A closer look at WNT/CTNNB1 signaling

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Publication date
2023

Citation for published version (APA):
CHAPTER 6

General discussion
The importance of WNT/CTNNB1 signaling for all metazoan life on earth is evident from its evolutionary conservation across a wide variety of multicellular animals, its versatile roles in tissue development and maintenance and its frequent involvement in oncogenesis. As laid out in Chapter 1, the overarching aim of this thesis was to gain a better understanding of the working mechanism, and specifically the dynamics of WNT/CTNNB1 signaling. One of the main outstanding questions in the field, which we addressed in Chapter 2, goes to the very core of the WNT/CTNNB1 signaling mechanism – how is the destruction complex, i.e. the cellular machinery that controls CTNNB1 turnover, inhibited? Although a full answer to that question might still take years of research from the entire field, it is becoming apparent that small and dynamic changes, which we can now just about measure with the available state-of-the-art technologies and experimental approaches, are essential. Our own data in Chapter 3 and 4 indicate that CTNNB1 levels and complex formation only change within a small, two- to fourfold range over the vast scale of biological outcomes and cellular phenotypes induced by WNT/CTNNB1 signaling in development, homeostasis and oncogenesis. In Chapter 5, we combined our fluorescent endogenous CTNNB1 reporter cell lines from Chapter 3 with an unbiased forward genetic screening approach. This allowed us to identify and distinguish between regulators functioning up- and downstream of CTNNB1 stability, based on small changes in CTNNB1 levels, comparable to those we observed in the previous chapters. In this final chapter, I will discuss the importance of subtle biological changes for WNT/CTNNB1 signaling specifically, and for cell signal transduction in general.
SMALL CHANGES GOVERN WNT/CTNNB1 SIGNALING

Levels, localization and complexes

As a master student, I read in the fifth edition of Molecular Biology of the Cell that without a WNT signal “Phosphorylated β-catenin is ubiquitylated and degraded in proteasomes” and that with WNT “Unphosphorylated β-catenin accumulates, migrates to nucleus […] and associates with coactivator”. From this description, we might expect a clear and binary switch from low CTNNB1 levels, to high levels of only nuclear CTNNB1. However, our microscopic measurements of endogenous CTNNB1 highlighted the nuances of this process.

First of all, changes are more subtle than might be expected from the textbook description that reads “β-catenin accumulates”. In endogenously tagged HAP1 cells (Chapter 3 and 5) or primary mouse embryonic fibroblasts (Chapter 4), the increase in intracellular CTNNB1 was approximately two-fold upon treatment with purified WNT3A as measured with both confocal microscopy and FACS (relative changes) and FCS (absolute levels). This is also consistent with observations from other studies of CTNNB1 dynamics (Jacobsen et al. 2016; Kafri et al. 2016; Massey et al. 2019). We also found similar small differences in vivo in Chapter 4, detecting a two-fold increase in nuclear CTNNB1 between the more posterior presomitic mesoderm – where WNT/CTNNB1 signaling is known to be active – and the more anteriorly located recently formed somites – where WNT/CTNNB1 is largely inactive (Sonnen et al. 2018). The same holds true along the axis of the intestinal crypt, where the differences in nuclear CTNNB1 were even less pronounced – despite the fact that the importance of WNT signaling for the stem cells in the intestinal crypt has been shown beyond doubt (reviewed in Kretzschmar and Clevers 2017). Finally, oncogenic mutation of CTNNB1 at position S45, which prevents phosphorylation of CTNNB1’s N-terminus and subsequent degradation, elevated CTNNB1 levels by only four-fold compared to unstimulated wildtype cells. Since this mutation is found in many cancers (reviewed in Polakis 2000), this suggests that a four-fold difference in CTNNB1 encompasses the broad biological bandwidth of non-WNT responsive cells, WNT/CTNNB1 activated (stem) cells, and tumor cells. The fact that we could separate upstream and downstream regulators in the WNT/CTNNB1 pathway based on small differences in SGFP2-CTNNB1 fluorescence (Chapter 5), further supports that these small changes are biologically relevant.

