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

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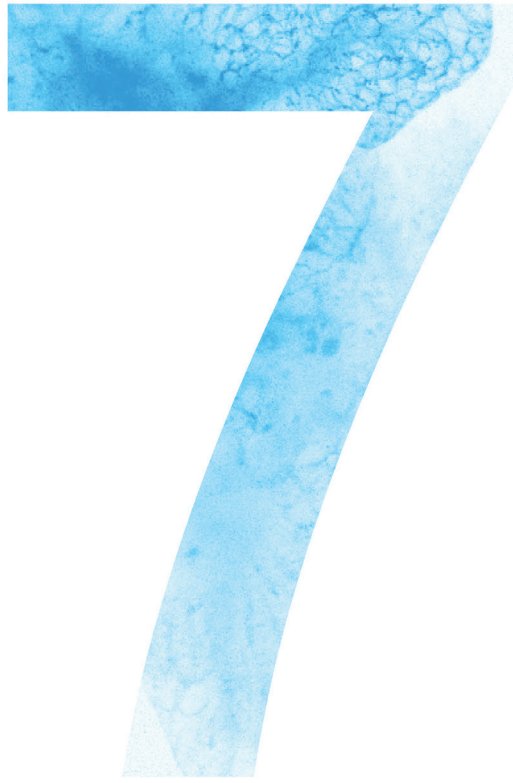
Publication date
2021



Chapter 7

Discussion

General discussion

The WNT signalling pathway is important in the development and homeostasis for all multicellular animals. Understanding the fundamentals of this pathway is indispensable for knowing how tissues are maintained, as well as repaired in case of injury. This knowledge has the potential to open up new avenues of treatment in regenerative medicine. On the other hand, hyperactivation of the WNT/CTTNB1 signalling pathway can contribute to cancer initiation and progression. Knowing the exact role of pathway components in the oncogenic process, will help us to find ways to fight it.

While these clinical applications may seem far removed from the work described in this thesis, I would argue that the development of new therapeutics is in some ways ahead of our biological understanding. For instance, in recent years surrogate WNT agonists have been engineered (Janda et al., 2017; Tao et al., 2019), thereby potentially overcoming one hurdle typically associated with using WNT proteins – namely the fact that they are lipid modified and thus prone to aggregate while being poorly compatible with an aqueous environment (Willert et al., 2003). Similarly, drugs that inhibit WNT/CTNNB1 signalling have shown promising results in pre-clinical studies and have, in some cases, entered clinical trials (Diamond et al., 2020; Gurney et al., 2012).

The fact that WNT/CTNNB1 signalling balances cell proliferation and differentiation in so many different contexts, yields opportunities as well as potential problems. For instance, one unwanted side effect of WNT inhibitors is that they can lead to a loss in bone density, resulting in fractures (Davis et al., 2020; Diamond et al., 2020; van Schie and van Amerongen, 2020). This particular example illustrates that to make personalized medicine a reality, we not only need to have access to targeted therapeutics, but also need to have detailed knowledge about the tissue-specific activities of the pathways we aim to target. Because injury and disease are part of a larger architecture, it is essential that we study fundamental properties of WNT signalling with this in mind as well.

The study of complex living systems presents two distinct challenges. First, both tissue morphogenesis and tissue homeostasis are highly dynamic processes in which cell identity and position change over time. Second, to understand the behaviour of the system as a whole, it needs to be studied at different scales: at the molecular, cell and tissue level (Cohen and Harel, 2007; Setty, 2012). In this thesis, we take important steps towards dissecting the role of WNT/CTNNB1 signalling in this context.

Studying gene expression changes in a dynamically changing environment

One of the challenges in developmental biology is to understand how subtle changes in gene expression drive growth and differentiation of complex tissues. While in recent years we have seen the development of many 3D culturing techniques that allow us to study cell signalling interactions, the use of mouse models is indispensable to understand signalling in a complex *in vivo* environment, such as the mouse mammary gland. This highly dynamic tissue serves as a prime example of a dynamic and complex environment with many cell types. The tissue develops postnatally and during every estrus cycle the mouse mammary gland epithelium undergoes outgrowth followed by regression. These changes are driven by a range of signalling pathways, including the WNT pathway (Fu et al., 2020). Multiple WNT pathway genes have previously been shown to be expressed in the mammary gland, but often at relatively low levels, making it challenging to measure gene expression changes (Yu et al., 2016).

A method to reliably measure subtle changes in gene expression across multiple experimental conditions is quantitative reverse transcription polymerase chain reaction (qRT-PCR). To compare samples, the technique requires normalization by one or more reference genes. However, it turns out that finding genes suitable as reliable references in a complex tissue over the course of developmental time is not that easy. In **chapter 2**, we developed and tested novel candidate reference genes and found that a set of three, *Prdx1, Phf7* and *Ctbp1,* outperform traditionally used reference genes such as *Gapdh* in the mammary gland. Using this validated set of mammary-specific reference genes allowed us to better pick up subtle gene expression changes, such as the subtle rise in *Wnt4* gene expression that occurs during puberty (Brisken et al., 2000; Rajaram et al., 2015).

