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TGIF Governs a Feed-Forward Network That Empowers Wnt Signaling to Drive Mammary Tumorigenesis

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SUMMARY

Many types of human cancers having hyperactivated Wnt signaling display no causative alterations in known effectors of this pathway. Here, we report a function of TGIF in Wnt signaling. TGIF associates with and diverts Axin1 and Axin2 from the β-Catenin destruction complex therefore allowing β-Catenin accrual. Intriguingly, activation of Wnt signaling induces the expression of TGIF, which unveils a feed-forward loop that ensures effective integration of Wnt signaling. In triple negative breast cancers (TNBC), elevated levels of TGIF correlate with high Wnt signaling and poor survival of patients. Moreover, genetic experiments revealed that *Tgif1* ablation impeded mammary tumor development in MMTV-Wnt1 mice, further underscoring a requirement of TGIF for oncogenic Wnt signaling.

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AUTHOR CONTRIBUTIONS

M.-Z.Z., O.V., R.B., and A.A. designed the experiments. M.-Z.Z., O.V., and A.A. performed the in vitro experiments with the assistance of Z.W., C.P., and L.L. and the in vivo experiments with the assistance of M.O., M.R. and G.T. D.D. performed the analysis of human datasets. F.C. performed the yeast two-hybrid screen. M.-Z.Z., O.F., and A.A. wrote the paper with the help of W.C.H. and R.B.

INTRODUCTION

The Wnt signaling pathway regulates a plethora of important cellular processes, such as specification of developmental fate, proliferation, and differentiation. For example, Wnt signaling coordinates proliferation and differentiation of stem/progenitor cells in a variety of epithelial tissues, including the mammary gland (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004). The broad theme that has emerged from extensive examination of Wnt signaling is that any perturbation in this circuitry disturbs tissue homeostasis, culminating in many diseases, most prominent among them cancer. Broadly recognized mechanisms behind Wnt signaling hyperactivation in cancers include mutations of pathway components and elevated autocrine signaling due to constitutive production of Wnt ligands (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004).

Wnt ligands initiate signaling by binding to two types of cell surface receptors: the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), and the Frizzled (Fz) family of serpentine proteins (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004). Over the past few years, there have been tremendous advances in elucidating the molecular mechanisms operating downstream of Wnt receptors to trigger activation of canonical β-Catenin signaling. The most prevalent model implies that engagement of Wnt with the Fz/LRP5/6 receptor complex provokes squelching of Axin1, which functions in resting cells as a scaffold for the β-Catenin destruction complex, bringing together adenomatous polyposis coli (APC), β-Catenin, casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3), and ubiquitin E3 ligase βTRCP, among others. Within this destruction complex, CK1 and GSK3 sequentially phosphorylate β-Catenin, resulting in its recognition by βTRCP, and subsequent degradation by the proteasome. Upon ligand binding, recruitment of Axin1 to LRP5/6 leads to disassembly of the destruction complex, resulting in increased β-Catenin accumulation (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004). An alternative model emerged recently is that activation of Wnt signaling does not merely affect assembly or composition of the destruction complex but instead hinders ubiquitination and clearance of β-Catenin within an intact complex, leading to saturation and inactivation of the destruction complex by phosphorylated β-Catenin (Li et al., 2012). Consequently, newly synthesized, non-phosphorylated β-Catenin escapes degradation by the saturated destruction complex and accumulates in the nucleus. Irrespective of the molecular mechanisms by which activated Wnt signaling achieves accumulation and translocation of β-Catenin into the nucleus, recruitment of β-Catenin to promoters of target genes via interaction with enhancer factor/T cell family (TCF) transcription factors stands as the main apparatus fulfilling the canonical Wnt transcriptional program (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004).

Transforming growth-interacting factor (TGIF) belongs to the superfamily of TALE homeodomain proteins that control many biological processes, including embryonic development, cell proliferation, and differentiation. Genetic ablation of *Tgif1* in mice resulted in a large spectrum of phenotypes, including placental defects, craniofacial abnormalities, and runting (Bartholin et al., 2008; Taniguchi et al., 2012). Perhaps the most extensively studied function of TGIF is suppression of transforming growth factor beta (TGF-β) signaling, which has prominent and widespread roles in cancer (Massague, 2008;

Seo et al., 2006; Wotton et al., 1999). TGF-β initiates responses by binding to and activating a cell surface receptor complex composed of TβRI and TβRII serine threonine kinases, which then propagate signals by phosphorylating the downstream effectors Smad2 and Smad3 (Feng and Derynck, 2005; Massague, 2008). The mechanism of TGIF-induced suppression of TGF-β signaling has been primarily attributed to its ability to recruit a complex of general transcriptional corepressors to DNA-bound Smad transcription complexes (Wotton et al., 1999). However, our recent findings argued that TGIF can also function through mechanisms unrelated to transcriptional repression (Faresse et al., 2008; Seo et al., 2006; Seo et al., 2004). Together, these observations highlighted an ability of TGIF to antagonize TGF-β signaling in multiple fashions.

Besides its ability to restrict the TGF-β tumor suppressor network, there is evidence that TGIF may function as an oncogenic protein in multiple settings, as exemplified by its ability to confer a full malignant phenotype upon ectopic expression in cell systems (Ettahar et al., 2013; Seo et al., 2006). Notwithstanding these intriguing hints, it is becoming increasingly clear that additional efforts should be put into discovery of molecular mechanisms of TGIF-induced carcinogenesis, as they remain mostly enigmatic. In this study, we sought to investigate whether TGIF contributes to oncogenic Wnt signaling in the mammary gland.

