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Regulation of Tcf711 DNA Binding and Protein Stability as Principal Mechanisms of Wnt/ β -Catenin Signaling

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SUMMARY

Wnt/ β -catenin signal transduction requires direct binding of β -catenin to Tcf/Lef proteins, an event that is classically associated with stimulating transcription by recruiting coactivators. This molecular cascade plays critical roles throughout embryonic development and normal postnatal life by affecting stem cell characteristics and tumor formation. Here, we show that this pathway utilizes a fundamentally different mechanism to regulate Tcf711 (formerly named Tcf3) activity. β -catenin inactivates Tcf711 without a switch to a coactivator complex by removing it from DNA, which leads to Tcf711 protein degradation. Mouse genetic experiments demonstrate that Tcf711 inactivation is the only required effect of the Tcf711- β -catenin interaction. Given the expression of Tcf711 in pluripotent embryonic and adult stem cells, as well as in poorly differentiated breast cancer, these findings provide mechanistic insights into the regulation of pluripotency and the role of Wnt/ β -catenin in breast cancer.

INTRODUCTION

Canonical Wnt/ β -catenin signaling impacts a wide range of biological activities, including stem cell self-renewal, organ morphogenesis, and tumor formation (Clevers and Nusse, 2012; Nusse, 2012). Regulation of the pathway centers on the stability of β -catenin, which is targeted for proteasome-mediated degradation by a complex containing adenomatous polyposis coli (APC), Axin structural proteins, and glycogen synthase kinase 3 (GSK3) (Stamos and Weis, 2013). Phosphorylation of β -catenin by GSK3 stimulates degradation dependent upon APC, Axin, and the β -TrCP E3 ligase (Aberle et al., 1997; Hart et al., 1999; Yost et al., 1996). Wnt signaling inhibits degradation of β -catenin by blocking its ubiquitination (Li et al., 2012). Pharmacological GSK3 inhibitors similarly inhibit β -catenin degradation by blocking β -catenin phosphorylation.

An important downstream mechanism of the Wnt/ β -catenin pathway occurs as β -catenin binds to the amino terminal of Tcf/Lef proteins, thereby displacing corepressor proteins bound to the Tcf/Lef (Cavallo et al., 1998; Daniels and Weis, 2005; Roose et al., 1998). Tcf-

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SUPPLEMENTAL INFORMATION

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β -catenin binding subsequently recruits transactivator proteins to the genomic sites that were previously occupied by corepressors (Brannon et al., 1997; Molenaar et al., 1996; van de Wetering et al., 1997). This accepted model of canonical Wnt/ β -catenin signaling is consistent with observed effects of Tcf/Lef proteins in many contexts (Cadigan and Waterman, 2012); however, it is not consistent with recent observations for mammalian Tcf711 (formerly Tcf3). In cells where Lef1 and Tcf7 (formerly Tcf1) act as β -catenin-dependent transactivators, only transcriptional repressor activity for Tcf711 was detected (Merrill et al., 2001; Wu et al., 2012a). Here, we show that β -catenin binding to Tcf711 does not form a transactivation complex, but instead initiates a fundamentally distinct mechanism. β -catenin binding inactivates Tcf711 by reducing its chromatin occupancy and secondarily stimulates its protein degradation. Mouse genetic experiments demonstrate that this inactivation is the only necessary function of the Tcf711- β -catenin interaction. These molecular and genetic findings provide insights into the role of Wnt/ β -catenin signaling in cells where Tcf711 expression is prominent, including embryonic stem cells (ESCs) and poorly differentiated breast cancer.

RESULTS

β -Catenin Reduces Tcf711 Protein Levels by Stimulating Protein Degradation

Molecular support for a conversion into transactivators by β -catenin includes the ability of a β -catenin-Tcf7 fusion protein to activate target genes without Wnt pathway stimulation (Staal et al., 1999). If Tcf711 were switched to a transactivator by β -catenin, one would expect a β -catenin-Tcf711 fusion protein to similarly activate target genes. In ESCs, the β -catenin-Tcf711 fusion was unable to activate TOPFlash and LRH-1 reporters, and instead repressed Wnt3a-stimulation of reporter genes (Figure 1A). Rather than converting Tcf711 to a transactivator, Wnt/ β -catenin stimulation notably decreased Tcf711 protein in ESCs treated with recombinant Wnt3a or the GSK3 inhibitor, Chiron99021 (CHIR; Figure 1B). These results indicate a significant difference in the downstream effects of Tcf7- β -catenin and Tcf711- β -catenin interaction.

To elucidate the transactivation-independent effects of β -catenin on Tcf711, we investigated how Tcf711 protein levels were reduced. Wnt3a- and CHIR-treated ESCs displayed increased *Lef1* and *Tcf7* messenger RNA (mRNA) levels that correlated with increased protein levels (Figure 1B), consistent with *Lef1* and *Tcf7* being Wnt/ β -catenin target genes (Filali et al., 2002; Hovanes et al., 2000; Roose et al., 1999; Waterman, 2004). In contrast, decreased Tcf711 protein was not paralleled by a significant change in mRNA levels (Figure 1B), indicating that β -catenin regulation of Tcf711 does not occur transcriptionally. Because Dgcr8 is a required component of the microprocessor complex, which is necessary for biogenesis of microRNAs (Wang et al., 2007), the CHIR-stimulated reduction of Tcf711 in *Dgcr8*^{-/-} ESCs showed that reduction of Tcf711 protein was also not microRNA mediated (Figure 1C). Treatments with the proteasome inhibitors MG-132 and MG-115 effectively blocked the CHIR-stimulated reduction of Tcf711 protein (Figures 1D and S1A), demonstrating that reduction of Tcf711 required protein degradation. Finally, reduction of Tcf711 was blocked by leptomycin B, indicating that it required Exportin1-mediated nuclear transport (Figure 1E).

