Wnt-induced transcriptional activation is exclusively mediated by TCF/LEF

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Abstract

Active canonical Wnt signaling results in recruitment of β -catenin to DNA by TCF/LEF family members, leading to transcriptional activation of TCF target genes. However, additional transcription factors have been suggested to recruit β -catenin and tether it to DNA. Here, we describe the genome-wide pattern of β -catenin DNA binding in murine intestinal epithelium, Wnt-responsive colorectal cancer (CRC) cells and HEK293 embryonic kidney cells. We identify two classes of β -catenin binding sites. The first class represents the majority of the DNA-bound β -catenin and co-localizes with TCF4, the prominent TCF/LEF family member in these cells. The second class consists of β -catenin binding sites that co-localize with a minimal amount of TCF4. The latter consists of lower affinity β -catenin binding events, does not drive transcription and often does not contain a consensus TCF binding motif. Surprisingly, a dominant-negative form of TCF4 abrogates the β -catenin/DNA interaction of both classes of binding sites, implying that the second class comprises low affinity TCF-DNA complexes. Our results indicate that β -catenin is tethered to chromatin overwhelmingly through the TCF/LEF transcription factors in these three systems.

Keywords β -catenin; ChIP-Seq; TCF; transcriptional regulation; Wnt pathway

Subject Categories Transcription; Signal Transduction

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Introduction

To initiate canonical Wnt signals, the extracellular Wnt ligand binds to the Frizzled (Fz) receptor and LRP5/6 co-receptor on the cell surface. This prevents the cytoplasmic axin complex from ubiquitinating and degrading β -catenin (Li *et al*, 2012). As a consequence, β -catenin accumulates and translocates to the nucleus, where it drives Wnt-specific transcriptional programs. In several forms of

cancer, components of the Wnt pathway such as Axin (Liu *et al*, 2000), APC (Rubinfeld *et al*, 1993; Su *et al*, 1993; Korinek *et al*, 1997), or RNF43 (Koo *et al*, 2012) are mutated, leading to inappropriate activation of the pathway (Morin *et al*, 1996; Korinek *et al*, 1997). In the majority of sporadic colon cancers, this constitutive activation is the first step of their development (Kinzler & Vogelstein, 1996; CGAN, 2012). Oncogenic point mutations in β -catenin prohibiting its degradation have similar outcomes (Morin *et al*, 1997; Polakis, 1997).

When β -catenin is translocated to the nucleus, it is recruited to Wnt-responsive elements bound by TCF transcription factors. β -catenin and Groucho/TLE compete for TCF binding to activate or repress target genes (Behrens *et al*, 1996; Huber *et al*, 1996; Moleenaar *et al*, 1996; Roose *et al*, 1998; Daniels & Weis, 2005). At the chromatin level, many interacting partners for the β -catenin/TCF complex have been identified suggesting complex mechanisms of transcriptional regulation of Wnt target genes. Generally, nuclear β -catenin serves as a link in a chain that spans from the target enhancer via TCFs to RNA polymerase II (Stadeli *et al*, 2006; Mosimann *et al*, 2009; Valenta *et al*, 2012).

The majority of the transcriptional co-activators bind to the last ARM repeat of β -catenin at the C-terminus of the protein. Preventing the interaction between β -catenin and its C-terminal co-activators inhibits Wnt/ β -catenin transcriptional activation (van de Wetering *et al*, 1997; Cox *et al*, 1999; Valenta *et al*, 2011). Some β -catenin transcriptional co-activators bind N-terminally to the first ARM repeats, such as BCL9 (Kramps *et al*, 2002; Mosimann *et al*, 2009). Many of the transcriptional co-activators of β -catenin affect chromatin structure by modifying histones, such as the histone acetyltransferases CBP (Wolf *et al*, 2002), p300 (Levy *et al*, 2004), and Tip60 (Kim *et al*, 2005a), or by rearranging nucleosomes, such as SWI/SNF and ISWI (Song *et al*, 2009). Other binding partners promote the association of TCF/ β -catenin with the RNA polymerase II complex such as members of the Mediator complex (Kim *et al*, 2006; Carrera *et al*, 2008) and components of the Paf1 complex (Mosimann *et al*, 2006; Parker *et al*, 2008).

