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### Developing and analysing novel tools to study endogenous WNT signalling in mice

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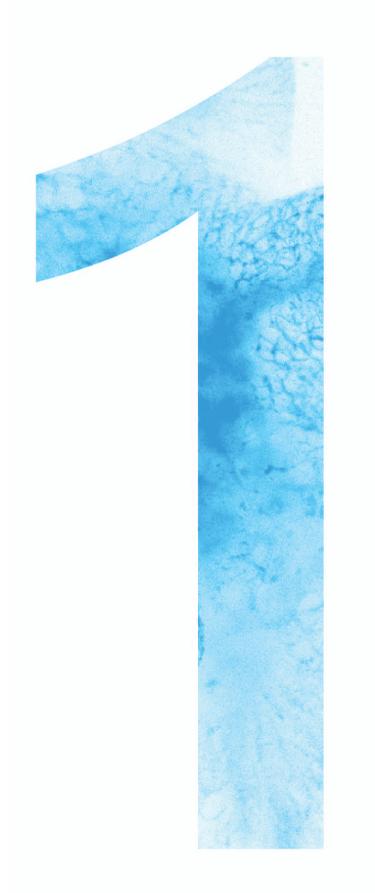
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# Chapter 1

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## General introduction

# General introduction

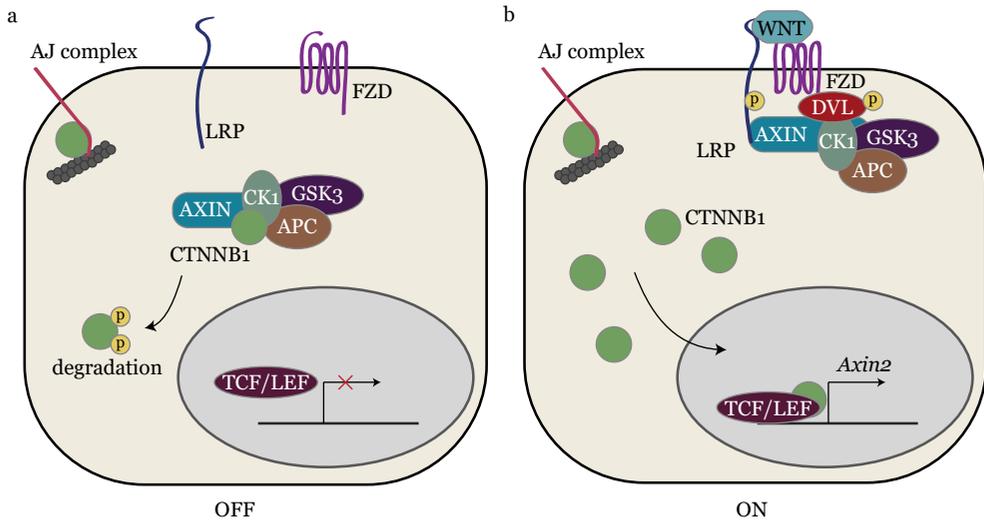
## The CTNNB1-dependent WNT signalling pathway

WNT signalling is involved in tissue development and homeostasis of all multicellular animals and is often mutated in various types of cancer. Initially discovered in both fruit flies and mice (Nusse and Varmus, 1982; Nüsslein-volhard and Wieschaus, 1980), ~40 years since the initial description of the first *Wnt* genes there is still much to be learned about the many molecular and cell biological events of this multifaceted and dynamic pathway. Nineteen different *Wnt* genes have been found in the mouse and human genome (Clevers and Nusse, 2012). One of the key downstream players in the WNT signalling pathway is beta-catenin (CTNNB1). The part of the pathway that uses CTNNB1 as its effector often referred to as ‘canonical’ or CTNNB1-dependent WNT signalling. Additionally, WNT responses also happen without the involvement of CTNNB1. These are referred to as ‘non-canonical’ or ‘beta-catenin independent’ responses. I will focus on the WNT/CTNNB1 signalling pathway in this thesis.