For best practice, prior to starting any qRT-PCR experiment on a developmental time series, it is important to select and validate a set of reference genes specific for the organism and tissue under study. In practice, this is not always done as it is easier to use a single reference gene that has been demonstrated to work in a different context. **Chapter 2** illustrates the feasibility of finding such reference genes by scrutinizing previously published genome wide expression datasets: While microarray or RNAseq analyses are typically used to look for gene expression differences, these same data can also be used to select genes that show highly stable (i.e. the least variable) expression across multiple different conditions. When selecting reference genes using this approach, it is important to consider whether a set of genes that show the same expression pattern over all tested developmental timepoints are truly stably expressed or show a similar pattern because they might be co-regulated. To this end, we performed gene ontology analysis to ensure that our final set of reference genes are involved in different molecular processes, which makes co-regulation less likely.

Since performing the work described in chapter 2, our lab has increasingly been using published RNAseq, ChIPseq and ATACseq datasets in our research - both bulk and single cell - to generate new hypotheses for follow up research (van de Grift et al., 2020) and to complement and validate our own experimental work.

Of course, while qRT-PCR provides fast, sensitive and reliable analysis when studying gene expression, it is less suitable when many genes or transcriptional changes between defined cell populations are of interest: in that case, high-throughput methods such as single-cell RNA sequencing (scRNAseq) provide effective measurement of the whole transcriptome of many individual cells. However, scRNAseq analyses typically require more expertise and bioinformatic processing and, as such, is unlikely to completely replace qRT-PCR in the near future. One particular caveat of scRNAseq is that broad coverage (i.e. sequencing many different cells from a given population or tissue) typically comes at the expense of sequencing depth (i.e. the number of reads obtained for the transcriptome of a single cell). At present, this limits the detection of lowly expressed genes (Grün and Van Oudenaarden, 2015; Zhang et al., 2020), which we know from our own experience to include *WNTs* and *FZDs*. As such, when studying subtle gene differences of a few genes, including lowly expressed ones, qRT-PCR remains quicker, more reliable and, not unimportantly, more accessible to most labs.

Mouse models to visualize, quantify and track endogenous WNT/CTNNB1 signalling *in situ*

A distinct disadvantage of many techniques, including genome-wide next generation sequencing efforts and more classical biochemical methods, is that they require the isolation of RNA, proteins or cells prior to analysis. This not only means that the endogenous context is lost, but for bulk analysis methods it also entails a loss of information regarding cell-to-cell heterogeneity. One goal of the work described in this thesis was to improve the detection of endogenous WNT/CTNNB1 signalling in complex tissues. To this end, we combined genetic knock-in and fluorescent protein technology, resulting in the generation of three new genetically engineered mouse strains. For all strains, we included specific design criteria to not only ensure their improvement over existing models, but also so that they would allow us to investigate WNT/CTNNB1 in a physiological context, with as little perturbation of endogenous gene expression as possible.

As one example, in **chapter 4** we targeted a reporter gene encoding SGFP2 to the 3' end of the *Axin2* coding sequence, in an effort to leave endogenous *Axin2* expression unaffected. This is important, given that *Axin2* is a universal negative feedback target gene that is expressed in most WNT/CTNNB1-responsive tissues (Jho et al., 2002; Lustig et al., 2002) and, depending on the strain background, *Axin2* loss-of-function mice present with developmental phenotypes or be born at sub-Mendelian ratios as discussed in chapter 4. As a second example, while many

WNT reporters exist, none combine expression of an endogenous WNT reporter with a lineage tracing driver. Therefore, the knock-in cassette in our *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ mouse strain allows both direct visualization and doxycycline-dependent lineage tracing of WNT/CTNNB1 responsive cells. Our model not only faithfully recapitulates the endogenous *Axin2* expression pattern, while leaving normal *Axin2* expression intact, but also serves as a proof-of-principle for generating similar cassettes separated by multiple 2A sequences in other contexts. As a third example, while the models in **chapters 4 and 5** were built and (are being) analysed separately, they were designed as a pair, keeping in mind that the colours of the PRIME lineage-tracing reporter would be compatible with as many other reporter alleles as possible, given that most scientists will incorporate a green fluorescent protein as a first and obvious choice.