Nuclear accumulation of CTNNB1 was also less prominent than expected from the textbook model “β-catenin accumulate and migrates”. We did measure a preferential increase in nuclear CTNNB1 compared to cytoplasmic CTNNB1 upon pathway activation, but the level of enrichment we measured was modest (1.1-1.4 times). However, in order to obtain this modest enrichment, our computational model indicated that three processes are required: accumulation; nucleocytoplasmic shuttling and nuclear retention (Chapter 3). When we performed insertional mutagenesis screens specifically for regulators downstream of CTNNB1 abundance (Chapter 5), we therefore specifically looked out for
regulators of nuclear translocation and retention. We identified several known and novel nuclear regulators of CTNNB1 activity in the nucleus, such as CREBBP, LEF1, and SETD1B. Transcriptional coactivators such as TCF have previously been show to retain CTNNB1 in the nucleus (Krieghoff et al. 2006). Therefore, it would be interesting to see if knock-down of these factors would indeed reduce nuclear accumulation in the endogenous setting. We did not identify clear candidates for the regulation of nucleocytoplasmic shuttling of CTNNB1 in these insertional screens. This could be due to a number of reasons, including the technical limitations that prevent identification of redundant as well as essential genes. In addition, it is possible that that CTNNB1 abundance and its nuclear translocation are inherently linked. There is a large body of research that shows the importance of posttranslational modification (PTM) and conformational changes for the localization of CTNNB1 (Gottardi and Gumbiner 2004; Wu et al. 2008; Sayat et al. 2008; Valenta et al. 2012; van der Wal and van Amerongen 2020). Since the inhibition of the destruction complex inhibits phosphorylation and ubiquitination of CTNNB1 (Mukherjee et al. 2018), it is possible that this differential PTM of CTNNB1 directly contributes to its nuclear translocation as well as its accumulation.

In addition to its accumulation and localization, the textbook model simplifies the complexing state of CTNNB1. In the “WNT off” condition CTNNB1 is thought to be in the cytoplasmic destruction complex and in the “WNT on” condition it is in a transcriptional complex. Using FCS, we were able to measure the diffusion dynamics of CTNNB1 in live cells. Indeed, we found diffusion speeds that are compatible with these complexes. In the nucleus, we observed the expected increase in complexed CTNNB1 after pathway activation, which – if we assume that this complexed CTNNB1 is the chromatin associated fraction – is also in line with the function of transcriptional co-activators in its nuclear retention. However, in the cytoplasm, CTNNB1 was part of a large, slow-moving complex in both unstimulated and stimulated conditions (be it through WNT3A, CHIR99021, or oncogenic mutation). As described in Chapter 2, there are currently many models of destruction complex inhibition. Our data supports a model where CTNNB1 increase is not caused by flipping of a binary switch, such as a complete dissociation, relocalization or inhibition of the destruction complex, but rather a partial inactivation and/or disintegration of the destruction complex in combination with changes to nuclear shuttling and retention of CTNNB1.

**Sensitivity to levels and fold-changes in CTNNB1**

The data in this thesis clearly show that changes in CTNNB1 in levels and localization are relatively mild. Then how does a two-fold change in CTNNB1 levels induce meaningful biological effects? Part of the answer is that the signal is amplified while it is propagated. While overall CTNNB1 levels only rose two-fold, we measured the most prominent increase of CTNNB1 (four-fold) in its nuclear complex, where it acts to promote gene transcription. The transcriptional activity of CTNNB1containing TCF/LEF complexes further amplifies the signal. The two-fold change in overall CTNNB1 levels corresponded to a 10–50 fold increase.
of target gene expression in cells (Chapter 3-5) and changes in AXIN2 reporter signal in vivo far exceeded changes in CTNNB1 levels (Chapter 4). This amplification has also been predicted and validated in multiple computational models of the WNT/CTNNB1 pathway (for example Goentoro and Kirschner, 2009; Hernández et al., 2012).