To determine the role of β -catenin binding to Tcf711, we used *Tcf711 Δ N/ Δ N* knockin ESCs. In contrast to wild-type Tcf711, Tcf711 Δ N was not degraded in response to CHIR or Wnt3a (Figures 1D and S1B), indicating that the Tcf711- β -catenin interaction was necessary for degradation. To determine whether the interaction was sufficient for degradation, we expressed Δ N β -catenin in ESCs and measured the Tcf711 levels by quantitative immunofluorescence. Δ N β -catenin expression was sufficient to reduce nuclear Tcf711 levels in *Tcf711*^{+/+} but not in *Tcf711 Δ N/ Δ N* cells (Figures 1F and S1C). Interestingly, several

recent studies showed that a mutant form of β -catenin (β -catenin Δ C) supported self-renewal of mouse ESCs and complemented defects caused by ablation of β -catenin despite the lack of the C-terminal transactivation domain in the β -catenin Δ C mutant (Kelly et al., 2011; Lyashenko et al., 2011; Wray et al., 2011). Therefore, it is notable that expression of Δ N β -catenin Δ C was also sufficient to reduce nuclear Tcf711 protein levels ESCs (Figure S1D). Given the substantial effects of altering Tcf711 levels in ESCs, the reduction of Tcf711 protein provides a mechanism for the poorly understood pro-self-renewal effects of β -catenin Δ C in ESCs.

Reduction of Tcf711 Is Sufficient to Replace the Tcf711- β -Catenin Interaction

If a principal mechanism of Wnt/ β -catenin signaling functions through inactivation of Tcf711, and not conversion to a Tcf711- β -catenin transactivator complex, reducing the level of Tcf711 should be sufficient to replace the Tcf711- β -catenin interaction. We first tested this hypothesis in ESCs, where reducing the amount of Tcf711 Δ N by small interfering RNA (siRNA) stimulated the reporter gene response to Wnt3a (Figures S2A and S2B). To examine the broader effects of reducing Tcf711 in mice, we reduced the level of Tcf711 by breeding for hemizygous mice (i.e., *Tcf711*^{+/-} or *Tcf711*^{-/ Δ N}; Figure S2C). It is important to note that *Tcf711*^{-/-} mice die shortly after gastrulation (Merrill et al., 2004). *Tcf711* Δ N/ Δ N embryos progress normally through gastrulation, but later develop a constellation of morphogenetic defects that result in death for all *Tcf711* Δ N/ Δ N mice at or before birth (Hoffman et al., 2013; Wu et al., 2012a). Mating *Tcf711*^{+/-} with *Tcf711*^{+/ Δ N} mice produced the Mendelian-expected ratio of *Tcf711*^{-/ Δ N} offspring, despite the genetic absence of a Tcf711 protein capable of interacting with β -catenin (Figures 2A and S2D). Moreover, *Tcf711*^{-/ Δ N} mice did not display any of the morphogenetic defects observed in *Tcf711* Δ N/ Δ N mice, including poor vascular integrity, edema, oligodactyly, and opened eyelids (Figures 2C–2D", S2E, and S2F). Indeed, *Tcf711*^{-/ Δ N} mice advanced to adulthood and appeared indistinguishable from *Tcf711*^{+/+} littermates throughout their ostensibly normal lifetimes (Figure 2B). Thus, removing one copy of *Tcf711* Δ N genetically rescued the defects caused by ablating the Tcf711- β -catenin interaction. These results demonstrate that inactivation of Tcf711 by β -catenin is the necessary effect downstream of Tcf711- β -catenin interaction for mouse embryogenesis and postnatal viability.

To determine the effects of reducing Tcf711 at the target gene level in mice, tissues that were previously shown to be affected in *Tcf711* Δ N/ Δ N embryos were examined in *Tcf711*^{-/ Δ N} embryos harboring the BAT-Gal reporter. Compared with *Tcf711*^{+/+} embryonic day 14.5 (e14.5) eyelids (Figures 2E–E'''), *Tcf711* Δ N/ Δ N displayed a restricted domain of BAT-Gal activity and decreased expression of Lef1, a Wnt/ β -catenin target, in the mucocutaneous junction of the eyelid (Figures 2F–2F'''; Wu et al., 2012a). The domain of Lef1 expression and BAT-Gal activity was increased in *Tcf711*^{-/ Δ N} relative to *Tcf711* Δ N/ Δ N, and BAT-Gal activity was detected only in cells expressing Lef1 (Figures 2F–2G''', S2G, and S2H). Given the inability of Tcf711 Δ N to respond to β -catenin, the rescue of BAT-Gal activity in *Tcf711*^{-/ Δ N} embryos shows that activation is mediated by Lef1, and attenuation of this activation depends on the level of Tcf711 repressor.

β -Catenin Stimulates TCF7L1 Inactivation in Human Breast Cancer

In addition to pluripotent cells in the early mammalian embryo, and ESCs in vitro, *TCF7L1* mRNA expression has been noted in several types of adult stem cells and in poorly differentiated cancers (Ben-Porath et al., 2008; Ivanova et al., 2002; Tumber et al., 2004). We reasoned that the aspects of the Tcf711-destabilization mechanism could provide insights into the effects of Wnt/ β -catenin in these important contexts. Breast cancer was chosen for further analysis because (1) Wnt/ β -catenin has been known to affect mammary tumors since it was first discovered by Nusse et al. (1984), (2) despite its long history, the underlying

mechanisms of Wnt/ β -catenin's effects in this disease remain poorly understood (Alexander et al., 2012), (3) poorly differentiated mammary tumors express high levels of *TCF7L1* mRNA (0.81 mean *TCF7L1* mRNA \pm 0.91 SD for 270 basal tumors, -0.32 ± 0.67 for 941 nonbasal tumors; $p < 0.0001$; Figure 3A; Ben-Porath et al., 2008), and (4) altering the level of Tcf7l1 caused significant effects in xenograft tumor-formation experiments (Slyper et al., 2012).