Ongoing research on the Wnt-pathway has identified a large number of β -catenin-interacting proteins. A BIND search (Bader *et al*, 2003) for β -catenin-interacting proteins resulted over 270 hits, including proteins that interact with β -catenin, APC and TCF family

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Table 1. Alternative β -catenin recruiting factors

Recruiting factor	References
cJun	Nateri <i>et al</i> (2005)
ER α	Kouzmenko <i>et al</i> (2004)
Foxo1/4/3a	Essers <i>et al</i> (2005)
Hif1 α	Kaidi <i>et al</i> (2007)
LRH-1 (NR5A2)	Botrugno <i>et al</i> (2004)
Oct4	Kelly <i>et al</i> (2011), Zhang <i>et al</i> (2013)
Sox17	Sinner <i>et al</i> (2004)

members directly or through multi-subunit complexes and are supported by data from *C. elegans*, *X. laevis*, *M. musculus*, *R. norvegicus* and *H. sapiens*. Besides TCF, multiple alternative transcription factors have been suggested to recruit β -catenin to enhancer sites (Table 1).

However, none of these studies have provided systematic approaches to substantiate the significance of TCF-independent β -catenin recruitment. In this study, we have performed a systematic genome-wide analysis of potential TCF-independent β -catenin recruitment sites. Our results imply that all β -catenin-DNA binding involves TCF.

Results

Two classes of β -catenin recruitment

Tcf4 and β -catenin have been repeatedly shown to interact and, together, activate transcription. However, little is known about the extent to which these two factors interact in the nucleus of a Wnt-activated cell. To globally characterize the extent of β -catenin-Tcf4 interactions, we performed chromatin immunoprecipitation coupled to massive parallel sequencing (ChIP-Seq) for both Tcf4 and β -catenin in the crypts of the murine small intestine. This tissue is known to be strictly dependent on Wnt signals for its homeostatic proliferation, as removal of Wnt components (Korinek *et al*, 1998; van Es *et al*, 2012) or introduction of Wnt inhibitors (Pinto *et al*, 2003; Kuhnert *et al*, 2004) abrogates this proliferation. Introduction of the Wnt potentiator R-spondin1 induces crypt hyperplasia (Kim *et al*, 2005b). Briefly, intestinal crypts were isolated from murine small intestines by EDTA-elution after removal of the intestinal villi. These crypts were then exposed to a crosslinking agent and subjected to normal ChIP-Seq analysis. We identified 2,302 β -catenin peaks and 10,086 Tcf4 peaks. Only 1,183 peaks coincided (Fig 1A and B).

The large number of β -catenin sites where no Tcf4 was detected led us to inspect the β -catenin sites more closely. When the β -catenin peaks were aligned and the raw Tcf4 data from the same

Figure 1. There are two classes of DNA-bound β -catenin in mouse small intestinal epithelium.

- A Venn diagram showing the number and extent of overlap of β -catenin and Tcf4 peaks that were obtained from mouse intestinal crypt epithelium. Cisgenome software package (Ji *et al*, 2008) was used for the identification of binding peaks from the ChIP-seq data.
- B UCSC Genome browser excerpts showing peaks of both classes of β -catenin peaks. Top panel shows the promoter of the Sp5 gene, a peak of the β^{T-HI} class. Bottom panel shows part of the gene body of the Cxcr7 gene, a peak of the β^{T-LO} class. Numbers indicate the minimum and maximum reads mapped to a single base-pair. Scale bars indicate 1 kb.
- C Heat map of the β -catenin and Tcf4 ChIP-seq data. Left column shows a heat map profile of all 2,302 β -catenin peaks. The peaks were centered and 2.5 kb upstream and downstream of every peak is also shown. The right column shows the same DNA coordinates as the β -catenin column, but displays a heat map profile of the raw Tcf4 ChIP-Seq data.

coordinates were displayed, more β -catenin sites appeared to be co-occupied by Tcf4. We observed a bimodal distribution suggesting two categories of β -catenin peaks (Fig 1C). The goodness-of-fit measured by Bayesian Information Criterion increased when two-component models were fit, supporting the visual observation. We therefore divided β -catenin peaks into two classes based on the Tcf4 signal intensity distribution: β -catenin-Tcf4-high ($\beta^{\text{T-HI}}$) and β -catenin-Tcf4-low ($\beta^{\text{T-LO}}$). The majority (77%) of the β -catenin peaks was co-occupied by Tcf4 and most likely represented the nuclear components of canonical Wnt signaling, but the $\beta^{\text{T-LO}}$ class appeared to lack a TCF/LEF counterpart and might have a separate function.

The crypts of the intestinal epithelium consist of several specific cell types (van der Flier & Clevers, 2009) and thus represent a mixture of cell type-specific binding patterns. To evaluate Tcf4/ β -catenin/chromatin interaction in a more homogeneous cell population, we performed ChIP-Seq in LS174t colon cancer cells, and HEK293 embryonic kidney cells. The LS174t cell line harbors a stabilizing mutation in the *CTNNB1* (β -catenin) gene, allowing for the accumulation of high levels of β -catenin in the nucleus of these cells. In contrast, HEK293 cells carry a wild-type (WT) version of the Wnt pathway: β -catenin only translocates into the nucleus in the presence of exogenous Wnt ligands. For the ChIP experiment, we stimulated the HEK293 cells with Wnt-conditioned medium (Wnt-CM) for 3 h.