Each design choice obviously also comes with consequences. For instance, for generating the *Axin2* knock-in allele in chapter 4, we had to decide between being able to monitor endogenous *Axin2* gene expression dynamics (which would have meant the inclusion of a destabilized fluorescent protein as also reported by (Sonnen et al., 2018) or increasing our chances of detecting reporter signal in the first place. We knew that *Axin2* expression levels are relatively low, and this is further underscored by the activity of the knock-in allele as we have so far failed to detect any SGFP2 signal or rtTA3 activity in the mammary gland. Therefore, in hindsight this choice seems warranted as we would likely have had even more difficulty detecting the *Axin2* reporter signal despite concentrating it in the nucleus by means of the 3xNLS targeting sequence in front of the SGFP2. In contrast to our findings, a recent publication describing a new 5' *Axin2* GFP allele (Wang et al., 2021) reports prominent *Axin2* expression in mammary fibroblasts and endothelial cells, as well as in many other tissues where we did not detect any signal of our 3' *Axin2* SGFP2 reporter. So far, neither of these fluorescent reporters appear to recapitulate the epithelial expression previously detected with the *Axin2*^{lacZ} and *Axin2*^{CreERT2} strains. These discrepancies remain puzzling and further illustrate that our understanding of how knock-in alleles behave with respect to endogenous gene activity is far from complete. All we can say at this point, is that our *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ model leaves the *Axin2* allele intact apart from the knock-in cassette, thereby preserving full transcriptional and translational control of the allele, as also illustrated by the fact that homozygous mice are viable without any aberrant phenotypes.

Initially, our CRISPR/Cas mediated tagging efforts were also focused on *Axin2*. Unfortunately, these attempts have remained unsuccessful. Whether this is due to technical or practical reasons or whether the efficiency of gene editing is reduced for lowly expressed genes remains to be determined. We then realized that while ultimately activity of the WNT/CTNNB1 pathway converges on TCF/LEF-dependent target gene expression, the most upstream and direct way to monitor cells with active WNT/CTNNB1 signalling is to track the subcellular redistribution of CTNNB1 itself.

As detailed in **chapter 6**, we therefore redirected our attention to CTNNB1, which we luckily found feasible to tag. We ultimately managed to optimize CRISPR/Cas9 tagging in zygotes towards a protocol for tagging *Ctnnb1* in an efficient and scarless manner, with 13% of the pups born having a correct *mTq2-Ctnnb1* knock-in.

Conceptual and biological implications of our work

On CRISPR/Cas genome editing

In **chapters 4 and 5** we used traditional gene targeting. For the *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ knock-in model, targeted ES cells were injected into goGermline blastocysts (Koentgen et al., 2016). Due to a mutation, the cells from the recipient blastocyst will not be able to produce viable sperm, thus ensuring that only the ES-cell derived cells will generate functional sperm cells in founders, thereby greatly improving germline transmission, which can otherwise be more or less efficient depending on the mosaicism of the founder.

The occurrence of mosaicism is a common phenomenon in genome editing strategies, and CRISPR is no exception to this. In this case, mosaicism is due to the precise time and efficiency at which genome editing occurs in the early embryo - generally between the two and eight cell stage (Kueh et al., 2017; Plaza Reyes and Lanner, 2018). There are strategies to minimize mosaicism, but in practice the CRISPR editing events will not happen before the zygote divides, so there will always be a distinct possibility that founders contain both edited and wildtype alleles – and may thus produce wildtype and edited sperm or oocytes. However, with high targeting efficiencies this should not pose a problem; the higher the efficiency, the higher the chance for the majority of cells to be targeted or sufficiently large numbers of founder animals being available to achieve germline transmission reasonably effectively and comparable to the rates achieved with knock-out and transgenic strains generated by more conventional approaches. Therefore, our goal with the generation of knock-in mice by CRISPR/Cas9 was to reach a high enough efficiency to allow quick and efficient generation of knock-in mice.

We were the first research group with the desire to generate a scarless knock-in of a larger cassette (in our case: a gene encoding a fluorescent protein) at the NKI/MCCA targeting core. In a collaborative effort, it took us ~2 years to adapt their existing protocols and optimize this procedure – similar to what was happening at other places around the world during that time. Since we were interested in inserting a fluorescent protein in the *Ctnnb1* locus, we first made an inventory of studies successfully reporting the introduction of large (>200bp) DNA stretches into endogenous loci. Several labs reported the use of long (>1kb) homology arms introduced using plasmid DNA, with efficiencies varying from 0% to a maximum of 50% (Chu et al., 2016; Ménoret et al., 2015; Yang et al., 2013). Using a different approach, trials had been performed *in vivo* in frog and zebrafish embryos using

very short homology arms of only 5-25bp that harnesses a repair pathway independent of Homology Directed Repair (HDR) called microhomology-mediated end-joining (MMEJ) (Sakuma et al., 2016). The maximum efficiency of 85% found sounds promising but was only described for one locus. For other loci, the efficiency varied between 0 to 10, 30 or around 60%, so targeting efficiency with MMEJ seems to be highly locus specific. More recently, the use of long single-stranded DNA as repair template has also come into view with the Easi-CRISPR protocol (Quadros et al., 2017). Over a dozen loci were tested and correctly targeted in 8.5% - 100% of live offspring, again showing a high variety between loci. The fact that multiple different approaches describe a wide range of targeting efficiencies depending on the specific locus, underscore that the generation of genetically engineered mouse models will likely always suffer from this aspect.

