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# NOT JUST A PIPE'S DREAM

# PLUMBING THE CONTRIBUTION OF CELL DIVERSITY TO OESOPHAGEAL HOMEOSTASIS

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Cover illustration: Abstract illustration of the mouse oesophagus.

# Not just a pipe's dream
# Plumbing the contribution of cell diversity to oesophageal homeostasis

Thesis for Doctoral Degree (Ph.D.)

By

**David Grommisch**

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To my family, nearest and dearest.

*"We know how cells work,*
*but can't predict the poetry*
*that will be written*
*by a human made up of them."*

– David Epstein*, Range*

# Popular science summary of the thesis

The skin, intestine, and food pipe cover our body's surfaces and exert a range of crucial functions. They form a barrier between the inside and outside of our body assist in nutrient absorption and protect us from water loss and external threats like bacteria and UV light. Thereby, they permit the formation of a continuously stable environment within our body despite changing external conditions. This stable environment is referred to as homeostasis.

Homeostasis is not a passive state but an actively regulated process. Surface epithelia undergo constant renewal throughout our lifetime in order to maintain their various functions. A multitude of finely adjusted biological mechanisms perpetuate and regulate homeostasis. Errors in the processes sustaining homeostasis can lead to impaired wound repair or tumour development. Thus, a detailed understanding of the mechanisms operating in homeostasis aids in a better understanding of disease onset.

How homeostasis of the food pipe is regulated and maintained is not fully understood and therefore the focus of this thesis. The mouse oesophageal epithelium overlays connective tissue and consists of several superimposed layers. Cells compose the subunits and building blocks of tissues. Cells of the layer positioned directly on top of the connective tissue possess the ability to renew and divide and are referred to as progenitor cells. Progenitor cells can remain within the basal layer or shift into superimposed layers upon which they change and lose their ability to divide. This process is called differentiation. The finely adjusted balance of progenitor cell fate between renewal and differentiation is the key to sustained homeostasis.

In one part of this work we show that a subset of progenitor cells exhibits an altered division rate and cell fate. In another part we discovered that cells of the epithelial and connective tissue as well as immune cells are unevenly distributed along the oesophageal axis. The changes in cell distributions imply altered cell-cell communication and influence the behaviour of epithelial progenitor cells in a regional fashion. Thereby, we demonstrated that cell-cell communication is a pivotal mechanism in the maintenance of oesophageal homeostasis. In addition, we established a system that allows the investigation of cell-cell communication outside of the living mouse.

In conclusion, this work sheds light on mechanisms regulating oesophageal homeostasis with specific focus on epithelial progenitor cells. Ultimately, our insights might aid in the identification of processes that account for tumour onset or provide the starting point for improved therapeutics targeting oesophageal diseases.

# Populärwissenschaftliche Zusammenfassung

Der Darm, die Haut, und die Speiseröhre sind Beispiele für Oberflächenepithelgewebe. Oberflächenepithelgewebe üben eine Vielzahl wichtiger Funktionen aus. Sie bedecken unsere Körperoberfläche, sind an Verdauung und Nährstoffaufnahme beteiligt, und schützen uns unter anderem vor Austrocknung, Licht und Bakterien. Kurz gesagt: Oberflächenepithelgewebe formen eine Barriere zwischen uns und unserer Umgebung. Dadurch ermöglichen sie unserem Körper das dauerhafte Aufrechterhalten eines stabilen, ausgeglichenen Zustandes, trotz sich verändernder äußerer Einflüsse. Dieser Zustand wird auch Homöostase genannt.

Oberflächenepithelien erneuern sich fortwährend im Verlauf unseres Lebens, um ihre Funktionen zu erfüllen. Daher ist Homöostase kein passiver Zustand unseres Körpers, sondern ein aktiv regulierter Prozess. Eine Unmenge biologisch fein abgestimmter Mechanismen reguliert Homöostase und wird unentwegt angepasst, um sie konstant und zuverlässig zu erhalten. Fehler in diesen regulatorischen Prozessen können zum Beispiel zur Entstehung von Krankheiten wie Krebs beitragen oder zur Beeinträchtigung von Wundheilung führen. Wie die Homöostase der Speiseröhre aufrechterhalten wird ist weitestgehend unerforscht und bildet daher den Fokus dieser Arbeit.

Oberflächenepithelgewebe bestehen aus einzelnen Bausteinen, den vielen, dicht beisammen liegenden und eng miteinander verbundenen Epithelzellen. Die Speiseröhre besteht aus mehreren übereinanderliegenden Schichten von Epithelzellen, die auf einer weiteren Schicht andersartiger Zellen, dem Bindegewebe, aufliegen. In der Hausmaus besitzt nur die direkt dem Bindegewebe aufliegende Zellschicht, Vorläuferzellen genannt, die Fähigkeit zur Vermehrung durch Zellteilung. Vorläuferzellen können entweder in der dem Bindegewebe aufliegenden Zellschicht verbleiben oder in höher gelegene Zellschichten ausweichen. Wenn Vorläuferzellen in höher gelegene Zellschichten treten verändern sie sich und verlieren die Fähigkeit der Zellteilung. Dieser Vorgang nennt sich Differenzierung. Die fein justierte Balance des Schicksals von Vorläuferzellen ist die Grundlage der Homöostase in der Speiseröhre.

In einem Teil dieser Arbeit fanden wir, dass nicht alle Vorläuferzellen der Speiseröhre identisch sind, sondern sich in der Häufigkeit der Zellteilung sowie ihrem Schicksal unterscheiden. In einem weiteren Teil entdeckten wir, dass die Zellen des Epithels, des Bindegewebes, und die darin befindlichen Abwehrzellen des Körpers unterschiedlich entlang der Speiseröhre verteilt sind. Diese ungleiche Verteilung führt zu veränderter Zell-Zell Kommunikation und beeinflusst das Verhalten der Epithelzellen. Damit ist Zell-Zell Kommunikation ein wichtiger Mechanismus zur Erhaltung von Homöostase in der Speiseröhre. Darüber hinaus entwickelten wir ein System, dass die zielgerichtete Untersuchung von Zell-Zell Kommunikation außerhalb der Maus ermöglicht.

Im Ganzen verdeutlicht diese Arbeit, dass eine Vielzahl biologischer Prozesse das Verhalten Vorläuferzellen beeinflusst. Dieses Wissen ermöglicht ein besseres Verständnis

der Vorgänge die zur Krebsentstehung beitragen. Schlussendlich können die hier gewonnen Einblicke der verbesserten Erkennung und Behandlung von Beschwerden in der Speiseröhre dienen.

# Abstract

Epithelial barrier tissues like the skin, intestine, and oesophagus form a physical barrier that protects our body from external threats. To accurately fulfil their function, epithelial barriers are subject to constant epithelial cell renewal throughout our lifespan. Recurrent tissue turnover requires the precise control of epithelial cell proliferation and differentiation to maintain homeostasis and health. Adult tissue stem cells residing in epithelial barriers are of pivotal importance for tissue homeostasis and repair. The stem cell niche, composed of a variety of cells, mechanical and chemical elements, provides decisive signalling cues that influence stem cell behaviour and fate.

The major part of this thesis investigates behaviour of the mouse oesophageal epithelial progenitor cell and sets out to uncover and characterise the contribution of cellular diversity to oesophageal homeostasis.
In **paper III**, we demonstrated that a subpopulation of oesophageal progenitor cells expressed *Tnfrsf19* (Troy) and contributed long-term to oesophageal homeostasis. Using lineage tracing in combination with mathematical modelling we proposed that *Troy* progenitor cell fate is predominantly symmetrical. In addition, functional TROY knockout *in vivo* suggested that TROY regulates progenitor proliferation and facilitates differentiation. Thus, TROY might be involved in context dependent cellular decision making processes providing a basis for behavioural progenitor heterogeneity.
In **paper IV**, we characterise regional oesophageal cell composition utilising single cell RNA sequencing. Combining cell-cell communication inference and organoid culture we reveal regionally diverse contributions of fibroblasts and immune cells as well as signalling pathways such as BMP and IGF that differently influence epithelial cell behaviour. In **paper II**, we developed an organoid co-culture system of oesophageal epithelial cells and fibroblasts that allows for detailed functional investigations of cell-cell communication *in vitro*.

The generation of the stratified squamous epithelium of the skin is governed by intricate and interwoven processes of proliferation, cell cycle exit, differentiation, and stratification. In **paper I**, we probed the function of ID1 in epidermal development and demonstrated ID1 binding to the transcription factor TCF3. We propose that ID1-CEBPA crosstalk regulates epidermal cell fate decision within a ID1-TCF3-CEBPA axis.

The work provided within this thesis demonstrates molecular mechanisms and signalling cues that impinge on epithelial cell behaviour during homeostasis and development.

# List of scientific papers

I. Kantzer, C. G*., Yang, W*., **Grommisch, D.**, Patil, K. V., Mak, K. H. M., Shirokova, V., & Genander, M. (2022). ID1 and CEBPA coordinate epidermal progenitor cell differentiation. *Development*, *149*(22), dev201262. doi: 10.1242/dev.201262
* These authors contributed equally to this work.

II. Eenjes, E., **Grommisch, D.**, & Genander, M. (2023). Functional Characterization and Visualization of Esophageal Fibroblasts Using Organoid Co-Cultures. *JoVE (Journal of Visualized Experiments)*, (191), e64905. doi: 10.3791/64905

III. **Grommisch, D.**, Wang, M., Eenjes, E., Svetličič, M., Deng, Q., Giselsson, P. & Genander, M. Symmetrically fated progenitors dynamically accommodate tissue maintenance in the esophagus. (*Manuscript*).

IV. **Grommisch, D.**, Hagemann-Jensen, M., Lund, H., Eenjes, E., Sandberg, R. & Genander, M. Regional heterogeneity impacts oesophageal homeostasis. (*Manuscript*).

# List of scientific papers not included in this thesis

I. Parsa, R., Lund, H., Georgoudaki, A. M., Zhang, X. M., Ortlieb Guerreiro-Cacais, A., **Grommisch, D.**, Warnecke, A., Croxford, A.L., Jagodic, M., Becher, B., Karlsson, M.C.I., & Harris, R. A. (2016). BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis. Journal of Experimental Medicine, 213(8), 1537-1553. doi: 10.1084/jem.20150577

II. Lund, H., Pieber, M., Parsa, R., Han, J., **Grommisch, D.**, Ewing, E., Kular, L., Needhamsen, M., Espinosa, A., Nilsson, E., Överby, A.K., Butovsky, O., Jagodic, M., Zhang, X.M., & Harris, R. A. (2018). Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells. Nature communications, 9(1), 4845. doi: 10.1038/s41467-018-07295-7

III. Lund, H., Pieber, M., Parsa, R., **Grommisch, D.**, Ewing, E., Kular, L., Han, J., Zhu, K., Nijssen, J., Hedlund, E., Needhamsen, M., Ruhrmann, S., Ortlieb Guerreiro-Cacais, A., Berglund, R., Forteza, M.A., Ketelhut, D.F.J., Butovsky, O., Jagodic, M., Zhang, X.M., & Harris, R. A. (2018). Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling. Nature immunology, 19(5), 1-7. doi: 10.1038/s41590-018-0091-5

# Contents

# List of abbreviations

| | |
|---|---|
| AKT | *Unclear* (discussed here [1]) |
| ASCL2 | Achaete-Scute Family BHLH Transcription Factor 2 |
| BARX1 | BARX Homeobox 1 |
| bHLH | Basic helix-loop-helix |
| BMI1 | BMI1 proto-oncogene, polycomb ring finger |
| BMP | Bone morphogenic proteins |
| BMPR | BMP receptor |
| bZIP | basic leucine zipper |
| C/EBP | CCAAT-enhancer-binding protein |
| CCR2 | C-C Motif Chemokine Receptor 2 |
| CD | Cluster of differentiation |
| ChIP-seq | chromatin-immunoprecipitation with sequencing |
| Co-IP | Co-immunoprecipitation |
| CRE | Causes recombination |
| CSF1 | Colony stimulating factor 1 |
| CSL | CBF-1/RBP-j κ,Su(H),Lag-1 |
| CX3CR1 | CX3C motif chemokine receptor 1 |
| DKK | Dickkopf-related protein |
| DLL | Delta-like protein |
| DNA | Deoxyribonucleic acid |
| DNMAML1 | dominant negative mastermind-like 1 |
| DPP4 | Dipeptidyl peptidase-4 |
| E | Embryonic day |
| E-box | Ephrussi-box |
| ECM | Extracellular matrix |
| EdU | 5-ethynyl-2'-deoxyuridine |
| EGF | Epidermal growth factor |
| EGFP | Enhanced green fluorescent protein |
| EpCAM | Epithelial cellular adhesion molecule |

| | |
|---|---|
| ERK | extracellular signal-regulator kinase |
| FACS | Fluorescence-activated cell sorting |
| FGF | Fibroblast growth factor |
| FLG | Fillagrin |
| GLI | Zinc finger protein GLI |
| GO | Gene ontology |
| GSK3β | Glycogen synthase kinase-3 beta |
| HHIP | HH-interacting protein |
| HLH | Helix-loop-helix |
| HSC | Haematopoietic stem cell |
| ID | Inhibitor of DNA binding |
| IFE | Interfollicular epidermis |
| IGF | Insulin-like growth factor |
| IGFBP | Insulin-like growth factor-binding protein |
| IL | Interleukin |
| ILC | Innate lymphoid cell |
| ISL1 | ISL LIM Homeobox 1 |
| JAG | Jagged |
| JAK | Janus kinase |
| JNK | c-Jun $NH_2$-terminal kinase |
| KLF | Krüppel-like factor |
| KRT | Keratin |
| LGR5 | Leucine-rich repeat-containing G-protein coupled receptor 5 |
| LOR | Loricrin |
| loxP | Locus of crossing (x) over, P1 |
| LRIG1 | Leucine Rich Repeats And Immunoglobulin Like Domains 1 |
| LXR/RXR | Retinoic acid receptors |
| Ly6a (SCA-1) | Lymphocyte antigen 6A-2/6E-1 |
| MMVL | Moloney Murine Leukemia Virus |

| | |
|---|---|
| mTERT | telomerase reverse transcriptase |
| NICD | Notch1 intracellular domain |
| NKX2.1 | NK2 Homeobox 1 |
| OA | Oesophageal atresia |
| p63 | Tumour protein 63 |
| PDGF | Platelet derived growth factor |
| PDGFR | Platelet derived growth factor receptor |
| Pparγ | Peroxisome proliferator activated receptor gamma |
| PTCH1 | Patched-1 |
| RA | Retinoic acid |
| RKIP | Raf kinase inhibitory protein |
| RNA | Ribonucleic acid |
| ROR2 | Tyrosine-protein kinase transmembrane receptor ROR2 |
| sFRP | Secreted Frizzled-related proteins |
| SHH | Sonic hedgehog |
| shRNA | Small hairpin RNA |
| SMO | Smoothened |
| SOX | sex determining region Y-box |
| STAT | Signal transducer and activator of transcription |
| TAZ | Transcriptional coactivator with PDZ-binding motif |
| TCF | T-cell factor |
| TGF-β | Transforming growth factor beta |
| THY1 | Thymocyte differentiation antigen 1 |
| TNF | Tumour necrosis factor alpha |
| TNFRSF | Tumor necrosis factor receptor superfamily |
| TOF | Tracheooesophageal fistula |
| UMI | Unique molecular identifier |
| WNT | Wingless and Int-1 |
| YAP | Yes-associated protein |

# Chapter 1

# 1 Introduction

You are truly busy doing nothing. In fact, right now, doing nothing but reading, you interpret visual stimuli, you send nerve impulses, you inhale and exhale, you pump and clean blood, you repair minor damage and fight cancer, you grow hair, and you replace and shed cells of epithelial barriers like the skin and oesophagus.