The number of β -catenin peaks observed in LS174t cells was similar to that in murine crypts (2,241) and the HEK cells exhibited slightly more β -catenin binding sites (4,338) (Fig 2A and F). The two classes of β -catenin binding sites were clearly observed in both cell lines (Fig 2E and J), the $\beta^{\text{T-LO}}$ class representing 40 and 34% of the β -catenin peaks in LS174t and HEK293 cells, respectively (Fig 2A and F). However, when the β -catenin binding patterns from the two cell lines were compared, only a minimal overlap was observed: 189 peaks of the $\beta^{\text{T-HI}}$ class and 24 of the $\beta^{\text{T-LO}}$ class were detected in both datasets (supplementary Fig S1). This demonstrated that β -catenin binding is highly dependent on tissue type. Yet, the two classes of binding events were observed irrespective of this difference. Interestingly, quantification of the average read intensity of the peaks in both classes showed that $\beta^{\text{T-LO}}$ peaks were of much lower intensity than the $\beta^{\text{T-HI}}$ peaks, possibly indicating a lower affinity protein-DNA complex (Fig 2D and I).

A consensus TCF/LEF motif has been previously identified (van de Wetering *et al*, 1997). To investigate the paucity of TCF4 in

the $\beta^{\text{T-LO}}$ class, we performed a motif analysis probing for the consensus TCF/LEF motif in both classes (Fig 3A and B). Between one and thirteen motifs per peak were observed in the $\beta^{\text{T-HI}}$ peaks, with 57% of the peaks containing at least one consensus motif. In contrast, only 18% of the $\beta^{\text{T-LO}}$ peaks contained a TCF motif, with a maximum of 3 motifs per peak. Similar results were obtained when the HEK293 dataset was probed.

As reported previously (Bottomly *et al*, 2010), we found a significant number of β -catenin-occupied elements that do not contain a consensus TCF motif. However, TCF family members have been shown to bind to degenerate, 'secondary' TCF motifs *in vitro* (Yochum *et al*, 2007; Badis *et al*, 2009). To investigate if the peaks of the $\beta^{\text{T-LO}}$ class are enriched in degenerate TCF motifs, we probed for the consensus motif with decreasing stringency (Fig 3C and D). As expected, lower stringency increased motif enrichment. However, the $\beta^{\text{T-HI}}$ class was more enriched in TCF motifs irrespective of the stringency used in the analysis. Thus, TCF recruitment through degenerative motifs is not more prevalent in the $\beta^{\text{T-LO}}$ class than in the $\beta^{\text{T-HI}}$ class. Combined, these data show that a large proportion of the $\beta^{\text{T-LO}}$ peaks did not contain a consensus or degenerate TCF motif and explained why these peaks appear to not be co-occupied by a TCF family member.

Because of the lower frequency of consensus and degenerate TCF motifs in the peaks of the $\beta^{\text{T-LO}}$ class, we investigated the prevalence of other known DNA motifs. We probed for the enrichment of the 146 motifs described in the TRANSFAC database (<http://www.gene-regulation.com/pub/databases.html>) in the two classes and sorted for the motifs that are more enriched in the $\beta^{\text{T-LO}}$ class versus the $\beta^{\text{T-HI}}$ class ($\beta^{\text{T-LO}}/\beta^{\text{T-HI}}$ ratio) and contain at least a motif in 10% of the peaks of the $\beta^{\text{T-LO}}$ class. We found 98 and 89 motifs in the LS174t and HEK293 datasets respectively (supplementary Fig S2A and B). The ten motifs with the highest $\beta^{\text{T-LO}}/\beta^{\text{T-HI}}$ ratio of both data sets contain the motifs of the ETS family members ELK4, ELK1, SPI1, GABP α as well as that of the insulator CTCF (supplementary Fig S2C and D, full list in supplementary Table S1). With the exception of the minimal enrichment of the HIF1A/ARNT motif only in the LS174t dataset, none of the top ten most enriched factors have been previously reported to recruit or interact with β -catenin. The relatively low $\beta^{\text{T-LO}}/\beta^{\text{T-HI}}$ ratio indicated that they are not specifically recruited to the $\beta^{\text{T-LO}}$ elements, but are rather generally associated with these elements.

Figure 2. The same two classes of DNA-bound β -catenin are also observed in LS174t and HEK293 cells.

