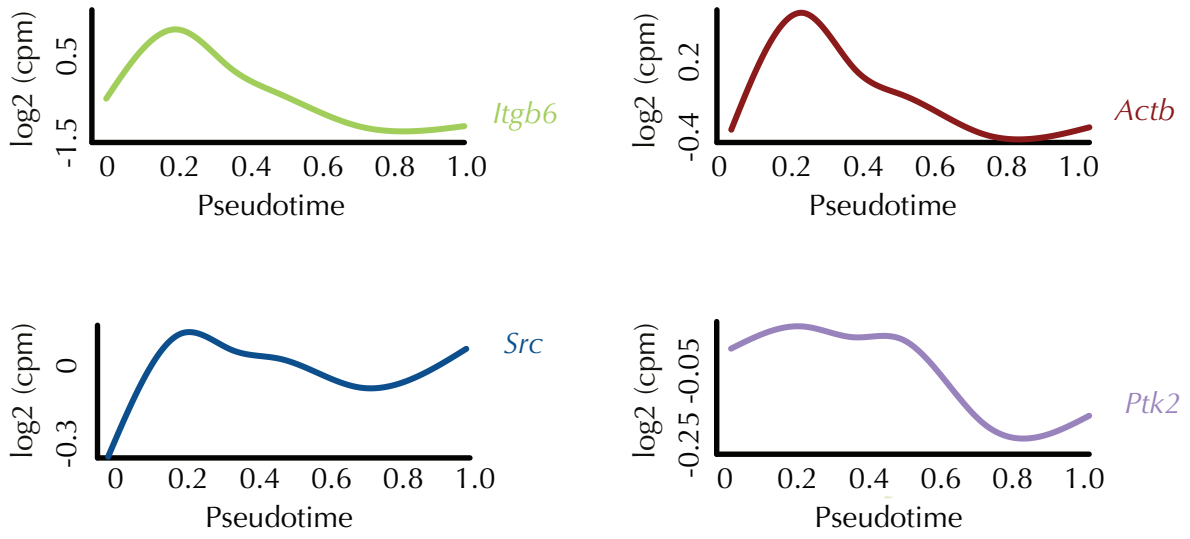


*Figure 9. Bulk-RNA seq Focal adhesion. Expression profiles of Focal adhesion signalling cascade elements mapped on pseudotime. As an example of integrin gene: Itgb6. Furthermore, Actb (Actin Beta), Src, Ptk2 (corresponding to FAK1 protein).*

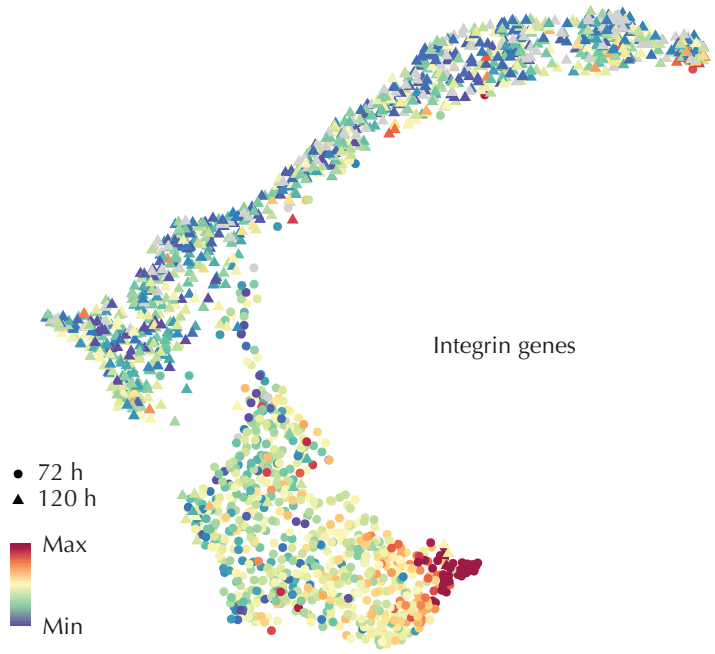


*Figure 10. scRNA-seq integrin genes. Visualisation of single cells at 72 and 120 hours of organoid growth. Colours are based on summed expression of integrin genes (Itga10, Itgb6, Itgb5, Itgb4, Itgb1, Itgav, Itgam, Itga9, Itga7, Itga6, Itga5, Itga3, Itga2, Itga1)*