Something else might have happened. The opening sentence is confusing. It contradicts itself. It might have caused slight irritation, and in doing so, confounded many processes in your body. It put you out of balance. The balance within our body that is actively maintained by regulatory processes is referred to as homeostasis. Homeostasis is believed to be a prerequisite for health and thus became a central concept of modern physiology. It is defined by the Oxford's English dictionary as:

> *"The tendency towards a relatively stable equilibrium between interdependent elements, especially as maintained by physiological processes."*

This thesis addresses homeostasis in epithelial tissues and specifically the oesophagus of the house mouse (*mus musculus)*. Biologists study homeostasis to understand the molecular processes underlying and maintaining cells, tissues, and organs within our body. Deciphering homeostasis is fundamental to identify and understand the detrimental changes occurring in diseases like cancer. But how did homeostasis become such a central concept in physiology?

## 1.1 The concept of homeostasis and beyond

The belief that a "balance of opposites" ensures health is with us since the ancient Greeks and was first recorded by Alcmaeon of Croton (500 BC). A more tangible concept of health was introduced in 1542 by the French physician Jean François Fernel. Contrary to the term *pathology*, the study of disease, he introduced the phrase *physiology*, the study of the functions of the healthy body. The thought that all components of an organism cooperate to sustain a balance we refer to as health was developed and formed in the following centuries mainly based on his ideas [1].

However, our current understanding of physiological regulation of the body rests heavily on the shoulders of two Physiologist of the 19$^{th}$ and 20$^{th}$ century, Claude Bernard and Walter Cannon (**Figure 1**). The French Physiologist Claude Bernard, who had worked on the pancreas, the glycogenic function of the liver, and the sympathetic nervous system, proposed the idea of an actively regulated "*milieu intérieur*" (internal environment) counterbalancing variations of the external environment our body is subject to [2,3]. Decades later, having worked on the digestive system and proposing the adrenaline

driven *Fight or Flight* response, American Physiologist Walter Cannon had a shot at the idea of a steady-state of the body. The result of his work and thought process was the invention of the term homeostasis derived from the ancient Greek terms for ὅμοιος (hómoios, "similar, the same") and στάσις (stásis, "standing, state"). Like Bernard, he concluded that the stable "*milieu intérieur*" is the prerequisite condition for a free and independent – healthy - life. Cannon built on Bernard's work and emphasised the importance of active controlling processes. He proclaimed that an interplay of active factors and corresponding antagonists exists. Together, the active and their complementary counteracting factors sustain homeostasis of the body [1,3,4].

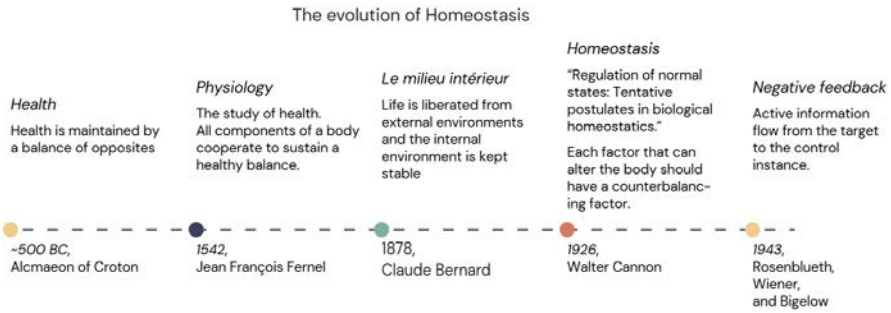



**Figure 1.** Timeline displaying the main characters and thoughts contributing to our current understanding of homeostasis.

Thereupon, Rosenblueth, Wiener, and Bigelow set out to "…define the behavioristic study of natural events and to classify behavior." [5]. Their finding of negative feedback as a control mechanism to preserve homeostasis shifted the at the time prevailing idea of homeostasis [3]. Previous to their discoveries, homeostasis was thought to be maintained by counterbalancing control instances. Now, it was understood that the target product of a biological process itself could regulate the source process it arose from. Homeostasis was viewed as an actively regulated process of dynamic information exchange between different components.

While this understanding of homeostasis is still prevalent today much of our knowledge and insights into the workings and processes of the body have advanced and the need for refined concepts emerged. For example, Austrian Physiologist Hans Selye, while studying the body's response to certain stressors, made the observation of General Adaption Syndrome, a state in which an organism fails to revert back to homeostasis and instead enters a new state [6]. Derived from ancient Greek ἕτερος (héteros, "other, another, different") and στάσις (ÿtasis, "standing, state") Selye named this new state heterostasis [7]. British scientist Kelvin J.A. Davies suggested to expand our understanding of homeostasis to Adaptive Homeostasis based how cells deal with oxidative stress. The Keap1-Nrf2 oxidant defence system allows for a transient (adaptive) upregulation of antioxidant response element target genes in order to protect cells from antioxidant damage [8]. Thus, despite scientists mostly agreeing that the maintenance of homeostasis

is prerequisite for health, it becomes apparent that the detailed understanding of homeostasis is still subject to debate. In fact, for many, if not all, tissues the intricate mechanisms maintaining homeostasis remain incompletely understood.

To gain thorough insights into the processes that maintain homeostasis scientists often study tissues in perturbed situations like injury or tumour onset or investigate specific organs during development. In recent years, the tool of lineage tracing emerged to study homeostasis in relatively unperturbed conditions [9–11]. Due to easy accessibility, the rather simple design, and advanced knowledge of the tissue, epithelial barriers present an ideal system to investigate the behaviour of cells or biological processes in homeostasis. Epithelial tissues like the skin, intestine, and oesophagus exhibit various architectures but ultimately fulfil a similar functions ranging from protection of external threats, to secretion of mucus or sweat, or nutrient uptake [12,13]. Most importantly, epithelial barriers present the enclosure and intersection of an interior with an exterior milieu, thereby providing the foundation of homeostasis. To ensure their protective barrier function epithelial tissues are subject to a constant steady-state turnover [14,15]. Frequent tissue turnover is typically maintained through stem or progenitor cells that constantly renew and have the ability to produce more specialised tissue specific cells [16].

## 1.2 Stem and progenitor cells

More than 150 years ago, German zoologist Ernst Haeckel used the phrase "stem cell" to describe the cell of origin capable of generating multicellular organisms (the zygote) [17]. However, our current understanding of the "stem cell" derives from the work of James Till and Ernest McCulloch, who adopted the expression "stem cell" to describe the existence of a cell residing in the bone marrow that has the potential to give rise to all cells of the hematopoietic system [18]. Thereafter, stem cells have been discovered in many more tissues including but not limited to the intestine, stomach, skin, and brain [15,19–24]. Nevertheless, the qualifications defining a stem cell are based on the discoveries made on haematopoietic stem cells (HSCs). Therefore, multipotent stem cells are defined by their ability to bring about a range of specialised (differentiated) cell types, and their ability to self-renew [25]. Typically, stem cells can divide symmetrically or asymmetrically [26]. Symmetric cell division results in two daughter cells of the same potential, either two stem cells or two specialised tissue cells. Asymmetric division gives rise to two cells of different potential, one stem cell and one more specialised daughter cell.

However, it is becoming increasingly clear that adult stem cells of many tissues operate in a different manner from HSCs to replace lost tissue and cells. For example, the interfollicular epidermis (IFE) and oesophagus exploit proliferative basal layer cell populations that do not possess the potential to give rise to multiple specialised cell types and are thus considered unipotent [27]. Therefore, they are often referred to as progenitor cells. While their naming might be a matter of semantics, frequent self-renewal capacity of progenitor cells has been reported [28]. Ultimately, tissue resident stem or

progenitor cells serve to maintain the structure and function of an organ during homeostasis and to renew tissues after injury or trauma. With this purpose, stem and progenitor cells establish a delicately calibrated equilibrium of cell loss and replacement, differentiation and proliferation. If perturbations to this finely adjusted homeostasis occur, tissue maintenance is hindered and injury responses fail or tumours develop [29]. Thus, stem cells are of crucial importance during development, the maintenance of homeostasis, and pathological onset like cancer.

## 1.3 Development – stretching to homeostasis

The stem cell described by Haeckel, the totipotent fertilised egg, simply made of two cells, has the remarkable ability to assemble an entire organism. In the mouse, at embryonic day 6.25 (E6.25) the multipotent cells of the single-layered epiblast begin to reorganise into a three-layered structure [30]. This reorganisation process is called gastrulation and continuous until E8.5. A flawless symphony of morphogens, timely attuned, will accomplish the formation of an entire organism [31,32]. Driven and modulated mainly by Wingless and Int-1 (WNT), nodal, Bone morphogenic protein (BMP), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β) signalling, the three germ layers ectoderm, endoderm, and mesoderm (as well as neural crest cells) give rise to the various tissues of an organism [33–37]. During organogenesis the mesoderm generates bones, muscle, cartilage, the circulatory system and many internal organs (i.e. kidney and spleen). The endoderm produces the gastrointestinal organs (i.e. oesophagus and intestine), the respiratory system, and other inner organs (i.e. thymus and bladder). Lastly, the ectoderm gives rise to the nervous system as well as the skin and all its epithelial appendages, hair follicles, sweat and sebaceous glands [38].
In the coming paragraphs, development of the epidermis and oesophagus will serve as examples to discern crucial elements necessary for the successful generation of tissues and ultimately the establishment of adult homeostasis.

### 1.3.1 Development of the epidermis

Mice are born with an intact barrier protecting them from dehydration, pathogens, and injury. The skin, a multi-layered (stratified) squamous epithelium, undergoes a sophisticated developmental program to fulfil this multitude of functions. After gastrulation, in a series of yet undefined molecular events, the surface ectoderm is primed to become the epidermis [39,40]. A series of neatly programmed events and the synergistic progress of proliferation, cell cycle exit, and layer formation (stratification) controlled by growth factors ultimately results in the stratified squamous epithelium of the epidermis (reviewed in detail in [41–45]) (**Figure 2**).

Changes in the expression of keratins (KRT), the major structural proteins of keratinocytes, accompany and are considered the hallmark of epidermal development [46].

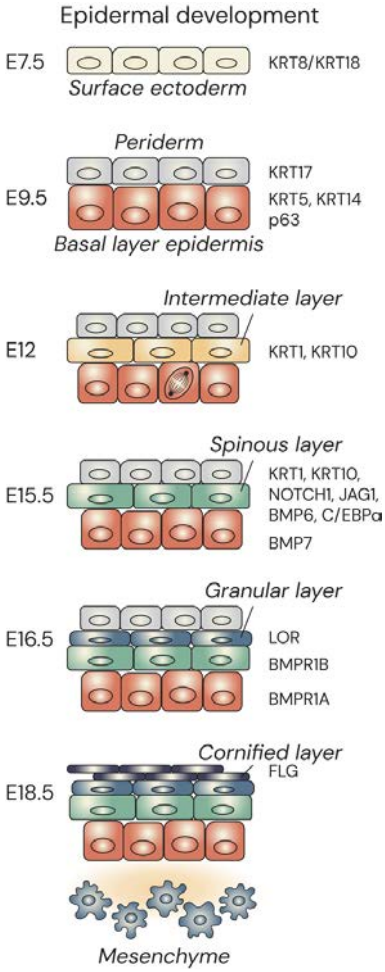



**Figure 2. Illustration of epidermal development**. At E7.5 the surface ectoderm consists of a single layer of cells expressing KRT8 and KRT18. At E9.5 the single basal layer expresses KRT5 and KRT14 as well as transcription factor p63 and is overlaid by the KRT17 periderm. At E12 asymmetric cell divisions of the basal layer cells are observed and stratification begins, leading to the formation of an intermediate layer labelled by KRT1 and KRT10 expression. At E15.5 the intermediate layer becomes the spinous layer expressing NOTCH signalling, C/EBPα and BMP6, while the basal layer gains expression of BMP7. At E16.5 expression of BMPR is observed and the granular layer forms labelled by LOR expression. At E18.5 stratification is mostly finished and the cornified layer expressing FLG is apparent. Throughout development mesenchymal cells underlying the epithelium aid in developmental processes and are crucial in the postnatal instruction of epithelial appendage formation such as hair follicles and sebaceous glands.

At the onset of skin epidermal development, the surface ectoderm displays a single layer of multipotent epithelial cells expressing KRT8 and KRT18 [47]. In addition, many other molecular effectors act alongside the modifications in KRT expression. One of the master regulators of epidermal development is tumour protein 63 (p63) [48–51]. In the developing epidermis p63 isoforms are found to be expressed as early as E7.5 and are able to induce the expression of *Keratin14* [51,52]. E9.5 is considered to be the onset of stratification. Even though the epidermis is still comprised of a single cell layer, E9.5 marks the time point at which expression of KRT5 and KRT14 are detectable [53]. In p63 compromised epidermis proper stratification and the expression of KRTs associated with stratification fails which highlights the crucial importance of p63 for proper epidermal development [54]. Furthermore, a layer of adhesive cells, called the periderm, overlays the surface ectoderm from E9 onwards and is shed around E16 to E17 [55].

At E12, the actual process of stratification begins. Stratification has been shown to coincide with a rotation of the cell division plane and is thus believed to be connected to differentiation, the cellular process of acquiring specialised features [56]. The epidermal intermediate layer marked by KRT1 and KRT10 expression is formed [40].

By E15.5 the epidermis is already around three cell layers thick, proliferation is restricted to the basal layer and suprabasal cell layers are marked by the

expression of KRT1 and KRT10 [47,57]. NOTCH1 and JAG1 expression can be detected and NOTCH signalling has been shown to drive stratification by inducing the expression of *Loricrin*, *Involucrin*, Peroxisome proliferator activated receptor gamma (*Pparγ*) and *nuclear factor kappa B* (*Nf-κB*) which in turn are able to modify epidermal behaviour during development [58–60]. In addition, NOTCH signalling downregulates p63 upon stratification [61]. Moreover, Bone morphogenic protein (BMP) signalling is crucial for epidermal development [62]. BMPs represent the largest family of secreted proteins within the transforming growth factor beta (TGF-β) pathway. At E15.5 expression of BMP6 is detected in suprabasal cell layers, while BMP7 expression is restricted to the basal cell layer [63–65].

Furthermore, the CCAAT-enhancer-binding proteins (C/EBPs) like C/EBPα, C/EBPβ, and C/EBPδ are expressed during epidermal development. C/EBPs present a family of transcription factors possessing a basic leucine zipper (bZIP) domain utilised to bind DNA (CCAAT) that enables them to modulate proliferation and differentiation of a cell [66,67]. In a *Keratin14* driven double knockout of C/EBPα and C/EBPβ, increased progenitor cell proliferation, defects in keratinocyte differentiation, and an overall loss of the epithelial barrier function were reported [68].