RESULTS

TGIF Interacts with Axin1 and Axin2

To identify TGIF interacting partners, we undertook a yeast two-hybrid approach using human TGIF as bait. Screening of a human placental cDNA library repeatedly recovered Axin2, a prominent antagonist of Wnt signaling (Behrens et al., 1998). To determine whether TGIF binds Axin2 in mammalian cells, we initially conducted coimmunoprecipitation experiments using transfected HEK293T cells. As shown in Figure 1A, TGIF interacted with Axin2 at steady states, and exposure of cells to canonical Wnt3a markedly increased this interaction. By contrast, stimulation of cells with non-canonical Wnt5a failed to increase this interaction (Figure S1A). A more in-depth examination of the TGIF/Axin2 interaction using mammalian two-hybrid and coimmunoprecipitation assays mapped the Axin2-binding domain to the TGIF central region, and the major TGIF-binding domain to the Axin2 DIX domain (Figures 1B, 1C, S1B, S1C). To determine whether this interaction is specific to Axin2, we conducted yeast two-hybrid and coimmunoprecipitation experiments encompassing major components of Wnt signaling (i.e., LRP5/6, Dvl, Fz, Axin1, APC, β-Catenin, TCF). The results revealed that TGIF associated with Axin2 and its close homologue Axin1 but not other components (Figures 1D, S1D, and data not shown). Similar to Axin2, the interaction of Axin1 with TGIF was selectively increased by canonical Wnt3a (Figures 1D, S1D). The interaction of TGIF with Axin1 or Axin2 is physiologically relevant, as stimulation of MCF7 (human breast cancer) or HMLE (human immortalized mammary epithelial) cells with Wnt3a also increased the interaction between endogenous TGIF and Axin1 or Axin2, as evidenced by reciprocal coimmunoprecipitation assays using specific antibodies (Figures 1E, S1E). A time course experiment using HMLE cells showed that Wnt3a stimulation provoked a rapid increase in the interaction of TGIF and Axin1 or Axin2, which became apparent 15 min post-stimulation and persisted for at least 1 hr

(Figure 1F). Under these experimental conditions, Wnt3a had little or no effect on Axin2 expression, most likely because this short-term stimulation was not sufficient to induce expression of Axin2, similar to what reported in the literature (Jho et al., 2002; Lustig et al., 2002). Quantification of the results indicates that 18 to 33% of Axin1/2 associate with 28 to 37% of TGIF (total pool) upon treatment of cells with Wnt3a (Figures 1E,1F). Together, these results revealed that TGIF and Axin1 or Axin2 form physical complexes, which appeared to increase upon activation of Wnt signaling.

TGIF Interferes with Axin1/2 Nucleocytoplasmic Shuttling

To begin unraveling the physiological role of TGIF in Wnt signaling, we took advantage of the literature that TGIF localizes mostly to the nucleus, whereas Axin2 shuttles between the cytoplasm and nucleus (Seo et al., 2006; Wotton et al., 1999; Yook et al., 2006). We found that overexpressing TGIF enforced Axin2 to accumulate in the nucleus (Figure 2A), which confirmed their physical interaction and provided an initial indication that TGIF might retain Axin2 away from the cytoplasm, where it coordinates assembly of the β-Catenin-destruction complex. In agreement with published data (Krieghoff et al., 2006), overexpressed Axin2 localized mostly to the cytoplasm (Figure 2A), perhaps because endogenous TGIF is a rate-limiting factor for Axin2 nuclear localization. Furthermore, we found that overexpressing TGIF disrupted the interaction between Axin2 and β-Catenin (Figure S2A), which is known to take place in the cytoplasm. We also conducted immunofluorescence and cell-fractionation assays using mouse embryonic fibroblasts (MEFs) from wild-type or *Tgif1*$^{-/-}$ mice, and found that *Tgif1* deletion culminated in redistribution of endogenous Axin2 into the cytoplasm as well as increased association of Axin2 with β-Catenin (Figures 2B, 2C, 2D, S2B). These results are not restricted to Axin2, as TGIF deficiency was also associated with redistribution of endogenous Axin1 into the cytoplasm and concomitant association with β-Catenin (Figures 2C, 2D). As a control, *Tgif1* deletion did not affect the interaction of β-Catenin with E-Cadherin (Figure 2D), indicating that TGIF specifically targeted the β-Catenin pool that is dedicated to the destruction complex. Mechanistically, TGIF appeared to engage Axin1 and Axin2 in a complex that prevented their association with the nuclear export receptor CRM1 (Cong and Varmus, 2004; Yook et al., 2006), since deletion of *Tgif1* resulted in increased association of endogenous CRM1 and Axin1 or Axin2 (Figure 2E). A similar conclusion could be drawn from an in vitro reconstitution experiment using purified proteins (Figure S2C), corroborating the ability of TGIF to block access of CRM1 for association with Axin1 or Axin2.