A schematic representation of the WNT/CTNNB1 signalling pathway (hereafter referred to as WNT pathway) is depicted in Figure 1. The receptors Frizzled (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) are present at the cell membrane, but they are not active in the absence of WNT ligands (Figure 1a). A destruction complex consisting of AXIN, Adenomatous Polyposis Coli (APC), Glycogen Synthase Kinase 3 (GSK3) and Casein Kinase 1 (CK1) (Li et al., 2012; Stamos and Weis, 2013) phosphorylates CTNNB1. As the name suggests, this destruction complex actively targets CTNNB1 for degradation. Progressive phosphorylation of S45, T41, S37 and S33 labels it to be targeted by the proteasome. In the presence of WNT ligands (Figure 1b), the destruction complex gets inactivated. Several models exist on the precise details of how this occurs, one of them being that AXIN and its binding partners are targeted towards the membrane together with Dishevelled (DVL) (Tortelote et al., 2017). With the destruction complex inactivated, newly synthesized CTNNB1 no longer becomes phosphorylated and accumulates in the cytoplasm. As a result, CTNNB1 relocates to the nucleus where it binds TCF/LEF (T-cell factor-lymphoid enhancer factor) transcription factors to act as transcriptional activators of downstream signalling events (Behrens et al., 1996).

Most cellular CTNNB1 is located at the cell membrane, where it is tethered to E-cadherin in the adherens junctions (Figure 1) (Oda and Takeichi, 2011). Since the non-E-cadherin bound, cytoplasmic pool of beta-catenin is targeted for destruction via the destruction complex, the levels in the cytoplasm are relatively low. Only upon activation of the WNT pathway, higher cytoplasmic levels are found (Behrens et al.,

1996). Whether the two pools of E-cadherin bound and unbound CTNNB1 show crosstalk and at what level, is still being debated (van der Wal and van Amerongen, 2020).



**Figure 1. Schematic overview of WNT/CTNNB1 signalling.** a) Without WNT stimulation, a destruction complex containing AXIN, CK1, GSK3 and APC phosphorylates CTNNB1, leading to destruction by the proteasome. CTNNB1 is also a key component of the adherens junction (AJ) complex, regulating tissue stability. b) Upon stimulation with a WNT ligand, the LRP5/6 and FZD receptors become activated and LRP5/6 and DVL get phosphorylated, binding the destruction complex at the membrane rendering it inactive, so that it doesn't target CTNNB1 for destruction anymore. As a consequence, CTNNB1 levels rise. In the nucleus, CTNNB1 together with TCF/LEF transcription factors activates the transcription of downstream genes, such as *Axin2*.

Different cell types express a unique set of WNT proteins, receptors and downstream effector (TCF/LEF) and target genes, although these tissue-specific aspects remain understudied. Additionally, the WNT pathway is well known for its role in cancer, but the type of alteration and the players involved differ between tissues (Nusse and Clevers, 2017; Wiese et al., 2018). Different tumour types harbour different mutations, and it is important to consider species-specific differences as well. For example, while *Wnt-1* was initially discovered in the mammary gland as an oncogene driving mammary tumour formation in mice (van Amerongen, 2020; Nusse and Varmus, 1982), mutations in the WNT pathway are relatively rare in human breast tumours, though hyperactive WNT signalling is found in several subtypes (Lamb et al., 2013; Polakis, 2012; van Schie and van Amerongen, 2020). Dissecting the precise nature, dynamics and activity of the WNT pathway at the molecular level in different cell types is therefore not only of interest from a

fundamental scientific perspective, but might also lead to a better understanding of the involvement of these processes in disease and reveal specific therapeutic opportunities.

### **WNT signalling and stem cells**

Stem cells are cells that have the capacity to both self-renew, as well as to give rise to more differentiated progeny. The clearest example of this is the pluripotent embryonic stem cell, which can generate all tissues of the body. Over the last decades it has become clear that most adult tissues are actively maintained by populations of cells with stem cell functions and a range of cell type-restricted potency (Clevers and Watt, 2018).