composition may play an additional role in modulating the protein functionality. Laminin, a key element in the matrix, has been shown to activate YAP1 through focal adhesion signalling. The relative cascade contains integrins, FAK, actin, and SRC that are highly expressed within 24 hours of organoid culture, as detected by bulk RNA-sequencing. Strong expression of integrin genes is also present in the subpopulation of cells with active YAP1 signalling in the 72 hours scRNA-sequencing sample and could be responsible for the variability of YAP1 subcellular localization (Figure 9 and Figure 10). ECM remodelling and FA signalling are known inducers of YAP1-mediated cell reprogramming and repair of the damaged colon *in vivo* (Yui et al., 2018). Administration of FAK or SRC inhibitors in the organoid culture could reveal whether a similar regulation is responsible for the activation of YAP1 in single cells. Furthermore, a better understanding and control of the ECM where organoids are growing could facilitate the study whether and how YAP1 activity is influenced by FA and ECM elements.

### 7.1.5. Extracellular matrix for *in vitro* culture of organoids

Organoids grow in Matrigel, an extracellular matrix that is derived from a tumour cell line. Consequently, Matrigel composition can vary from lot to lot. Considering that different mechanical signals can induce distinct YAP1 responses, distinct cell behaviours are to be expected by changes in extracellular structure (Panciera et al., 2017). This unknown variable in the physical environment works against our wish for the cells to be exposed to a similar environment. In our study, we make use of the same batch of Matrigel to circumvent this artefact. Synthetic hydrogels are becoming a good alternative to Matrigel since their exact composition can be tightly controlled. Using such matrix Gjorevski showed that hydrogel stiffness regulates the proliferative capacity of intestinal stem cell by modulating YAP1 activity (Gjorevski et al., 2016). Whereas soft matrix causes YAP1 to re-localise to cytoplasm and thereby arrest cell division, stiff matrix sustains YAP1 in nucleus, causing cell expansion. A constant rigid extracellular matrix, however, does not allow for the development of a mature budding organoid. Indeed, a homogeneous drop in nuclear YAP1 is detected once the cyst increases in size, probably because of cell compactness. When dynamic matrices are used, designed to soften over time, variability in YAP1 localisation and mature organoids are observed (Gjorevski et

al., 2016). These results support our hypothesis that local variation of extracellular matrix density is needed to induce variability in YAP1 activity and thereby enable symmetry breaking.

## 7.2. YAP1 TRANSIENT AND VARIABLE ACTIVITY ALLOWS THE BREAK OF THE SYMMETRY

In our work we observe that YAP1 abundance and subcellular localization change while organoids progress in their development, and that this is important for the break of the symmetry. Intercellular variability in nuclear YAP1 is observed in cysts after the 4-cell stage and precedes the specification of DLL1 positive cells at the 8-16-cell stage, which corresponds to the symmetry breaking event, and initiation of Notch-Delta lateral inhibition. DLL1 positive cells have active YAP1 signalling, but lose dependency on YAP1 once they become Paneth cells. Homogeneous activation or inactivation of YAP1 in the cysts prevent Paneth cell differentiation and lead to enterocysts formation, highlighting the essential role of a transient and variable YAP1 signalling for the occurrence of symmetry breaking (Fig. 4a, g, h, Fig. 5, Extended Data Fig. 7g, h, Extended Data Fig 10 (Serra et al., 2019)).