- A Heat map of the β -catenin and Tcf4 ChIP-seq data of LS174t cells. Left column shows a heat map profile of all 2241 β -catenin peaks. The peaks were centered and 2.5 kb upstream and downstream of every peak is also shown. The right column shows the same DNA coordinates as the β -catenin column, but displays a heat map profile of the raw TCF4 ChIP-Seq data.
- B, C UCSC Genome browser excerpts showing peaks of both classes of β -catenin peaks. In (B) is shown the promoter of the *LGR5* gene, a peak of the $\beta^{\text{T-HI}}$ class. In (C) is shown the gene body of the *NOP10* gene, a peak of the $\beta^{\text{T-LO}}$ class. Numbers indicate the minimum and maximum reads mapped to a single base pair.
- D, E Quantification plots of the different classes of β -catenin and TCF4 peaks showing the relative read intensity per base pair of the different classes in arbitrary units. Red lines represent the reads from peaks of the $\beta^{\text{T-HI}}$ class, black lines those of the $\beta^{\text{T-LO}}$ class.
- F Heat map of the β -catenin and Tcf4 ChIP-seq data of HEK293 cells. Left column shows a heat map profile of all 4,338 β -catenin peaks. The peaks were centered and 2.5 kb upstream and downstream of every peak is also shown. The right column shows the same DNA coordinates as the β -catenin column, but displays a heat map profile of the raw Tcf4 ChIP-Seq data.
- G, H UCSC Genome browser excerpts showing peaks of both classes of β -catenin peaks. In (G) is shown the promoter of the *LGR5* gene, a peak of the $\beta^{\text{T-HI}}$ class. In (H) is shown the promoters *C10RF174* and the *LOC100133612* transcripts, a peak of the $\beta^{\text{T-LO}}$ class. Numbers indicate the window size of minimum and maximum reads mapped to a single base-pair.
- I, J Quantification plots of the different classes of β -catenin and TCF4 peaks showing the relative read intensity per base-pair of the different classes in arbitrary units. Red lines represent the reads from peaks of the $\beta^{\text{T-HI}}$ class, black lines those of the $\beta^{\text{T-LO}}$ class.

β^{T-LO} peaks do not correlate with Wnt-dependent transcription

The β -catenin/TCF4 complex activates transcription because β -catenin acts as a scaffold protein to recruit histone modifiers (reviewed in Mosimann *et al*, 2009). We probed if the β^{T-LO} -binding sites represent transcriptional regulatory sites, even though they appear to lack TCF4 and display β -catenin binding. In brief, stable LS174t clones containing a dominant-negative form of TCF4 (Δ NTCF4) expressed under an inducible promoter (van de Wetering *et al*, 2002) were expression-profiled by microarray in the presence and absence of Δ NTCF4. This truncated form of TCF4 lacks the β -catenin interaction domain, but retains normal DNA affinity, thus inhibiting the Wnt pathway by competition with TCF4 for its cognate DNA site. The experiment gave us a profile of TCF4-dependent gene expression. We then compiled sets of genes with either a β^{T-HI} or β^{T-LO} peak within 5 kb of the transcriptional start site. These gene sets were used in a Gene Set Enrichment Assay (GSEA) (Mootha *et al*, 2003; Subramanian *et al*, 2005) correlating them with transcriptional changes upon repression of the Wnt pathway in LS174t cells (Fig 3D and E). The β^{T-HI} gene set correlated well with the genes that were down-regulated in the presence of Δ NTCF4. However, genes associated with the β^{T-LO} class did not significantly correlate with either up- or downregulated genes (normalized enrichment scores -1.76 and 1.10 respectively). Thus, the β^{T-LO} targets appear not to be part of the Wnt/TCF signature as it was defined in LS174t cells. Similarly, we stimulated HEK cells with Wnt-CM and obtained a microarray expression profile of Wnt target genes in these cells. When probed with β^{T-HI} and β^{T-LO} genes in a GSEA, the β^{T-HI} genes correlated with the up-regulated genes whereas the β^{T-LO} genes did not show a correlation (supplementary Fig S3). We concluded that also in the HEK293 system, β^{T-LO} peaks did not demarcate Wnt target genes.

To evaluate the transcriptional activity of β^{T-LO} elements more closely, we selected eight β^{T-LO} elements from both LS174t and HEK293 datasets and tested these in a luciferase reporter assay. The selection contained promoters, introns and intragenic elements with and without consensus TCF motifs (supplementary Table S2). As a control, we also tested eight elements selected from the β^{T-HI} class that were not characterized previously. Seven out of the eight β^{T-LO} elements were not able to induce significant expression of the luciferase reporter gene, regardless of the presence of a TCF/LEF motif (Fig 4A). One element showed a minor transcriptional activation (5.7 fold) but this activation was not significantly diminished in the presence of Δ NTCF4 when compared to the TOP10 positive control reporter. In comparison, 4 out of the 8 elements from the β^{T-HI} class were able to induce luciferase expression; this activity was significantly reduced in the presence of Δ NTCF4 (Fig 4A).