We also observed heterogeneity within the basal levels of CTNNB1 between single cells, tissues and developmental stages. Therefore, the question arises: do these differences in basal CTNNB1 level then also reflect the activation status of the pathway? To some degree this might be the case, as changes in CTNNB1 levels during the cell cycle or due to changes in adhesion have been shown to induce gene transcription (Olmeda et al. 2003; Davidson and Niehrs 2010; Howard et al. 2011; Ding et al. 2014; Gayrard et al. 2018; van der Wal et al. 2020). Nevertheless, there is evidence that absolute CTNNB1 levels do not determine downstream signaling. Rather cells respond to fold-changes in CTNNB1, which can be explained by a so-called incoherent feed-forward loop (Goentoro et al. 2009a, b). This means that a cell responds not to an absolute level of CTNNB1 (e.g. 100 or 150 nM), but rather to the amplitude of its dynamic increase (e.g. two-fold – either from 50 to 100 nM or from 75 to 150 nM). Thus two cells with different absolute levels (e.g. 100 and 150 nM) will have the same transcriptional output. This mode of CTNNB1 sensing provides both robustness to population differences, as it ensures that differences in absolute levels due to environmental variations do not induce downstream signaling, while at the same time cells have a high sensitivity to small dynamic changes in CTNNB1 that are specifically induced by WNT ligand binding.

The tools developed in this thesis could be further exploited to address the contribution of environmental factors on absolute levels and fold-changes in CTNNB1 and whether this influences the eventual signaling outcome. For example, the live-imaging datasets of Chapter 3 could be further mined to correlate cell cycle stage and adhesion status on CTNNB1 levels and responses to pathway activation. Unfortunately, at the time we were unable to track individual cells in CellProfiler. The close proximity of neighboring HAP1 cells and their respective nuclei, and mobility of the cells resulted in aberrant cell tracks. However, new and smarter cell tracking software is continuously being developed (see for example Kucinski and Gottgens, 2020) and could be used to reanalyze old datasets like our own. Subsequently, different responses to WNT stimulation could be clustered (see for example Chavez-Abiega et al., 2022), and linked to features, such as cell cycle stage, cell size or shape or neighbor fraction, to see if and how these factors shape the CTNNB1 response. In addition, using the HAP1-FACT cell lines developed in Chapter 5, which express both SGFP2-CTNNB1 and mCherry under the influence of the 7TCF promotor, can help directly link the levels and subcellular accumulation of CTNNB1 as well as cell shape, size, and cell cycle stage to transcriptional output.

Although fold-changes might determine signaling outcome within a certain range of absolute concentrations, absolute levels do seem to dictate target gene expression directly
when CTNNB1 levels are further elevated due to overexpression or oncogenic mutation (Goentoro et al. 2009a). To return to our example, while within a range from 0-200 nM, dynamic fold-changes might dictate the level of gene expression (i.e. a change from 50 to 100 nM has the same output as a change from 75 to 150 nM), levels outside of this range (e.g. 400 nM) induce transcriptional activity even in the absence of dynamic changes. However, the tolerated level of absolute CTNNB1 appears to be tissue specific as can be seen by specific alterations to the pathway in cancer. In most colon cancers where APC is mutated and CTNNB1 levels are increased, there seems to be a selection of specific mutations that retain some degree of CTNNB1 regulation. This has been termed the ‘just-right’ hypothesis, and proposes that a certain level of CTNNB1 in the intestine is favorable for growth, while excessively high levels cause cell death (Albuquerque et al. 2002). However, this ‘just-right’ level in colon appears to be already too high for breast tissue, where we see more modest alterations of WNT signaling amplification, presumably leading to lower overall CTNNB1 levels (reviewed in van Schie and van Amerongen 2020). Part of this tissue specificity could be explained by the large difference in transcriptional partners and transcriptional programs that are activated by WNT/CTNNB1 signaling (reviewed in Söderholm and Cantú 2020). To further study how CTNNB1 levels vary across organs, tissues and cell types, our mTq2-CTNNB1 mouse model characterized in Chapter 4 in combination with imaging or FACS could be used. There are considerable technical challenges in normalization of the signal, as many properties such as autofluorescence and scattering of light are highly tissue and cell type specific and the levels of mTq2-CTNNB1 might be exceedingly low in some tissues. From our experiences with the Axin2P2ArtTA3T2A3xNLSSGFP2 mouse, which carries an SGFP2 reporter as an Axin2 3’ UTR knock-in, it has become clear that detecting the very low levels of WNT/CTNNB1 signaling in for example the mammary gland is on the boundary of what is technically achievable (van de Moosdijk et al. 2020). However, the determination of the tissue-specific ranges of CTNNB1 levels in combination with transcriptional read outs, could be instrumental in understanding the ‘just right’ levels in specific tissues as well as in understanding how these levels correlate to transcriptional output.

