Beta-catenin

A molecular multiplayer

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General Discussion

This thesis focusses on a long outstanding question in both the field of cell adhesion and WNT signalling; how does a cell balance CTNNB1 between its function in cell adhesion and WNT signalling? I like to call CTNNB1 a molecular multiplayer. In this analogy, player number one is cell adhesion. Here, CTNNB1, as part of the core cadherin-catenin complex, links the cadherin proteins in the membrane to the intracellular cytoskeleton. This stabilizes the junctions through tension, making CTNNB1 a crucial link in the chain of cell adhesion. Player number two is WNT signalling, where CTNNB1 fulfils its role as main effector. Via receptor/ligand binding, the WNT pathway gets activated, which ultimately leads to build up of CTNNB1. CTNNB1 can then travel to the nucleus where it acts as a co-transcription factor, crucial for activating WNT target gene transcription. To summarize, this multiplayer single-handedly fulfils both a structural and transcriptional role in all multicellular animals. It remains a conundrum how cells regulate these functions of CTNNB1 and whether or how these are actively separated, integrated or both.

Separating function and location: technical challenges

As discussed in chapter 1, separating CTNNB1 function and location is one of the most important and challenging aspects of researching the CTNNB1 balance. In the absence of WNT stimulation, CTNNB1 is mainly present at the membrane (see images in chapter 4). It can even be used as an excellent membrane marker in adherent cells (chapters 3-5 and other unpublished data from our lab). According to the classic description of WNT signalling, CTNNB1 levels rise in the cytoplasm upon WNT stimulation, which allows its increase in the nucleus (as confirmed in chapter 4). As we are used to thinking of CTNNB1 in pools, i.e. a cell adhesion and WNT signalling pool, pools based on CTNNB1 localization are easily distinguished into a membrane, cytoplasmic, and nuclear pool. It is tempting to directly link the functional pools to the localization pools, i.e. a membrane cell adhesion and nuclear/cytoplasmic WNT signalling pool. However, the separation of localization based on function remains uncertain due to cellular trafficking and knowledge gaps in, for example, the mechanisms and place of destruction complex deactivation (see figure 2 in chapter 1).

Taking this into account, to capture the balance of CTNNB1 and its dynamics in both of its functions, studies are ideally designed to 1) have high temporal resolution, 2) provide high spatial resolution, and 3) contain a functional readout for its two functions. In chapter 4, 5, and 6 of this thesis, we attempted to tweak the CTNNB1 balance by modifying either WNT signalling (chapter 4), cell adhesion (chapter 5), or the interactions of CTNNB1 with its partners (chapter 6) and study the effects on the CTNNB1 dynamics and distribution in the cell. However, in all of the experiments discussed in this thesis, it remained difficult to definitively link changes in CTNNB1 distribution to its functions in cell adhesion or WNT signalling, whilst maintaining the high spatial-temporal resolution obtained with our imaging methods. Here, I discuss the lessons learned from these setups and suggest possible improvements.

Finding the right tools – adding directionality and binding specifics to the localization measurements

In chapter 4, we examined CTNNB1 distribution, dynamics and kinetics in the nuclear, cytoplasmic, and membrane compartments upon WNT stimulation via live-imaging of an endogenously tagged CTNNB1 HAP1 cell line. In chapter 5, we studied CTNNB1 distribution in the cellular compartments in MCF10A cells plated on a range of stiffness substrates. Although these approaches provided a readout for CTNNB1 distribution (i.e. spatial resolution) and for the live imaging dynamics as well (i.e. temporal resolution), in both studies we were unable to draw definitive conclusions on a functional level. Moreover, although we could visualize

the dynamics, we were unable to determine its directionality. When CTNNB1 redistributes, between which pools does this redistribution occur?