### 7.2.1. YAP1 perturbation

In conventional biological studies, the role of a gene or protein is investigated by perturbing its expression or function. In this project many different approaches are taken to study the function of YAP1 in the event of symmetry breaking. First, we genetically induce *Yap1* or *Lats1/2* knock out or *Yap1* overexpression. Because they are essential genes, we use conditional systems. Since these modifications require that the genes are floxed out or overexpressed via chemical stimulation or lentivirus infection (and not all the cells may respond equally well to this treatment) we do not obtain full penetrance of the phenotypes. Nevertheless, our results obtained with the different approaches are consistent with each other. We further confirm these findings with several alternative experiments, such as: YAP1 inhibition by the compound Verteporfin, removal of the positive regulator Wnt, and simulation of YAP1 activity by the exogenous addition of the purified downstream effector EREG. The combined results obtained with the different experiments allow us to model the requirement of a transient and variable YAP1 activity for the occurrence of the symmetry breaking event.

The amount of *Yap1* mRNA detected by bulk RNA-sequencing does not change during the course of organoid development, but YAP1 protein abundance varies, suggesting that the protein is modulated post-translationally. Therefore, the use of a constitutively active or phosphorylation-mutant protein would be more appropriate than a YAP1 overexpressed protein. In addition, the post-translational regulation on YAP1 could be followed by monitoring the phosphorylated form of the protein by imaging. Unfortunately, none of the commercially available antibodies that we tested worked. Nevertheless, since we observe the expected increase in nuclear YAP1 after YAP1 overexpression, we do not find it necessary to employ additional methods to modulate YAP1 activity.

### 7.2.2. Alternative methods to study YAP1 functionality and the symmetry breaking event

A YAP1-fluorescent cell line represents another useful tool to investigate the role of YAP1 in self-organization and symmetry breaking, however, no YAP1 reporter is available and working in organoids at the moment. By live imaging, for instance, we could follow dynamics of YAP1 subcellular localisation and activity, and study the mechanisms driving YAP1 cell-to-cell variability and, hence, symmetry breaking. In particular, we could test whether there is a correlation between organoid size, nuclei number, or asynchronous cell divisions, and onset of YAP1 heterogeneity. But what induces intercellular YAP1 variability remains an open question. Looking at genes that correlate with YAP1 activity, we can speculate that local alterations in the cell environment can generate local changes in mechanical cues that modify YAP1 localisation (Extended Data Fig. 10g (Serra et al., 2019)).

Potentially, we could also examine the ability of YAP1 in conferring a survival or proliferative advantage to single cells and directly compare the efficiency of organoid formation between YAP1 positive and negative cells. Not being able to sort the two populations with a fluorescent signal, we address the issue by isolating cells from organoids that on average have high nuclear YAP1 (cysts at 72 hours) and mature organoids with reduced nuclear YAP1 (organoids at 120 hours).

Another way to perturb and study YAP1 would be optogenetics, a technique that allows to regulate proteins activity through light signals. In this setting, heterogeneity in YAP1 state could be induced prematurely, with controlled illumination of specific cells in a light sheet microscope, at a time when the co-transcriptional activator normally is homogeneously nuclear. Following the growth of such organoids via live imaging could bring new insights into whether YAP1 cell-to-cell variability is causal and sufficient to initiate symmetry breaking, or whether other factors are needed. Similar results could be obtained by injecting siRNA against *Yap1* or *Lats1/2* in selected cells of a small cyst (4-8 cell stage) and observing the occurrence, or lack, of DLL1 positive cells. However, this approach is time-consuming and labour-intensive. siRNA injection requires the use of a small needle able to puncture the cyst. Our system, unfortunately, poses some restrictions, since the needle gets clogged by crossing the Matrigel and Matrigel removal is not compatible with proper organoid growth. While we are actively trying to set up such technologies in our laboratory we take an approach where heterogeneous YAP1 activity is triggered by mixing single cells isolated either from inducible *Yap1* or *Lats1/2* knock-out organoids with single cells isolated from wild type organoids. Such an experimental design requires a tight control of the amount of cells assembling to form a new organoid. Preliminary data lead to inconclusive phenotype.