We also tested eight HEK β^{T-LO} elements and eight control HEK β^{T-HI} elements in a luciferase assay in HEK293 cells in the presence or absence of Wnt-CM. Additionally, we generated a HEK293 line expressing Δ NTCF4 under the control of a tet-ON promoter (H29, supplementary Fig S4) and tested the elements in the presence and absence of Δ NTCF4. None of the β^{T-LO} elements demonstrated significant Wnt-dependent activation (Fig 4B), nor was the observed activation significantly affected by Δ NTCF4 co-expression, while 4 out of 8 β^{T-HI} elements showed Wnt-induced activation that could

be abrogated by Δ NTCF4. This indicated that the Wnt pathway does not act through these elements.

β^{T-LO} peaks are mediated by TCFs

β^{T-LO} peaks are of lower intensity, do not correlate with Wnt-driven expression and lack TCF4 co-occupancy. To study the dependency of β -catenin-DNA interactions on the presence of TCF4, we performed ChIP-seq of β -catenin, TCF4 and a flag-tagged Δ NTCF4 before and after induction of the latter. The TCF4 antibody used in these studies is directed against the N-terminal part of the protein. This epitope is absent in Δ NTCF4. In brief, cell lines were grown in the presence or absence of doxycycline for 16 h. HEK293 cells were then stimulated with Wnt-CM for an additional 2 h before fixation and harvesting. In the absence of doxycycline, no flag-tagged Δ NTCF4 was observed, while Δ NTCF4-signal was readily detected in both the HEK and LS174t cells after induction (Fig 5C and supplementary Fig S5). TCF4 intensity was slightly lower in the presence of Δ NTCF4 in LS174t cells (Fig 5E), but was unchanged in the HEK293 cells (supplementary Fig S4E). As expected, β -catenin occupancy of the β^{T-HI} class of elements was significantly diminished when Δ NTCF4 was expressed in LS174t as well as HEK293 cells (Fig 5D, supplementary Fig S5D). Surprisingly, β -catenin was also lost from the β^{T-LO} peaks. This revealed that Δ NTCF4 was able to disrupt all β -catenin-DNA interactions, not just those interactions where TCF4 was previously detected. Additionally, Δ NTCF4 was observed on DNA elements where WT TCF4 did not bind (Fig 5C and F). Thus, Δ NTCF4 was able to compete with the factor that is responsible for β -catenin recruitment in both the β^{T-LO} and β^{T-HI} class and disrupt the tethering of β -catenin to DNA.

We observed that some β -catenin peaks are diminished to a lesser extent than others. To investigate if the magnitude of decrease is reflected in the transcriptional change of correlated genes, we performed GSEA analysis with subsets of the β^{T-HI} and β^{T-LO} element-associated genes and found that there is no correlation (supplementary Fig S6 and Table S3).

To evaluate the effect of over-expression of Δ NTCF4 on other transcription factors that bind to the same DNA elements, we performed a ChIP-qPCR experiment with HNF4 α as bait. The motif for this transcription factor has previously been observed to be enriched in TCF4-bound elements (Hatzis *et al*, 2008). We have observed the consensus motif for HNF transcription factors in several of our β -catenin bound elements (supplementary Table S1) and this transcription factor is expressed in our LS174t cell line. We selected sites that are bound by β -catenin in the absence of Δ NTCF4 but disappear after Δ NTCF4 over-expression. We detected comparable HNF4 occupancy levels in the absence and presence of Δ NTCF4 (supplementary Fig S7A). We conclude that over-expression of Δ NTCF4 does not disrupt the binding of HNF4 α to these elements.

Other TCF family members (TCF1 and LEF1) with similar DNA affinities are expressed in the LS174t cell line (Brantjes *et al*, 2001). To investigate if the β^{T-LO} class of elements are occupied by these TCF family members, we performed ChIP-qPCR with TCF1 and LEF1 as bait. We studied several elements of the β^{T-LO} class that are diminished when Δ NTCF4 is over-expressed and found that a number of these elements, but not all, are occupied by TCF1 or LEF1 (supplementary Fig S7B,C). This suggests that the TCF family

Figure 3. β^{T-LO} peaks have less TCF/LEF motifs and do not correlate with Wnt-sensitive transcription.