Consistent with previous analyses, we noted a very high frequency of the basal molecular subtype among invasive mammary tumors expressing the highest levels of *TCF7L1* mRNA (83% of tumors with *TCF7L1* mRNA > 1.1 were basal, and 22% of all tumors assessed were basal; Figure 3A; Ben-Porath et al., 2008; Slyper et al., 2012). To examine patterns of *TCF7L1* protein expression, we used a *TCF7L1*-specific antibody for immunohistochemistry (IHC) staining of an array of breast cancer tissue samples (Figure S3A; Khramtsov et al., 2010). In contrast to *TCF7L1* mRNA, nuclear *TCF7L1* protein was not significantly higher in basal subtype tumors (55 ± 60 , $n = 23$) relative to nonbasal tumors (44 ± 74 , $n = 47$; Figures 3B and S3A), and the frequency of basal tumors displaying strong nuclear *TCF7L1* (i.e., nuclear IHC score > 180) was lower than the overall frequency of basal tumors on the array (25% with high nuclear *TCF7L1* versus 32% of all tumors; Figures 3C, S3B, and S3C). Thus, although *TCF7L1* mRNA is highly elevated in basal subtype tumors, *TCF7L1* protein is not.

Previous analyses of β -catenin protein in patient samples demonstrated that nuclear and cytoplasmic β -catenin was strongly associated with basal subtype tumors and poor prognosis (Geyer et al., 2011; Khramtsov et al., 2010; López-Knowles et al., 2010). To determine whether the disparity between *TCF7L1* mRNA and protein levels could be caused by elevated β -catenin, we compared the *TCF7L1* IHC results with the β -catenin IHC results among identical patient samples. Remarkably, tumors with strong nuclear *TCF7L1* had predominantly no nuclear or cytoplasmic β -catenin (β -catenin IHC score of 0; Figure 3D), and tumors with cytoplasmic and/or nuclear β -catenin (β -catenin IHC score of 2 or 3) displayed predominantly diffuse or low levels of *TCF7L1* protein (Figure 3D). These data indicate that in human mammary tumors, stimulation of Wnt/ β -catenin is strongly correlated with decreased nuclear *TCF7L1*.

To test for a causal relationship between β -catenin and *TCF7L1* levels in breast cancer, we used several cancer cell lines. Endogenous *TCF7L1* protein was reduced by CHIR in all cells examined (MCF7, MDA-MB-231, MDA-MB-468, HS578T, and HCC38; Figure 3E). As in the ESCs, *TCF7L1* mRNA was not significantly diminished (Figure S3E), and the reduction of *TCF7L1* protein was blocked by the proteasome inhibitor MG-132 (Figure S3F). Treating cells with CHIR or CHIR + MG-132 increased the cytoplasmic levels of *TCF7L1* detected by immunofluorescence (Figure S3G). As in the ESCs, Tcf7 was endogenously expressed and stimulated TOPFlash activity (Figures 3F and S3H), whereas overexpression of Tcf7l1 or β -catenin-Tcf7l1 fusion repressed TOPFlash activity (Figures 3F and 3G). Repression by Tcf7l1 Δ N and Tcf7l1 HMG* mutants indicated that inhibition of TOPFlash was caused by a combination of β -catenin-binding- and DNA-binding-dependent activities of Tcf7l1 (Figure S3I). These results suggest that the mechanism of β -catenin-mediated inactivation of Tcf7l1 that was previously observed in ESCs and mouse embryos also occurs in human breast cancer.

Tcf7l1 Inactivation Occurs Independently of Phosphorylation by HIPK2 or NLK

Treating human breast cancer cells with CHIR caused a substantial shift in the mobility of *TCF7L1* as analyzed by SDS-PAGE (Figure 3E). Although it is more difficult to detect in mouse ESCs, likely because of the multiple Tcf7l1 isoforms expressed in ESCs (Salomonis et al., 2010), this shift also affected endogenous Tcf7l1 in mouse ESCs treated with CHIR or

Wnt3a (Figures 4A, 4B, and S4). Interestingly, work in other systems demonstrated mobility shifts caused by β -catenin-dependent phosphorylation of Tcf711/Tcf3 proteins by homeodomain-interacting protein kinase 2 (HipK2) (Hikasa et al., 2010; Hikasa and Sokol, 2011) and nemo-like kinase (NLK) (Ishitani et al., 1999, 2003). In particular, HipK2 has been proposed to serve as a primary mediator of Tcf711 regulation by reducing chromatin binding after phosphorylation at conserved residues (Hikasa et al., 2010; Hikasa and Sokol, 2011). We previously showed that Tcf711 chromatin occupancy is reduced in ESCs by Wnt3a, and the reduction required the Tcf711- β -catenin interaction (Wu et al., 2012a). Therefore, we tested whether phosphorylation of Tcf711 at conserved residues was needed for inactivation by using the Tcf711-P2/3/4 mutant, which harbors mutations at the residues phosphorylated by HipK2 and NLK (Hikasa et al., 2010). Surprisingly, the Tcf711-P2/3/4 mutation did not affect CHIR-induced degradation of Tcf711 or Tcf711 repression of target gene expression in ESCs (Figures 4C and 4D). Thus, it is unlikely that Wnt/ β -catenin inactivation of Tcf711 in ESC requires phosphorylation by HipK2 or NLK. In addition, although Tcf711 was indeed phosphorylated, we detected no change in phosphorylation in the absence or presence of CHIR stimulation. The CHIR-induced mobility shift of Tcf711 was not phosphatase sensitive, suggesting that it was not mediated by increased phosphorylation (Figure 4B). The nature of this posttranslational modification is not known; however, it was blocked by MG-132 (Figure 4E), indicating that it required an active proteasome.