TGIF Promotes β-Catenin Abundance

The results outlined so far suggest that TGIF associates with and diverts Axin1 and Axin2 from the transport machinery that coordinates their nuclear export and thus prevents assembly of the destruction complex. In so doing, TGIF could conceivably hinder β-Catenin phosphorylation by GSK3β, and its subsequent degradation by βTRCP. We obtained several lines of evidence supporting this hypothesis. *Tgif1*$^{-/-}$ MEFs displayed increased phosphorylation of β-Catenin at the GSK3β phosphorylation sites Ser33/37 as compared to wild-type MEFs (Figure 3A). Expression of TGIF increased the abundance of wild-type β-Catenin, but was ineffective against β-Catenin.S33Y, a degradation-resistant mutant (Figure 3B). Ectopic expression of TGIF inhibited the interaction between β-Catenin and βTRCP

(Figure 3C). Induction of TGIF expression in MCF7 cells harboring a doxycycline-inducible TGIF increased the abundance of endogenous β-Catenin, concurring with decreased interaction of β-Catenin with βTRCP (Figure 3D). Similar experiments using MEFs showed that *Tgif1* ablation decreased the abundance of β-Catenin, concurring with increased association of β-Catenin with βTRCP (Figure 3A). However, *Tgif1* deletion did not affect the abundance of β-Catenin mRNA (Figure S3A). Stimulation of *Tgif1*$^{-/-}$ MEFs with Wnt3a failed to promote β-Catenin accumulation as compared to wild-type MEFs (Figure 3E). Exposure of *Tgif1*$^{-/-}$ MEFs to the proteasome inhibitor MG132 for 12 hr increased the β-Catenin abundance to the level detected in wild-type MEFs (Figure 3F). Analysis of mammary glands from *Tgif1* knockout mice showed that TGIF deficiency decreased the abundance of total and active (non-phosphorylated) β-Catenin without altering the accumulation of its mRNA (Figures 3G, 3H, S3B). Extending our analysis to human breast tissue, we noticed a correlation between the expression of TGIF and β-Catenin that is mainly confined to the whole epithelial duct structure (Figure S3C); we return to this observation later (Figure 5F). Collectively, these findings strongly suggest that a physiological function of TGIF is to divert Axin1 and Axin2 from the β-Catenin destruction complex, thereby leading to β-Catenin accrual.

TGIF as a Mediator of Wnt Signaling

Owing to its ability to promote β-Catenin stability, one would surmise that TGIF functions as a key component of Wnt signaling. In fact, expression of TGIF enhanced the sensitivity of MCF7 and HMLE cells to Wnt3a-induced TOPFlash (Figure S4A), a surrogate readout of Wnt signaling (Korinek et al., 1997). Likewise, expression of TGIF synergized with β-Catenin in eliciting increased TOPFlash activity (Figures S4B, S4C), in line with the hypothesized function of TGIF to foster β-Catenin stability through nuclear sequestration of Axin1 and Axin2. In further support to this notion, expression of Axin1 or Axin2 blunted the synergism between TGIF and β-Catenin on TOPFlash; yet this antagonistic effect was reversed by raising the levels of TGIF (Figure S4B). In an alternative approach, expression of TGIFΔAxin2 (TGIFΔ148-177), which no longer interacted with Axin2 (Figures 1B, S1B), was void of any effect on β-Catenin-induced TOPFlash (Figure S4C). Finally, expression of TGIF failed to enhance β-Catenin.S33Y-induced TOPFlash as compared to wild-type β-Catenin (Figure S4C), providing further support to the notion that TGIF contributes to Wnt signaling by fostering β-Catenin stability.

In our attempts to validate this scenario under physiological conditions, we found that depleting TGIF in MCF7 or HMLE cells suppressed Wnt3a-induced expression of six endogenous Wnt target genes: *CCND1*, *MYC*, *LEF1*, *DKK1*, *AXIN2*, and *LGR5* (Figures 4A, 4B). This inhibitory effect was not due to changes in cell proliferation, since depletion of TGIF did not affect progression through the cell cycle under these experimental conditions (Figure S4D). Similar gene expression profiles and cell cycle progression results were obtained using MEFs from wild-type and *Tgif1*$^{-/-}$ mice (Figures S4E, S4F, S4G). To further substantiate our findings in vivo, we crossed *Tgif1* knockout mice with *MMTV-Wnt1* mice, which harbor constitutive Wnt signaling in the mammary gland (Tsukamoto et al., 1988). Using eight mice in each group, we found that *Tgif1* deletion markedly blocked Wnt1-induced gene expression (Figures 4C, 4D). During the course of these in vivo analyses, we

generated two mammary cancer cell lines (MWT23 and MWT37) from *MMTV-Wnt1* mice bearing a floxed *Tgif1* allele along with a Tamoxifen-inducible Cre recombinase (*MMTV-Wnt1;Tgif1*$^{fl/fl}$*;CAG-Cre-ER*^{T2}) (Shen and Walsh, 2005). We found that *Tgif1* deletion in MWT23 or MWT37 cells challenged with Tamoxifen (Tam) decreased expression of *Ccnd1*, *Myc*, *Lef1*, *Dkk1*, *Axin2*, and *Lgr5* (Figures 4E, 4F, S4H, S4I). This effect is likely due to *Tgif1* deletion, as add-back of TGIF in these cells restored Wnt signaling to levels similar to those seen in the parental MWT23 and MWT37 cell lines (Figures 4E, 4F, S4H, S4I). Together, these data strongly suggest that TGIF functions as a key component of Wnt signaling, most likely by restricting movement of Axin1 or Axin2 into the cytoplasm and thereby enforcing β-Catenin accumulation.