A Bar graphs showing the percentage of peaks in each class that have at least one consensus TCF/LEF motif.
B Matrix-based logo of the TCF/LEF motif that was used in the motif analysis.
C, D Motif analysis probing for the consensus TCF motif with different stringencies to allow degenerate motifs to be included in the analysis. Motif matrices were mapped against the genome with decreasing stringency (parameter -r 20, 100 or 500, using motifmap_matrixscan_genome command from Cisgenome v2 package). Motif frequency per kilobase of β -catenin occupied elements is plotted for both the β^{T-HI} and β^{T-LO} class of the LS174t (C) and the HEK293 (D) datasets.
E, F GSEA probing gene lists with gene sets that have at least one peak of either the β^{T-HI} or β^{T-LO} class within 5 kb of the TSS. Used gene lists were sorted for gene change upon over-expression of Δ NTCF4 dominant-negative TCF4 transcription factor. NES, normalized enrichment score, based on the enrichment score at maximum. P-values are calculated based on 1,000 iterations.

members display partially overlapping binding patterns, probably as a result of a degree of individual specificity. As not all the tested β^{T-LO} elements were occupied by TCF1 or LEF1, β -catenin recruitment through these family members cannot account for the full extent of the β^{T-LO} elements.

Taken together, these results show that β -catenin occupancy is lost over the elements of both the β^{T-HI} and β^{T-LO} class when Δ NTCF4

is over-expressed, that this over-expression does not interfere with other transcription factors that bind to these elements and that β -catenin recruitment to the elements of the β^{T-LO} class can in part be explained by the presence of other TCF family members. Finally, the recruitment of Δ NTCF4 to elements of the β^{T-LO} class shows that also these β^{T-LO} class sites are genuine, yet low-affinity TCF binding sites.

Figure 4. β^{T-LO} elements are not able to induce transcription in a luciferase assay.

A Luciferase assay performed in LS174t cells in the presence or absence of Δ NTCF4. Eight elements from each class were tested.

B Luciferase assay performed in HEK193T cells in the presence or absence of Wnt-CM and Δ NTCF4. Eight elements from each class were tested.

Data information: Means of 3 individual experiments are shown. Values show fold enrichment over control vector. Unaltered pGL4.10 vector was used as negative control with a CMV-driven Renilla as transfection control. The positive control consists of 10 consensus TCF/LEF motifs followed by a minimal promoter. Individual elements were cloned in front of the Luciferase gene combination with or without a TATA-box depending on the location of the original element. The amount of consensus TCF/LEF motifs present in every reporter is depicted on the X-axis. Asterisks denote the elements that show Wnt and Δ NTCF4 sensitive activation.

Discussion

Here, we explore the DNA occupancy signature of β -catenin, a transcriptional coactivator that relies on DNA-binding proteins to be recruited and tethered to the DNA and we investigate its relation to TCF4. Surprisingly, not all observed β -catenin-bound elements appeared to be co-occupied by TCF4. However, when a dominant-negative form of TCF4 was introduced, it bound to these β^{T-LO} sites and displaced β -catenin. This suggests that the minimal binding of TCF4 to these sites in normal conditions is below the ChIP-Seq detection limit, yet enough to tether some β -catenin to chromatin. When artificially high levels of Δ NTCF4 are induced, the accumulated protein passes the detection threshold. However, the β^{T-LO} sites still exhibit lower Δ NTCF4 signals than the high-affinity β^{T-HI}

sites, perhaps reflecting the number of consensus TCF/LEF motifs present in these elements.

The binding pattern of Δ NTCF4 was sufficient to explain all β -catenin binding events and did not support the existence of an alternative β -catenin recruiting factor. Additionally, the β -catenin displacement by Δ NTCF4 was based on its ability to compete with WT TCF/LEF family members for occupancy of the TCF/LEF motif. On the elements where no or little TCF4 was detected but from which β -catenin was displaced by Δ NTCF4, the factor responsible for β -catenin recruitment must have also bound the TCF/LEF motif. Hence, it is most likely a TCF/LEF family member. Indeed, when we probed for DNA occupancy of TCF1 and LEF1, a subset of these elements appeared to bind these elements, further supporting the need for a TCF family member in recruiting β -catenin to the DNA.

Figure 5. β^{T-HI} and β^{T-LO} peaks are displaced by Δ NTCF4 in LS174t cells.

A, B Heat maps showing ChIP-Seq signals of β -catenin, TCF4 and flag-tagged Δ NTCF4 of the coordinates defined by β -catenin peaks in the presence or absence of doxycycline induced Δ NTCF4.
D–F Quantification plots of the different classes of β -catenin and TCF4 peaks showing the relative read intensity per base-pair of the different classes in arbitrary units. Red lines represent the reads from peaks of the β^{T-HI} class, black lines those of the β^{T-LO} class. Each plot shows the quantification of the corresponding heat map column.