In the end, we succeeded in generating our *mTq2-Ctnnb1* knock-in strain and, in parallel, allowed the transgenic and knockout core to apply this knowledge to the generation of multiple other strains after ours was finished. Seamless integration of the reporter offers a distinct advantage over traditional targeting methods, which invariably leave a 'scar' in the form of a selection cassette (or at the very least a *loxP* or *FRT* site even if the selection cassette is ultimately removed). Moreover, if this protocol works for other loci, it will shorten the time needed to create knock-in mouse lines to several weeks instead of months. As detailed in **chapter 6**, the combination of Cas9 protein and Long Single-stranded (LSS) oligo as repair template as described by Quadros and colleagues (Quadros et al., 2017) seemed most promising. This has since been validated for different loci tested at the same facility, with targeting efficiencies generally reaching over 50% (Ivo Huijbers, personal communication).

Even though there are ample studies promising highly efficient protocols for CRISPR editing that should work, specific experimental pipelines should still be carefully designed and optimized for each experimental application. As we empirically determined, many small steps contribute to the overall success, starting with a crucial first step: the availability of gRNAs that work efficiently, providing the location of the PAM site. Ideally, these guides should be located close (≤8 bp) to the designed integration site and should not be present in the repaired locus to prevent the chance of re-cutting. If the locus of interest lacks a PAM site close to the desired integration site, it might be possible to switch Cas proteins. A whole family of Class II Cas-proteins, derived from different prokaryotes, is currently available for editing in mammalian cells. The different Cas-proteins have different gRNA characteristics such as stability and what PAM-sequences are recognizes (Hajizadeh Dastjerdi et al, 2019; Karvelis et al, 2017). On top of that, Cas proteins have been genetically altered for specific purposes such as lower off-targets, nickase activity, or dead Cas9 (dCas9), creating new genome engineering tools and opportunities when "standard" CRISPR/Cas9 editing does not seem to be best choice for the locus of interest. For

example, a deaminase fused to a dCas9 proved efficient for specific base editing in mouse oocytes (Kim et al., 2017; Liang et al., 2017a), and Cas12a from *Eubacterium rectale*, with its different PAM motif (CTTN or TTTN), can be used to generate transgenic mice when a locus does not provide a suitable PAM motif for the widely used Cas9 (Liu et al., 2020).

Secondly, Cas9 delivery should be efficient and allow good timing of Cas9 activity (Aida et al., 2015; Chen et al., 2016; Lin et al., 2014; Plaza Reyes and Lanner, 2018). Injecting Cas9 protein instead of mRNA is more efficient when creating knock-in mice (Jung et al., 2017; Ménoret et al., 2015). It is suggested that this difference is because no translational step is needed for the protein to become active, leading to quicker activity and less time for mRNA or repair constructs to get degraded. Since the genetic fragment to be knocked in is inserted via HDR, a Cas9-induced double-stranded break is best induced from late S to G2-phase, when HDR is most active (Hustedt and Durocher, 2017). During the 2-cell stage, the mouse embryo has an exceptionally long G2 phase. If Cas9, linked to a biotin-streptavidin complex to draw it towards the DNA quickly, is injected during the 2-cell stage, this induces KI efficiencies up to 10-fold as compared to injecting Cas9 protein during the 1-cell zygote (Gu et al., 2018). However, injecting at the 2-cell stage is technically more challenging, so this procedure may not become widely adapted.

Thirdly, the repair template can be delivered with short or long arms and as double- or single stranded DNA, mRNA or protein. The most efficient repair system can differ per model organism or cell type. In our hands, double-stranded DNA with long arms (800bp and 2kb) worked well for tagging both human and mouse cells, while this did not result in knock-in mice in our hands.

Finally, it is impossible to ignore an important feature of the locus to be targeted: If the locus has a sequence that does not allow manufacturing of homology arms and a repair template, for example because of long repeating sequences, it might mean that tagging the locus will remain highly challenging. To overcome this, micro-homology repair approaches such as the CRISPR/Cas9-based precise integration into the target chromosome (PITCh) system could be an option (Aida et al., 2016), but this appears more challenging than the Easi-CRISPR approach in zygotes (Quadros et al., 2017). The CRISPR/Cas field remains in active and rapid development and provided that protocols will be found that work more generally for different loci, will likely replace conventional targeting strategies in the future.

Overall, an efficient protocol was set up to insert a fluorescent protein in the *Ctnnb1* locus in mouse zygotes. This tagging protocol has an important bottleneck: it depends on pronuclear zygote injections. Pronuclear injection is preferred over cytoplasmic because the gene editing components are directly delivered close to the DNA, lowering the needed concentration and volume of the gene editing components. However, these injections are technically challenging and require skilled personnel to ensure high survival rates. Therefore, a next step of optimization

that would make the approach wider available should be focussed on the delivery method of the CRISPR components.