The first adult stem cells defined were hematopoietic stem cells (HSCs). They were initially discovered as ‘radiation sensitive cells’ in the bone marrow (Till and McCulloch, 1961). HSCs exist in a defined hierarchy, where a rare stem cell divides infrequently and in an asymmetric fashion, giving rise to another stem cell and a dedicated progenitor cell that continues to divide and differentiate into lineage restricted cells. This model is static, and cells can only go one way; towards differentiation. This hierarchical stem cell model became the blueprint for other tissues. However, in many tissues the populations of stem and progenitor cells described over the years behave much more dynamic, allowing plasticity in cell populations, so that daughter cells retain the potential to revert to a stem cell state. This is for example seen upon wound repair of the skin, where differentiated *Gata6*-expressing cells in the sebaceous duct of the hair follicle move into the epidermis and change into self-renewing stem cells (Donati et al., 2017). Experiments on the potency of cells upon tissue healing, *in vitro* colony formation and transplantation experiments further showed that stem cells are not always a dedicated and well defined pool of cells, but can gain stem cell function from their direct environment or niche (Donati et al., 2017).

Since a large body of literature supports the involvement of WNT signalling in many developmental processes and because of its link to regulating cell proliferation, it is not surprising that WNT signalling is one of the major pathways controlling stem cell self-renewal and differentiation. WNT proteins have proven to be essential for maintaining pluripotency in embryonic stem cell cultures (Berge et al., 2011) and a variety of adult stem cell populations are WNT/CTNNB1 responsive, including those in the intestine (Barker et al., 2007), skin (Huelsken et al., 2001; Lim et al., 2013; Snippert et al., 2010b), mammary gland (van Amerongen et al., 2012; Wang et al., 2015b; Zeng and Nusse, 2010), hematopoietic system (Nemeth et al., 2009) liver (Wang et al., 2015a) and many more tissues.

WNT proteins are lipid-modified and therefore hydrophobic (Willert et al., 2003). This means that after secretion, WNT proteins cannot traverse far through the extracellular space. Indeed, WNT proteins function mainly as short-range signals

between adjacent cells (Clevers et al., 2014). The short-range nature of WNT signals suits the view of a cellular micro-environment forming a stem cell niche.

### **The use of mouse models to study WNT signalling**

Despite an ever-increasing collection of tools to study WNT signalling in 2D and 3D cell culture, these experimental systems cannot fully recapitulate the complex interactions and processes at work in the body. Therefore, the use of *in vivo* models has been crucial, and remains necessary, for deciphering the full complexity of WNT signalling (as reviewed in (Wiese et al., 2018)). Researchers have extensively explored WNT signalling events all over the animal kingdom, using model organisms such as Planarians, sea anemones, fruit fly, frog, zebrafish and chicken. For the mammalian system, the mouse serves as the most-used model organism. Like humans, the mouse genome encodes 19 WNT ligands and 10 FZD receptors. In addition, it has been amenable to genetic modification since the 1980s, allowing the generation of knock-out and transgenic strains. Finally, mice allow multiple ways to investigate stem cells.

The first *in vivo* studies with mice containing a WNT reporter were performed with so-called TOPGAL reporter mice, a transgenic mouse model in which three TCF/LEF binding sites control expression of a  $\beta$ -galactosidase reporter gene (*lacZ*) (DasGupta and Fuchs, 1999). As a result, cells with active WNT signalling can be stained blue using a standard enzymatic assay. Using this mouse model, DasGupta and Fuchs found a regulatory role for WNT signalling during hair follicle development and differentiation. Following this, several WNT reporter mice have contributed to painting the picture of WNT-responsive cell populations *in vivo* (Cho et al., 2017; Ferrer-Vaquero et al., 2010; Lustig et al., 2002; Maretto et al., 2003; de Roo et al., 2017).

Although expression information alone is highly informative, not every WNT-responsive cell is necessarily a stem cell. To really be classified as stem cells, they should meet distinct criteria: They should not only divide and thereby give rise to differentiated cells in the tissue, but they also need to self-renew efficiently to survive long past the turnover time of the tissue.

Historically, the developmental potential of stem cells has been determined via transplantation studies of isolated cells into recipient mice (Etzrodt et al., 2014). These experiments reveal the differentiation potential of the transplanted population, but they do not necessarily reflect their normal potency, since transplantation can alter the behaviour of the transplanted cells (van Amerongen et al., 2012; van Keymeulen et al., 2011; Morris et al., 2004; Oshima et al., 2001; Prater et al., 2014). Therefore, in the last 10-15 years there has been a movement towards studying stem cells in their physiological environment. This has been made possible with the use of mouse strains that allow *in situ* lineage tracing.