We observe that β^{T-LO} class consists of low-coverage β -catenin binding events and displays only a minor enrichment of TCF/LEF motifs. On the single element level, this translates into the presence or absence of a single consensus TCF/LEF motif. This suggests that the binding of a limited amount of TCF/LEF proteins to a single motif allows for the recruitment of only a limited amount of β -catenin. The presence of β -catenin on elements without a consensus TCF/LEF motif suggests that TCF/LEF binds to degenerate motifs in these cases. Indeed, examples of alternative TCF/LEF splicing variants with different affinities have been described (van de Wetering *et al*, 1997; Atcha *et al*, 2003, 2007; Hecht & Stemmler, 2003) in addition to the ability of some TCF/LEF family members to bind degenerate motifs with lower affinity in protein binding arrays (Badis *et al*, 2009). When we surveyed the two classes of β -catenin binding DNA for these degenerate motifs, we found that these occur in both β^{T-HI} and β^{T-LO} elements. Thus, this type of interaction could account for the β -catenin binding events where no consensus motif is detected. However, we cannot exclude the possibility of TCF/LEF family members being recruited to the DNA indirectly through other proteins, foregoing the need for a TCF motif.

The β^{T-LO} class is not correlated to the changes in gene expression upon manipulation of the Wnt pathway. Additionally, they are unable to activate luciferase reporter expression. They are, however, co-occupied by over-expressed Δ NTCF4 and are displaced by this truncated form of TCF4. This suggests that the amount of TCF4 that is bound to a particular enhancer is dependent on the available motifs in that element. The amount of TCF4 in turn decides the

amount of β -catenin that is recruited. Together, these factors decide the strength of the transcriptional activation that is achieved by the enhancer in question. The transcriptional activity of a particular enhancer is unlikely to be defined solely by the Wnt pathway. Perhaps other transcription factors need minimal TCF-mediated β -catenin recruitment to drive transcription using the same DNA element even though the low levels of TCF/ β -catenin in itself are not sufficient to do so. Here, we show the existence of two classes of TCF/ β -catenin complexes *in vivo* and *in vitro* and their difference in Wnt-mediated transcriptional output.

Combined, these data imply that DNA-bound β -catenin is overwhelmingly recruited to chromatin by a TCF/LEF family member in our model systems. Although many co-factors have been described to recruit β -catenin to the DNA, Δ NTCF4 was sufficient to diminish β -catenin recruitment to all types of elements, co-occupied by either high or low levels of TCF4.

Materials and Methods

Cells

We used LS174T human colon cancer cells carrying an activating point mutation in β -catenin and LS174t-pcDNA4TO- Δ NTCF4 cell line carrying a doxycycline-inducible Δ NTCF4 cDNA (van de Wetering *et al*). Cells were grown in the presence or absence of doxycycline (1 mg/ml) for 24 h. A HEK293T clone was

created using the same pcDNA4TO- Δ NTCF4 vector. Cells were transfected using pPEI and single cells were allowed to grow clones under simultaneous Neomycin and Kanamycin selection to retain the TET-repressor system and the Tet-ON Δ NTCF4 vector. Individual clones were screened for their ability to express high levels of Δ NTCF4 only in response to doxycycline by western blot using the anti-FLAG (M2) antibody (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained directly to judge the homogeneity of Δ NTCF4 expression using the same M2-anti-flag antibody. For analysis HEK293-pTER- Δ NTCF4 cells were grown in the presence or absence of doxycycline for 24 h and subsequently exposed to 50% Wnt3a conditioned medium for 2 h. Wnt3a-CM was produced using stably transfected L cells after 1 week of conditioning in medium as previously described (Sato *et al*, 2011) containing 10% fetal bovine serum.

Microarray analyses

We used previously described microarray analysis of LS174t-pcDNA4TO- Δ NTCF4 in the absence and presence of doxycycline performed using the Agilent 4 $\times$ 44K whole human genome array system according to the manufacturer's protocol. Similarly, HEK293-pTER- Δ NTCF4 cells were grown in the presence or absence of doxycycline for 24 h prior to 2 h of Wnt-CM stimulation. RNA was harvested and purified using the Qiagen (Hilden, Germany) RNeasy Spin Column kit and then prepared for microarray hybridization according to the Agilent protocol.

ChIP-seq, ChIP-qPCR

Freshly isolated small intestines were incised along their length, and villi were removed by scraping. The tissue was then incubated in phosphate-buffered saline (PBS)/EDTA (5 mM) for 5 min. Gentle shaking removed remaining villi, and intestinal tissue was subsequently incubated in PBS/EDTA for 60 min at 4°C. Vigorous shaking yielded free crypts that were used for ChIP-seq analysis. Alternatively, approximately 30 $\times$ 10⁶ LS174T-pcDNA4TO- Δ NTCF4 cells grown 24 h in the presence or absence of doxycycline (1 mg/ml) or HEK293-pcDNA4TO- Δ NTCF4 grown for 24 h in the presence or absence of doxycycline followed by a 2 h Wnt3a incubation were used for ChIP-seq procedure.