TGIF is a Wnt Target Gene

We noticed in our earlier experiments that Wnt3a stimulation resulted in increased accumulation of the TGIF protein in MEFs, MCF7, and HMLE cells (Figures 3E, 4B). We confirmed this observation in vivo by demonstrating increased TGIF abundance in two mouse models of activated Wnt signaling: *MMTV-Wnt1* and *Dkk1*$^{+/-}$ (Figure 5A) (Mukhopadhyay et al., 2001; Tsukamoto et al., 1988). Stimulation of MEFs with Wnt3a or expression of Wnt1 in *MMTV-Wnt1* mice induced accumulation of *Tgif1* mRNA (Figures S5A, S5B). A ChIP experiment demonstrated that stimulation of HMLE cells with Wnt3a increased binding of β-Catenin or TCF4 to a TCF-binding element in the *TGIF1* promoter (Figure 5B). Consistent with this finding, Wnt signaling activation in HMLE cells expressing a Dox-inducible β-Catenin.S33Y increased the expression of the TGIF protein and mRNA (Figure 5C). Conversely, suppressing Wnt signaling in HMLE cells expressing an inducible-dominant negative form of TCF4 (DN-TCF4) (Korinek et al., 1997) blunted Wnt3a-induced accumulation of the TGIF protein and mRNA (Figure 5D). Additionally, depleting β-Catenin by two independent Dox-inducible shRNAs in the human mammary cancer cell line MDA-MB231, which harbors constitutive autocrine Wnt signaling (Bafico et al., 2004), was associated with decreased expression of the TGIF protein and mRNA (Figures S5C, S5D). Finally, we detected a significant decrease in TGIF expression in mice bearing conditional deletion of β-Catenin (Figure 5A) (Tan et al., 2008), further emphasizing *Tgif1* as a direct Wnt target gene.

The aforementioned findings suggest that TGIF orchestrates a feed-forward loop to propagate Wnt signaling once activated. To further explore the involvement of TGIF in Wnt signaling under normal homeostatic conditions, we assessed the long-term effects of Wnt signaling on TGIF expression in MCF7 cells. In a time-course experiment, Wnt3a stimulation induced a robust increase in the TGIF protein level after 24 hr, but this induction gradually declined, returning to the basal level at 96 hr (Figure 5E). Similar results were obtained when the expression of the *TGIF1* mRNA was analyzed (Figure S5E). During the course of these experiments, we also evaluated the expression of *CNND1*, *MYC*, *DKK1*, and *LEF1* and observed similar transient responses to Wnt signaling (Figure S5E). In light of our earlier findings that TGIF promotes Wnt signaling, it is tempting to suggest that the decline in TGIF expression driven by prolonged Wnt3a stimulation might enable downregulation of Wnt signaling to an extent that is suitable for maintaining normal cell function and homeostasis.

Expression of TGIF Recapitulates Activation of Wnt Signaling in TNBC

To further investigated the physiological interplay between TGIF and Wnt signaling, we examined whether TGIF expression in human cancer correlated with activation of Wnt signaling, focusing on TNBC, which has been shown to display recurrent hyperactivation of Wnt/β-Catenin signaling (Cowin et al., 2005). We initially interrogated the Cancer Genome Atlas (TCGA) breast cancer database and found that TGIF expression increased with tumor stages when compared to normal tissues (Figure S5F). To confirm these results, we conducted immunohistochemistry staining using human tissue microarrays (TMA) containing 173 normal and breast cancer samples. Relative to adjacent normal tissues, an increase in TGIF expression was detected in tumors at any stages examined, although the amplitude of such change tends to increase in more advanced stages, similar to our TCGA analysis (Figures 5F, S5F, S5G). Intriguingly, we detected a correlation between the expression of TGIF and β-Catenin in TNBC as compared to other breast cancer subtypes (Figure 5F), indicating that TGIF overexpression is associated with constitutive activation of β-Catenin in TNBC, which is in line with recurrent activation of Wnt signaling in TNBC (King et al., 2012). To determine whether TGIF overexpression per se could trigger constitutive Wnt signaling, we analyzed two derivatives of MCF7 harboring a Dox-inducible TGIF. Inducing TGIF expression resulted in increased β-Catenin protein without affecting its mRNA level (Figures 5G, S5H), supporting our concept that TGIF promotes β-Catenin accumulation by preventing its clearance by the destruction complex. Noteworthy, inducing TGIF expression also elicited increased expression of several Wnt target genes (Figure S5H), confirming that TGIF overexpression is sufficient to trigger constitutive activation of Wnt/β-Catenin signaling in human breast cancer cells.

To cross-validate our patient-oriented findings, we analyzed four published gene expression datasets of human breast cancers (Miller et al., 2005; Pawitan et al., 2005; Wang et al., 2005; Yu et al., 2008) using combined expression of the Wnt target genes *CCND1*, *MYC*, *DKK1*, *LEF1*, *AXIN2*, and *LGR5* as a minimal signature. Here again, we found a strong correlation between the expression of *TGIF1* and activation of Wnt signaling in all datasets examined (Figure S5I).

Finally, we investigated whether the TGIF expression level correlated with the outcome of patients with TNBC. For this we made use of a dataset containing exclusively TNBC, which enabled us to generate two groups with “high” and “low” TGIF expression and divide these two groups into subtypes of TNBC (see supplemental experimental procedures). Elevated expression of TGIF trended toward relapse and patient death (Figures 5H, S5J). Of note, this trend holds true for all TNBC subtypes, including mesenchymal, basal-like 1, basal-like 2, immunomodulatory, mesenchymal stem–like, and luminal androgen receptor (data not shown).