Reduction of Chromatin Occupancy Provides the Critical Upstream Point of Tcf711 Regulation

To elucidate how the reduction of chromatin occupancy is causally linked to protein degradation, we conducted quantitative chromatin immunoprecipitation (ChIP) experiments to measure Tcf711 chromatin occupancy following the combined CHIR + MG-132 treatment. As expected from the changes in Tcf711 protein levels (Figure 1B), CHIR reduced Tcf711 occupancy on target genes (*Axin2*, *Cdx1*, and *Mycn*), and MG-132 increased occupancy (Figure 4F). Importantly, CHIR treatment reduced chromatin occupancy even when destabilization of Tcf711 was blocked by MG-132 (Figure 4F), indicating the reduction in DNA binding was upstream of degradation. Interestingly, since MG-132 prevented the mobility shift of Tcf711 (Figure 4E), this result also indicates that the reduction of chromatin binding does not require the posttranslational modification. Combined with the increased cytoplasmic Tcf711 staining after CHIR + MG-132 treatment (Figure S3G), these data indicate that Tcf711 is likely degraded after export from the nucleus.

To examine the role of chromatin occupancy in regulating Tcf711 stability, we used the Tcf711 HMG* mutation, which affects the DNA-binding HMG domain and disrupts DNA binding (Merrill et al., 2001). The HMG* mutation was sufficient to reduce Tcf711 protein stability in the absence of CHIR (Figure 4G). Moreover, stability of the mutant Tcf711 HMG* protein was not substantially decreased by CHIR (Figure 4H), indicating that destabilization of Tcf711 requires a change in chromatin occupancy. In support of the model focused on reduction of chromatin occupancy, coimmunoprecipitation (coIP) experiments showed that Tcf711- β -catenin interaction was stimulated by CHIR + MG-132 (Figure 4I), and β -catenin chromatin occupancy increased (Figure 4J) while Tcf711 occupancy decreased (Figure 4F). Together, these data are most consistent with the notion that the primary effect of the Tcf711- β -catenin interaction is to inhibit chromatin occupancy. The secondary effect of Tcf711 degradation provides an additional mechanism by lowering Tcf711 levels, which further reduces the amount of Tcf711 available to bind to chromatin. Thus, the combination of reduced DNA binding and Tcf711 degradation leads to an additive reduction of Tcf711 repression in response to Wnt/ β -catenin activity.

DISCUSSION

The molecular effects of Wnt/ β -catenin and Gsk3 inhibition on Tcf711 described here indicate that Tcf711 primarily functions outside of the classic model of canonical Wnt/ β -catenin signaling. In ESCs, inactivation of Tcf711 did not require phosphorylation of Tcf711 at conserved sites, and β -catenin was sufficient to reduce Tcf711 levels without exogenous pathway stimulation. These results are consistent with a mechanism of inactivation wherein β -catenin binding inhibits Tcf711-repression by reducing chromatin occupancy, consequently stimulating its degradation. This mechanism provides a simple explanation for the controversial pro-self-renewal effects of the β -catenin Δ C mutant in ESCs (Kelly et al., 2011; Lyashenko et al., 2011; Wray et al., 2011): the critical effect of inactivating Tcf711 is stimulated by the β -catenin Δ C form, thus making β -catenin's C-terminal transactivation domain dispensable in ESCs.

Together, the results from experiments using human breast cancer tumors, breast cancer cell lines, and mouse genetics indicate that inactivation of Tcf711 is the predominant mechanism whereby Wnt/ β -catenin signaling interacts with this mammalian Tcf/Lef protein. The viability of *Tcf711*- Δ N mice genetically demonstrates that inactivation of Tcf711 is the only effect of Tcf711- β -catenin binding that is required for normal mouse development and life. That said, additional activities downstream of Tcf711- β -catenin interaction likely exist. Indeed, reporter gene assays support rare Tcf711- β -catenin transactivator activity in some cell types (e.g., 293T, COS7, and human keratinocytes; Merrill et al., 2001; Slyper et al., 2012; Wu et al., 2012a); however, the biological significance of this effect remains to be determined.

Human breast cancer is one important context in which Tcf711-based activation has been suggested (Slyper et al., 2012). Basal subtype tumors are particularly relevant because they have been noted to share a gene-expression signature with ESCs (Ben-Porath et al., 2008) and fetal mammary stem cells (Spike et al., 2012). These tumors, which express high levels of Tcf711 mRNA, have been suggested to arise following reprogramming to an earlier embryonic stage (Mizuno et al., 2010), and thus it is important to understand how Wnt/ β -catenin and Tcf711 function. Previous direct experiments showed that ectopic Tcf711 expression and Wnt3a both stimulate xenograft tumor formation, mammosphere formation, and colony formation in Matrigel from breast cancer cell lines (Slyper et al., 2012). Although it is formally possible that Tcf711- β -catenin complexes may act as transactivators for a set of target genes critical for breast cancer cells, the data presented here do not support this possibility. Tcf711 displayed only repressor activity in reporter assay experiments, and Tcf711 was degraded following CHIR treatments. We propose two non-mutually-exclusive possibilities: (1) Tcf711 and Wnt/ β -catenin signals mediate parallel effects, each stimulating tumor cells, and (2) Tcf711- β -catenin complexes have a biochemical activity that is distinct from the classical transactivator activity. The former possibility is supported by the recent demonstration of a Wnt/Gsk3/Slug/Snail signaling axis affecting triple-negative breast cancers (Wu et al., 2012b).

The effects of β -catenin on Tcf711 are most parsimoniously explained by a mechanism of β -catenin directly inhibiting Tcf711 binding to chromatin. Experiments examining the effects of β -catenin on the Tcf/Lef interaction with naked DNA showed little or no effect on binding in vitro, whereas β -catenin interaction significantly affected binding to chromatin by Lef1 (Tutter et al., 2001). Interestingly, mutational analysis of Lef1 indicated that an amino terminal region provides intramolecular inhibition of chromatin binding. β -catenin binding blocks the intramolecular inhibition, thereby stimulating Lef1 binding to chromatin (Tutter et al., 2001). Although the effect of β -catenin on Lef1 is different from that predicted for Tcf711, these previous findings demonstrate both positive and negative regulation of

chromatin binding via the β -catenin interaction region of Tcf/Lef proteins. Further research is necessary to elucidate the biophysical and biochemical nature of β -catenin's effects on the chromatin binding properties of Tcf711.