For example, electroporation of zygotes results in successful CRISPR tagging using the CRISPR-EZ protocol (Chen et al., 2016). Interestingly, electroporation of fertilized oocytes even works without taking them out of the mouse. With a method named improved-Genome editing via Oviductal Nucleic Acids Delivery (i-GONAD), CRISPR RNPs were delivered to E0.7 embryos via *in situ* electroporation (Ohtsuka et al., 2018). Efficiency of editing via i-GONAD was found to be similar to microinjection methods, while technically it is less challenging. Moreover, in the same year a study was published showing another *in utero* method (Yoon et al., 2018), showing proof-of-principle evidence that *in vivo* CRISPR/Cas9 gene editing can be achieved by injecting recombinant adeno-associated viruses into the oviduct of pregnant females. This is an interesting method without the need for injection or electroporation and showed high survival rates. However, since *in utero* genome editing is relatively new, it is important to check for possible side effects and the rate of exposure to Cas9. But overall, they show promise for future application making easy and efficient CRISPR genome editing available for most labs.

An important draw-back to CRISPR/Cas genome editing in general should not be forgotten: the lack of detailed understanding of off-target editing events and their possible effects. In cell-lines off-targets are passed on to daughter cells in division, so it is important to create clonal cell lines that can be thoroughly checked or prevent off-targets where possible. One strategy to lower the chance of getting off-targets can be to minimize exposure to Cas9; both in the timing of its presence in the cell and its concentration. The lower the exposure, the lower chances are for off-targets, although a balance should be found because it can also lower the chance for correct on-target integration. Another strategy is the use of nickase instead of nuclease Cas9, lowering the chance for double-stranded breaks at possible off-target sites. Yet, nickases are rarely used for *in vivo* editing because of the even lower HDR efficiency found (Lee and Lloyd, 2014; Ran et al., 2013b).

Luckily, in mice, off-targets will eventually be crossed out over future generations. Therefore, we did not check for off-targets events in our *mTq2-CTNNB1* mice. However, developing a mechanistical understanding of off-targets and screening of off-target sites is needed to be absolutely sure that no unwanted biological effects are inadvertently created by CRISPR engineering.

On imaging

While recent developments in gene editing have made many non-model organisms tractable to genetic manipulation and studies in the lab, the mouse remains an important model system for mammary gland biology research. One challenge that comes with working with mammalian model organisms such as mice, is that the

biological processes of interest are relatively difficult to study because they often occur hidden deep inside the body.

To study them, the tissue of interest will typically need to be isolated, allowing only a snapshot of the tissue to be generated at the given time. These static images provide a lot of information on the number, localization, and differentiation status of the progeny of the labelled cells but can fail to describe the full dynamics of a complex tissue. For some tissues such as intestine, brain and mammary gland, intravital imaging is an option that allows to follow cell fate live over time for several hours per imaging session (Dawson et al., 2021; Fumagalli et al., 2020; Jacquemin et al., 2021; Messal et al., 2021; Scheele et al., 2017). However, this is technically challenging, which hampers wide adoption of the technique, in addition to the fact that not all tissues may be suitable for this approach.

As mainly shown in **chapters 4 and 6**, it also remains challenging to image isolated wholemount tissues, which are typically opaque and need to undergo clearing to become transparent. In recent years, the rapid development of organoid cultures for many mammalian tissues has provided the opportunity to image cells in a 3D context that resembles the original tissue complexity. Even though a 3D culturing system cannot fully recapitulate the systemic environment of the body, they may complement *in vivo* studies. First, organoids should allow live-cell imaging over longer periods of time. Second, signalling can be easily manipulated by the use of chemical compounds. Third, since they can be expanded in culture, organoids allow high numbers of cells to be analysed per experiment, allowing more quantitative conclusions to be drawn. In this respect, it is promising to see that both the endogenous *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ reporter and the mTq2-CTNNB1 fusion protein can be imaged in living intestinal organoids, although it is yet to be determined if they also report WNT/CTNNB1 signalling in organoids of different tissue origins, including the mammary gland.

In our new mouse models, we inserted several fluorescent proteins that are not yet widely used as genetic reporters *in vivo*. This includes mScarlet-I, mTurquoise2, IRFP670 and SGFP2. While multiple single, dual and multicolour fluorescent reporter lines are available for lineage tracing purposes, fluorescent protein development is far from finished. Different labs worldwide work on improving already existing fluorescent proteins, engineering mutations that can lead to higher brightness, maturation, or other properties. As an example, this recently led to the record-bright green fluorescent protein variant mGreenLantern, which is up to 6 times brighter then EGFP (Campbell et al., 2020) and a novel mVenus variant, mGold, that is reported to be 5 times more photostable (Lee et al., 2020).

Next to optimizing existing variants, researchers are also designing completely synthetic FPs to build in specific desirable properties, as was done for the mScarlet family (Bindels et al., 2016). And while we tend to think that much of the biological world is known by now, completely novel FPs are still being discovered, which

harbour unique properties that could in the future turn out very interesting to use *in vivo* (Lambert et al., 2020). Many of these improved and new FPs are highly promising to be tested *in vivo*, but it will require dedicated efforts and collaborations between scientists with different expertise to do so.