TGIF Contributes to Wnt-Induced Mammary Tumorigenesis

Having established a link between TGIF and Wnt signaling in TNBC, we investigated if expression of TGIF affected growth of mammary tumors harboring hyperactive Wnt signaling and found that *Tgif1* ablation abolished Wnt1-induced tumorigenesis (Figures 6A, S6A). During the observation period, all 25 *MMTV-Wnt1* mice developed tumors, whereas

none of the 20 *MMTV-Wnt1;Tgif1*$^{-/-}$ did (Figure 6B). Interestingly, only 5 out of 23 *MMTV-Wnt1;Tgif1*$^{+/-}$ mice developed one tumor each that became palpable markedly later (Figures 6C, S6B), indicating that the *Tgif1* gene dosage is sufficient to affect tumor fate. Noteworthy, *Tgif1* deletion in the wild-type background did not affect normal mammary development or architecture, supporting the hypothesis that TGIF deficiency hampers mammary tumor formation in *MMTV-Wnt1* mice by impeding oncogenic Wnt signaling (Figure 6D, and data not shown). To further appreciate the impact of TGIF on Wnt1-induced tumorigenesis, we investigated whether *Tgif1* ablation could override the hyperplasic mammary phenotype typically seen in *MMVT-Wnt1* mice. Vis-à-vis the wild-type counterpart, *MMTV-Wnt1* mice exhibited widespread hyperplasia characterized by extensive ductal side branching and aberrant lobular structures (Figure 6D), as previously described (Tsukamoto et al., 1988). *Tgif1* ablation in *MMTV-Wnt1* mice blocked the development of the hyperplasic phenotype, yielding mammary gland with almost normal morphogenesis (Figure 6D). Further histopathological analyses documented that all *MMTV-Wnt1;Tgif1*$^{+/+}$ mice developed lobuloalveolar adenocarcinoma, whereas all *MMTV-Wnt1;Tgif1*$^{-/-}$ mice remained free of such tumor (Figure 6E).

To determine whether overexpression of TGIF could phenocopy the mammary tumor phenotype in *MMTV1-Wnt1* mice, we used our MWT23 and MW37 cell lines, which develop tumors upon transplantation into mammary fat pad of syngeneic mice. To generate cells overexpressing TGIF, we used a Flp-In-dependent recombination system to achieve integration of one or two copies of TGIF in MWT23 and MWT37 cells at the same genomic loci, thereby avoiding any clonal heterogeneity that could arise owing to random integration of variable copy numbers or silencing after integration within the same cell population. Western blot analysis showed that integration of a single copy yielded an expression level approaching that of endogenous TGIF, whereas integration of two copies yielded nearly twice the endogenous level (Figure 7A). In transplantation assays, deletion of endogenous *Tgif1* (by Tam stimulation) suppressed mammary tumor formation, and this effect was reversed by expression of a single copy of TGIF (Figure 7B). More importantly, MWT23 and MWT37 cells harboring two TGIF copies exhibited an increase in mammary tumor growth as compared to the parental cells (Figure 7B), providing strong evidence that overexpression of TGIF promotes mammary tumor pathogenesis.

To investigate whether TGIF promotes mammary tumor formation by a mechanism dependent on its ability to interfere with assembly of the β-Catenin destruction complex, we performed in vivo epistasis experiments between TGIF and Axin1/Axin2 using MWT23 and MWT37 cells. The results showed that *Tgif1* deletion was ineffective in suppressing tumor growth of MWT23 and MWT37 cells stably expressing two different sets of shRNAs targeting Axin1 and Axin2, when compared to cells stably expressing control shRNA (Figures 7C, 7D). Accompanying biochemical and gene expression profiling experiments demonstrated that *Tgif1* deletion decreased the abundance of the β-Catenin protein and *Ccnd1*, *Myc*, *Lef1*, and *Dkk1* mRNAs in control cells, but failed to do so in cells depleted for Axin1 and Axin2 (Figures 7C, S7). Collectively, these results suggest TGIF contributes to Wnt1-induced mammary tumor formation by impeding the ability of Axin1/Axin2 to coordinate β-Catenin degradation.

TGIF Promotes Wnt Signaling by a Mechanism Independent of TGF-β Signaling

In light of its ability to restrict TGF-β cytostatic signaling (Seo et al., 2006; Wotton et al., 1999), we wondered whether *Tgif1* deletion could restrain Wnt1-induced mammary tumor pathogenesis merely by implementing sustained TGF-β signaling rather than suppressing Wnt signaling. However, under conditions in which six mock-treated *MMTV-Wnt1;Tgif1*$^{+/+}$ mice developed tumors, six *MMTV-Wnt1;Tgif1*$^{-/-}$ mice treated with a TGF-β receptor inhibitor (SB-505124) were still free of tumors, despite the apparent blockade in TGF-β signaling, as evidenced by the impaired phosphorylation of Smad2 (Figure S8A). To confirm this result, we conducted transplantation experiments using TGIF-deficient MWT23 or MWT37 cells stably expressing a dominant negative form of TβRII (DN-TβRII) or β-Catenin.S33Y as a control. In contrast to β-Catenin.S33Y, expression of DN-TβRII failed to rescue mammary tumor formation (Figure 8A). Rather, we noticed that expressing DN-TβRII exacerbated the decrease in mammary tumor formation, which is in agreement with previous observations that activation of TGF-β signaling promotes MMTV-Wnt1-induced mammary tumorigenesis (Labbe et al., 2007). In control experiments, expression of DN-TβRII blocked Smad2 phosphorylation, attesting to the functionality of our design approach (Figure S8B).

To further investigate the relative importance of regulating the TGF-β signaling and the Wnt signaling, we used the TGIF mutant TGIFΔAxin2, which does not interact with Axin2 or activate Wnt signaling but inhibits TGF-β signaling (Seo et al., 2006) (Figure S8C, S8D). In reconstitution experiments using MWT23 or MWT37 cells, expression of wild-type TGIF restored Wnt1-induced mammary tumor formation, whereas expression of TGIFΔAxin2 was ineffective (Figure 8B). We conclude that TGIF contributes to Wnt-induced gene expression and mammary tumor formation by a mechanism dependent on its ability to foster Wnt signaling rather than suppress TGF-β signaling.