Finally, to prove that the establishment of a Notch-Delta lateral inhibition is necessary for differentiation of Paneth cells, the Notch signalling cascade is also chemically perturbed. Homogeneous inhibition of Notch receptor results in fewer symmetry breaking events. Here again, current efforts in the laboratory focus on developing molecular (siRNA), genetic and optogenetic modulators of the Notch pathway. Their

common aim is to locally enforce Notch-Delta activities on distinct neighbouring cells to study the formation of Paneth cells.

### 7.2.3. Which additional factor(s) contribute to the symmetry breaking event?

One key remaining question is to identify which additional factors cooperate with YAP1 to induce symmetry breaking in the cyst. Indeed, we observe that, once heterogeneity in YAP1 activity is manifested in the cysts, not all the cells with nuclear YAP1 become DLL1 positive cells, and not all the DLL1 positive cells become Paneth cells. Cell-to-cell variability might not induce directly a switch in cell behaviour, but initiates feedback loops that, in association with other signals, can trigger a more powerful response. The fact that DLL1 marks Paneth cells and early secretory precursors could explain the higher fraction of DLL1 positive cells, compared to Paneth cells. A fine balance of local signals and cell states can direct the differentiation of DLL1-expressing cells into one of the secretory lineage.

Here, Notch-Delta lateral inhibition is analysed by looking at patterns of HES1-DLL1 by immunostaining of fixed samples. *Hes1* is a well-known cycling gene, detected already at the 2-4 cell stage of organoid growth. Waves of expression of *Hes1* are due to the short half-life and capacity of HES1 to bind and repress its own promoter. HES1 represents a potential factor that might have a synergistic effect with YAP1 variability, in the control of DLL1 expression. One hypothesis is that cells of the cyst have asynchronous oscillating levels of HES1 protein; when YAP1 cell-to-cell variability is instituted, only a small group of cells will have both nuclear YAP1 and no HES1 expression. Consequently, cells with nuclear YAP1 and no HES1 expression could increase the basal levels of DLL1 expression and establish the Notch-Delta lateral inhibition. Such an hypothesis could be tested by following dynamics of HES1 turnover and YAP1 subcellular localisation. To do so, one would need to generate working reporters with a comparable half-life and functionality to the ones of the endogenous proteins.

### 7.2.4. How does YAP1 regulate *Dll1* expression?

*Dll1* has been reported to be a YAP1 target gene. In particular, YAP1 ChIP-sequencing and high-resolution chromatin conformation capture data (Hi-C) performed on epidermal cells show that YAP1 binds to enhancers associated with *Dll1* under high tension conditions (Totaro et al., 2017). Our laboratory is currently investigating whether a direct binding of YAP1 to *Dll1* regulatory regions occurs also in the intestinal organoid system, or whether other effectors link YAP1 activity and *Dll1* expression. A YAP1 chromatin interactome map can be generated by combining ATAC-sequencing, ChIP-sequencing, and Hi-C performed on cells with different YAP1 activity state. ATAC-sequencing enables the identification of DNA regions with increased accessibility and the mapping of nucleosome position and TF binding sites. This method would bring new insights on which chromatin domains are affected by YAP1 regulation. ChIP-sequencing provides a genome-wide description of protein-DNA interactions and histone modification. Such approach could capture the regions that are bound by YAP1 and highlight the connection to putative enhancers associated with Notch ligand genes. Hi-

C analyses the 3D conformation of the chromatin and reveals contacts between DNA loci that could be kilobases away. Enhancers are often found far from the relative associated promoters, hindering the prediction of the genes that they modulate. Hi-C 3D mapping could then verify the presence of a direct relation between putative YAP1-bound *Dll1* enhancers, and *Dll1* promoter. Finally, a joint profiling of chromatin accessibility and RNA transcripts at the single cell level can provide an improved molecular characterisation of the symmetry breaking event.