At E16.5 the epidermis is four cell layers thick and the expression of LOR becomes apparent [47,69]. BMP receptor 1A (BMPR-1A) is expressed in the basal layer of the murine epidermis while expression of BMPR-1B appears restricted to the suprabasal epidermis [70]. Subsequently, at E17.5 the expression of Filaggrin appears [71]. Finally, by E18.5 epidermal stratification is completed and the epidermal architecture is composed of the basal, spinous, granular, and cornified layers.

Finally, epidermal development is not finished at birth. The epithelial skin appendages, hair follicles, sweat and sebaceous glands, are only fully developed postnatally and are formed in processes involving the master regulator p63 [72]. Morphogenesis of epithelial appendages is teamwork and occurs in concerted localised processes involving the interplay between epidermal epithelial cells and the underlying dermal mesenchymal cells [57,73–75].

### 1.3.2 Oesophageal development

In mice, the foregut tube derives from a ventral folding of endodermal epithelial sheet during gastrulation at E8.0 [76]. The oesophagus, trachea, and stomach all originate from the anterior foregut endoderm [36]. Thus, development of the oesophagus and trachea are tightly connected. Their intertwined development is highlighted by rather common developmental defects affecting their separation like oesophageal atresia with or without tracheooesophageal fistula (OA/TOF) that occur when signalling pathways go awry [77]. The signalling pathways ensuring proper gut development and oesophageal-tracheal separation depend on endodermal-mesenchymal interactions and their specifics have recently been revealed using sequencing techniques [78–81].

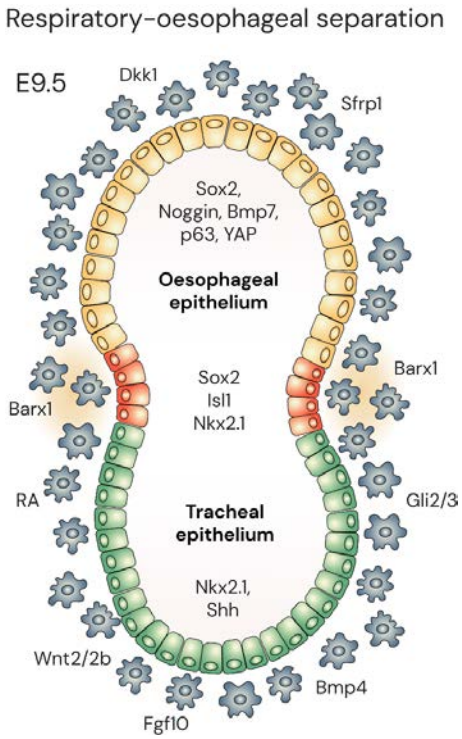



**Figure 3. Illustration of respiratory-oesophageal separation at E9.5.**
Respiratory-oesophageal separation occurs from E9.5 to E11.5. Dorsal-ventral patterning of signalling pathways and transcription factors, as well as crosstalk between the foregut endoderm and mesoderm ensure the accurate separation into the future oesophagus and trachea. SHH, BMP, FGF, RA, and WNT signalling instruct cell identity and separation. The dorsal foregut endoderm (yellow) will become the future oesophagus. Inhibition of WNT and BMP signalling maintains SOX2 expression in epithelial precursors of the oesophagus. The ventral foregut endoderm (green) will become the future trachea. Active WNT, BMP, SHH, and FGF signalling maintain NKX2.1 expression in epithelial precursors of the trachea. Midline endoderm (red) is located at the dorsal-ventral boundary and expresses both oesophageal precursor marker SOX2 and tracheal precursor marker NKX2.1 in addition to ISL1.

From E8.5 to E9.5 Schwann and neural crest cells migrate to the foregut and innervate its mesenchyme [82,83]. Proliferation and differentiation of these sex determining region Y-box 10 (SOX10) expressing precursor cells generates the enteric nervous system of the

oesophagus and finishes around two weeks after birth. However, their detailed contribution to oesophageal development has not been studied, yet. In contrast, the role of mesenchymal cells during oesophageal development has been investigated.

Mesenchymal expressed *Foxf1* haploinsufficiency has been shown to impair respiratory-oesophageal separation and foregut growth [84]. From E9.5 until E11.5 the dorsally oriented SOX2 expressing oesophagus and ventrally oriented NK2 homeobox 1 (NKX2.1) expressing trachea will ultimately split in a process called respiratory-oesophageal separation. Precise exposure, interplay, and timing of morphogens and core signalling pathways does not only direct proper foregut separation but additionally steers the expression of transcription factors that are known to direct oesophageal transformation. Retinoic acid (RA), Sonic hedgehog (Shh), transforming growth factor beta (TGF-β), BMPs, and WNT signalling, as well as a group of receptor tyrosine kinases such as fibroblast growth factors (FGFs), platelet derived growth factor (PDGFs), and epidermal growth factors (EGFs), have all been demonstrated to guide respiratory-oesophageal separation or oesophageal morphogenesis (reviewed in [85–90]).

For example, *Shh* expression is restricted to the endoderm during foregut development. In contrast, expression of active SHH signalling components, like the

Hedgehog-mediating GLI zinc finger (GLI) family transcription factors, is confined to the mesenchyme surrounding the foregut tube. GLI exerts positive regulation of BMP signalling in the ventral mesenchyme and inhibition of WNT signalling in the dorsal endoderm via BARX1 and is therefore crucial for respiratory-oesophageal separation [91–94]. In addition, active WNT signalling promotes the expression of *Fg10* that in turn elevates *Nkx2.1* expression while simultaneously repressing *Sox2* expression [88,93]. Opposite to the ventral foregut endoderm, the dorsal foregut endoderm, that will later generate the oesophagus, requires antagonising BMP signalling to ensure correct oesophageal development [92,95]. (**Figure 3**).

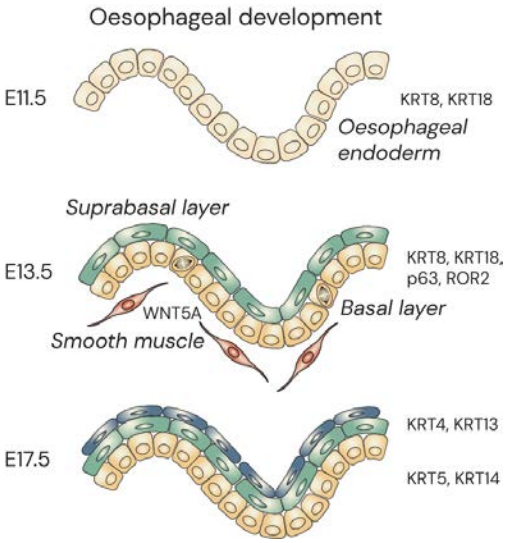



**Figure 4. Illustration of oesophageal development.** Following respiratory-oesophageal separation the oesophageal epithelium (beige) consists of a single layer of KRT8 and KRT18 expressing cells. From E13.5 asymmetric cell divisions of the basal layer can be observed and the epithelium begins to stratify instructed by p63. A WNT5A-ROR2 axis between the surrounding smooth muscle cells and oesophageal epithelium aids in this process. At E17.5 the oesophageal basal layer (yellow) starts to express KRT5 and KRT14 and expression of KRT4 and KRT13 is observed in suprabasal layers (green, blue).

Despite the established signalling pathway networks, the actual mechanism of respiratory-oesophageal separation is not fully understood. Live imaging studies using dorsally epithelial expressed (*Sox2*) driven GFP expression in *in vitro* organotypic cultures suggest that a "splitting and extension"-model is the most likely mechanism of separation [87]. Accordingly, the formation of an epithelial saddle that moves in a caudal to cranial manner occurs simultaneously with the continued growth of oesophagus and trachea and ultimately results in the separation of oesophagus and trachea [89,96].

After successful separation from the trachea the oesophagus consists of pseudostratified columnar cells that express KRT8 and KRT18 [97]. From E12 vagal sensory fibres begin to innervate the oesophagus [86,98]. While the contribution of vagal sensory fibres to peristalsis of the oesophagus is partly understood their potential influence on epithelial morphogenesis is unclear. From E13.5 the oesophagus gradually becomes a multi-layered stratified epithelium, coinciding with a rotation of cell division plane [99]. Similar to the developing epidermis p63 acts as a master regulator during oesophageal development and *p63* deletion results in stratification failure of the oesophagus [100,101]. Notably, p63 instructs the epithelial morphogenesis from cuboidal cells to a squamous cell type in a proximal-to-distal (craniocaudal) manner [102]. Similarly, the smooth muscle

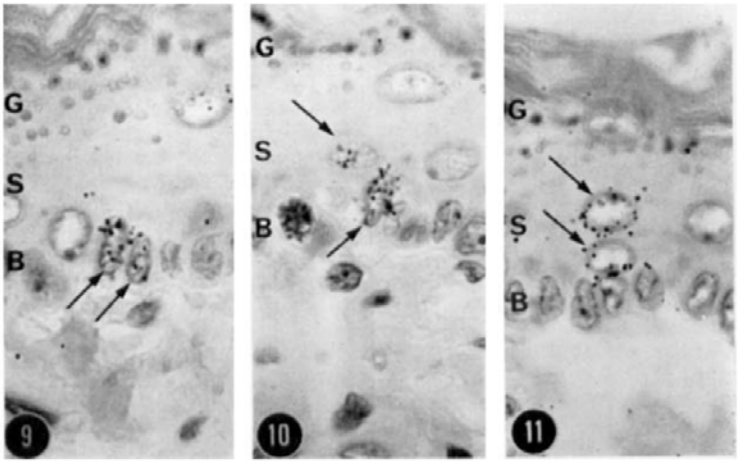
cell layers, that surround the oesophageal tube, are replaced in a craniocaudal fashion in between E15.5 and postnatal day 21 by striated muscle originating from cranial mesoderm [103–106]. Unlike the contribution of the nervous system, the contribution of smooth muscle cells to oesophageal development has been studied. Correct radial polarisation of smooth muscle cell progenitors has been shown to affect oesophageal elongation in a WNT5A-ROR2 dependent manner [107]. From E17.5 onwards the basal epithelial layer undergoes a gradual conversion from KRT8 and KRT18 towards KRT14 expression that is mostly finished by birth [108] (**Figure 4**).

However, oesophageal epithelial development as well as development of striated muscle and the enteric nervous system continues postnatally. From the first postnatal day onwards expression of the differentiation marker KRT10 appears [88]. By postnatal day 8 the epithelium becomes keratinised and changes in the oesophageal epithelium occur until at least up to postnatal day 70, to accommodate for ongoing tissue expansion in response to stretching forces [109,110]. Important signalling pathways and transcription factors at play during development potentially provide important cues to the mechanisms regulating adult oesophageal homeostasis. Knowledge of how the balance between proliferation and differentiation is maintained during adult homeostasis might ultimately aid in the understanding of tumour onset. Vice versa, factors that are deregulated during cancer onset might aid in recognition of factors that are important for sustaining oesophageal homeostasis.

## 1.4 Oesophageal homeostasis

Due to its simple architecture, the adult oesophagus presents an ideal system to interrogate adult epithelial homeostasis. The first comprehensive overview of the mammalian oesophagus was provided by Emil Goetsch. Describing the remarkable differences in oesophagi of various species, he noted that the rodent oesophagus consists of a "..layer of stratified squamous cells presenting [...] different members [...] of two main types." [111]. The plain architecture of the oesophagus is met by an equally simple function - the transportation of food from the pharynx to the stomach. In mice, only the basal cells of the four to five cell layer thick epithelium can proliferate in normal conditions. Upon differentiation the unipotent epithelial basal cells undergo stratification and are eventually shed off into the lumen. In contrast to the epidermis, the mouse oesophagus is devoid of any epithelial appendages. Therefore, questions concerning tissue dynamics, like the proliferative, differentiating, and stratifying behaviour, can directly be addressed to the exclusive and unrivalled oesophageal progenitor cell population.

In 1965, Marques-Pereira and Leblond investigated incorporation of the radiolabelled DNA precursor thymidine-$H^3$ into oesophageal cells (**Figure 5**) [14]. Previous observations had suggested that frequently proliferating cells of the oesophagus reside in the basal layer and mostly (but not exclusively) undergo symmetrical cell divisions [112]. Their




Figs. 9, 10 and 11 Radioautographs of esophageal epithelium of 200 g male rat sacrificed 48 hours after injection of 1 $\mu$c of thymidine-H$^3$ per g of body weight. × 875.

Fig. 9 Basal pair.
Fig. 10 Mixed pair.
Fig. 11 Differentiating pair

**Figure 5.** Thymidine-H$^3$ labelling experiments of Marques-Pereira and Leblond.
(Marques-Pereira, J. P., and C. P. Leblond. "Mitosis and differentiation in the stratified squamous epithelium of the rat esophagus." American Journal of Anatomy 117, no. 1 (1965): 73-87.) © *John Wiley and Sons, 2005.* Reprinted with permission.

observations, later confirmed by Smart, suggested that oesophageal progenitor cell fate choices are random and can best be described stochastically following cell division [14,99,112].

Their insights allowed for a general understanding of oesophageal homeostasis which presented continuous cell replacement achieved by active cell production and simultaneous cell loss. In addition, their observations on the equal probability of daughter cells to enter the suprabasal layer after division suggested that oesophageal progenitor cells are of equivalent potential [14].

However, the molecular mechanisms functionally instructing the basal cell population to proliferate or differentiate remained unknown and could only be investigated decades later. The interdisciplinary work of Jaenisch and Mintz who combined molecular biology and embryology, enabled the study of specific genes *in vivo* [113]. Their work enabled the generation of transgenic mice and facilitated monitoring or abrogation of gene expression *in vivo* [114].

### 1.4.1 Transcription factors regulating oesophageal homeostasis

Transcription factors are crucial determinants of cell fate decisions [115]. Correct development of the oesophagus largely depends on transcription factors like SOX2 and NKX2.1 [116–118]. Since the deletion of genes often causes phenotypic defects during development much of our current knowledge about influential transcription factors during adult oesophageal homeostasis is a combination of discoveries made in development

and pathologies like cancer. In addition, recent advances in organotypic cell culture allow for the refined understanding of the function of transcription factors in development and homeostasis in human settings [119].

SOX2 is not only of crucial function during development but equally important in the maintenance of oesophageal homeostasis. Que and colleagues discovered that hypomorphic $Sox2^{EGFP/Cond}$ mice, despite developing intact oesophagi, suffer from impaired postnatal oesophageal maturation [117]. Conversely, overexpression of SOX2 in the basal layer of the oesophagus led to increased progenitor cell numbers and defective differentiation [120]. The significance of adequate SOX2 levels for oesophageal homeostasis was underlined by studies associating squamous cell carcinoma with increased SOX2 expression [121,122]. Using co-immunoprecipitation (Co-IP) and chromatin-immunoprecipitation with sequencing (ChIP-seq), Watanabe and colleagues demonstrated the interaction of SOX2 with other transcription factors such as p63, C/EBPβ, and KLF5 [123]. Consequently, SOX2 might be decisive in coregulating gene expression of transcription factors that govern physiological oesophageal progenitor cell fate. Indeed, SOX2 was identified to coregulate the expression of the oncogene *Etv4* in combination with p63 in squamous cell carcinoma [123]. Intriguingly, SOX2 hypomorphic mice exhibited a lack of p63 within the oesophageal epithelium, strengthening the potential coregulatory function of SOX2 and p63 [117].

p63 knockout mice display differentiation defects in several epithelia [100,108]. In the epidermis, p63 was shown to govern the proliferative potential and accurate stratification of epidermal keratinocytes [51]. In the oesophagus, p63 deletion led to defects in stratification. In addition, p63 deletion resulted in the appearance of ciliated and goblet cells in the oesophagus. Altogether, these findings establish a function of p63 for the formation of stratified epithelia, differentiation, and cell identity [101,102,124,125]. Last, deletion of p63 in organotypic cell culture inhibited the self-renewal of the oesophageal epithelium [126]. In conclusion, SOX2 and p63 demonstrate paramount influence on proliferation, differentiation, and stratification of oesophageal progenitor cells.