DISCUSSION

A key regulatory step in Wnt signaling is the regulation of β-Catenin abundance by a cytoplasmic destruction complex composed of Axin1 or Axin2, APC, GSK3, CK1, and βTRCP. Axin1 and Axin2 function as scaffolding platforms for this β-Catenin destruction complex, coordinating consecutive phosphorylations of β-Catenin by CK1 and GSK3 that marks β-Catenin for ubiquitination by βTRCP, leading to β-Catenin degradation by the proteasome (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004). Because Axin1 and Axin2 are deemed to be the rate-limiting constituents of the destruction complex, the vast majority of the destruction complex-oriented studies were devoted to delineating the mechanisms governing the availability and/or abundance of Axin1 and Axin2 in the cytoplasm, including sequestration by LRP5/6, ADP-ribosylation by tankyrase, or phosphorylation by GSK3 (Huang et al., 2009; Kim et al., 2013; Mao et al., 2001). However, since Axin1 and Axin2 shuttle between the cytoplasm and nucleus (Cong and Varmus, 2004; Yook et al., 2006), one would envision that other mechanisms might also impose a control over assembly of the destruction complex. Our present study reveals an interaction between TGIF and Axin1 or Axin2 in the nucleus that seems to play an important role in Wnt signaling.

Our results clearly demonstrate that TGIF sequesters Axin1 or Axin2 into the nucleus and thereby interfering with the assembly of the β-Catenin destruction complex, leading to β-Catenin accrual. Consistent with this observation, TGIF is essential for activation of the Wnt transcriptional program both in vitro and in vivo. Intriguingly, activation of Wnt signaling induces expression of TGIF, suggesting that TGIF might function to coordinate a feed-forward loop that ensures effective integration of Wnt signaling. Congruent with our findings, *Tgif1* knockout mice display phenotypes similar to those observed in *Wnt2* knockout mice, including abnormal placental defects, premature death, and runting (Bartholin et al., 2008; Monkley et al., 1996). Likewise, both *Tgif1* and *Axin2* knockout mice display craniofacial abnormalities (Taniguchi et al., 2012; Yu et al., 2005), further supporting the link of TGIF to canonical Wnt signaling.

Based on our findings, we propose a model in which TGIF plays active roles in both the absence and presence of Wnt signaling by limiting the cytosolic pools of Axin1 or Axin2 that are typically dedicated to the assembly of the β-Catenin destruction complex (Figure 8C). Taking into consideration the current prevailing models of Wnt signaling (Clevers and Nusse, 2012; Li et al., 2012; MacDonald et al., 2009; Moon et al., 2004), we speculated that this antagonistic mechanism might function to fine tune the destruction complex assembly to enable β-Catenin to accumulate to an extent that ensures integration of suitable levels of endogenous Wnt signaling needed to maintain normal cell function and tissue homeostasis. Alternatively, TGIF could function at steady state conditions to facilitate saturation of the destruction complex by newly synthesized β-Catenin, which in turn enables cells to respond efficiently to an acute activation of Wnt signaling. Finally, it is also conceivable that TGIF might function to implement sustained Wnt signaling depending on the pathophysiological setting, cell behaviors, or alterations in components of the destruction complex other than Axin1 or Axin2. As such, the molecular framework that we propose will likely contribute to the elucidation of mechanistic paradigms of the destruction complex irrespective of its fate (i.e., saturation, disassembly) subsequent to activation of Wnt signaling.

Our study also underscores an ability of TGIF to promote Wnt-driven mammary tumor pathogenesis. Interestingly, both pharmacological and genetic experiments demonstrate that TGIF promotes mammary hyperplasia in MMTV-Wnt1 mice by activating Wnt signaling rather than repressing TGF-β signaling. Recent xenograft studies showed that activation of Wnt signaling can either promote growth or trigger squamous differentiation of human breast cancer cells depending on the cellular context (Green et al., 2013). Since deletion of *Tgif1* in *MMTV-Wnt1* mice was effective in blocking mammary tumor, one would suggest that TGIF might function as a key component of oncogenic Wnt signaling, although we cannot rule out the possibility that TGIF also contributes to Wnt-driven squamous cell differentiation under other conditions. Further studies will be required to clarify this issue.

Although our analyses revealed increased TGIF expression in TNBC, we did not find *TGIF1* amplification. Moreover, we found only one missense mutation (substitution R228 to I) in *TGIF1* in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database, further ruling out the existence of significant genetic alterations that could account for the deregulation of TGIF expression in this malignancy. Recently, we reported that the gene encoding PHRF1, an E3 ubiquitin ligase that drives TGIF degradation, was deleted in 21.6% of human breast

cancers (Ettahar et al., 2013). On the basis of this observation, it is appealing to speculate that recurrent deletion of *PHRF1* in breast cancer could represent one potential mechanism that governs TGIF accumulation. However, initial analysis of datasets available in public databases failed to reveal any significant correlation between *PHRF1* deletion and *TGIF1* gene expression in TNBC or other breast cancer subtypes, suggesting that accumulation of TGIF may involve additional mechanisms. Thus, an in-depth understanding of the molecular mechanisms behind the increased TGIF expression in TNBC will require more extensive and comprehensive investigations.