In order to investigate the heterogeneity of single cells preceding the appearance of DLL1 positive cells, our scRNA experiments should be repeated at earlier time points. As emergence of DLL1 positive cells precedes our 72 hours and 120 hours time points, it would be necessary to include and compare cysts from the 4-8 cell stage and cysts at 16 cell which correspond to the onset of YAP1 miscellaneous activity and DLL1 occurrence, respectively.

Efforts should also be put in identifying genes that are enriched in YAP1-DLL1 positive cells but are not YAP1 target genes. This could unveil additional signalling cascade(s) contributing to DLL1 expression.

To fully comprehend the state of a cell it is important to consider its microenvironmental and neighbour-interaction context, as discussed in the review attached to this thesis (Mayr et al., 2019). Unfortunately, most sequencing techniques lack spatial resolution. To overcome this limitation, one could use a double labelling strategy where both the single cells and the organoid of origin would be marked and could be detected by scRNA sequencing. Multiplexed-smFISH imaging would then allow for direct visualization of selected mRNAs abundance and localisation in single cells within their local conditions. This should greatly contribute to the understanding of the mechanisms driving cell state variability and symmetry breaking in the cyst.

### 7.2.5. Paneth cells, Wnt gradient and bud formation

The present work suggests a strong correlation between development of enterocysts and the absence of Paneth cells. Our current hypothesis is that Paneth cells are required for a fully mature budding organoid to form, and that a failure to do so (in other words a failure to break the symmetry) results in uniform round cysts. As described in the introduction, Paneth cells constitute a great source of Wnt, as well as other factors that allow for generation and sustaining of a stem cell niche. Paneth cells are thus thought to be responsible for the establishment of the crypt. However, Paneth cells are not enough to induce bud formation, *per se*, but a Wnt gradient is necessary to induce local hyperproliferation and subsequently crypt outgrowth. This is nicely shown by our experiments with the compound CHIR99021, a GSK3 inhibitor and therefore Wnt pathway activator. Organoids grown with CHIR99021 are homogenously exposed to Wnt, and they show enrichment of Paneth cells, scattered all over the structure, but they do not develop a crypt-villus structure. These organoids do not become enterocysts (Extended Data Fig. 1g (Serra et al., 2019)), have only few enterocytes, and are enriched in TA and LGR5 positive cells. Of note, this outcome is analogous to the one induced by *Apc* mutation, where organoids are enlarged, have a thin epithelium, and big lumen (Yin et al., 2014). Similar results have been obtained also by keeping Wnt in the organoid

culture for more than 72 hours, which is when it is usually removed from the medium (data not shown).

Next, studies are needed to identify whether a minimum number of Paneth cells clustering in a given area of the cyst is required to form a gradient of Wnt signalling and induce the formation of a bud. Furthermore, it is still unknown whether Paneth cells that have differentiated into distinct domains of the cyst are able to move and group together, and whether a defined distance between different groups of Paneth cells is necessary to allow the multiple budding events.

## 7.3. MOUSE INTESTINAL ORGANOIDS AS A MODEL SYSTEM

One of the advantages of intestinal organoids as a model system is their expansion capacity. Each experimental condition comprises at least a hundred of them. When derived from single cells, such a high number of organoids can capture phenotypic variability of biological processes such as symmetry breaking. Moreover, it enables the inference of functional relationships, and confers statistical power.