While the functions of SOX2 and p63 appear to involve a broad spectrum of progenitor cell fate decisions, the Krüppel-like factor (KLF) transcription factors KLF4 and KLF5 present more precise but not minor important functions for oesophageal homeostasis. KLF5 expression is restricted to the oesophageal basal layer while KLF4 is highly expressed in suprabasal layers and occasionally detected in basal layer cells of the oesophagus [127]. The sole reported effect of KLF5 overexpression in the oesophageal epithelium was increased progenitor proliferation. Surprisingly, increased progenitor proliferation did not lead to hyperplasia or other abnormalities in the oesophageal epithelium suggesting that KLF5 exclusively controls proliferative potential of basal layer cells [127]. In contrast, deletion of KLF4 deletion led to hyperproliferation and delayed oesophageal epithelial maturation resulting in hypertrophy and dysplasia [128]. In addition, KLF4 was demonstrated to regulate *Klf5* expression and to bind KLF5. Hence, KLF4 might

be able to repress the action of KLF5 upon differentiation and deletion of KLF4 induces proliferation in suprabasal cells via loosening the pro-proliferative mechanism of KLF5. Recently, KLF4 has been shown to mark committed basal layer cells that govern the transition from oesophageal development to adult homeostasis in response to YAP mediated mechanical stretch [110].

### 1.4.2 Core signalling pathways regulating oesophageal homeostasis

Transcriptions factors are pivotal in the regulation of cell fate. Besides, morphogens present another crucial factor in maintaining oesophageal homeostasis. Morphogens are secreted soluble proteins and their spatiotemporal expression patterns guide accurate developmental pattern formation [32]. The cellular interpretation of morphogen gradients dictates the expression of genes such as transcription factors that determine cell fate and ultimately control homeostasis [31]. RA, SHH, BMP, NOTCH, WNT, and HIPPO signalling have been established in influencing oesophageal development and homeostasis. In the following, a comprehensive overview of their respective functions is given (**Figure 6**).

*Retinoic acid signalling*

RA signalling is crucial in early development and closely related to SHH signalling. In mouse whole embryo culture, chemical inhibition of RA signalling led to the complete abrogation of *Shh* and *Ihh* signalling in the foregut epithelium, a severe reduction of *Wnt2* and *Wnt2b* in foregut mesenchyme [129]. Retinoic acid receptors (LXR/RXR) are found to be upregulated or activated in oesophageal adenocarcinoma and oesophageal squamous cell carcinoma, respectively [130]. RA signalling has been shown to promote glandular differentiation without affecting proliferation in human *ex vivo* oesophageal epithelial cultures suggesting that RA signalling influences cell identity and contributes to the condition of Barrett's oesophagus [131]. However, RA treatment of otherwise unmodified mice has been demonstrated to increase oesophageal tissue turnover [10,132]. Since RA treatment did not result in transformed epithelial tissue, it is likely that RA signalling alone is not sufficient to induce malignant tissue transformation.

*Sonic hedgehog signalling*

Complementary to observations of increased RA signalling, mouse models of surgical chronic-reflux displayed an activation of SHH signalling that transformed epithelial progenitor cells into columnar cells via a dedifferentiated embryonic-like progenitor cell state [133]. In another study van Dop and colleagues utilised two different mouse models to investigate SHH signalling in oesophageal homeostasis [134]. Under normal conditions, *Gli1*, Smoothened (*Smo*), and HH-interacting protein (*Hhip1*) expression was found to be restricted to basal layer cells suggesting that SHH signalling serves as an autocrine signal for oesophageal progenitor cells. Increased SHH signalling was either achieved by conditional whole-body deletion of the SHH inhibitor receptor protein patched homolog 1 (Ptch1) or forced *Krt5* driven overexpression of SHH transcriptional effector protein GLI1.

Both mice exhibited prominent expansion of the progenitor cell compartment, increased p63 and KRT14 expression, as well as a corresponding decrease of epithelial differentiation. In combination, these findings imply that SHH signalling is important for a oesophageal progenitor cell identity. However, since the *Ptch1* deletion affects the entire body and *Gli1*-reporter activity is low, it is difficult to discern if the observations originate in epithelial or mesenchymal cells of the oesophagus. Thus, the possibility remains that the reported phenotype is due to a cascade of indirect effects [134]. Conditional epithelial specific *Shh* deletion would enable a better understanding of the necessity of SHH signalling in oesophageal homeostasis. Thus, the importance of SHH signalling for oesophageal homeostasis warrants further research. Notably, another study revealed that constitutive *Shh* expression in the oesophageal epithelium resulted in BMP4 upregulation of the underlying mesenchyme. In turn, the epithelium transformed into a columnar phenotype displaying atypical expression of SOX9, KRT8, KRT18, and columnar cell cytokines showcasing a close connection of SHH and BMP signalling in the oesophageal derived epithelium [135].

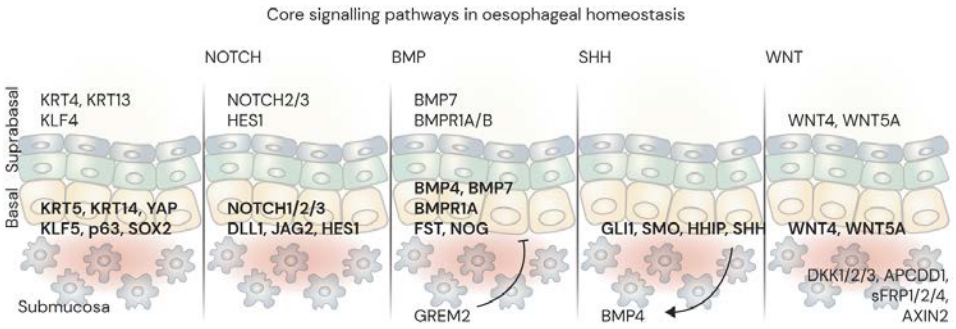



**Figure 6. Core signalling pathways in oesophageal homeostasis.**
Overview of the expression of components in general, in NOTCH, BMP, SHH, and WNT signalling (from left to right). Position of text indicates expression in either the submucosa (red), basal epithelial layer (yellow), or suprabasal epithelial layer (green, blue).

*BMP signalling*

During development, the BMP antagonist *Noggin* is expressed in the dorsal endoderm and epithelial stratification and differentiation are impaired upon *Shh* driven endodermal *Bmpr1a* deletion [95]. In a comprehensive study, Jiang and colleagues reported expression of BMP4 and BMP7 in the adult oesophageal epithelium [136]. However, BMP reporter expression (BRE-lacZ) was limited to suprabasal cell layers. Similar to foregut development, the expression of the BMP antagonists *Follistatin* and *Gremlin2* was restricted to oesophageal progenitor cells or the underlying mesenchyme respectively, ensuring BMP signalling specificity in suprabasal cell layers. In subsequent experiments, *Krt5* driven overexpression of a constitutively active BMP receptor (BMPR1A) led to increased epithelial differentiation. In turn, *in vitro* progenitor cells displayed reduced

proliferation and increased differentiation when treated with BMP4. In conclusion, BMP signalling likely influences epithelial cell fate during development and adult homeostasis. The importance of BMP signalling is strengthened in *Shh* driven endodermal *Bmpr1a;b* double-mutant mice in which displayed foregut separation defects [118]. The separation defects could partially be rescued by additional *Sox2* deletion and constitutively active BMPR1A was shown to directly repress promotor activity of *Sox2 in vitro* illustrating the synergy of morphogens and transcription factors.

*WNT signalling*

While the role of WNT signalling in adult oesophageal homeostasis is largely unexplored, SOX2 and WNT signalling synergy has been reported during foregut development. SOX2 suppressed WNT signalling via enhancing the expression of WNT inhibitors *Sfrp1*, *Sfrp2*, and *Dkk1* in the surrounding mesenchyme ensuring accurate oesophageal development [119]. On the other hand, Barx1 deletion led to a loss of WNT inhibitors sFRP1 and sFRP2 in the foregut mesoderm resulting in a respiratory fate of the dorsal foregut endoderm [137]. In addition, WNT signalling transducer β-catenin mutants develop normal oesophagi emphasising that active WNT/ β-catenin signalling is not needed for oesophageal fate decisions [93,137]. BAT- and TOP-Gal reporter mice surprisingly demonstrated active WNT in the developing oesophageal epithelium while oesophageal organoids develop independent of WNT promoting factors such as R-spondin 1 [138,139]. Most studies indicate the importance of WNT signalling suppression to develop an oesophageal epithelial fate despite that WNT reporter mice suggest active WNT signalling. Thus, similar to BMP signalling that is usually repressed in oesophageal progenitor cells, repression of WNT signalling in oesophageal progenitor cells might play an important role in sustaining tissue homeostasis during adulthood.

*NOTCH signalling*

NOTCH signalling is as another essential pathway in oesophageal homeostasis regulating correct differentiation. NICD1 induced *Notch3* transcription and expression of *Hes5* and early differentiation markers *Krt13* and *Ivl* through complex formation with DNA binding transcription factor CBF-1/RBP-j κ,Su(H),Lag-1 (CSL), thereby initiating oesophageal progenitor differentiation. Additionally, *in vivo* NOTCH inhibition via *Krt14* driven DNMAML1 inhibited epithelial differentiation and resulted in basal cell hyperplasia [140]. Concomitantly, an upregulation of NOTCH pathway components led to decreased proliferation and an accompanying increase in differentiation that correlated with NOTCH ligands DLL3 and JAG2 as well as the expression of NOTCH target gene *Hes5* [141]. A recent study reported a competitive advantage of wild type oesophageal progenitor cells with *Notch1* deletion. However, Notch1 deletion impaired tumour growth a discrepancy that is likely due to altered cell dynamics of wild type and tumour cells [142]. Last, chemical inhibition of NOTCH in human epithelial endoscopy derived organoid cultures resulted in deregulated differentiation with increased progenitor cell numbers [143]. Collectively, these findings

establish NOTCH signalling as an important driver of oesophageal epithelial differentiation. Strikingly, NOTCH signalling was shown to integrate with YAP/TAZ mediated mechanical transduction signalling to regulate epidermal cell fate emphasising synergies of core signalling pathways in determining cell fate and suggesting a potential role for HIPPO signalling in oesophageal homeostasis [144].

*HIPPO signalling*

A connection of NOTCH and YAP has also been shown in the developing oesophagus. Endodermal driven YAP deletion in $Shh^{Cre};Yap^{fl/fl}$ mice, led to reduced epithelial stratification while constitutively active YAP expression resulted in abnormal epithelial expansion of both progenitor and differentiated cells. Yap deletion or overexpression resulted in a reduced or elevated expression of NICD1, respectively [145]. In postnatal development, YAP regulates mechanical stress induced emergence of a KLF4 expressing progenitor population thereby coordinating the transition to adult oesophageal homeostasis [110]. YAP/TAZ signalling has not only been shown to integrate with NOTCH signalling but also with WNT emphasising the potential co-regulation of core signalling pathways during oesophageal development and homeostasis [146].

Despite the large body of work which has provided insights into oesophageal homeostasis, many more studies are still required to better understand the intricate control of oesophageal homeostasis. While transcription factors and core signalling pathways such as BMP and SHH are comparably well understood during foregut development their precise functions during adult homeostasis remain largely elusive. In particular, signalling pathway crosstalk such as the synergistic NOTCH and YAP signalling or cell-cell communication in oesophageal homeostasis are not understood in detail. However, cell-cell interactions and the reciprocal signalling of foregut endoderm and mesenchyme are of crucial importance during foregut development [79,107].

## 1.5 Cellular plasticity and the stem cell niche

Potential cell-cell communication or signalling pathway interplay between the oesophageal epithelium and underlying mesenchyme that influences cell states and fate decisions of oesophageal progenitor cells have not been explored in homeostasis yet. However, RA and SHH signalling are able to alter oesophageal epithelial cells from a squamous to columnar identity showcasing the importance of epithelial cell plasticity in malignant tissue transformation [131,133].

A century ago, Spemann and Mangold made fascinating discoveries. In tissue transplantation experiments they were the first to show that developmental cell fates can be influenced by soluble factors from other cell populations [147,148]. Afterward, experiments in adult rodents displayed that large vibrissae bulbs became short vibrissae after transplantation into the outer ear and were able to grow over generations [149]. However, cellular fate is not only influenced by soluble factors. By inserting an epidermal nucleus

into an amphibian enucleated oocyte, Gurdon obtained an entire organism [150]. Beyond proving that each adult nucleus contains the entire genome the nucleus insertion additionally demonstrated the enormous plasticity of epithelial cells. Finally, Takahashi and Yamanaka were able to reprogram fibroblasts into cells with pluripotent potential, the induced pluripotent stem cell (iPSC), solely via ectopic expression of transcription factors [151]. Since then, fibroblasts and other cells have been converted to other lineages and tissue cell types – all relying on cellular plasticity and the ability of a cell to be reprogrammed.

Eventually, *in vivo* epithelial cell plasticity following injury became apparent. One prominent example is the intestinal crypt which is organised in a hierarchical manner [152]. While slow cycling *Lgr5* positive intestinal stem cells are thought to hold the highest stem cell potential, *Bmi1* positive '*+4*' as well as *mTert* positive cells bear stem cell potential as well [23,153–155]. Surprisingly, upon deletion of *Lgr5* stem cells the gut was observed to be stable for at least one week suggesting that homeostasis can equally be temporarily achieved without conventional intestinal stem cells [156]. Subsequently, various depletion studies demonstrated that cells of the secretory or enterocytic lineage were able to revert back to become *Lgr5* positive cells [157–159]. Similar observations have been reported in other tissues including the trachea and stomach. Depletion of tracheal basal cells resulted in the de-differentiation of committed secretory 'Clara' cells to basal stem cells [160]. In the stomach crypt, a *Troy* positive quiescent cell population was able to restore crypt cells of all lineages following tissue damage [161]. Finally, even the formerly supposed rigid lineage hierarchies of the HSC are beginning to blur [162–164]. Altogether, experiments demonstrating cell transformation *in vivo* fuelled the quest of identifying responsible factors that dictate cell fate and plasticity. Due to the discovery of soluble factors the microenvironment a stem cell resides in was a likely instructor of epithelial cell choices.

*The stem cell niche*

The idea that a niche influences stem cell decisions was brought about by Schofield who suggested that haematopoietic stem cells (HSCs) are instructed by other cells to maintain their stem cell potential and undergo differentiation if unable to occupy this specific environment [165]. Subsequently, extensive research has established the importance of the milieu a stem cell resides in. This specific microenvironment, encompassing many components including the extracellular matrix (ECM), associated supportive cells, secreted signalling factors, or the microbiome, is referred to as the stem cell niche. The ECM influences stem cell behaviour via its stiffness, cell adhesion properties, or mechanical forces [166–169]. Moreover, stem cells often reside in hypoxic milieus indicating that cell metabolism and oxygen levels are important aspects regulating tissue homeostasis [170–173]. Especially intriguing considering gastro-oesophageal reflux conditions, intracellular pH levels demonstrated crucial importance in cell fate decision making [174]. The entirety of the stem cell niche generates and sustains a milieu that enables stem cells to exert the accurate balance of self-renewal and differentiation necessary to maintain homeostasis [175–178].