Photoconvertible fluorescent proteins provide an avenue for adding directionality to imaging data. Since the discovery of the first fluorescent protein in the jellyfish species *Aequorea Victoria* (Shimomura et al., 1962), new varieties of fluorescent proteins have been discovered and optimized through genetic engineering (Bindels et al., 2020; Thorn, 2017) (1001 proteins listed on FPbase to date (Lambert, 2019)). Photoconvertible proteins belong to the family of phototransformable proteins, which were first engineered through mutagenesis two decades ago. These proteins are able to activate or switch their excitation and emission spectra upon controlled excitation with a different wavelength of light (as reviewed by Adam (2014)). On the sub-cellular level, these properties could be used to activate certain regions of interest through precise laser activation, switching the protein pool in that specific location to another colour. After switching, this pool can then be tracked over time, visualizing its starting point and destination. Alternatively, a whole cell can be switched, allowing visualization and tracking of newly produced protein that will have the original colour (Nemet et al., 2015).

In (low level) overexpression studies, CTNNB1 was successfully tagged and tracked using phototransformable proteins (Gayrard et al., 2018; Kam & Quaranta, 2009). Upon migration, photoconvertible CTNNB1 was shown to move from the membrane to the nucleus (Gayrard et al., 2018) and similar results were obtained with a photoactivatable form of CTNNB1 upon junctional dissociation (Kam & Quaranta, 2009). Although these studies disrupted junctional stability severely, they provide a proof-of-concept that directional CTNNB1 movement between different cellular compartments can be measured. Whether more subtle changes in distribution in response to smaller changes in adhesion or WNT signalling and at endogenous CTNNB1 levels instead of in an overexpression setting can be visualized as well, remains to be determined. Of course, such an experiment comes with its practical limitations. First, it would require three laser lines, removing freedom in the number of additional options for measurements. Furthermore, by testing green-to-red photoconvertible proteins mEos4B (Paez-Segala et al., 2015), mEos3.2 (Zhang et al., 2012), and Dendra2 (Gurskaya et al., 2006) during this project, we empirically found that the bleaching times and brightness of these proteins were not yet optimal for prolonged live tracking at low endogenous levels. Moreover, this approach is still based on localization and does not provide an answer to the question of functionality at a given location.

It remains difficult to create an experimental setting that has the high spatial resolution of the microscopy techniques used in this thesis and functional readouts for, preferably

both, cell adhesion and WNT signalling. Most functional readouts, such as qRT-PCR, RNA-sequencing, and luciferase assays fall short as they are incompatible with microscopy. Moreover, they are in bulk, meaning that separation of the localization pools (even with cellular fractionation) and directly linking the functional results to changes in CTNNB1 distribution remains difficult. An indirect manner of measuring CTNNB1 function would be to measure CTNNB1 binding to its partners that relate to its function in either cell adhesion or WNT signalling: e.g. the CDH1 bound fraction of CTNNB1 will likely be functioning in junctions or traveling to junctions.

In chapter 2, we discussed several advanced microscopy techniques and their usage in studying a complex signalling pathway such as WNT/CTNNB1 signalling. While we can determine proximity using confocal microscopy (as used in chapter 3 and 4), the resolution is not high enough to determine if proteins are actually bound. Super resolution, possibly combined with techniques such as Bimolecular Fluorescence Complementation (BiFC), could help distinguish the membrane CTNNB1 that is bound to junctional components from membrane CTNNB1 that is possibly bound to WNT components (as reviewed by Sigal et al. (2018), Schermelleh et al. (2019) and Valli et al. (2021)). A BiFC sensor to visualize CTNNB1/TCF interactions already exists (Ding et al., 2014). Obtaining this binding information in relation to the size of the full membrane fraction could provide important data to use for modelling onto our existing data, possibly adding an additional layer to the model presented in de Man et al. (2021).

Another way to achieve an indication of protein binding, whilst retaining full spatio-temporal resolution, would be to create Förster Resonance Energy Transfer (FRET) sensors. FRET sensors use the energy transfer properties of compatible fluorescent proteins ("FRET pairs"), in which a donor fluorescent protein will excite the acceptor fluorescent protein when they are in close vicinity to each other. When proteins of interest are tagged with these donor/acceptor pairs, fluorescent signal of the acceptor fluorescent protein will reflect binding (as reviewed by Pietraszewska-Bogiel & Gadella (2011)). Using FRET sensors requires careful quantification (Kedziora & Jalink, 2015) and their development can be time-consuming. FRET sensor design comes with many considerations, including that these rely on close proximity of fluorescent proteins, so often sensors will need to be placed within protein loops instead of C- or N-terminally to achieve required proximity after binding (Hamers et al., 2014; Lindenburg & Merkx, 2014; Marx, 2017). In our case, an obvious starting point would be to make a FRET sensor for CTNNB1 binding to CDH1 (or CDH2, depending on the cell line used) and one for binding to TCF. Although several tension sensors for cadherins exist (Borghi et al., 2012; Cai