From a translational perspective, we validated the relationship between TGIF and Wnt signaling in human breast cancer. Notably, we found a correlation between the TGIF expression levels and activation of Wnt signaling in breast cancers (Miller et al., 2005; Pawitan et al., 2005; Wang et al., 2005; Yu et al., 2008). Moreover, our analyses revealed that TGIF and β-Catenin are overexpressed in a high proportion of TNBC samples as compared to normal tissue or other breast cancer subtypes. Finally, we detected a trend toward a poorer outcome for the TNBC with a higher expression of TGIF. Besides these findings with potential prognostic impact, our data also highlighted a requirement of TGIF for Wnt-driven mammary tumor formation in vivo, giving rise to another framework for future investigation of TGIF as a potential target for anti-cancer drug design to curb aberrant Wnt signaling in TNBC, and possibly other malignancies harboring hyperactive Wnt signaling.

In summary, our data unveil a functional interplay between TGIF and Axin1 or Axin2 that appears to evolve into a feed-forward loop to sustain Wnt signaling. Since TGIF appeared to contribute to Wnt-induced mammary hyperplasia, this amplification mechanism could conceptually deepen oncogenic Wnt signaling capabilities to an extent that ultimately compromises tissue homeostasis, culminating in neoplasia. Thus, by identifying TGIF as an essential component of Wnt signaling, our findings provide important insights into mechanistic paradigms of aberrant Wnt signaling in human malignancies.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screen

The yeast two-hybrid screen was performed using a fragment of human TGIF (amino acid 1-192) as bait, essentially as previously described (Colland et al., 2004). The TGIF fragment was obtained by PCR and cloned into the pB27 plasmid as a C-terminal fusion to LexA. Subsequently, the bait construct was transformed into the L40ΔGAL4 yeast strain. As a prey, a random-primed cDNA library from human placenta poly(A^+)-RNA was constructed into pP6. The library was transformed into the Y187 strain and mated to the bait strain. The screening conditions were adapted in order to test a minimum of 50 million interactions. The selectivity of the His3 reporter was modulated with 3-aminotriazole (3AT). His+ colonies were selected on a medium lacking tryptophan, leucine, and histidine and the prey fragments were sequenced. Axin2 was one of the preys that exhibit strong and specific binding to TGIF.

Cell Lines and Culture

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. MCF7 and HMLE cells were chosen to study the link of TGIF to Wnt signaling because they were found to express detectable levels of components of this pathway that were investigated in this study.

Mice

MMTV-Wnt1 and CAG-Cre.ER^{T2} mice were obtained from the Jackson Laboratory. *Tgif1*$^{fl/fl}$, *Tgif1*$^{+/-}$, *Dkk1*$^{+/-}$, and liver-specific *Ctnnb1* knockout (*Ctnnb1*$^{fl/fl}$*;Foxa3.Cre*) mice were described previously (Mukhopadhyay et al., 2001; Shen and Walsh, 2005; Tan et al., 2008). Experimental animals were kept as virgins throughout the study.

For transplantation experiments, MWT23 and MWT37 cells were injected into mammary fat pad of 8-week old FVB mice and tumor volumes were measured every week. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Harvard Medical School or University of Mississippi Medical Center.

Immunoprecipitation and Immunoblotting

Cell extracts were prepared as previously described (Demange et al., 2009; Ettahar et al., 2013) and subjected to immunoprecipitation with the appropriate antibody for 2 hr, followed by adsorption to Dynabeads® Protein G (Invitrogen) for 1 hr. The samples were then analyzed by SDS-polyacrylamide gel and immunoblotting. We used 500 to 1,000 μg and 50 to 100 μg of total proteins for immunoprecipitation and immunoblotting, respectively, depending on the cell line. Quantification of the association of Axin1/2 with TGIF was determined by scanning laser densitometry.

Immunofluorescence and Immunohistochemistry

Cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.1% triton X-100, and incubated with the primary antibodies or IgG-matched isotype control antibody at room temperature for 2 hr. Then cells were incubated with the secondary antibodies conjugated to Alexa-Fluor®568 or Alex-Fluor®448 and stained with DAPI. Slides were viewed on a fluorescence microscope.

For analysis of mouse mammary glands by immunohistochemistry, tissues were fixed in 10% formalin and embedded in paraffin. For human TMA analysis, we used slides containing sections from paraffin embedded normal or breast cancer samples. Mouse or human sections were deparaffinised with xylene and rehydrated in a graded series of ethanol. Antigen-retrieval was performed for 10 min at high temperature in citrate buffer. Then, slides were blocked and incubated overnight with anti-TGIF, anti-β-Catenin or IgG-matched isotype control antibody (negative control) at 4°C. Finally, slides were incubated with the secondary antibody coupled to peroxidase for 30 min, incubated with peroxidase substrate, and mounted. The expression of TGIF or β-Catenin was visualized using a bright-field microscope.

The paraffin embedded human samples (173) were purchased from US, Biomax, Inc. The use of these human samples was exempted from approval by the Institutional Review Board of INSERM or University of Mississippi Medical Center.

Real-Time PCR

cDNA synthesized with RNeasy-isolated RNA and SuperScript 3 (Qiagen and Invitrogen, respectively was mixed with iQ SYBR green supermix (BioRad) and sense and antisense oligonucleotides (500 nM). After cDNA amplification (40 cycles), samples were normalized to GAPDH or Cyclophilin A and data were expressed as mean ± SD of 3 to 8 independent samples. The primer sequences are available upon request.