*Cell-cell communication within the stem cell niche*

An essential discovery of the intestinal stem cell niche were Paneth cells that reside in the intestinal crypt and secrete various WNTs, which are indispensable signalling factors promoting intestinal self-renewal [179,180]. After Durand and colleagues demonstrated that Paneth cell ablation did not result in crypt destruction an important role for mesenchymal cells beyond regulating accurate development emerged [181]. Subsequently, mesenchymal fibroblast populations were identified as alternative sources of WNT signalling in the intestine and colon contributing to sustain crypt functionality [182–184]. In the skin, distinct fibroblast lineages were shown to influence epithelial cell fate after injury via ECM deposition and diverse responsiveness to epithelial derived WNT signalling [185]. In addition, the dermal papilla, a condensation of mesenchymal cells underlying the hair follicle, serves as an invaluable signalling centre regulating hair formation and cycling. Interaction of mesenchymal and epithelial cells is not only responsible for activation of hair follicle stem cells but additionally the lineage heterogeneity among transit amplifying cells of the hair follicle [186,187]. In the lung, fibroblasts derived WNT signalling was demonstrated to be of critical importance for the maintenance of alveolar AT2 cells [188]. In conclusion, fibroblasts present an essential component of stem cell niches that are able to provide molecular signalling cues and aid tissue repair after injury.

Further, various immune cells, either tissue resident or recruited following injury, compose parts of stem cell niches. Eosinophilic oesophagitis (EoO) patient derived organoids present normal epithelial structure. The addition of cytokines typically expressed in EoO, like IL-13 and TNFα, led to reduced differentiation and increased progenitor cell numbers resembling EoO patient tissue [143]. In another study, wound infiltrating macrophages were shown to regulate hair follicle neogenesis via TNFα-AKT/β-catenin signalling in *Lgr5* positive hair follicle stem cells. Accordingly, deletion of macrophages led to defects in wound-induced hair anagen [189]. Likewise, macrophages were shown to be a necessary niche component for regenerative responses after injury in the colon. *Csf1* deletion did not show any effects on colon crypt maintenance presumably because specific CFS1-dependent macrophage subsets exert homeostatic functions [190–192]. In the mammary gland, macrophages have been shown to influence the regenerative capacity of stem cells [193]. However, immune cells do not only exert vital functions following injury. Wang and colleagues were able to identify *Trem2* positive macrophages that maintain hair follicle quiescence via cytokine induced JAK-STAT5 signalling in homeostatic conditions [194]. While immune cells are able to influence stem cell behaviour they have also been shown to support homeostasis by affecting other niche cells. A group of self-maintaining macrophages assisted intestinal homeostasis by sustaining submucosal endothelial cells and enteric nerves [195]. Another experiment revealed that γδ T-cells within the epidermis interact directly with epithelial cells and influence hair follicle regeneration after wounding. In addition, γδ T-cells produced fibroblast growth factor 9 (FGF9) urged WNT secretion of

fibroblasts that in turn induce wound-induced hair neurogenesis suggesting that cellular networks exist to re-establish homeostasis after injury [196].

Notably, cell-cell interactions are not unidirectional. The presented examples display the influence of fibroblasts and immune cells on epithelial cells. In turn, epithelial cells are able to affect fibroblasts and immune cells. In the thymus, epithelial cells provide distinct environments steering thymocyte selection and T cell development [197]. Moreover, tissue resident macrophages of various tissues including liver, lung, and intestine, exhibited enhancer profiles at a level of distinction that could not be explained by differences in their developmental origin. Instead, it is likely that distinct tissue- and definite lineage-specific transcription factors control the chromatin landscape of tissue-resident macrophages [198]. In addition to fibroblasts and immune cells, neurons influence epithelial homeostasis. In the absence of a perineural niche, lineage plasticity of SHH signalling responsive bulge stem cells was inhibited [199,200]. The focus of this paragraph has been to expose the contribution of diverse cell types within the stem cell niche to epithelial cell behaviour during homeostasis and repair. Nonetheless, it is worth noting that reciprocal interactions of macrophages and fibroblast are essential during tissue repair and tissue maintenance i.e. by ECM deposition or secretion of growth factors [201–203].

It becomes evident that homeostasis is sustained due to the interplay of cells within the stem cell niche that generate an instructive milieu. In addition, epithelial cell-cell contact dependent mechanisms determine homeostatic behaviour. Originally, epithelial proliferation was suggested to cause local crowding in combination with delamination that ensured homeostasis [204,205]. In cell culture experiments, autocrine WNT/β-catenin signalling was demonstrated to influence the cell state of neighbouring cells. Neighbouring cells were proposed to be asymmetrically coupled in a contact-dependent mechanism that ultimately ensures homeostasis [206]. However, live imaging based experiments indicated that local differentiation drives proliferation and the expansion of nearby stem cells thereby counteracting the fate of their differentiating neighbours [207]. Additionally, direct contact to a basal cell prevented the de-differentiation of secretory lineage cells upon basal cell depletion in the trachea [160]. In addition, epithelial cell migration might present an additional feature employed by epithelial cells to ensure tissue homeostasis [208].

To date, a detailed description of a potential oesophageal stem cell niche is missing. The absence of obvious architectural features like the hair follicle in the epidermis or the crypt in the intestine and colon hamper the identification further. However, indications of progenitor heterogeneity within the oesophageal progenitor population and observations of irregular *Bmp4* and *Gli1* expression in the oesophageal epithelium as well as irregular expression of *Gremlin2* in the oesophageal fibroblast population suggest the presence of various microenvironments in the oesophagus [134,136,209–211].

## 1.6 Implications for the human oesophagus

The human and mouse oesophagus differ in their general architecture. The mouse oesophagus displays prominent keratinisation which is completely absent in the human oesophagus. While the human epithelial mucosa consists of up to 40 cell layers, the mouse oesophageal epithelium is comprised of three to four cell layers only. In human, four to five cell layers possess proliferative capacity whereas only the basal cell proliferates in mouse. Moreover, prominent mesenchymal invaginations, so called papillae, fold the human oesophagus and separate it into distinct papillary and interpapillary regions [212]. It was shown that epithelial progenitor cell proliferation and cell fate differ depending on their placement in papillary or interpapillary zones, indicating either a potential stem cell hierarchy or the existence of an instructive microenvironment [213]. While the interpapillary zone is supposed to contain rarely proliferating, asymmetrical cell division and low cell adhesion, the papillary zone displays frequent cell divisions that occur predominantly symmetrical. However, the clone forming efficacy of human oesophageal epithelial cells isolated based on CD34 and EpCAM was equal suggesting that a broad range of cells from varying differentiation stages bears equal progenitor potential [214,215]. Furthermore, the human oesophagus contains submucosal glands that harbour cells with proliferative potential that are able to change fate in metaplasia [216]. On a final note, label retaining cells were identified in the human oesophageal basal layer after oesophagectomy and within tissue affected by Barrett's oesophagus. However, this studies did not exclude the possibility that cell populations of non-epithelial origin comprise the labelled entities [217]. Overall, the mouse and human oesophagus display a range of anatomical differences which might limit the transferability of findings utilising mice as a model system.

Nonetheless, due to the overall similarity of the human and mouse genome and many conserved functions in between mice and human biology, mouse models present the best system to study the underlying molecular mechanisms of oesophageal homeostasis to date. I*n vivo* knock-outs or *in vivo* lineage tracing present powerful tools to understand the behaviour of cells in a tissue [218]. Regardless of the benefits mouse models hold, extensive verifications of insights obtained from mouse research are absolute prerequisites before the knowledge obtained in animal studies can be translated and used as potential human therapeutics.

The emergence of organoid cultures might enable a better understanding of human oesophageal physiology. Studies utilising organoids derived from pluripotent stem cells or human oesophageal cell lines reflect many discoveries obtained conducting mouse oesophageal research [119,143,219,220]. For example, NOTCH inhibition in human oesophageal cell derived organoids revealed similarities to cytokine mediated epithelial structure changes in EoO patient-derived organoids [143]. Furthermore, similar to the mouse oesophagus *Sox2* mutations have been determined to cause abnormalities in the human oesophagus and *Sox2* amplification and overexpression have been reported in human oesophageal

squamous cell carcinoma [77,122]. In human pluripotent derived oesophageal organoids faithfully recapitulated oesophageal developmental processes and might therefore present a powerful tool to model human pathologies [119]. However, so far adult human oesophageal progenitor derived organoids show limited passaging capacity despite a comparable transcriptome to oesophageal tissue either implying limited proliferation potential of human oesophageal progenitors or insufficient growth factor supplements in the *in vitro* setting [221].

## 1.7 Aim: Characterising oesophageal homeostasis

The overarching aim of the work presented here is to investigate the cellular networks and mechanisms that maintain homeostasis in the mouse oesophageal epithelium. The introduction presents examples of signalling pathways that ensure accurate oesophageal development and are beginning to be understood during oesophageal homeostasis, Collectively, it is supposed to present the enormous amount of regulatory mechanisms that operate to sustain homeostasis. Currently, the regulatory tools governing homeostasis in the oesophagus remain poorly understood. To close this gap in knowledge, the work presented here is mostly concerned with oesophageal progenitor cell behaviour, cellular diversity, and cell-cell interactions occurring in oesophageal homeostasis.

A range of studies demonstrates equal potential and stochastic behaviour of oesophageal progenitor cells [222–224]. However, regional confinement and the random targeting employed in these studies do not necessary exclude the existence of rare specialised cell types within the oesophageal epithelium, as for example was recently discovered with specialised KRT8 expressing taste bud cells in the proximal oesophagus [10,223–226].

In **paper III**, we combined lineage tracing experiments and single cell sequencing to understand and describe the contribution of *Troy* expressing progenitor cells to oesophageal homeostasis. Further, we established an oesophageal organoid co-culture system that facilitates the investigation of specific cell-cell communication between oesophageal fibroblasts and epithelial cells in **paper II**. In addition, in **paper IV** we set out to characterise and identify cellular components of the mouse oesophagus during homeostasis. Uneven cellular distributions or specific localisations of distinct cell populations might reveal basic insights into a potential oesophageal progenitor cell niche. In **paper I**, we aimed to determine the role of HLH protein ID1 during epidermis development.

# Chapter 2

# 2 Methods

**Cre/lox site-specific recombination**

In 1988, Sauer and Henderson reported site-specific recombination in mammalian cells by the Cre recombinase of bacteriophage P1, advancing the work of Sternberg and Hamilton of 1981 [227,228]. Their findings were the foundation of a powerful tool widely used today. In essence, the site-specific recombinase Cre excises (or turns) any DNA that is found between two specific 34 bp long DNA recognition sites, so called locus of crossing [x-ing]-over bacteriophage P1 (loxP) sites. Subsequently, an inducible Cre-loxP system in which Cre is activated by 4-Hydroxy tamoxifen, the metabolised form of Tamoxifen, was reported [229]. The key of the system is crossing mice that express Cre recombinase under a time, tissue, or cell specific promotor with mice that carry loxP sites. Depending on the positioning of these loxP sites the system can be utilised in a wide variety of ways. Originally, loxP sites flanked genes or exons of interest enabling the deletion of the targeted genes or exons.

In this work, the Cre/lox system is for example used to trace *Troy* and *Sox2* expressing cells and all their respective offspring within the oesophagus. In our setup, Cre recombinase excises a transcriptional stop signal called STOP cassette [230]. Excising this STOP cassette allows the transcription of reporter constructs like the fluorescent proteins EGFP or tdTomato. Once excised the offspring of cells that underwent recombination will carry the reporter expression, so will its offspring and so forth. This method is called lineage tracing since it allows to track the behaviour of cells originally expressing a gene of interest and all their offspring. In addition, the Cre/lox system is used to label cells of PDGFRα$^{H2BeGFP}$ [231], IL-17A$^{IRES-eGFP}$, Lgr5$^{eGFP-IRES-CreERT2}$;R26R$^{tdTomato}$, Cx3Cr1$^{GFP}$;Ccr2$^{RFP}$, and Cx3cr1$^{CreER-eYFP}$ mice.

However, the system (like any other) is not entirely perfect. Low amount of Cre recombinase can result in partial excision of the three identical SV40-derived poly(A) signal repeats constituting the STOP cassette resulting in variability of marker expression [232]. Moreover, the application of Tamoxifen needed to induce the Cre/lox mechanism, can cause effects. Tamoxifen is an estrogen receptor agonist and as such bears the ability to modulate estrogen signalling. For instance, oral and intraperitoneal administration of Tamoxifen has been shown to deplete gastric parietal cells at a dose of >3 mg/20 g body weight [233]. Most importantly, Cre recombination has been shown to occur independent of Tamoxifen administration [234]. Therefore, titration of Tamoxifen administration and control of potential baseline recombination are crucial for a proper assessment of the obtained results.

**Single cell sequencing**

The origins of single-cell sequencing go back a long way. In 1953, Franklin, Watson, and Crick unveiled the double helix structure of the DNA consisting of the four bases adenine, cytosine, guanine, and thymine. In 1977, "Sanger sequencing", the first sequencing method was reported using radiolabelled dideoxynucleotides that terminate DNA synthesis during replication. The labelled fragments could be separated by size and thereby revealed the DNA sequence [235]. The most widely used method today, the Illumina CRT system, adopted a similar approach to Sanger sequencing. Cyclic reversible termination employs fluorescent-labelled 3'-blocked deoxynucleotides that abrogate DNA synthesis and allow for uncovering DNA sequences base-by-base [236].

In this work, we use bulk RNA sequencing of sorted tdTomato positive cells in **paper III**. Bulk RNA sequencing allows for a detailed comparison of the global transcriptional landscape between defined conditions of pooled cells, biopsies, or tissue sections. However, highly organised tissues, organs, and even entire organism are comprised out of many different cell types. After several years of advancing RNA sequencing it became possible to capture the transcriptome of individual cells. In 2009, the first single-cell RNA sequencing method was reported and continuously improved in the following years regarding cellular throughput, captured genes, and sensitivity [236–238]. Single-cell RNA sequencing allows for a detailed exploration and discovery of the heterogeneity within and between individual cells and enables the decoding of tissue complexity on a transcriptional level. The technology led to the identification of rare but functionally important cell types, like ionocytes in the airways, and improved our understanding of developmental processes and tissue composition, exemplified by the generation of the tabula muris [239,240].

In this work, we use the 10x Genomics Chromium method, a commercialised droplet-based platform, in **paper III**. The platform exploits microfluidic droplets to capture the gene expression information contained in a single cell in an affordable and high-throughput manner. Cell and gene specific quantification of mRNA is facilitated by unique molecular identifiers (UMIs). UMIs enable a direct comparison of the gene expression level as they are considered to mirror the original number of mRNA molecules and can counteract amplification biases introduced by error prone PCR [241]. However, the method is limited to capturing only the 3'-end or 5'-end fraction of the transcriptome.