et al., 2014; Conway et al., 2013; Eder et al., 2017; Gayrard & Borghi, 2016), few attempts at creating a FRET sensor for measuring the cadherin-CTNNB1 interaction have been made (Röper et al., 2018). To our knowledge, none such sensors exist for TCF binding to CTNNB1.

For WNT signalling, a wider range of functional readouts exist. As mentioned in chapter 2, 7x TCF-GFP reporters mainly used for luciferase assays can also be used for live imaging. However, in my experience, these may be less suitable for revealing subtle differences over time in a quantitative manner due to the build-up of fluorescent protein during the response. Technological advances for live transcriptional reporters to follow endogenous transcriptional dynamics are rapidly developing and might provide better options to measure TCF/CTNNB1 transcription activity in a time-resolved single-cell manner (as reviewed by Meeussen & Lenstra (2024)).

CTNNB1 Interacting partners – a way to experimentally direct CTNNB1 functionality or not?

Tweaking the functions of CTNNB1 to research its redistribution proved to be challenging as well. Although WNT signalling can be easily stimulated using WNT ligands or small molecule inhibitors/chemical compounds such as CHIR99021 (see chapter 4), subtly affecting cell adhesion can be a challenge. In chapter 5, we attempted to affect cell adhesion via adjusting the matrix stiffness. While we were able to measure a WNT response and a stiffness response, we were unable to specify if these responses were indeed due to changes in cell adhesion. In chapter 6, we therefore made a start with introducing several point mutations for CTNNB1 in chapter 6 to affect CTNNB1 binding to its partners in either function, thereby hopefully affecting the balance of functional CTNNB1 pools.

We proposed point mutations on critical residues Y142 and Y654 of CTNNB1, affecting CTNNA1 and cadherin binding to CTNNB1, respectively. These point mutations, mimicking post-translational modifications, could help elucidate order, structure, and regulatory mechanisms behind the maintenance of the CTNNB1 balance in a subtle manner. However, even with these point mutations their effects may not be straight forward. First, to prevent cadherin binding to CTNNB1, there are indications that different residues can be of importance for the binding of different cadherins. For example, Y489 has been shown to regulate CDH2 binding, releasing CDH2 after phosphorylation by Abelson (Abl) tyrosine kinases (Rhee et al., 2007). Due to the high homology of CDH1 and CDH2 its seems likely that this binding site affects CDH1 binding and vice versa for the Y654 residue, which others have shown to affect CDH2 binding (Chen et al., 2017), although the respective contributions of Y489 and Y654

have not been determined conclusively. However, as some of the kinases required for site-specific modifications might be cell type dependent, it is something to keep in mind when designing mutations for different cell lines with different predominant CDH types.

Secondly, it is uncertain to what extent the point mutations suggested will also affect cell adhesion as a function or CTNNB1 localization (as demonstrated by van Veelen et al. (2011)). For example, for Y654E, which should disrupt cadherin-CTNNB1 binding, homozygous Y654E mice were not viable. However, this seemed to be due to WNT/CTNNB1 signalling related developmental issues, rather than abrupted cell adhesion (van Veelen et al., 2011). Moreover, in cell lines containing the Y654E mutant, CTNNB1^{Y654E} was not always found to decrease cell adhesive capacity (Tominaga et al., 2008). Of course, it is possible that when mutating a single residue on CTNNB1 alone, cells will bypass this by upregulating JUP, which in desmosomes fulfils a similar role in cell adhesion as CTNNB1, to keep cell adhesion intact (Knudsen & Wheelock, 1992).