**Destruction complex composition and inactivation**

As extensively discussed in Chapter 2 and the discussion of Chapter 3, the exact composition, stoichiometry and mode of inactivation of the destruction complex remain a topic of active investigation. Our FCS data was consistent with a larger multimeric destruction complex, but we measured a low occupancy of CTNNB1 within these complexes. Related to the inactivation of the destruction complex, we measured that a large portion of CTNNB1 also remained complexed upon WNT stimulation, although the complex size did decrease. Our data added some necessary nuance to our understanding of destruction complex functioning. However, with our current techniques we were unable to determine what the exact dynamic composition of the destruction complex is.
To answer this question, we believe that maintaining endogenous concentrations is essential. It is clear that complex formation can be easily skewed upon overexpression. As an example, we see a range of different localizations for AXIN1 and APC across different expression levels. Overexpression of AXIN1 and APC leads to formation of very large punctate structures (for example, Faux et al., 2008; Mendoza-Topaz et al., 2011). At near-endogenous levels in Drosophila the puncta appear smaller (Schaefer et al. 2018), and in vitro studies predict an even lower number of molecules per complex (Kan et al. 2020; Ranes et al. 2021). To visualize endogenous destruction complexes within cells we expanded our CRISPR/Cas9 tagging efforts to AXIN1, APC, GSK3B and DVL2 (described in the addendum, Chapter 7). While we were successful for APC, GSK3B and DVL2, we were unable to tag AXIN1. Generally, CRISPR/Cas9 mediated homology directed repair is limited in many cell lines (He et al. 2016). Tagging becomes even more challenging for lowly expressed genes like AXIN1, but additional strategies might be able to overcome these hurdles (for example Gabriel et al., 2021).

In our preliminary data of endogenous DVL2 and GSK3 we observed again more subtle phenotypes than have been previously described. For example, DVL2 only localized to puncta upon overexpression of exogenous DVL, but was otherwise diffuse. In addition we were unable to visualize recruitment of DVL2 to the plasma membrane upon WNT3A treatment, which was consistent with data from others that employed Total Internal Refraction Fluorescence microscopy in order to visualize the dynamic recruitment of small DVL polymers to the membrane in an endogenous setting (Kan et al. 2020; Ma et al. 2020; Schubert et al. 2022). For endogenously tagged GSK3B, we did not observe localization to puncta in the absence of WNT, or accumulation in multivesicular bodies upon pathway activation with confocal microscopy, shaping again a more nuanced picture compared with localization in overexpression studies of destruction complex components and constitutively activated LRP (Taelman et al. 2010; Mendoza-Topaz et al. 2011).