To truly test how FPs will perform in mice, they need to be measured in their relevant biological environment, because the anticipated performance of FPs can differ from their actual performance *in vivo* (Heppert et al., 2016). For example, while mNeongreen is predicted to be brighter than GFP (Shaner et al., 2013), this is not the case in the animal model *C. elegans* (Heppert et al., 2016). The results of a direct comparison of several *C. elegans* genetic reporter strains carrying either GFP or mNeongreen are surprising: in some experiments the FPs perform similarly, but in others one FP is clearly brighter than the other. We do not know the exact biological aspects that cause these differences and to what extend they vary between organisms. Until we get a better understanding of these aspects, it is important to assess context-specific performance of FPs *in vivo*. Unfortunately, the mouse is technically not as easily manipulated and imaged to predict FP performance *a priori* as *C. elegans*. To keep up with the development of novel FPs and maintain sufficient innovation, it would be convenient to have an optimized pipeline to screen for novel fluorescent reporters in mice as well. Clearly, the time and effort associated with generating new mouse models can be prohibitive to taking the risk of trying out new options as we have done for the *Rosa26*^{PRIME} reporter in **chapter 5**.

On in situ lineage tracing analyses

WNT signalling is involved in the proliferation and differentiation of multiple stem cell populations, including those of the mouse mammary gland (van Amerongen et al., 2012; Fu et al., 2020; Plaks et al., 2013; Rios et al., 2014; Wang et al., 2015b). There is much debate about the identity of these WNT-responsive mammary stem cells. These are mainly due to the unresolved complexity of the mammary stem cell hierarchy, combined with differences in experimental setup and interpretation. In **chapter 3** we describe best practice methods for lineage tracing of stem and progenitor cells in the mouse mammary gland.

To date, most inducible lineage tracing models use CreERT2/loxP technology. Here, spatiotemporal control over genetic labelling is provided by combining a tissue-specific promoter with the administration of tamoxifen. Although it should be stressed that information gleaned from these experimental systems continues to be highly relevant for understanding the complex biology of both healthy and cancerous tissues, recent studies have revealed important drawbacks to the use of tamoxifen based systems, especially in the mammary gland: While tamoxifen is required to induce CreERT2-mediated recombination, it also acts as an estrogen antagonist in this tissue,

thereby potentially impairing normal development and function (Shehata et al., 2014). This is an unwarranted side-effect that might also affect tracing in other estrogen-sensitive tissues such as the endometrium or ovaries, since *in situ* lineage tracing experiments are explicitly designed to interfere with normal biology as little as possible. It may also preclude a proper analysis of the sex differences in (hormonal) cancer susceptibility between males and females (Van Den Berg et al., 2015; Silva and Swerdlow, 1993).

A main goal of this project, covered in **chapters 3, 4 and 5**, was to improve existing models for *in vivo* lineage tracing of WNT-responsive stem cells in the mammary gland. Specifically, we wanted to *(i)* build a novel, well-characterized set of driver and reporter lines to allow sensitive, specific and physiologically relevant labelling of WNT-responsive mammary stem cells and *(ii)* test if a doxycycline-inducible rtTA3 approach could replace existing tamoxifen-inducible CreERT2 strains. The short answer to this last question, unfortunately, is "no". While our new driver strain (**chapter 4**) is fully functional, it did not allow tetO-Cre mediated recombination of a reporter allele in the mammary gland even in combination with the highly efficient *Rosa26*^{mTmG} reporter allele. At present, the reasons behind this negative result remain unknown, although one or more of the following could explain our findings. First, given the low expression levels of *Axin2*, the levels of rtTA3 might be limiting. This might be bypassed by using homozygous animals, although this does not guarantee that the threshold for activation would be met in this case. Second, it could be due insufficient induction of the *tetO-Cre* strain used for these experiments (Schönig et al., 2002). Since wrapping up the experiments described in chapter 4, our lab has tested a second independent *tetO-Cre* strain (Perl et al., 2002), which did not obviously result in enhanced recombination frequencies – making this explanation less likely. While our current approach was unsuccessful, moving away from the use of tamoxifen as an inducing agent still seems the most logical step forward and is especially important in tissues with a known hormone dependency.

It should be noted that it was our original plan to build two new doxycycline-inducible rtTA3 drivers – a 5' and a 3' *Axin2* knock-in allele. For practical and logistical reasons, we only succeeded in generating the 3' *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ strain. In hindsight, however, generating both strains would still have been the perfect academic exercise: the 5' *Axin2 rtTA3* allele would have allowed the only proper and direct comparison of our existing 5' tamoxifen-inducible *Axin2*^{CreERT2} strain (van Amerongen et al., 2012), whereas a comparison of the 5' and 3' *rtTA3* knock-in allele would likely have revealed any differences in *rtTA3* expression due to the different integration site of the knock-in cassette.