EXPERIMENTAL PROCEDURES

IHC Staining and Scoring of Mammary Tumor Microarray

Quantitative analysis (i.e., scoring) of IHC was performed without knowledge of specimen identification. Scoring was based on a combination of the intensity of stained cells and the percentage of tissue. Separate values for nuclear and cytoplasmic Tcf711 immunoreactivity were determined for each sample. Total Tcf711 IHC scores were calculated using a modified Reiner scoring system (Reiner et al., 1990) by multiplying the intensity of staining (0–3 value) by the percentage of positive cells. Ranking and scores for β -catenin levels and localization were previously described in Khramtsov et al. (2010).

Statistical Analyses of Tumor RNA and Protein Expression Data

The Kruskal-Wallis test was used to compare the overall effects of stage on nuclear, cytoplasmic, and total Tcf711. The Wilcoxon rank-sum test with Bonferroni correction was used to conduct pairwise comparisons among four stages. A two-sample t test was used to compare Tcf711 mRNA between basal and nonbasal groups. The Wilcoxon rank-sum test was used to compare Tcf711 nuclear, cytoplasmic, and total IHC scores between basal and nonbasal groups and between luminal A and nonluminal A groups. Spearman correlations determined the relationships between nuclear Tcf711 and cytoplasmic β -catenin; p values < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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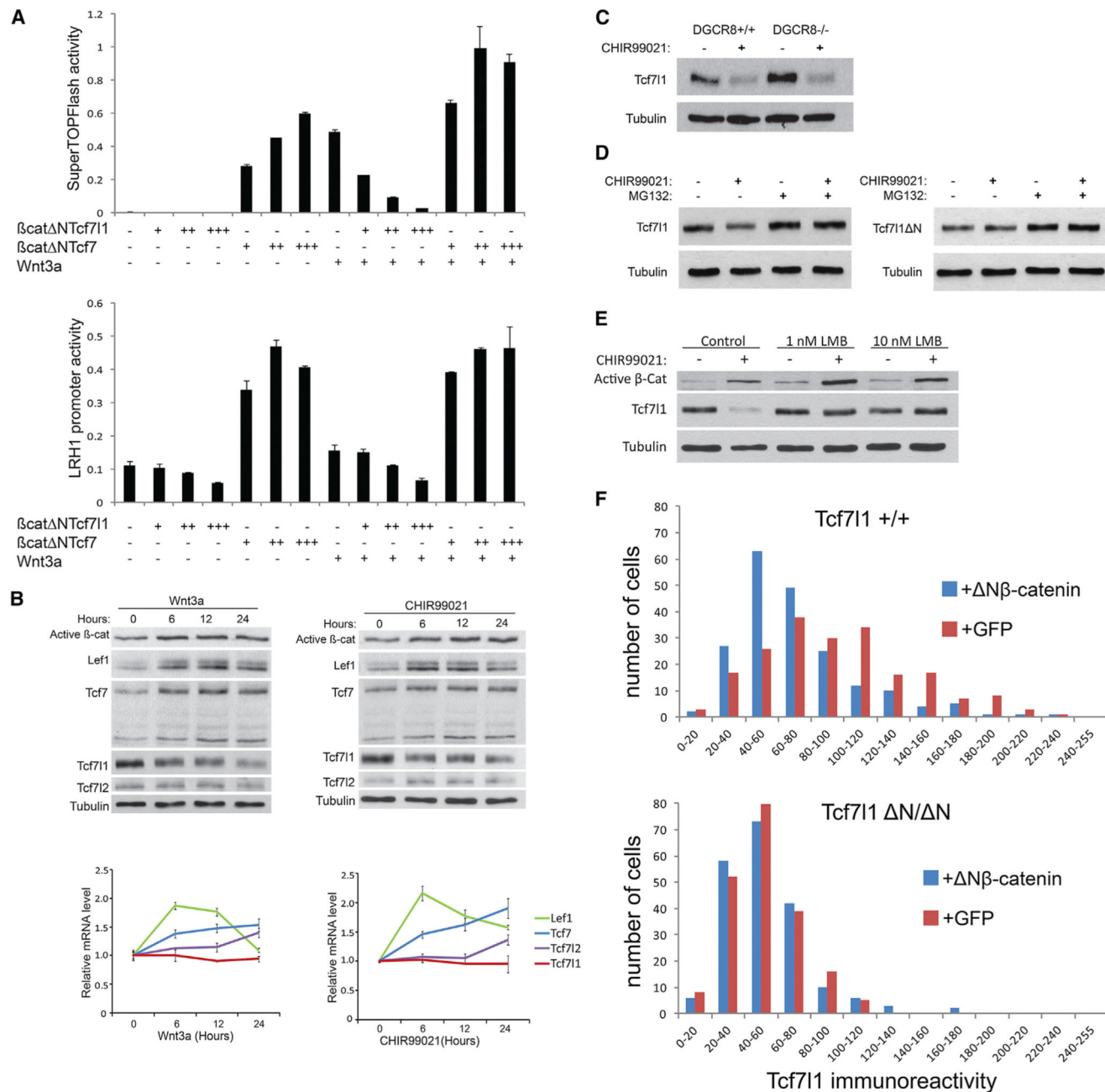


Figure 1. Wnt/ β -Catenin Stimulates Tcf711 Protein Degradation

(A) Transient transfection of *Tcf711*^{-/-} ESCs with β -catenin-Tcf fusion plasmids and SuperTOPFlash (top) or LRH1 promoter (bottom) luciferase reporter plasmids. Values represent mean \pm SD for triplicate transfections.