In **paper IV**, we utilise plate-based Smart-seq3 and Smart-seq3xpress that offer accurate RNA molecule counting in combination with full-length transcriptome coverage [242,243]. We wanted to enrich for rare intraepithelial CD45 positive cells, therefore we turned to plate-based method that allowed us to sort selected single cells by FACS. In addition, Smart-seq protocols outperform the 10X Chromium platform regarding the detection of lowly expressed genes and enable the detection of splice variants due to full-length sequencing. In detail, the methods are excellently described here [244].

Briefly, common to both methods is that they rely on capturing the poly(A) tail of mRNAs using an oligonucleotide containing a poly(T) stretch to generate first strand cDNA [241]. Generation of full length cDNA is achieved by template switching during reverse transcription. A template switching oligo enables specific reverse transcriptase to surpass and extend through and beyond the 5'-end cap of mRNA [242,243]. Subsequently, the cDNA is amplified by PCR. Further, both methods rely on mechanical fragmentation or tagmentation of the final cDNA molecules since they rely on short read sequencing [245]. An important distinction between the two methods is that the 10X Genomics method introduces cell-identifying barcodes as well as the UMI when the poly(A) tail of mRNA is captured while Smart-seq methods introduce barcode and UMI following cDNA fragment creation via tagmentation. In doing so, a cell specific assignment of the fragmented full length transcriptome is achieved. After a final PCR amplification using primers containing cell or sample specific barcodes the obtained cDNA library is ready to be sequenced. Following sequencing, the data is aligned to a reference genome and can be analysed using a wide range of available software like R-based RaceID, Seurat, or Python-based Scanpy [246–248].

**Organoids**

In 2009, Sato and colleagues reported the formation of crypt-villus presenting structures *in vitro*. These structures could be derived from single cells and remained stable over extended periods of time [249]. Importantly, these structures exhibited many features of the *in vivo* intestinal crypt including the generation of cells of secretory and absorptive lineages as well as Paneth cells. By now, organoids have become a widely used system and are derived from basically every organ of mouse, pig, or human. The first mouse oesophageal organoids were reported in 2014 [132]. However, to date deriving human organoids from adult cells remains challenging and they usually cease after five to six passages for yet undefined reasons [221]. Despite this drawback, they have been established from human pluripotent stem cells or patient derived cancer cell lines [119,143,145,220]. Organoids are useful tools that enable the establishment of biobanks, large scale drug-screening approaches, and can even be used in tissue transplantation [250–254]. In **paper II**, we establish an oesophageal epithelial-fibroblast co-culture system. In this work, organoids are used to understand the effect of morphogens or distinct cell types such as fibroblasts on oesophageal epithelial organoid formation and behaviour.

# Chapter 3

# 3 Paper I

# Intricate regulation of epidermal development via BMP, ID1, and CEPBα

In **paper I**, we investigated the role of DNA-binding protein inhibitors (ID1) in the context of epidermal development. The generation of the stratified squamous epithelium of the skin is governed by intricate and interwoven processes of proliferation, cell cycle exit, differentiation, and stratification. Thus, development of the epidermis presents an ideal model system to study the precise role of specific molecular effectors. ID1 is of special interest since it has been implied in the maintenance of the adult epidermis and is responsible for the upkeep of progenitor states in many other contexts [255–259].

## 3.1 Background

Inhibitor of DNA binding proteins (IDs) are helix-loop-helix (HLH) proteins that lack a basic domain essential to bind to DNA [260,261]. By binding to basic helix-loop-helix (bHLH) transcription factors, IDs are able to abrogate transcription programs. Thereby, IDs can affect proliferation and differentiation. Upon differentiation of stem or progenitor cells IDs are often downregulated allowing bHLH transcription factors to exert their action [262–264]. ID proteins are typically expressed in stem and progenitor cells where they come into contact with E proteins, that present one class of bHLHs. E proteins, like TCF3, TCF4, and TCF12, bind the specific DNA sequence CANNTG (E-box) and are expressed ubiquitously but in tissue-specific manners [264,265]. ID proteins are expressed in the developing and adult epidermis [262,266,267]. In addition, ID proteins are found to be upregulated following skin injury and in psoriatic skin suggesting a role in regulating proliferation and differentiation of keratinocytes [268,269].

Accurate formation of the epidermis during development requires a precisely conducted orchestra of a variety of molecules such as BMPs and C/EBPs [270–272] [62,273]. The promotor of *Id1* contains BMP-specific response elements and BMPs are able to drive the expression of IDs [62274]. BMP regulated ID1 is important for hair follicle stem cell quiescence and hair shaft progenitor specification as well as the reestablishment of epidermal homeostasis after injury [273,275].

C/EBPα and C/EBPβ are both upregulated in differentiating keratinocytes suggesting a role in keratinocyte differentiation [270–272]. *In vivo* experiments targeting basal cell layers revealed a role for C/EBPs during skin homeostasis. Epidermal specific *Keratin5* driven C/EBPα knockouts displayed no alterations in skin homeostasis, despite an increased susceptibility to skin tumorigenesis [276]. Importantly, double C/EBPα/C/EBPβ knockout

showed increased expression of *Id1* suggesting either and increased amount of cells usually expressing *Id1* or a compensatory role of ID1 upon C/EBPα/β deletion [68].

However, the role of ID1 during skin development is still incompletely understood.

## 3.2 Aims

The overall aim of this work was to understand the role of ID1 in the development of mouse epidermis. We exploited both, *in vivo* mouse models to study the role of ID1 during epidermal development, and *in vitro* primary epidermal keratinocyte cultures.

**1. Establish a functional relevance for ID1 during development of the epidermis**

We aimed to study the loss of ID1 *in vivo* using ultrasound-guided *in utero* injections of small hairpin RNA (shRNA) into wild type mice or CRE-expressing lentivirus into conditional transgenic Id1 mice. In cultured keratinocytes, we utilised shRNA to inhibit the expression of *Id1* as well as doxycycline induced ID1 overexpression. Transcriptional profiling (bulk RNA sequencing) of progenitor and differentiated keratinocytes would further advance our understanding of the role of ID1.

**2. Identify ID1 binding partners during epidermal development**

We aimed to identify the interaction of ID1 with other proteins during epidermal development using co-immunoprecipitation and subsequent mass spectrometry.

**3. Identify epidermal stem cell properties regulated by ID1**

Based on aims 1 and 2, we intended to perform ChIP-seq using antibodies against identified binding partners. Thereby, we would be able to identify ID1 regulated target genes allowing for mechanistic insights into the potential function of ID1 in cell cycle control or differentiation. Last, luciferase reporter assays allow for a functional verification of the identified binding partners *in vitro*.

## 3.3 Results

We made use of an already available early epidermal development (E9, E13, and E15) dataset and, in line with the authors, identified two clusters of epidermal cells at E13 [277]. *Id1* displayed a significant enrichment in the smaller cluster that exhibits expression of differentiation related genes like *Klf4* and *Krtdap*. Embryos that received *in utero* lentiviral *shId1* injections showed reduced epidermal thickness and proliferative cells in the usually non-proliferative suprabasal cell layer. Altering ID1 expression *in vitro* in keratinocytes in combination with RNA sequencing analysis suggested that ID1 is involved in maintaining a keratinocyte progenitor cell state. On the one hand, we observed increased expression of keratinocyte differentiation associated genes and reduced proliferation upon ID1 knockdown. On the other hand, proliferation of keratinocytes increased upon ID1 overexpression. Mass spectrometry analysis of Co-IP using a FLAG antibody against

FLAG-tagged ID1 revealed the bHLH proteins TCF3, TCF4, and TCF12 as ID1 binding partners that were all expressed during epidermal development. Keratinocytes transfected with shTcf3 showed increased proliferation opposite to keratinocytes transfected with shId1. Upon silencing of *Tcf3* expression of *Cebpa* decreased. In addition, we showed that TCF3 can bind the *Cebpa* promotor and enhancer regions using ChIP-qPCR. Finally, *in vitro* CEBPα overexpression led to a reduction of *Id1* and led to a decreased susceptibility of keratinocytes to BMP signalling as indicated by reduced *Id1* expression.

## 3.4 Conclusions

Our observations place ID1 into a complex network of the finely regulated skin development. The work presented here proposes a regulatory role for a BMP-TCF3-C/EBP axis in epidermal development. First, we establish a crucial role of ID1 in the maintenance of an epithelial progenitor state. ID1 upregulation increased epithelial cell proliferation and differentiation markers are upregulated upon ID1 knockdown. Subsequently, established TCF3 as an ID1 binding partner during skin development and demonstrated the binding of TCF3 to *Cebpa* promotor and enhancer regions. Last, we showed that overexpression of C/EBPα results in decreased *Id1* promotor activity and *Id1* expression upon BMP4 facilitated BMP signalling. Thus, ID1 and C/EBPα likely hold antagonising roles in keratinocytes. While ID1 is important to maintain a progenitor state, C/EBPα might render keratinocytes more susceptible to differentiation cues. Indeed, Klein and colleagues place ID1 in a reciprocally regulated network of transcription factors involved in keratinocyte transition (differentiation and migration) partly guided by BMP signalling [278]. In conclusion, this study uncovered an important role for ID1 during epidermis development. However, more research is needed to understand the proposed ID1-TCF3-C/EBP axis in detail.

# Chapter 4

# 4 Paper II, III, and IV

# Regulation of oesophageal homeostasis – a holistic view

**Paper II, III**, and **IV** focus on the investigation of oesophageal homeostasis.

**In paper II**, we establish a co-culture system of oesophageal epithelial cells and fibroblasts that enables detailed functional investigations of their reciprocal interactions *in vitro*. Often, *in vivo* studies take a considerable amount of time and resources, and their analysis proves challenging due to a vast amount of convoluting parameters. Thus, *in vitro* systems generally allow for a simplified understanding of biology in a more defined setting. In addition, organoids are used for important therapeutic processes like drug screenings and viability assays.

**In paper III**, we investigated the contribution of *Troy* positive progenitor cells to oesophageal homeostasis. The simple architecture of the epithelium makes the murine oesophagus an ideal system to ask questions addressing progenitor cell behaviour and fate. We identified that *Troy* expression labels a subset of epithelial progenitor cells. Since *Troy* is an established stem cell marker in the intestine and stomach it posed a relevant target to investigate. Using a combination of single cell RNA sequencing, lineage tracing, and mathematical modelling we establish that *Troy* is predominantly expressed in progenitor cells gradually increasing from proximal-to-distal. Additionally, *Troy* positive cells exhibit differentially expressed genes when compared to *Troy* negative cells. Last, we demonstrated that progenitor cells display altered lineage tracing behaviour upon *Troy* deletion. Conclusively, our data suggests that *Troy* is involved in context dependent decision making processes of oesophageal progenitor cells.

**In paper IV**, we explored regionalisation of the mouse oesophagus. To date, no study examined the mouse oesophageal microenvironment. Utilising single cell RNA sequencing and organoid cultures we revealed regional gradients in diverse cell populations and gene expression. We use bioinformatic tools to infer regional cell-cell communication and proposed that regionality bears implications for tissue homeostasis. Our results suggest differences in regional environments that might have implications for pathological conditions such as tumour development.

## 4.1 Background

To accurately maintain homeostasis a finely calibrated balance between cell proliferation and differentiation of stem or progenitor cells is essential. The epithelial basal layer of the adult oesophagus harbours progenitor cells that frequently self-renew and give rise to differentiated cells of the stratified squamous epithelium. Cancer stem cells employ many of the mechanisms that preserve an undifferentiated cell state of stem or progenitor cells [279]. Thus, investigating mechanisms underlying stem or progenitor cell dynamics is not only crucial to understand homeostasis but in addition might lead to a better understanding of tumour onset and tissue repair. Nonetheless, how squamous stratified epithelia are maintained in detail is not fully understood.

*Oesophageal epithelial progenitor heterogeneity*

During the 1960's Messier, Marques-Pereira, Leblond, and Smart showed that the oesophageal basal layer contains frequently dividing cells whose cell fate was best described randomly [14,99,112]. Since then, lineage tracing in combination with mathematical modelling has revealed important insights into cellular behaviour during homeostasis and additionally allows for a better understanding of epithelia during growth or under perturbed conditions [225,280–282]. In doing so, Doupé and colleagues revealed that all oesophageal progenitor cells bear equal potential [10]. Similar, investigation of progenitor cells in the interfollicular epidermis of the mouse tail concluded that tail epithelial progenitor cells had equal potential [9]. In conclusion, these studies suggest that a single rapidly proliferating progenitor cell population maintains the oesophageal and tail skin epithelia.

Subsequent studies challenged this view for the skin epithelium and identified a hierarchical organisation of progenitor cells in the tail epidermis instead [11]. *Krt14* expressing progenitor cells were found to give rise to transit amplifying cells expressing the differentiation marker *Ivl*. While *Ivl* labelled cells contributed to homeostasis long-term they did not contribute to wound healing proposing progenitor cells of distinct potential and function. Following it was demonstrated that the interfollicular tail epidermis is composed of two distinct cell populations maintaining parakeratotic and orthokeratotic epidermis, respectively. These two epidermal regions where suggested to be regulated by WNT and LRIG1 signalling from both the epidermal and dermal compartments [283]. A similar situation is present in the oral mucosa, in which coexisting IGFBP5 and LRIG1 progenitor cells ensure tissue homeostasis [284]. Importantly, none of the mentioned studies detected label retaining epithelial cells, indicating the absence of a quiescent stem cell population. In the intestinal epithelium a clear difference between label retention and stem cell functionality suggested that the absence of label retaining cells does not necessarily imply equal potential of all progenitor cells [285]. In conclusion, the epidermis likely harbours progenitor subpopulations with distinct potential in terms of proliferative kinetics and regenerative capacity [286]. Indeed, slow-cycling *Thy1* positive progenitors in

the interfollicular epidermis indicated a possible co-existence of distinct progenitor cells similar to the tail epidermis [287].

In the mouse oesophagus, several studies investigated the possible existence of a proliferative or lineage hierarchy. Based on differences in cell cycling activity and surface marker expression of oesophageal progenitor cells a putative stem cell population was proposed [209,288]. In addition, the Hoechst 33342 dye efflux test, that labels HSCs, in combination with cell surface markers identified a side population of oesophageal progenitor cells [210,289]. Since none of these studies was able to verify previously identified surface markers that are supposed to label putative stem cells, it is likely that the mere usage of surface markers is insufficient to identify stem cells. Lineage tracing studies utilising the suggested markers (CD71, CD49f) could give insights into the long-term contribution of presumptive subpopulations. More recently, three subgroups of oesophageal progenitor cells that exhibit differences in cell cycle phase and respond differently *in vivo* to environmental cues like all trans retinoic acid were described [132]. Finally, experiments investigating *Krt15* expressing oesophageal basal layer cells demonstrated that *Krt15* cells give rise to long term clones and have an increased organoid formation capacity [211]. In conclusion, similar to the skin epithelium the oesophagus might harbour a heterogeneous progenitor population, despite a multitude of studies demonstrating oesophageal progenitor unipotency. Studies using single-cell RNA sequencing can aid in determining cellular heterogeneity. In fact, a recent study displayed three transcriptionally distinct oesophageal basal cell populations underlining potential cell heterogeneity of oesophageal progenitor cells [290]. In addition, rare KRT8 expressing taste buds were discovered within the oesophageal epithelium illustrating cellular heterogeneity within the oesophageal epithelium [226].