To further study the dynamic composition of the destruction complex, a range of imaging techniques could be employed. Fluorescence Resonance Energy Transfer (FRET) could be used to study direct interactions in the destruction complex and even conformational changes in its constituent components. With FRET it was shown that AXIN1 undergoes a conformational change upon WNT ligand binding, thereby reducing CTNNB1 binding (Kim et al. 2013). While for FRET measurements direct interactions and correct orientation of fluorophores is required, Fluorescence Cross Correlation Spectroscopy (FCCS) can also quantify protein-protein interaction based on their joint diffusion, which - like FCS - also contains further information on complex sizes. In the WNT field, FCCS has already been employed to study the dynamic of FZD receptor complexes and receptor-ligand interactions (Petersen et al. 2017; Veerapathiran et al. 2020; Eckert et al. 2020). However, FCCS is a sensitive technique that can bring many technical challenges (Foo et al. 2012), and we were so far unable to obtain reliable data on destruction complex composition using this methodology (Chapter 7). Another promising possibility is to use...
super resolution techniques such as DNA-PAINT to study protein complex compositions (for example Simoncelli et al., 2020). Although time resolution in combination with super resolution techniques remains limited, detailed information on the variability on numbers of molecules and stoichiometry per complex can be obtained to gain further understanding in the functioning of the destruction complex.

CONCLUDING REMARKS

Subtle signaling beyond WNT/CTNNB1

When reading Biology of the Cell at the start of my studies, signals that determine cell fate and behavior appeared to me to be simple on and off switches. Now, after conducting my PhD research, it has become abundantly clear that cell signaling is made up of heterogeneous, temporary and spatially determined changes in protein levels and interactions that are difficult to measure. Naturally, these properties are not confined to the WNT/CTNNB1 pathway. For example, noisy signaling is well documented in GPCR and MAPK signaling (e.g. Filippi et al., 2016; Norris et al., 2021). Further levels of complexity are introduced by crosstalk between the many cell signaling pathways (Rowland et al. 2017).

So why have signaling pathways evolved noisy networks? One explanation can be found in the fact that, counterintuitively, noise gives robustness to signaling systems (Ladbury and Arold 2012). A single event of ligand-receptor interaction, cellular heterogeneity in gene expression or even slight changes in protein interactions from mutant proteins will not switch the system on or off, if its effects don’t exceed the noise that is inherently present in the signaling network. Thus, by having noise, noise can also be tolerated in biological systems. Moreover, the stochastic nature of interactions within signaling cascades allows cells to have distinct reactions to different signals and signal strengths, thus increasing the amount of information that can be transmitted in the cell (Azpeitia et al. 2020). This information is often preserved in the dynamic reaction of the response (Selimkhanov et al. 2014).

The fact that signaling events are noisy, heterogeneous and dynamic poses challenges for cell biologists. Tools to study cell signaling thus ideally should [1] have high sensitivity to capture small changes, [2] be spatially resolved to both measure relevant cell compartments as well as capture cellular heterogeneity, [3] have sufficient temporal resolution to measure dynamic events and [4] maintain endogenous expression levels. For this reason, we selected genome editing and advanced microscopy. This provided excellent resolution and quantitative measurements of endogenous WNT/CTNNB1 signaling both in vitro and in vivo. However, endogenous tagging and advanced microscopy studies require large time investments and report on few proteins or genes at a time. Further developments in gene editing, high content imaging and automated analysis will surely provide many
new insights into cell signaling. In addition, other techniques are also moving to better understand heterogeneity and dynamics of signaling networks. For example, many omics techniques have already moved into single cell modalities, which give great insight into cellular heterogeneity in an (epi)genome, transcriptome, proteome and metabolome wide fashion (Suvà and Tirosh 2019; Kucinski et al. 2020). Moreover, developments that combine imaging and omics techniques to increase spatial resolution are being actively developed to get the best of both worlds (Lewis et al. 2021). Finally, continual improvements in computational modelling to understand these complex networks will be required to understand the relevance of subtle perturbations in cell signaling (Kholodenko et al. 2012). These technical advances in measuring cellular states with high spatiotemporal resolution combined with predictive computational models for complex signaling networks, we will learn to understand how cells and organisms separate the signal from the noise.