Based on the localization of CTNNB1, van Veelen et al. (2011) showed *in vivo* that although less CTNNB1^{Y654E} is bound to CDH1, CTNNB1^{Y654E} is still present at the membrane in early homozygous embryos. Similar results were found in cell lines expressing fluorescent CTNNB1^{Y654E} mutants, where dominant membrane localization still occurred (Gayrard et al., 2018). However, as noted in chapter 1, remaining CTNNB1 membrane signal could be related to other interactions of CTNNB1 at the membrane.

Interestingly, both the Y654 (and Y489) and Y142 residues have been indicated in tumorigenesis and epithelial-mesenchymal transition (EMT) (Bonvini et al., 2001; Náger et al., 2015; Shang et al., 2019; van Veelen et al., 2011; Wadhawan et al., 2009; Xi et al., 2013). In many of these cases, reduced cell adhesion is observed in addition to activated WNT signalling, albeit often in a background of other WNT activating mutations. *In vivo* and *in vitro*, contradicting results have been found so far on the effect (if any) of these mutations on the transcriptional activity of CTNNB1 (Bonvini et al., 2001; Howard et al., 2011; van Veelen et al., 2011).

Choosing the correct system and approach for genetic editing

Many suggestions for improvement discussed above require some form of genetic editing. As mentioned throughout this thesis, CTNNB1 distribution is partially regulated by binding affinities for its interaction partners. Experimental settings using overexpression of either CTNNB1 or its binding partners will therefore saturate the system and are not recommended for studying the CTNNB1 balance. For endogenous gene editing, CRISPR/Cas9 quickly emerged

as the go-to technique after its discovery. However, as shown in chapter 6, implementing this technique to create endogenous fluorescent knock-ins and point mutations efficiently and effectively can still be challenging and time consuming. As CRISPR/Cas9 technology develops, improved approaches such as base editing (Rees & Liu, 2018) and prime editing (Anzalone et al., 2019) might lead to higher efficiency (as reviewed by Kantor et al. (2020)).

Choosing the right system for studying certain aspects of the CTNNB1 balance in isolation whilst maintaining physiological relevance and experimental feasibility is of importance here. During my PhD thesis research, we initially focused on breast epithelial cells (chapter 5 and 6). Considering the difficulties encountered with single cell sorting and transfecting MCF10A cells (chapter 6), and having characterised the adherens junctions in the HAP1 cell line (chapter 4) and I propose the HAP1 cell line as a fast direction for genetic editing. Even though this cell line is not epithelial, it is adherent and possesses CDH2 containing adherens junctions. Together with our observations of CTNNB1 increases at the membrane compartment in chapter 4, the HAP1 cell line would thus have been a good place to start to characterize the directionality and liabilities of the distribution of CTNNB1 and the role of its interacting partners through making point mutations. To get down to the molecular details of these interactions, mass spectrometry in combination with cellular fractionation will allow further investigation of what binding partners CTNNB1 encounters at the membrane.

In future studies assessing CTNNB1 in a more physiologically relevant system, embryonic stem cells (ESCs) might be an alternative option as this study would open possibilities for quantitative 3D measurements in a gastruloid model (McNamara et al., 2023; Suppinger et al., 2023), including the subsequent differentiation towards different cell types. This would be even more physiologically relevant in terms of cell adhesion and forces encountered than 2D epithelial sheets. Moreover, it would be interesting to see what happens during cell division in this setting under mutated conditions, as this might be a naturally occurring moment in which the distribution of CTNNB1 is shifted and needs to reform itself as adherens junctions dissolve and are reestablished.

Back to the origin

As a person, I always want to ask "why". As a biologist, however, one is not supposed to ask such questions. Still, what sparked my interest in this topic of the dual functionality of CTNNB1 is this why question: Why would a cell use a single protein for two such vital functions? In biology, the closest thing to asking that question would be to ask: How did a cell evolve to use one protein for two vital functions?