A good example of how we take advantage of the ease of growing large number of organoids is the derivation of the pseudo-time trajectory of organoid growth from images of fixed samples. Snapshots of temporal progression do not allow for identification of the exact moment of symmetry breaking, and therefore leave no record of morphological and phenotypic features of an organoid that is about to bud. Cells are asynchronous in the process of organoid formation, with some progressing faster than others. On the other hand, the current manuscript shows that continuous organoid trajectory can be inferred by imaging a large number of organoids and aligning each onto a trajectory based on their stage of development. Further analysis of molecular and morphological features in relation to the trajectory contributes to the description of the emergence of an organoid from a single cell (Fig 2, Extended Data Fig. 2, Extended Data Fig. 3 (Serra et al., 2019)).

## 7.4. ON THE IMPORTANCE OF GOOD ORGANOID LINES

One of the questions of this project is to determine whether LGR5 positive stem cells and differentiated LGR5 negative cells follow the same mechanism of organoid growth. This question was first addressed using sorted populations (positive or negative for LGR5) of the reporter line Lgr5-EGFP-ires-CreERT2. Data generated with this line suggested similar efficiency of organoid formation for both populations of cells. Of note, these results were in contradiction with the published literature showing higher efficiency for LGR5-GFP high cells (Sato et al., 2009). To rule out any artefacts coming from variegated expression of the reporter line itself, experiments were repeated with another reporter line LGR5::DTR-EGFP (Barker and Clevers, 2010; Tian et al., 2011). The LGR5 reporter is stably expressed in this line and allows us to address the initial query. This data show that both populations follow the same developmental trajectory, but the efficiency rate is higher for LGR5 positive stem cells (Fig. 1, Fig. 2d, f, h, Extended Data Fig. 1d, e,

Extended Data Fig. 2f, Extended Data Fig. 3 (Serra et al., 2019)). This also confirms that in the Lgr5-EGFP-ires-CreERT2, not all stem cells are GFP positive, therefore, this line is not being used further.

## 7.5. BENEFITS AND LIMITATIONS WITH IMAGING

Cells interacting with each other and with the environment, can give rise to structures of higher order of complexity. Signalling cascades and transcriptional modulation direct the state of a cell and, consequently, influence the process of self-organization. To understand how such systems evolve, it is important to follow their development spatially and temporally with single cell resolution, as we discuss in the review "Exploring single cells in space and time during tissue development, homeostasis and regeneration" (Mayr et al., 2019). Fluorescence imaging, can provide knowledge on spatial context, cell morphology, transcript and protein abundance, localisation and cell-cell contact. It represents a proven approach to obtain information on the state of a cell within a collective. Furthermore, time-lapse microscopy or pseudo-time trajectories, derived from imaging of thousands of entities in chronological time points, can bridge the spatial and temporal scales and track the progression of the biological phenomenon under study. In the work presented in this thesis, real-time and fixed-time imaging have been extensively used to chase the development of organoids and capture the symmetry breaking event. However, these methods do have some limitations. First, proper reporter cell lines for molecular and morphological readouts are often difficult to generate. A fluorescent tag can interfere with the functionality, stability, and/or localisation of the protein of interest, leading to abnormal behaviour of the target and eventually detrimental effects on the cell. Fluorescent or luminescent reporters, regulated by the promoter of the gene of interest, are not always stably expressed, which was for example the case with Lgr5-EGFP-ires-CreERT2 as was previously mentioned. Additionally, these reporters only reflect the presence of the protein, but tell nothing of its status. Proteins can also be detected by high affinity antibodies, but their binding is not always specific to the protein target of interest. Moreover, targets with non-uniform distribution within the cell might saturate the fluorescent signal in a specific subcellular compartment and thereby impede detection in compartments with lower concentration. β-cat, for example, is highly concentrated on the cell membrane, rendering the nuclear fraction hardly observable. For some genes/proteins of interest all of these restrictions may apply. A common alternative is to study such a target by investigating its downstream effectors as indirect readout of its functionality. In the work described here, for instance, *Hes1* expression is a proxy of active Notch receptor, and *Lgr5* expression is an indication of active canonical Wnt/β-catenin signalling.