(B) Western blot (top) and quantitative RT-PCR (qRT-PCR, bottom) analyses of ESCs treated with 50 ng/ml recombinant Wnt3a (left) or 3 μ M CHIR (right). Values represent mean \pm SD for biological triplicates.

(C–E) Western blot analysis of ESCs treated with 3 μ M CHIR for 24 hr in *Dgcr8* mutant cells (C), for 6 hr with MG-132 (5 μ M) in *Tcf711*^{+/+} and *Tcf711* Δ N/ Δ N cells (D), and for 12 hr with leptomycin B (E).

(F) Distribution of nuclear Tcf711 immunoreactivity levels in *Tcf711*^{+/+} (top) and *Tcf711* $\Delta N/\Delta N$ (bottom) cells expressing either GFP (red bars) or $\Delta N\beta$ -catenin (blue bars). A total of 200 nuclei were counted for each condition. Data are representative of three separate experiments. See also Figure S1.

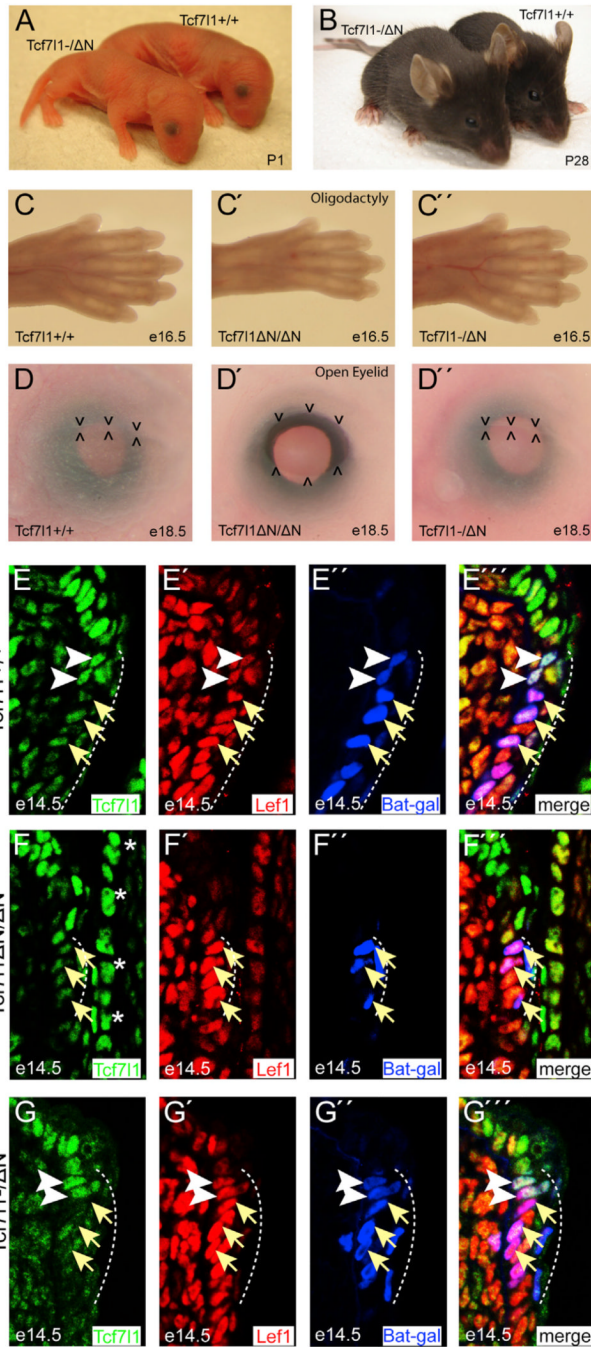


Figure 2. Reducing *Tcf711* Levels Replaces the Requirement for β -Catenin Interaction in Mice (A-D'') *Tcf711*^{-/ΔN} mice appear normal at birth (A) and through adult stages (B; see also Figure S2D). *Tcf711*^{-/ΔN} embryos do not develop the phenotypes observed in *Tcf711ΔN/ΔN* embryos (i.e., oligodactyly [C-C''] and opened eyelids at birth [D-D'']); see also Figures S2E and S2F).

(E-G'') *Tcf/Lef*- β -catenin activation of BAT-Gal reporter is restored in the *Tcf711*^{-/ΔN} eyelid. Immunofluorescent staining for *Tcf711* (green), *Lef1* (red), and β -galactosidase (blue) in e14.5 eyelids from BAT-Gal transgenics with the indicated *Tcf711* genotype. Arrows point to *Lef1*-high and *Tcf711*-low nuclei.

Arrowheads point to Lef1-high and Tcf711-positive nuclei. The dotted line denotes the BAT-Gal-positive region. Asterisks mark Tcf711-positive cells in the nearby cornea.