An interesting example to name here is that of *Caenorhabditis elegans*, which contains separate forms of CTNNB1 for each function (Korswagen et al., 2000; Natarajan et al., 2001). In *C. elegans*, three homologues can be distinguished, BAR-1, WRM-1 and HMP-2. HMP-2, the homologue that is most comparable to vertebrate and *Drosophila* CTNNB1 (Costa et al., 1998), solely binds to HMR-1, the cadherin homologue in *C. elegans* (Korswagen et al., 2000). The other two homologues, BAR-1 and WRM-1, function in WNT signalling, with WRM-1 functioning in a divergent WNT pathway specific to *C. elegans* (as reviewed by Robertson & Lin (2012)). A similar phenomenon is observed in the planarian species *Schmidtea mediterranea*, which contains two CTNNB1 homologues (beta-catenin-1 and -2), again with separate functions due to selective binding properties for interacting partners of cell adhesion or WNT signalling (Chai et al., 2010). It is strange that this phenomenon remains limited to these species and is not observed anywhere else across the evolutionary tree – as far as we know. Perhaps in other organisms, conformational changes create similar distinct forms of CTNNB1 (Gottardi & Gumbiner, 2004). The existence of separate forms of CTNNB1, however, does lean towards a system where separation of CTNNB1 functions is preferred over possible interaction.

CTNNB1 is highly conserved across all metazoan species (Zhao et al., 2011). Perhaps the simplest form of life where CTNNB1 can be found is *Dictyostelium discoideum*, a non-metazoan social amoeba species. This normally unicellular organism, forms multicellular structures under stress, in which Aardvark, its CTNNB1 homologue, plays a crucial structural role (Coates et al., 2002; Grimson et al., 2000; Murray & Zaidel-Bar, 2014). In addition, Aardvark functions as a transcriptional regulator dependent on a GSK3 ortholog (Grimson et al., 2000; Harwood, 2008). This goes to show that however simple a form of life may be, CTNNB1 and its duality of function can be found as soon as multicellular life comes into play. One could argue that due to its function in cell adhesion, it *enables* multicellularity. For its function in WNT signalling however, it *needs* multicellularity as paracrine signalling is a crucial part in the signalling cascade. This elucidates that CTNNB1 might have secondarily evolved its function in WNT signalling. Going back to origin of the catenin family though, both CTNNA1 and CTNNB1 appear to have evolved independently of cadherins. Although Aardvark performs a structural role in *D. discoideum*, this is not manifested by cadherins, which are not present in this species (Dickinson et al., 2011). Conversely, the colony forming, unicellular Choanoflagellates contain cadherins (Fairclough, 2015), but these do not bind CTNNB1 (Nichols et al., 2012). Interestingly, a similar story goes for CTNNA1 (Takeichi, 2018), which besides its structural role in cell adhesion has shown to be important in the regulation of cell

polarity together with RhoA (Vassilev et al., 2017). This means that one can exist without the other and makes the existence of a cadherin independent function of catenins more likely (as also addressed in the WNT2022 opening talk by Masatoshi Takeichi). Taken together, the dual function of CTNNB1 is found even in simple life forms, but separate evolutionary tracks for catenins and cadherins suggest that the duality in function arose through some shared benefit and is not based on a critical interaction between cell adhesion and WNT signalling.

This raises the question, if we follow Darwin's theory of survival of the fittest, why CTNNB1 evolved to have two functions. Or if truly, *C. elegans* is the fittest survivor, having separated the functions of CTNNB1. In chapter 4, we propose a model in which the membrane compartment functions as a buffer, capturing CTNNB1 at low concentrations to prevent activation of WNT target gene transcription in the nucleus when WNT signalling levels are still too low. If this membrane pool is indeed the adhesive junctional pool, then this additional role for cell adhesion in preventing WNT signalling activation at levels below a certain threshold could have been an evolutionary benefit for maintaining a single protein for its two functions. In the end, the occurrence of CTNNB1 and its functionality in metazoan (and non-metazoan) species can be used for speculation, but can be interpreted in many ways and is therefore not conclusive.

Regulation, interaction or both?