Reporter lines for *Hes1* and *Lfng* (LuVeLu), another Notch downstream gene, have been tested during the course of the project. Whereas the former emitted only a weak signal, the latter was not reliably expressed in organoids. Therefore, HES1 protein is studied by immunostaining. Similar results and conclusions have been made for DLL1 and LYZ1 proteins, after imaging their respective fluorescent lines.

Not all cell types of the murine intestine have been transcriptionally characterised yet, meaning that no marker genes or specific antibodies have been identified so far. Staining of proteins expressed exclusively in immature Paneth cells are required to further

understand the onset of symmetry breaking in intestinal organoid development. Unfortunately, an effective antibody, specific for this cellular state, is not yet available in our laboratory.

In conclusion, high content imaging and time-lapse microscopy provide information with temporal and spatial resolution at the single cell level that, combined with data generated through sequencing technologies, allow the study of the principle of self-organisation. Therefore, possible limitations of such methodologies should not bode ill for their future wide-spread use, rather, time should be invested in setting properly the experimental conditions.

## 7.6. FUTURE PERSPECTIVES

Organoids are great model systems that mimic the native tissue or organ, but they do not reproduce completely the *in vivo* scenario. Multiple tissues compose an organ and cooperate to sustain the organ structure and function, but often organoids are formed of a single tissue or do not contain all distinctive cell types. Therefore, systemic effects cannot be analysed. Moreover, not all the organoid systems reach the mature state of the respective organ (e.g. retinal or cerebral organoids) and this could be due, for instance, to lack of vascularization and restricted nutrient supply. To overcome all these issues, researchers are now trying to optimize co-culture conditions and bioengineer methods to allow vascularization, innervation, or fluid-flow in the organoids. Furthermore, existing protocols need improvement to enable the generation of organoids in a stereotypic manner and with reduced heterogeneity. The gain of such properties could extend the present use of organoids into translational research and drug screenings. Phenotypic reproducibility and tissue maturation could be facilitated by the reproduction of controlled environmental conditions that are often still missing. For instance, spatiotemporal control of soluble factors could help the differentiation of the organoids into mature phenotypes.

In general, future directions should be directed toward an in-depth characterization of the organoid systems to understand to which extend they recapitulate developmental events, tissue regeneration or homeostasis. This information will consent to unravel complex biological phenomena using the correct model system, and amplify current applications for organoids. Indeed, organoids have the potential to answer questions that are difficult to study *in vivo*, or which are specific to humans, e.g human neurodevelopmental processes (Lancaster et al., 2013). For some tissues, organoids are already used to model cancer, infectious diseases, developmental and genetic disorders, but for others it is required first a better understanding of the respective organoid system (Bigorgne et al., 2014; Dekkers et al., 2013; Finkbeiner et al., 2012; Lancaster et al., 2013; Yeung et al., 2010). Finally, organoids could find broader use in identification of efficient precision therapies in personalised drug-screens, and to enable autologous transplantation of organoid-derived tissues.

In the work presented in this thesis, we have characterized the process of intestinal organoid formation and observed that it recapitulates a regenerative response of the intestinal epithelium, initiated by the transcriptional regulator YAP1.

YAP1 can act as a general sensor of tissue damage, and driver of regeneration-specific programs in several tissues. Mechanical cues that activate YAP1 are likely changing once homeostasis is restored, leading to termination of the YAP1 response. In the future, it would be important to understand the means by which YAP1 is activated by tissue damage as well as the finalization of the healing that ensure a proper tissue restoration. Uncontrolled YAP1 activity could lead, for example, to the development of cancer (Zanconato et al., 2016). The comprehension of YAP1 regulation will potentially allow the development of better strategies to improve the regenerative capacity of our tissues and restrain cellular overgrowth. Furthermore, deeper insights into how organoid development is achieved, and how cell reprogramming in the foetal-like state is modulated, could enable the discovery of new targets for compound treatments with the ability of speeding up the process of recovery.