Chromatin immunoprecipitation was performed as previously described (Hatzis *et al*, 2008; Mokry *et al*, 2010). In brief, cells were cross-linked with 1% formaldehyde for 20 min at room temperature. For β -catenin ChIP-seq, cells were crosslinked for 40 min using ethylene glycol-bis(succinimidyl succinate) (Thermo Scientific, Waltham, MA, USA) at 12.5 mM final concentration, with the addition of formaldehyde (1% final concentration) after 20 min of incubation. The reaction was quenched with glycine and the cells were successively washed with phosphate-buffered saline, buffer B [0.25% Triton-X 100, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)] and buffer C [0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)]. The cells were then resuspended in shearing buffer [0.3% SDS, 1% Triton-X 100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)] and sheared using Covaris S2 (Covaris, Woburn, MA, USA) for 8 min with the following settings: duty cycle: max, intensity: max, cycles/burst: max, mode: Power Tracking. The sonicated chromatin was diluted to 0.15 SDS, incubated for 12 h at 4°C with 25 μ l of the anti-TCF4

(N20) antibody (Santa Cruz), 5 μ l of the anti-FLAG (M2) antibody (Sigma-Aldrich), 50 μ l of the anti- β -catenin (H102) antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), 40 μ l of the HNF4 α antibody (Santa Cruz SC8987), 40 μ l of the TCF1 antibody (Santa Cruz SC 8589) or 15 μ l of the LEF1 antibody (Millipore CS200635, Billerica, MA, USA) per IP with 100 ml of protein G beads (Upstate). The beads were successively washed two times with buffer 1 [0.1% SDS, 0.1% deoxycholate, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)], one time with buffer 2 [0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)], one time with buffer 3 (0.25 M LiCl, 0.5% sodium deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)], and two times with buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.6)] for 5 min each at 4°C. Chromatin was eluted by incubation of the beads with elution buffer (1% SDS, 0.1 M NaHCO₃). After washing and elution, the immunoprecipitated chromatin was de-crosslinked by incubation at 65°C for 5 h in the presence of 200 mM NaCl, extracted with phenol-chloroform, and ethanol precipitated. Immunoprecipitated chromatin was used as input material for qPCR analysis with the primers listed in supplementary Table S4, or it was additionally sheared, end-repaired, sequencing adaptors were ligated and the library was amplified by LMPCR. After LMPCR, the library was purified and checked for the proper size range and for the absence of adaptor dimers on a 2% agarose gel and sequenced on SOLiD/AB sequencer to produce 50-bp long reads. Sequencing reads were mapped against the reference genome (mm9 or hg19 assembly) using MAQ or BWA package. Cisgenome software package (Ji *et al*, 2008) was used for peak-calling with 0.1 FDR (supplementary Table S5).

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) was performed using the freely available software from the Broad Institute GSEAP 2.0 (<http://www.broadinstitute.org/gsea/index.jsp>) (Mootha *et al*, 2003; Subramanian *et al*, 2005). To create gene sets corresponding to the $\beta^{\text{T-HI}}$ and $\beta^{\text{T-LO}}$ classes of ChIP-seq peaks, individual peaks were correlated to nearest genes and when a gene was occupied by significant β -catenin-precipitated reads within 5 kb of the transcription start site, it was incorporated in the gene set. The gene lists consisted of all changed or unchanged genes from the microarray analyses (no cut-off), provided that their detected signal was 2 fold above background. The ranked gene lists were then probed with the different gene sets with a GSEA-Pre Ranked setting. Duplicate probes were collapsed to the highest probe. The expression array data that was used in the GSEA analysis is listed in supplementary Table S6.

Reporter assays

Genomic fragments encompassing typically about 1 kb of genomic sequence encompassing a β -catenin peak were amplified by PCR from human genomic DNA and cloned in front of the firefly luciferase gene in pGL4.10. In the case of TSS-proximal regions elements were cloned directly in front of the Luciferase TSS. In the case of non-TSS-proximal regions they were cloned in front of a minimal TATA box that was, in turn, cloned in front of the firefly luciferase gene in pGL4.10. The reporters were transfected with

pPEI (Sigma) all cell lines, with Renilla luciferase as a transfection control and their activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). As a positive control the previously described TOPFLASH reporter was used (Korinek *et al*, 1997). Empty pGL4.10 or pGL4.10TATA vectors served as negative controls.

Supplementary information for this article is available online: <http://emboj.embopress.org>

Author contributions

JS, PH and MM performed experimental procedures under the guidance of HC. JS and PH performed ChIP and MM analyzed the sequencing data. JS and HC wrote the manuscript. EC supervised and facilitated the DNA sequencing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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