*Troy in gastrointestinal tissues*

We identified a subpopulation of oesophageal progenitor cells that express the stem cell marker *Tnfrsf19*, or Troy. *Troy* was first described in late developmental stages of the murine embryo [291]. As part of the TNFR superfamily it represents a type I membrane glycoprotein. TNFR can trigger a variety of signalling pathways within a cell and act by activating caspases, induce translocation of nuclear factor-κB, activate mitogen-activated kinases such as c-Jun $NH_2$-terminal kinase (JNK), or activate extracellular signal-regulator kinase (ERK). As such, tumour necrosis factor receptors are important for immunomodulatory functions including cell proliferation, differentiation, cell survival, cell death, and inflammation [292,293].

*In vivo*, *Troy* is widely expressed during development. Among others *Troy* can be detected in adult neural stem cells, kidney papilla cells, and skin [294–298]. In addition, *Troy* expression has been demonstrated in a range of gastrointestinal tissues. *Troy* was identified as a co-expressed marker in LGR5 intestinal stem cells [299]. In the stomach, differentiated chief cells localised at the gland base express *Troy*. Upon ablation of the

proliferative stem cells in the upper part of the stomach gland, the previously differentiated *Troy* cells re-entered the cell cycle and replenished the entire gland structure.

*Troy as a target and modulator of WNT signalling*

*Troy* chief cells express stem cell marker genes like *Lgr5*, *Ascl2*, and *Lrig1,* as well as WNT signalling target genes *Axin2*, *EphB2*, and *Cd44* [161]. Thus, it has been proposed that WNT signalling regulates cell behaviour of *Troy* cells in the intestine and stomach [300]. TROY was shown to inhibit WNT signalling by suppressing the binding of R-spondin to LGR5 thereby preventing intracellular WNT signalling progression [301]. In human glioma, RKIP bound TROY strengthening the role of TROY as a negative regulator of WNT signalling [302]. Through stabilisation of GSK3β, a member of the β-catenin degradation complex, RKIP decreased WNT signalling [303]. The ability of TROY to antagonise WNT signalling suggests that TROY functionally impinges on cell proliferation and differentiation. In fact, *Troy* was shown to regulate differentiation of human mesenchymal stem cells in response to WNT signalling [304]. Recently *Troy* expression has been shown to correlate with tumour grade and types of gastric cancer and might serve as a prognostic marker of patient outcome [305]. Moreover, *Troy* is overexpressed in colorectal cancer cells and potentially provides a selective advantage for malignant cells [306]. Indeed, transfection of melanoma cells with an siRNA silencing *Troy* resulted in reduced proliferation and reinforced a growth promotion role of TROY [307]. Notably, β-catenin knockdown strongly reduced *Troy* expression in colorectal cancer suggesting that *Troy* itself is a WNT signalling target [306]. Another study demonstrated increased *Troy* expression upon WNT3A treatment in human mesenchymal stem cells and proposed a reciprocal regulation of TROY and C/EBPs or the induction of *Gremlin2* [304,308]. In conclusion, these findings indicate that *Troy* can be a WNT signalling target and in turn serves as a negative feedback regulator to ensure adequate cell balance. Last, ample evidence exists advocating a crucial role for TROY in the regulation of proliferation and differentiation of a variety of cells.
In addition to WNT signalling, *Troy* has been either directly or indirectly connected to NF-κB, EGF, JNK, RA, and TGF-β signalling indicating that the function of TROY is context dependent or embedded and responsive to signalling networks [298,306,307,309–311].

*Oesophageal regionalisation*

While several studies address oesophageal progenitor cell behaviour in perturbed and unperturbed conditions, a potential oesophageal progenitor cell niche remains completely unexplored to date. Since accurate development of the oesophagus requires synergy between cells of the foregut endoderm and mesenchyme, it is tempting to speculate that similar synergies influence progenitor cell behaviour in the adult oesophagus.

In mouse and human, the lung and intestine present uneven cell distributions and gene expression patterns [312–317]. In the intestine, distinct immune and mesenchymal cell populations were shown to inhabit the length of the intestine [182–184,312,318–320]. In addition, unequal placing of secretory cells or regionalised expression of Toll-like receptors instruct local defence mechanisms or processing of transitioning food [321–323]. During intestinal development, mesenchymal-epithelial interactions shaped regionalisation pinpointing at a decisive role of regionalised signalling pathways [316]. Importantly, crosstalk between various cell populations such as fibroblasts and ISCs is of critical importance for intestinal homeostasis [202,324]. While intestinal regionalisation is broadly established, scarce information suggests a regionalisation of the oesophagus.

The recently discovered, rare, KRT8 positive taste buds of the oesophagus are exclusively found in the proximal oesophagus [226]. In addition, a gradual increase of Langerhans cells in a proximal to distal manner was reported in the human oesophagus [325–327]. Further, regional differences in motor function and swallowing pressure exist [328,329]. Intriguingly, squamous cell carcinoma and inflammatory conditions like eosinophilic oesophagitis have increased incidences in the distal oesophagus [330–332]. Collectively, these findings suggest the existence of uneven cell distributions along the oesophageal length. It is likely that uneven cell distributions result in specific local signalling networks that influence oesophageal homeostasis and disease susceptibility.

## 4.2 Aims

*Troy* expressing cells exert important functions during gastrointestinal homeostasis and repair. Therefore, the expression of *Troy* in oesophageal progenitor cells prompted us to investigate implications for cell behaviour and fate. Despite a large body of well conducted studies investigating oesophageal progenitor cell potential, we deemed this important because rare cell populations could potentially evade the random labelling strategies applied.

**1. Characterisation of *Troy* progenitor cells in the mouse oesophagus**

We aimed to study the abundance and localisation of tdTomato (tdTom) labelled cells from Tnfrsf19-eGFP-ires-CreERT2;R26-floxed-stop-tdTomato transgenic mice. Further, we wanted to address *Troy* progenitor behaviour using lineage tracing and by comparing the obtained clone size data to already published statistical mathematical models that are based on randomly selected progenitor cells. Additionally, we aimed to use short term lineage tracing and EdU incorporation assays to specify potential cell cycle alterations and cell fate specifications of *Troy* progenitors compared to randomly labelled *Sox2* progenitors. Last, bulk RNA-sequencing of *Troy* positive progenitors and progenitors affected by functional *Troy* deletion would enable us to identify cellular mechanisms regulated via TROY.

**2. Characterise progenitor cell heterogeneity**

We aimed to investigate progenitor cell heterogeneity in an unbiased manner using single cell RNA-sequencing. Since transgenic mice contain alterations of their genome that could potentially influence gene expression, we wanted to conduct single-cell RNA sequencing on wild type mice.

**3. Determine regional oesophageal cell composition**

We aimed to investigate oesophageal cell composition and distributions using single-cell RNA sequencing of the epithelium and submucosa on regionally segmented tissue.

**4. Identify regional cell-cell communication in the oesophagus**

We aimed to infer cell-cell communication based on the obtained cell types from aim 3 using published bioinformatic tools such as CellChat. Thereby, we would be able to identify core signalling pathways involved in oesophageal homeostasis. In addition, we aimed to compare regional signalling pathways in order to gain insights into regional differences along the oesophageal lengths.

**5. Establish an oesophageal organoid co-culture system**

We aimed to generate an organoid culture system that would allow for the investigation of oesophageal epithelial cell behaviour in the presence of cells present in the *in vivo* oesophageal environment.

**6. Determine effect of oesophageal niche factors**

Based on **aim 3** and **aim 4**, and using the organoid culture developed in **aim 5**, we aimed to interrogate the effect of cell populations, growth factors, agonists and antagonists of morphogens on oesophageal epithelial behaviour.

## 4.3 Results

*Oesophageal progenitor subpopulations*

We observed an intriguing increase of tdTom cells from the proximal to distal oesophagus in Troy$^{CreERT2}$;tdTomato mice. Since this observation could be explained by altered recombination efficacies along the oesophagus we confirmed our finding with RNA *in situ* hybridisation. Following, we used *in vivo* EdU administration to determine the proportion of actively cycling *Troy* cells. Intriguingly, tdTom labelled cells incorporated EdU to a lesser extent than tdTom negative cells. In addition, differences of tdTom positive and tdTom negative cells in 2D colony formation assays and organoid formation pinpointed at a functional difference of *Troy* expressing cells. In order to gain a better understanding of *Troy* progenitor cell fate, we conducted lineage tracing experiments. This enabled the comparison of *Troy* progenitor derived clone sizes to previously published statistical

models of oesophageal progenitor cell behaviour. Strikingly, *Troy* cell derived clone sizes were much larger than could be expected based on the nominal model indicating a functional difference of *Troy* positive and negative cells. However, the overall contribution of tdTom positive cells remained stable over time. Together these findings argued for the existence of a distinct *Troy* progenitor cell albeit *Troy* cells appeared to possess the same potential as *Troy* negative progenitor cells.

In order to understand this phenomenon further, we began to adjust parameters of the nominal model and observed that a shift in the cell fate decision parameter *r* could resolve the observed clone size discrepancy. In addition, the adjusted model fit observations of increased clone loss of *Troy* progenitor cells compared to the nominal model. With regard to these observations, we wanted to scrutinise a potential role of TROY in directing cell fate. We compared short term clonal development of haploinsufficient tdTom *Troy* cells to tdTom cells suffering complete *Troy* deletion as well as EGFP positive clones in Sox2CreERT2;EGFP mice. Our findings revealed that *Troy* progenitor cells exhibited an enhanced symmetrical cell fate.

After a three months lineage tracing period *Sox2* derived clones matched the predicted clone size of the nominal model while the average clone size of *Troy* traced clones was increased confirming our previous observations and strengthening that *Troy* marks a distinct subpopulation of oesophageal progenitor cells with altered cell fate. In addition, the average size of *Troy* knockout derived clones exceeded *Troy* positive clone sizes suggesting that *Troy* itself might be involved in the regulation of progenitor cell behaviour. Using EdU incorporation we determined that *Troy* knockout cells displayed increased proliferation *in vivo*. To better understand differences between *Troy* expressing and *Troy* knockout cells we conducted bulk RNA sequencing on sorted tdTom cells. GO enrichment analysis unveiled that genes connected to epithelial proliferation are altered in *Troy* knockout cells matching our *in vivo* findings. Likewise, changes in basement membrane adhesion, differentiation markers, and a range of morphogen related genes were differentially expressed. In conclusion, our findings propose that *Troy*.

Finally, after having established a role for *Troy* progenitor cells in homeostasis we asked how *Troy* cells contribute to perturbations of tissue homeostasis. We used RA injections to alter oesophageal homeostasis and observed that tdTom positive cells displayed enhanced cell proliferation compared to tdTom negative cells. All in all, our observations indicate that *Troy* marks a subset of oesophageal progenitor cells, is involved in regulating cell fate decisions, and that *Troy* cells contribute to perturbations of homeostasis in a distinct manner. Due to the detected unequal expression of *Troy* along the oesophageal axis it is likely that the mouse oesophagus employs a context dependent expression of *Troy* to moderate local differences in the maintenance of tissue homeostasis.

*Regionality of the mouse oesophagus*

We began investigating potential oesophageal regionality by conducting single-cell sequencing on the separated epithelium and submucosa of regional oesophageal tissue segments. Aside from revealing the cellular heterogeneity within fibroblasts, immune cells, and epithelial cells our data pinpointed at a change in cellular composition between oesophageal regions. We verified our findings using immunofluorescence and detected different distributions of enteric neurons, endothelial cells, immune cells, fibroblasts, and altered smooth muscle layer thickness thereby establishing anatomical changes along the proximal-to-distal oesophageal axis.

Probing deeper into epithelial cells we detected changes in the transcriptome between the proximal and distal oesophageal epithelium that we could verify using *in situ* RNA hybridisation. We investigated the regional composition of basal and suprabasal cells in an attempt to explore functional implications of the altered transcriptome. The observed differences within the proximal and distal epithelium suggested regional influences on epithelial composition. In addition, regionally isolated progenitor cells exhibit altered organoid forming efficacy supporting that cell and transcriptional differences bear implications for tissue homeostasis.

Notably, one epithelial progenitor population was present almost exclusively within the distal epithelium. We identified the combined expression of *Igfbp5*, *Pappa*, *Lrig1*, and *Smoc2* as a gene signature separating this basal subpopulation from other basal cells and verified the combined expression *Igfbp5* and *Pappa* in the distal epithelium.

Following, we wanted to understand the contribution of fibroblasts to oesophageal regionality. Like epithelial cells fibroblast subpopulations showed uneven distribution along the proximal-distal oesophageal axis and proximal and distal fibroblasts exhibit gene expression differences. We confirmed gene expression differences of *Id3* and *Apcdd1* as well as *Lgr5* using in situ hybridisation and immunofluorescence. To investigate functional implications of regional fibroblast populations we made use of the organoid co-culture model established in **paper II**. The generated 3D *in vitro* model allowed for the combined culture of oesophageal epithelial cells and fibroblasts. We demonstrated that the presence of fibroblasts is sufficient to promote the growth of oesophageal organoids even in the absence of Noggin and R-spondin placing oesophageal fibroblasts as a key player in epithelial maintenance. This system enables a deeper understanding of presumptive cell-cell communication between epithelial cells and fibroblasts *in vitro*.

Utilising our co-culture system in **paper IV** we demonstrated that proximal fibroblasts exhibit enhanced support of epithelial organoid growth compared to fibroblasts derived from the distal oesophageal submucosa suggesting that fibroblasts display regional differences in instructing the oesophageal epithelium.

Since immune cells have been shown to influence homeostasis in various tissues we wanted to understand a potential contribution of immune cells to oesophageal homeostasis. Like epithelial cells and fibroblasts, we detected varying allocations of

immune cell types along the proximal-distal oesophageal axis within the submucosa and epithelium. We could confirm our findings using flow cytometry and immunofluorescence. We revealed a prominent proximal to distal gradual increase in CD207 positive Langerhans cells and T cell populations GATA3 positive ILCs were predominantly identified in the proximal submucosa. A CSF1R antibody treatment regime results depletion of CSF1 signalling dependent immune cells like macrophages and Langerhans cells. Employing this immune cell depletion system we detected altered organoid forming capacity specifically of distally derived epithelial organoids indicating that immune cell subpopulations contribute to oesophageal homeostasis.

Finally, we aimed to understand regional signalling pathways. We used CellChatDB to infer cell-cell communication patterns between cell types in the oesophageal regions. Comparing the inferred regional signalling patterns suggested altered BMP and IGF signalling along the proximal-distal oesophageal axis. We demonstrate a prominent BMP expressing fibroblast population in the distal oesophagus and showcased the effect of IGF in oesophageal organoid culture.

## 4.4 Conclusions

*Troy delineates oesophageal progenitor subpopulations*

Our data investigating *Troy* progenitor cells supports the notion of existing heterogeneity within the oesophageal epithelium. *Troy* expressing cells are unevenly distributed along the proximal-distal oesophageal axis and *Troy* deletion results in altered progenitor cell behaviour. Thus, our findings suggest a context dependent role of TROY that has implications for oesophageal progenitor cell behaviour. This is underlined by the observation that *Troy* cells react distinctively to tissue challenges like RA.

Nevertheless, our work bears a few caveats. We compared *Troy* derived clone sizes to already established statistical mathematical models. Discrepancies between our observations and those of others could partly be accounted to differences of mice in underlying parameters like the fraction of progenitor cells, cell cycle time, and proliferation and stratification rates [10,224]. However, in order to specifically study *Troy* progenitor populations we were in need of a specific mouse strain than. We addressed this issue in comparing the fraction of KI67 positive progenitor cells in between the various mouse strains used in our study, as well as incorporation of EdU and CyclinA2 staining. In doing so, we were able to demonstrate that cell cycle distribution is generally comparable in between the Troy and Sox2 strains.