Since we were not able to use the new *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ driver for lineage tracing purposes in the mammary gland, we have not been able to actively contribute to or settle the debate regarding the stem and progenitor cell hierarchy in this tissue. Many groups continue to work in this area, however, which has

underscored if not necessarily resolved the complex dynamics of (stem) cell behaviour in this tissue (Watson, 2021). As it stands, multiple lineage restricted populations have been identified using cell-type specific drivers (van Keymeulen et al., 2011; Lafkas et al., 2013; Lilja et al., 2018; Rodilla et al., 2015). At the same time, the mammary gland also shows prominent signs of plasticity, as can be revealed by transplantation (van Amerongen et al., 2012; van Keymeulen et al., 2011) or induced by oncogenic mutations (Van Keymeulen et al., 2015; Koren et al., 2015). At present, only *Procr*+ cells appear to have true bipotent potential (Wang et al., 2015b). Although also considered to be a WNT/CTNNB1 target gene, the *Procr*+ and *Axin2*+ populations do not appear to overlap, and it remains to be determined in how far our originally identified bipotent *Axin2*+ cells really stand to higher scrutiny. Definitive proof of their existence (or evidence to the contrary) was a major reason for designing and developing the multicolour *Rosa26*^{PRIME} strain.

Despite our failed attempt to improve the lineage tracing of WNT-responsive mammary stem cells, at least as far as the *Axin2* strain is concerned, we can conclude the following. First, the mammary gland field remains in dire need of strains that were designed and tested to function well in this particular tissue. Second, the future of *in vivo* lineage tracing likely lies in neutral tracing approaches rather than in the use of stem-cell specific (or other targeted) driver strains. The latter are unlikely to recapitulate endogenous gene expression and activity completely. Of course, this automatically implies that neutral drivers should, indeed, be neutral – which in lineage tracing terminology typically means that they should be globally expressed, as is generally assumed to be the case for the *Rosa26*^{CreERT2} (Ventura et al., 2007) and *Rosa26*^{rtTA} (Stadtfeld et al., 2010) lines, for instance. One might hypothesize that our newly identified reference genes from **chapter 2** could be employed for this purpose.

Another way forward would be to abandon experimental induction of reporter gene activity altogether, instead relying on stochastic recombination events (Kozar et al., 2013; Lloyd-Lewis et al., 2018). Currently available systems recombine too infrequently to be useful for deducing general principles of mammary gland growth and differentiation, however, especially given that the tissue does not show a spatially confined, stereotypic growth pattern (in contrast to other tissues, such as the intestinal epithelium or the epidermis). The dynamic growth properties of the tissue (including the massive expansion and remodelling during puberty, pregnancy and involution) are precisely what make these lineage tracing experiments so important and interesting to perform, but difficult to interpret. In the future, other strategies such as the recently reported FRAME-tag strategy employed in yeast (Anzalone et al., 2021), might prove to be better suitable. Other opportunities exist by including barcode and next-generation sequencing based lineage tracing techniques such as GESTALT (McKenna et al., 2016), or through simultaneous lineage tracing and cell type identification using CRISPR-Cas9 induced genetic scars

to allow combined lineage tracing and transcriptome profiling (Spanjaard et al., 2018). While this has recently been realized in thousands of single cells, it is not possible yet to achieve this for an entire tissue or, ideally, the whole mouse. The experimental challenge lies in ensuring that full lineage information, that is, every cell division, is recorded reliably. However, such approaches pose the additional problem of preserving positional information which is required to understand and reconstruct the 3D tissue architecture, and how this influences the identity and potential of mammary stem cells (Watson, 2021).

A final challenge with interpreting lineage tracing results comes from being able to confidently discriminate independent recombination events. The more fluorescent colours present in the lineage tracing reporter, the easier it is to distinguish separate clonal events. However, what appears to be a clonal patch of one colour can still emerge from separate switching events by chance. To minimize the chances of misinterpretation, tracing can be performed at very low clonal density, so that only a handful of cells in a tissue have switched. The downside of this is that only few cells can be followed and that many samples must be prepared and analysed to collect sufficient data for quantitative analyses. Higher switching frequencies can provide more information per tissue, but this requires careful biostatistical analysis of the prevalence of the different fluorescent recombination events (Wuidart et al., 2016). This involves modelling of expected stem cell behaviour such as that of unipotent and multipotent stem cells in the mammary gland epithelium. To properly model cell behaviour, it is important that switching of the reporter line is carefully analysed in each tissue, to include this factor in the analysis. For instance, by design the *Rosa26*^{Confetti} reporter should result in 1:1:1:1 of the four different fluorescent colours (hriGFP, EYFP, tdimer2 and mCerulean). Empirical evidence, including our own experience, shows that these colours do not occur with equal frequencies. Our *Rosa26*^{PRIME} reporter from **chapter 5** was also designed to result in 1:1:1 recombination frequencies of the red, yellow and cyan option. FACS analysis reveals that our allele may suffer from the same problem as *Rosa26*^{Confetti}, despite its different set up (variant Lox sites versus multiple LoxP sites). How the *PRIME* allele behaves *in vivo*, and whether this differs across tissues or cell types or the driver line used, remains to be tested.