Our findings reveal also that YAP1 transient and variable activity is required for the occurrence of the symmetry breaking event during intestinal organoid development. Symmetry breaking is an important process in biology that occurs at different scales, from molecular to cellular, tissue, and organism; and contributes to the proper formation of self-organizing systems. For instance, the break of the symmetry permits cell diversification and functional specification that characterizes many developmental processes. However, we lack complete understanding of the distinct underlying mechanisms. In the work presented in this thesis, we propose that local changes in cell crowding and in extracellular mechanical cues - perhaps dependent on asynchronous cell divisions and matrix degradation - lead to intercellular variability of YAP1 activity. This variable activity then further amplifies the divergence between cells by inducing different transcriptional responses, and results in the break of the symmetry. In the future, it will be interesting to study whether the same fundamental phenomenon guide diverse biological processes, such as self-patterning and self-driven morphogenesis. Advancements of 3D-culture technologies, and methods with high resolution will provide deeper knowledge on the matter, and allow to define general mechanisms that control the events of symmetry breaking.

In the past years it has become evident that cell fates are not hard-wired. Instead, cells change dynamically their fate depending on the cues they receive from the environment, and the nearby cells. Cell surroundings differ between homeostatic, disease, or regenerative conditions and cellular states also diverge. To appreciate how such transitions are regulated it will be beneficial to study cell behaviours within their natural context. The combination of several quantitative technologies with spatial and temporal resolution will help the formulation of predictive models for cell behaviours, and onset of cell-to-cell variability. Information gained by these means, will eventually support the comprehension of how tissue organization is determined and maintained.

In parallel to the increasing knowledge that can be achieved through the constant development of new methodologies, and integration of multiple data-types, researchers will have to face the exciting challenges to handle the big amount of data produced, reduce it to a meaningful level, analyse it and draw significant conclusion.

# 8. CONCLUSION

All together the work presented in this thesis investigates the ability of single cells to self-organise in multicellular asymmetric structures using mouse intestinal organoids as a model system. We examine the transcriptional program that characterises the initiation of organoid formation and identify an active regenerative response in single cells, induced by the transcriptional regulator YAP1. Our findings suggest that mouse intestinal organoids manifest the same plasticity and repairing capacity as cells of the intestinal epithelium under damage conditions. This holds a promise for further studies into the mechanisms of tissue restoration. The integration of single cell imaging and genomics approaches made it possible to study cell-to-cell variability as an inherent property of cellular populations; and further to characterise its role in driving the break of the symmetry of an initial homogeneous system. Our results show that cell-to-cell variability in YAP1 activity enables the break of the symmetry of the round cyst by initiating a Notch-Delta lateral inhibition and Paneth cell differentiation. Subsequently, this event permits the emergence of cellular diversification and functional compartmentalisation. It is still not fully elucidated how variability in YAP1 activity is established, but considering the function of YAP1 as mechanotransducer, it may be due to local variation in mechanical cues. On a technological level our study shows that it is possible to observe cellular behaviour both spatio-temporally via imaging and by transcriptional profiling, in order to investigate coordinated cellular interactions. With organoid systems it is possible to study the initiating conditions responsible for the fundamental symmetry breaking of identical cells into defined ordered structures.

Self-organisation is a common phenomenon observed not only at the rise of complex life forms, but also during tissue development, homeostasis and regeneration. Therefore, an in-depth knowledge of how self-organisation is achieved can be beneficial for the understanding of aberrant mechanisms driving diseases, and, consequently, for translational medicine. Our findings represent a starting point to direct future studies that aim to characterise and correct irregular events of tissue organisation *in vivo*.

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# 10. APPENDIX SECTION

## AMINO ACID ABBREVIATIONS

| | | |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic acid | Glu | E |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |
| Any amino acid | | x |