Ultimately, researching the balance of CTNNB1 between its two functions comes down to the question whether they are regulated to remain separate or to provide interaction, or a combination of both. Based on the vast number of binding partners for CTNNB1, possible conformational changes and evolutionary indications, regulatory mechanisms to separate the CTNNB1 must exist. Since binding sites for critical partners of either CTNNB1 function overlap, phosphorylation regulating any binding site will inadvertently affect overlapping binding sites as well. An example mentioned in this thesis is the conformational change of the C-terminal tail proposed by Gottardi & Gumbiner (2004). Another interesting candidate is BCL9, which is part of the WNT enhanceosome, but which binding domain overlaps with that of CTNNA1. Binding of both proteins is regulated through Y142 phosphorylation, forming a single regulatory node for crucial adherens and signalling partners (Brembeck et al., 2004).

Based on early findings in *Xenopus*, *Drosophila* and cell lines (Cox et al., 1996; Fagotto et al., 1996; Heasman et al., 1994; Orsulic et al., 1999; Sadot et al., 1998; Sanson et al., 1996), a sink model was already proposed by several investigators (Fagotto, 2013; Fagotto et al., 1996; Huang et al., 2015; Jeanes et al., 2008) in which cadherins provide a sink for

CTNNB1, capturing it to temper WNT signalling. Our findings in chapter 4 align with these early findings, mainly based on either overexpression or knockouts of cadherins and later confirmed in EMT studies (Gayrard et al., 2018; Howard et al., 2011). We provide the first visualization of the actual dynamics and kinetics confirming such a model in response to a range of WNT concentrations without altering cell adhesion, suggesting the presence of a threshold or "overflow" for the sink, if you will. Although this "sink model", or "membrane buffer" as I prefer to call it, relays some form of interaction between CTNNB1 function in cell adhesion at the membrane and WNT signalling, it does not directly couple morphology and transcription. I would rather call this functional facilitation.

There are examples to be named of interaction between cell adhesion and WNT signalling through CTNNB1. Recently, it was shown that during zebrafish development, cells with incorrect levels of WNT/CTNNB1 signalling along a morphogen gradient can be removed by a cadherin-dependent mechanism (Akieda et al., 2019). However, this does not point towards a general purpose of functional coupling of cell adhesion and WNT signalling, relevant in all cell types and situations. The regulation of CTNNB1 distribution and whether functional interaction occurs may be a very context dependent situation.

Concluding remarks

In this thesis, we showed that redistribution of CTNNB1 occurs in all cellular compartments upon either activation of WNT signalling or mechanical stimulation (potentially via junctional remodelling). This shows a fluent interplay between CTNNB1 pools based on location. The relevance of this redistribution in terms of a functional response remains debatable. In chapter 4, we observed CTNNB1 redistribution upon WNT stimulation, preferentially increasing in the nucleus at high levels and in the membrane at low levels. This led us to hypothesize that the membrane functions as a buffer for CTNNB1 at sub-threshold levels of WNT stimulation. However, we were unable to confirm whether this buffering effect of the membrane was linked to cell adhesion and perhaps even cell adhesion strength. Conversely, upon mechanical stimulation, redistribution of CTNNB1 takes place with proportionally more CTNNB1 in the nucleus at low stiffness. Subsequent RNA-sequencing, however, revealed no uniform effects of stiffness on expression of classical WNT target genes.

CTNNB1 seems highly dynamic across all of its cellular compartments in response to changes in either adhesion or WNT signalling. The increases in CTNNB1 levels we measured are often subtle and require a low signal to noise ratio to be measured. This confirms the need for the use of imaging at endogenous levels using sensitive and well controlled

setups (as described in chapter 3). Traditional biochemical methods, that can be subject to technical variation and function at a bulk level, might not be suitable for measuring these subtle differences, as seen in chapter 4. Although we identified notable changes in CTNNB1 distribution using these sensitive imaging methods, we were not able to link these to a functional response. As described in this general discussion, novel methods assessing binding or transcriptional activity while maintaining the high spatial-temporal sensitive setup used must be developed for a better time-resolved separation of location and function in future studies.

However complex, unravelling the duality in function of CTNNB1 remains relevant as both of its functions and their balance are important in development, adult homeostasis and disease. Although in healthy situations, the dual function of CTNNB1 might be beneficial, in disease CTNNB1 dysregulation can affect not only one but two crucial processes, making it a sensitive node in signalling and adhesion.

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