Outlook

There will likely always be some risk involved when creating novel genetic mouse models, with choices made at the design stage potentially having consequences years further down the road, no matter how carefully targeting constructs are designed on paper or even tested *in vitro,* the ultimate test of how an allele will behave *in vivo* can only be achieved when the mouse model has been created. This is illustrated by the fact that despite our best efforts we have not been able to use the lineage-tracing

driver developed in **chapter 4** to further delineate the mammary stem and progenitor cell hierarchy.

The generation of new mouse models also remains resource intense, requiring either a lot of time (when done in house) or a lot of money (when outsourced to a commercial partner). In view of the above, it was perhaps inevitable that functional analyses with the *PRIME* (**chapter 5**) and *mTq2-Ctnnb1* (**chapter 6**) models could not be completed within the timeframe of this PhD project.

Nevertheless, as described in **chapters 4 and 6**, we have successfully generated new mouse strains to visualize and measure endogenous WNT/CTNNB1 signalling *in vivo*. One might argue that in doing so, we have also reached a bit of an impasse: Through careful design, we are now capable of building the tools that allow us to measure WNT/CTNNB1 signalling at physiological levels, only to find out that these levels are so low that we run into multiple experimental and technical challenges when it comes to detecting and quantifying them. Put differently: while the technology now allows us to watch these biological processes in action, it turns out that biology operates at levels where we run into experimental limitations.

For instance, the current dogma still dictates that CTNNB1 becomes stabilized and translocates towards and accumulates in the nucleus in cells with an active WNT response. Yet in the past few years, different studies from our lab, including the work described in **chapter 6** of this thesis, have found that the levels of CTNNB1 increase 2-4 fold at most, either in the presence of a WNT stimulus or in the presence of constitutive hyperactivation (Jacobsen et al., 2016; de Man et al., 2021). Our *mTq2-Ctnnb1* knock-in mouse model also supports this notion, suggesting that commonly used detection techniques severely overestimate its abundance: The endogenous *mTq2-Ctnnb1* signal, which is highly expressed in epithelial adherens junctions, allowed easy detection of the fluorescent signal at cell-cell contacts. Still, we did not observe nuclear accumulation of CTNNB1 in Paneth cells in the intestinal crypt, as is observed and typically reported using immunohistochemistry (van de Wetering et al., 2002). We think this is unlikely to be due to the tagging itself, but rather a proper reflection of its subtle accumulation. While the output of WNT/CTNNB1 signalling is typically amplified at the transcriptional level, here too we find that the endogenous levels of *Axin2* expression are much lower than *lacZ* based reporter systems would suggest. Visualization of the relative fluorescent levels as shown in *Axin2*$^{P2A\text{-}rtTA3\text{-}T2A\text{-}3xNLS\text{-}SGFP2}$ organoids in **chapter 4** was useful for detecting subtle changes in *Axin2* expression. The heatmap colouring allows changes barely visible by eye to be depicted in such a way that the relative levels can easily be interpreted.

Despite these challenges, follow up work in the lab will remain dedicated to quantifying endogenous WNT/CTNNB1 signalling in both physiological and oncogenic circumstances. The work described in this thesis provides the basis for these lines of research, by demonstrating that tagging genes and proteins of interest with fluorescent proteins allows their endogenous expression to be visualized in real

time by fluorescence microscopy. This is a major advantage over many biochemical assays, but also over overexpression approaches. The latter can lead to unwanted side-effects, so measuring endogenous signals is preferred (Mahen et al., 2014). As we found out, however, when imaging the endogenously expressed proteins many practical and technical hurdles need to overcome before the biological questions themselves can be addressed. It not only requires sensitive microscopes and detectors, but also careful planning and design. For instance, tagging of diploid or even haploid cells is probably recommended if the goal is to perform functional, quantitative analyses of the entire protein pool. This approach was recently applied successfully by others in the lab to learn more about the exact mechanisms and interactions happening in the destruction complex upon WNT activation, which remains a topic of discussion in the field (de Man et al., 2021; Tortelote et al., 2017). Another line of investigation that was inspired by the work described here is the study of crosstalk between the pool of CTNNB1 bound at the membrane and the free-floating pool (van der Wal and van Amerongen, 2020).

In summary, combining CRISPR/Cas gene editing, lineage tracing, live imaging and organoid technology shows huge promises for studying stem cell biology, development and cancer *in vivo*. Together, they will help unravel the molecular events of WNT signalling at an unprecedented level of detail. This thesis has laid the foundation by describing the generation and first analysis of several novel tools that should be of use for the broader scientific community to help paint and complete the dynamic picture of the role of WNT signalling in regulating tissue development and homeostasis.

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