Lineage tracing with genetically engineered mice provides a sophisticated approach to study the progeny of stem cells in complex, intact tissues (Kretzschmar and Watt, 2012). It typically requires compound mouse models, in which two or more strains are intercrossed. Most often, a reporter strain carrying a Cre-lox inducible and heritable marker, such as a fluorescent protein, is crossed with a driver strain that expresses a Cre-recombinase under the control of a tissue- or cell type specific promoter. Frequently, Cre-recombinase activity can be controlled directly using Tamoxifen or indirectly using a doxycycline/rtTA system, depending on the genetic model used. With the use of inducible, tissue-specific drivers, spatiotemporal control is achieved to activate the reporter at a given developmental or life stage and in a cell type of choice. The heritable marker in the reporter line, the expression of which is typically under the control of a strong constitutive promoter once Cre/lox mediated activation has occurred, allows researchers to follow the progeny of recombined (or 'switched') cells over time *in situ*. A well-known example of lineage tracing of WNT-responsive stem cells is the multicolour labelling of intestinal stem cells with an  $LGR5^{CreERT2}$  driver and the  $Rosa26^{Confetti}$  reporter, allowing the researchers to determine how symmetrically dividing stem cells in the intestinal crypt give rise to the lining of the intestinal epithelium (Snippert et al., 2010a).

### **Key questions and challenges**

Despite the achievements mentioned above, many questions remain. Some of these have remained unanswered for decades and can only be answered now thanks to developments in genetic manipulation and imaging, among others. Others require a major investment in time and resources to build new tools.

At the molecular level, the textbook model of WNT/CTNNB1 signalling (as also depicted in Figure 1) dictates that CTNNB1 accumulates in the cytoplasm and then translocates to the nucleus. Yet many researchers have felt the frustration of not being able to detect clear nuclear enrichment of CTNNB1 in their experimental system. Does this mean that we should simply conclude that WNT/CTNNB1 signalling is not active unless nuclear translocation is black and white? Or does this mean that we should invest in building tools that will allow us to visualize and quantify the actual changes in WNT/CTNNB1 signalling activity at endogenous levels as best we can?

At the level of cells and tissues, other challenges come into play. For instance, how do we ensure that the right cell population of interest is labelled without disrupting the normal biology of the tissue? Many WNT reporter strains are conventional transgenics, containing a randomly integrated reporter cassette with multimerized TCF sites upstream of a minimal promoter (Barolo, 2006). It is well known that the activity patterns of these different reporters do not fully overlap, although this is not often exhaustively analysed and properly reported (Al Alam et al., 2011). Likely, these artificial reporters miss important contextual information

(such as the complete enhancer repertoire that would normally drive tissue-specific gene expression) and, as a result, they may not fully recapitulate WNT pathway activity or provide a complete overview of WNT signalling *in vivo*. Provided that we can properly label the relevant cell population, how can we track it inside different tissues of interest, which are typically opaque and often pose imaging challenges? And, with lineage tracing in particular and developmental biology becoming increasingly more quantitative (Dekoninck et al., 2020; Hannezo and Simons, 2018; Wuidart et al., 2016), how can we best ensure that we will be able to collect all of the relevant parameters – not only clone size and distribution, but also individual cell shape and positional information?

In summary, we still have much to learn about the WNT signalling pathway in health and disease. Studying fundamental properties of WNT signal transduction will hopefully help unlock both the therapeutic potential of WNT signalling in regenerative medicine and injury repair, as well as for targeted intervention of the WNT pathway in specific types of cancer. At the basis of these developments, however, lies the challenging task of designing and generating new tools to better quantify, visualize and track WNT/CTNNB1 signalling in complex mammalian tissues. The research in this thesis describes our efforts in this area, focusing on the dedicated readout of WNT/CTNNB1 signalling - and the developmental processes it controls - at the molecular, cell and tissue level.

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