In fact, it has been shown that oesophageal progenitors proliferate depending on the circadian rhythm [333,334]. Thus, the decreased fraction of proliferative *Troy* cells could indicate that *Troy* labels a specific cell state that in turn exhibits differences in cell behaviour and fate decisions. A recent statistical mathematical modelling approach incorporated pseudo-random proliferation and observed that it strengthens the possibility of a unipotent progenitor cell population [224]. However, a mathematical

modelling approach that considered reversible cell states established that the data obtained by lineage tracing of epithelia could equally be explained by dynamic heterogeneity among progenitor cells [335]. In combination with our observations this corroborates that *Troy* expression defines a distinct progenitor cells state in the oesophageal epithelium.

Other limitations include that general proliferation rates might differ in between mouse strains. However, these parameters could be adjusted accordingly within the model. In addition, due to the lack of a *Troy* driven H2BGFP we did not accurately determine the biological stratification rates within our mouse model. Nonetheless, our Sox2 mice determined clone size development mirrored published data and a comprehensive comparison of mouse strains which have been used in oesophageal lineage tracing revealed similar biological parameters between strains [224]. Since the Sox2 and Troy mice exploited in this study are both of a C57BL/6J background, large differences in proliferation and differentiation are thus unexpected.

Collectively, our clone size data suggests that *Troy* progenitor cells adhere to neutral drift and thus it is unlikely that they present a cell of elevated potential [336]. In single-cell sequencing *Troy* expressing cells are scattered among other basal layer cells strengthening that *Troy* expression labels a cell state rather than distinct cell identity. Of note, studies in the skin epithelium and the developmental closely related airway epithelium displayed that their respective epithelium is comprised of two different basal progenitor cell populations [287,337]. To ultimately dissect progenitor cell contribution within the oesophagus it could be interesting to employ a combined Cre-loxP and Flippase (Flp)-frt system to simultaneously investigate random and specific progenitor cell labelling. This method was recently used to study the contribution of *Sox2* and *Sox9* expressing cells in a model of gastric cancer [338]. Other approaches could use lineage tracing in combination with viral or loxP flanked barcode systems that would no longer depend on sparse labelling of progenitor cells to investigate clone dynamics [339].

Despite the discussed limitations important aspects of our study remain. We observed differences in organoid and colony formation assays advocating behavioural differences between *Troy* positive and *Troy* negative progenitor cells. In addition, we demonstrated alterations in cell behaviour in respect to proliferation and differentiation and detected differential gene expression comparing haploinsufficient TROY to insufficient TROY cells. In combination with the detected proximal-distal expression gradient of *Troy* expression *in vivo*, our results indicate a physiological relevance of TROY during oesophageal homeostasis. Notably, modelling approaches within the intestinal epithelium began to account for potential effects of the stem cell environment [340,341].

*Prominent cell distributions characterise oesophageal regionality*

Our observations of a gradually increasing expression of *Troy* suggests that changes reflected in gene expression occur along the oesophageal axis. Already in 1972 Itai and colleagues suggested that physiological changes along the oesophageal axis influence

homeostasis and impact pathological changes [342]. In their comprehensive study they described anatomical changes of the oesophageal axis in age and disease that might be the determining cause of disease onset.

Our work aimed to comprehensively characterise cell distributions along the oesophageal axis and gain a better understanding of oesophageal physiology along its axis. Uneven distributions of epithelial cells, fibroblasts, and immune cells likely result in regional signalling patterns. Recent advances in inferring cell-cell communication based on single-cell RNA sequencing gene expression data allowed us to investigate signalling patterns in oesophageal regions. Utilising an *in vitro* organoid system we could establish inhibitory effects of BMP signalling and growth promoting effects of IGF signalling on oesophageal epithelial cells, respectively. Therefore, it is likely that regional signalling patterns underly and influence oesophageal homeostasis *in vivo*.

To date, many aspects of adult oesophageal physiology remain unexplored including progenitor cell plasticity, potential mechanical forces, or stiffness of the ECM along the oesophageal axis.

Despite the absence of outstanding anatomical features like the crypt or hair follicle, the mouse oesophagus presents anatomical characteristics. Around its circumference the oesophagus presents 'folds' or 'dents' that are especially apparent in the proximal oesophagus. Mechanical forces greatly influence cell behaviour, as exemplified during postnatal oesophageal development [110]. In addition, mechanical forces due to cell-cell interactions were shown to regulate the expansion behaviour of yeast [343]. Intriguingly, the study demonstrated that mechanical forces could promote prolonged survival of clones with up to 90% reduced fitness. Thus, investigating the mechanical tension and compression forces that might originate from swallowing might be an important factor regulating progenitor cell behaviour and homeostasis.

I*n vivo* imaging techniques aided in a better understanding of progenitor cell behaviour. For example, elegant *in vivo* imaging studies within the crypt base revealed that stem cell division caused a relocation of neighbouring cells [344]. Additionally, it was shown that retrograde movement within the intestinal crypt is essential for effective stem cell numbers [345]. In combination, these studies emphasise the importance of cell location within a tissue. A recent study using *in vivo* imaging of differentiating KRT10 epithelial cells demonstrated a continued ability of differentiated cells to proliferate [346]. This finding highlights the importance of epithelial cell plasticity and location.

Here, we propose that immune-epithelial and mesenchyme-epithelial crosstalk is involved in sustained oesophageal homeostasis. However, more studies are needed to understand oesophageal homeostasis in detail. Nonetheless, a better understanding of oesophageal physiology might ultimately lead to improved understanding of oesophageal disease onset and aid in the identification of therapeutic targets.

# Chapter 5

# 5 Conclusions and Outlook

The concept of homeostasis has not only driven science but deeply infiltrated our everyday lives. We want to eat a balanced diet and search for work-life balance in our everyday hustle. The firm believe that our body is operating in an everlasting re-balancing roots deeply within us. The rich history in pondering over homeostasis paired with technological progress enabled us to recognise and examine homeostasis within the smallest building blocks of life.

However, how homeostasis is maintained and re-established upon injury in detail is not fully understood. Along the lines of this thesis I tried to elucidate how biologist (and related professions) view and study homeostasis.

In **paper III**, we studied the behaviour of epithelial *Troy* progenitor cells in oesophageal homeostasis. We applied a statistical mathematical modelling approach in combination with lineage tracing to examine the *Troy* behaviour and cell fate in homeostasis and under perturbed conditions. We demonstrate that *Troy* expression labels a progenitor state regarding its cell behaviour, cell fate, and transcriptional profile. In addition, we propose TROY as a regulator of progenitor behaviour.

In **paper IV** using the model established in **paper II,** we gave a comprehensive overview of oesophageal homeostasis with respect to proximal-to-distal cell distributions and signalling pathways.

Oesophagus development occurs in a craniocaudal fashion, including the respiratory-oesophageal separation mechanism, muscle and enteric nervous system development, and gene expression patterns. Therefore, our observations might represent reminiscent signalling pathways of developmental processes. Nonetheless, site-specific gene expression patterns have been reported in human fibroblasts [347]. Our findings highlight that the oesophageal progenitor cell milieu exhibits regional differences that might result in locally altered instructions of homeostasis [348]. Local microenvironments like Paneth cells of the intestine, fibroblasts, immune cells, and glial cells were shown to be of tremendous importance to maintain homeostasis in the skin, lung, mammary glands, and other tissues [183,349–351]. In addition, epithelial cell plasticity is increasingly recognised [281]. Since at least one century, it is known that cell states are plastic and can be altered after transplantation. In addition, It has been shown that pioneering factors like SOX9 in hair follicles highly influence cell fate [352]. The Yamanaka factors can revert cells into entirely different types and the oocyte environment can instruct an epithelial cell to create an entire organism [150,151]. All in all, cell behaviour appears to be primarily determined by the spatiotemporal context of a given biological situation.

*Context dependent cellular states*

An increasing number of work addresses cellular states embedded in complex networks [353–356]. These concepts expand on the ideas that transcriptional heterogeneity or transcriptional provide stochastic variability and thereby heterogeneity among cells [357,358]. Recently, Kramer and colleagues demonstrated that the current situation of a cell within a given environment is the strongest determinant of cell behaviour to cues from the environment. Adaptive information processing of cells was rooted in the distinct utilisation of cellular networks to integrate external information with their own current situation [359]. Overall, these findings indicate that cells process information depending on their current situation. Advances in cellular, spatial, or even temporal transcriptomics and proteomics will enable us to progress from investigating cellular identities to cellular transition states or entities [243,360–365]. Technological progress will allow us to investigate bigger pictures of biological contexts enabling us to deal with concerns that for example the thought-provoking commentary *The Unaimed Arrow Never Misses* brought up: "…cell biologists rarely zoom up to the organ and the organism levels, while the stem-cell biologist are not always interested to zoom forward in time to look at the differentiated cells or organ." [366]. Regardless, if one agrees with this statement or not, methods generating big-data will become more affordable and allow us to go beyond describing discrete actions in well-established paradigms and dogmas. Instead, studies addressing complex sets of interacting components and networks can be conducted [367,368]. Especially considering cell states, their location within niches, the reciprocal influence of cells, and their context dependent information processing I could not agree more with Morrisey and Rustgi who stated:

> *"…the definition for both 'niche' and 'stem cell' in the lung or other tissues with pseudostratified or true stratified epithelium is likely to require some rethinking."* [96,202,369–375].

Epithelial tissues might be the ideal system to reveal signalling or transcriptional networks *in vivo* due to established cell-cell communication during development and homeostasis, prominent epithelial plasticity, and the eminent feature to react to continuous stress [376]. Studies demonstrating epithelial plasticity and their ability to reverse to foetal-like cell states underline this thought [133,281,377]. More importantly, cellular plasticity has been shown to drive tumour development [378]. Therefore, insights into context dependent, signalling network determined heterogeneity are of crucial importance to recognise the best possible pharmacological targets and exploit therapeutic potentials.

In **paper I**, we investigated the role of ID1 and proposed a ID1-TCF3-C/EBPα axis regulating epidermal development. Our study showed that transcription factors cooperate or hinder each other to ensure the correct execution of biological processes. Likely, studies investigating changes in transcription factor networks in response to certain stressors or artificial stimulation will be prevalent [278]. The recognition of network

changes might allow for a much deeper understanding of transcriptions factors and their regulation during cell state transitions. Therefore, understanding of transcription factors such as p63 in the epidermis and oesophagus within context dependent networks is important and could aid in understanding when co-regulatory proteins like TROY are required.

*Cell memory*

Cellular context matters in other fascinating discoveries. Epigenetics – the modification of proteins and DNA – offers additional mechanisms contributing to heterogeneity and additionally equips cells with the ability to remember their past [379,380]. Moreover, cells appear to "remember" protein and gene expression patterns and carry them over several lineages [381,382]. If cells are able to uphold specific mechanistic patterns over time and pass them to their offspring one could argue that they theoretically possess a memory [383]. For example, stem cells of the epidermis develop epigenetic memory in order to remember assaults from the past [384]. In addition, immune cells show distinct placements in the intestinal epithelium and the epidermis. Astonishingly, following laser ablation the newly recruited immune cells appear to remember the positioning of the previously ablated cells suggesting that not only cells but even tissues my possess memory features [312,385].

*Cell memory and homeostasis*

In my opinion, the myriad of factors that control cell plasticity and cellular context, such as epigenetics, cell-cell communication, and cell-ECM interactions, do not only broaden but re-define our understanding of homeostasis. While it might be ridiculous to assign the concept of memory to a single cell or cellular lineage, the instruction of immune cells to specific places certainly suggests a memory embedded in biological context. Thus, our current idea of an actively balanced system maintaining health - while certainly true – might not cover the entire picture. Instead of maintaining a stable state – homeostasis – physiological settings might want to revert back to a state they actively remember ("reverting back to stability") stored within biological, physical, and chemical processes. Just as our neuronal memory often does not recall events from the past in detail, this potential biological memory might be inaccurate resulting in the accumulation of faulty processes leading to disease and aging. The identification of biological factors that are of crucial importance within a specific context will hopefully allow us to diagnose and treat imbalances of homeostasis. Such factors could advance our ability to revert cells into a state of "remembered" stability. Studies of biological context superintendents point in this direction [386]. To summarise, we need to understand intratissue specific differences and their implications for homeostasis in order to fully grasp elemental processes of malignant tissue transformation.

# 6 Ethical considerations

Mouse strains and performed procedures are entirely covered by ethical permits N243/14, N116/16, 14051-2019, and 735-2021 that comply with EU and Swedish national legislations. A crucial aspect in designing animal experiments is a careful consideration of the needed number. While the power of the experiment needs to be sufficient in order to gain valuable insights, the 3 Rs (Replacement, Reduction, Refinement) were always kept in mind. By proper experimental planning, sharing mice with colleagues, and careful breeding we try to reduce mouse numbers. Freezing down mouse lines instead of long term maintenance presents another way to reduce animal numbers.

However, in our studies the replacement of animal experiments is challenging. Processes that depend on the interaction of diverse entities and an elaborate tissue structure can so far only be based on *in vivo* studies. To understand clonal dynamics of progenitor cells the *in vivo* environment is essential. To our knowledge there is no *in vitro* system that fulfils requirements to answer those questions, yet.

For mechanistic insights and for validation purposes we tried to use oesophageal organoids as an *in vitro* alternative whenever possible. Nonetheless, the original setup of organoids involves cell isolation from animals as well. In addition, *in vitro* systems need to recapitulate observations we gained from *in vivo* experiments to act as use- and meaningful replacement. Despite these considerations, organoids present a valuable system that offers grounds to slowly replace and reduce *in vivo* experiments. Our established co-culture technique can aid in answering questions underlying specific cell-cell interactions within tissues outside animals.

Organoid culture techniques can be setup from human samples and therefore demonstrate a great technique to transfer insights gained from animal experiments to a clinically more relevant system. Organoids derived from human donors arguably entail even higher ethical considerations. According to the Health and Medical Service Act § (SFS 2017:30) health and medical research involving subjects shall particularly be based on the respect for the patient's self-determination and integrity and to a possible extend be designed and conducted in consultation with the patient.

Performed experiments as well as associated results are thoroughly documented. Generated raw data as well as processed data needs to be accessible at later time points and is stored accordingly. Moreover, once a study based on genetic data is published we aim to make the raw data and the processing pipeline available. All our projects will followed the publication ethics guidelines from Karolinska Institutet.

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bauen konnten. Unsere Matratzenlager waren immer super, **Simon**! Simon und Helena, ich bin unglaublich stolz auf euch und euren Weg und habe auch schon Pläne für ein Haus, **Helena** :p Vielen Dank, **Michael**, für das Grillen am Wildgehege, das viele köstliche Essen, und deine generelle Hilfsbereitschaft. Danke, dass ihr immer für mich da seid. **Mama**, deine Aufopferung und Hingabe für uns drei Aufsässige werde ich nie vergessen. Du bleibst eine Inspiration! Ich habe euch alle lieb!

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**Thank you all!**

"We tend to forget that happiness doesn't come as a result of getting something we don't have, but rather of recognizing and appreciating what we do have."

*– Friedrich Koenig*