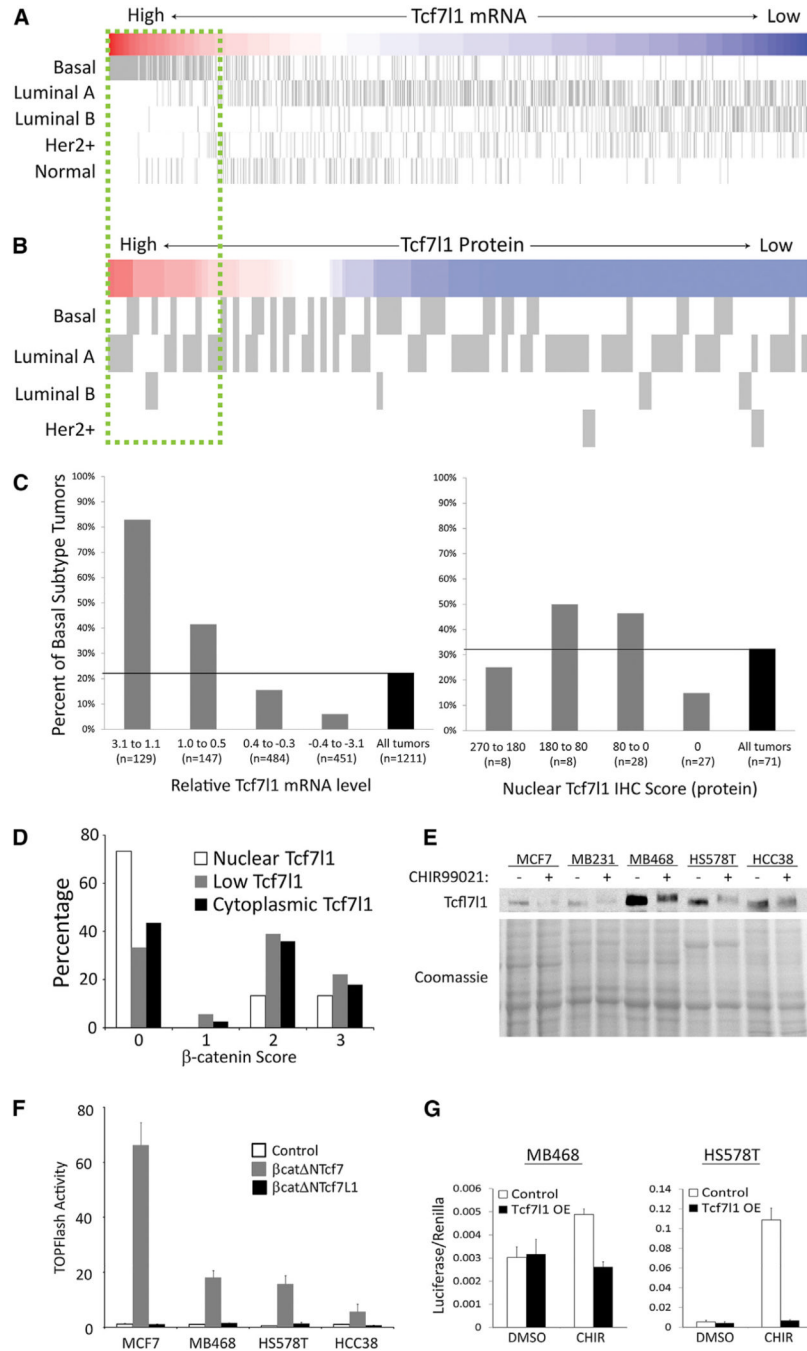


Figure 3. Wnt/ β -Catenin Inactivates TCF7L1 Protein in Poorly Differentiated Breast Cancer

(A) Heatmap showing relative *TCF7L1* mRNA levels and tumor subtype status from a compendium of 1,211 mammary tumors. Subtypes are displayed according to previous designations (Ben-Porath et al., 2008).

(B) Heatmap showing relative TCF7L1 protein nuclear immunoreactivity for all invasive tumors in an array of samples from 71 individual patients. Subtypes are displayed as determined previously (Khrantsov et al., 2010).

(C) Graphs show the distribution of basal subtype with respect to the level of *TCF7L1* mRNA (left) or nuclear TCF7L1 protein (right).

(D) Distribution of tumors with nuclear TCF7L1 (n = 15, white), diffuse TCF7L1 (n = 38, black), or low TCF7L1 (n = 18, gray) classification relative to the IHC score for cytoplasmic β -catenin for each individual tumor. Values represent the percentage of tumors for each TCF7L1 classification displaying the indicated cytoplasmic β -catenin IHC score.

(E) TCF7L1 protein (western) and total protein (Coomassie) for human breast cancer cell lines treated with vehicle (DMSO) or CHIR (3 μ M) for 12 hr.

(F and G) SuperTOPFlash luciferase reporter activity for breast cancer cell lines transiently transfected with the indicated β -catenin-Tcf fusion plasmid, wild-type Tcf711 plasmid, or empty vector. Values represent mean \pm SD of technical duplicates of biological duplicates. See also Figure S3.

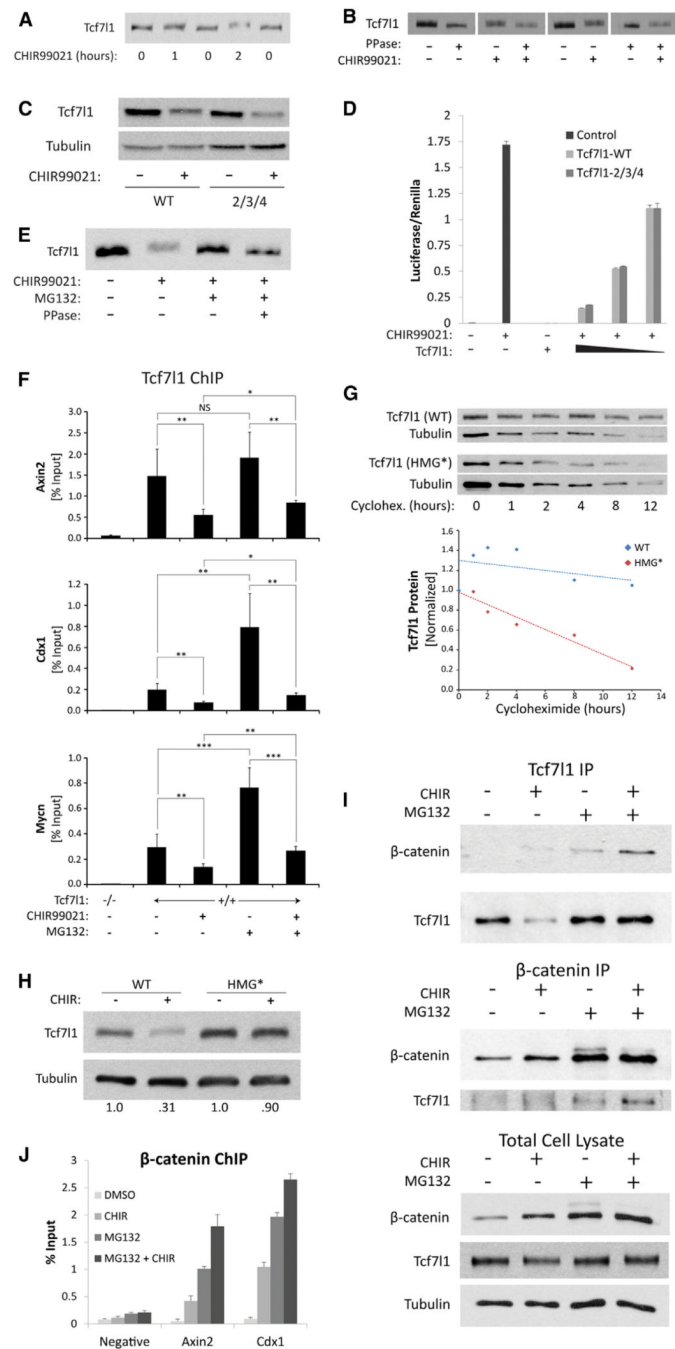


Figure 4. Inhibition of Chromatin Occupancy Is Upstream of Tcf711 Protein Degradation

(A and B) Western blot analysis of Tcf711 protein from ESCs.

(A) Cells were treated with 15 μ M CHIR for 0–2 hr.

(B) Cells were treated with 3 μ M CHIR for 12 hr and lysates were treated with 15 U/ μ L lambda phosphatase.

(C) Tcf711 protein from *Tcf711*^{-/-} ESC stably expressing either Tcf711-wild-type (Tcf711-WT) or Tcf711-2/3/4. Cells were treated with 6 μ M CHIR for 18 hr.

(D) SuperTOPFlash assay using *Tcf711*^{-/-} ESC transiently transfected with either Tcf711-WT or Tcf711-2/3/4. Cells were treated with 3 μ M CHIR for 12 hr. Values represent mean \pm SD of technical duplicates of biological duplicates.

(E) Tcf711 protein from *Tcf711*^{-/-} ESC transiently transfected with either Tcf711-WT or Tcf711-2/3/4. Cells were treated with 3 μ M CHIR for 12 hr, 100 nM MG132, or 10 U/ μ L lambda phosphatase. Values represent mean \pm SD of technical duplicates of biological duplicates.

(F) Tcf711 ChIP analysis of Axin2, Cdx1, and Mycn in *Tcf711*^{-/-} and *Tcf711*^{+/+} ESCs treated with 3 μ M CHIR for 12 hr with or without 100 nM MG132. Values represent mean \pm SD of technical duplicates of biological duplicates.

(G) Tcf711 (WT) and Tcf711 (HMG*) protein levels in *Tcf711*^{-/-} ESC transiently transfected with either Tcf711-WT or Tcf711-HMG* treated with 3 μ M Cycloheximide (Cyclohex.) for 0, 1, 2, 4, 8, or 12 hr. Values represent mean \pm SD of technical duplicates of biological duplicates.

(H) Tcf711 and Tubulin protein levels in *Tcf711*^{-/-} ESC stably expressing either Tcf711-wild-type (WT) or Tcf711-HMG* treated with 6 μ M CHIR for 18 hr with or without 100 nM MG132. Values represent mean \pm SD of technical duplicates of biological duplicates.

(I) Tcf711 IP, β -catenin IP, and Total Cell Lysate protein levels in *Tcf711*^{-/-} ESC stably expressing either Tcf711-wild-type (WT) or Tcf711-HMG* treated with 6 μ M CHIR for 18 hr with or without 100 nM MG132. Values represent mean \pm SD of technical duplicates of biological duplicates.

(J) β -catenin ChIP analysis of Axin2 and Cdx1 in *Tcf711*^{-/-} ESC treated with 3 μ M CHIR for 12 hr with or without 100 nM MG132. Values represent mean \pm SD of technical duplicates of biological duplicates.

- (E) Tcf711 protein from lysates of *Tcf711*^{-/-} ESC stably expressing wild-type Tcf711. Cells were treated with 3 μ M CHIR and 5 μ M MG-132 for 12 hr. Lysates were treated with 15 U/ μ L lambda phosphatase.
- (F) Quantitative ChIP using anti-Tcf711 antibody. Mouse ESCs were treated for 12 hr with 3 μ M CHIR and/or 5 μ M MG-132. qPCR measurements of Tcf711-bound DNA are shown for regions near *Axin2*, *Cdx1*, and *Mycn* genes. Values represent the mean + SD of percent of precipitated DNA relative to input for duplicate technical measurements of five biological replicates. **p < 0.01; ***p < 0.001; NS, p > 0.05.
- (G) Top: Tcf711 and Tubulin proteins from lysates of *Tcf711*^{-/-} ESC stably expressing either wild-type Tcf711 (WT) or the mutant Tcf711 HMG* (HMG*) protein. Cells were treated with 30 μ g/ml cycloheximide. Bottom: quantitation of western blot and normalization of Tcf711 protein levels was calculated for WT (blue) and HMG* (red) proteins. Each data point represents the mean of biological triplicates.
- (H) Western blot analysis comparing the CHIR-mediated reduction WT and HMG* Tcf711 proteins using the same ESCs as in (G).
- (I) CoIP experiments using anti-Tcf711 (top) or anti- β -catenin (middle). Protein was immunoprecipitated from lysates of cells treated with 3 μ M CHIR and 5 μ M MG-132 (bottom).
- (J) Quantitative ChIP using anti- β -catenin antibody. Chromatin was isolated from ESCs treated for 12 hr with 3 μ M CHIR and 5 μ M MG-132. qPCR measurements of β -catenin-bound DNA are shown for regions near *Axin2* and *Cdx1* genes. Values represent the mean + SD of percent of precipitated DNA relative to input for duplicate technical measurements of three biological replicates.
- See also Figure S4.