Chromatin Immunoprecipitation (CHIP)

To analyze binding of β-Catenin or TCF4 to the *TGIF1* promoter, cells were cultured under standard conditions, fixed, and sonicated using a standard protocol. Sonicated chromatin was then immunoprecipitated using antibodies against β-Catenin or TCF4, or isotype matched control IgG. PCR was run on the chromatin and the products were analyzed in a 2% agarose gel. To examine the effect of Wnt3a stimulation on β-Catenin or TCF4 binding to the *TGIF1* promoter, cells were treated with control or Wnt3a CM for 24 hr before being processed for chromatin sonication and immunoprecipitation with antibodies against β-Catenin or TCF4, or isotype matched control IgG. Relative DNA binding was determined by qRT-PCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANCE

Constitutive activation of Wnt signaling pathway is commonly observed in TNBC but the underlying mechanisms remain poorly understood. Here, we show that TGIF orchestrates a feed-forward network to sustain Wnt signaling in mammary epithelial cells. This mechanism could be physiologically meaningful to TNBC diagnosis, as elevated levels of TGIF are correlated with high Wnt signaling and a trend toward poor survival. We also provide data showing the requirement of TGIF for Wnt-driven mammary tumorigenesis in vivo. Thus, by demonstrating that TGIF functions as an essential component of the Wnt signaling pathway and documenting its implication in oncogenic Wnt signaling in the mammary gland, our findings shed light on the molecular mechanisms behind the constitutive activation of Wnt signaling in TNBC.

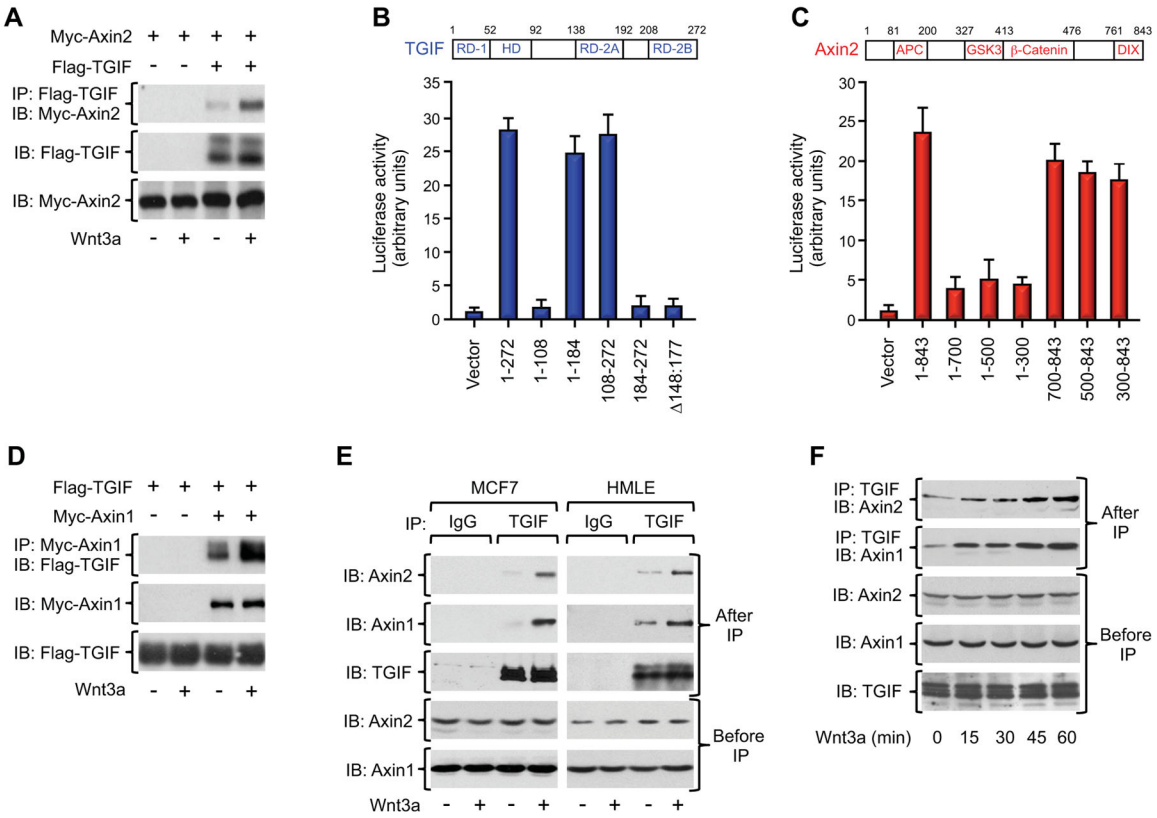

Figure 1. Interaction of TGIF with Axin1/2

(**A**) HEK293T cells were transfected with Myc-Axin2 in the presence or absence of Flag-TGIF and treated with control or Wnt3a conditioned media (CM) for 1 hr. Lysates were subjected to anti-Flag immunoprecipitation (IP) followed by immunoblotting (IB) with anti-Myc. In this and all the following experiments, expression of proteins under investigation was determined by direct immunoblotting.

(**B**, **C**) HEK293T cells were transfected with pG5E1β-Luc together with Gal4-Axin2 and VP16-TGIF mutants (B) or Gal4-TGIF and VP16-Axin2 mutants (C). Luciferase activity was measured and data were expressed as mean ± SD of 3 independent samples.

(**D**) HEK293T cells were transfected with Flag-TGIF in the absence or presence of Myc-Axin1 and treated with control or Wnt3a CM for 1 hr. Cell lysates were subjected to anti-Myc immunoprecipitation followed by immunoblotting with anti-Flag.

(**E**) MCF7 or HMLE cells were treated with control or Wnt3a CM for 1 hr and cell lysates were immunoprecipitated with IgG or anti-TGIF before being analyzed by immunoblotting using anti-Axin1, anti-Axin2, or anti-TGIF.

(**F**) HMLE cells were treated with Wnt3a CM for the indicated times and cell lysates were immunoprecipitated with anti-TGIF before being analyzed by immunoblotting using anti-Axin1 or anti-Axin2.

See also Figure S1.

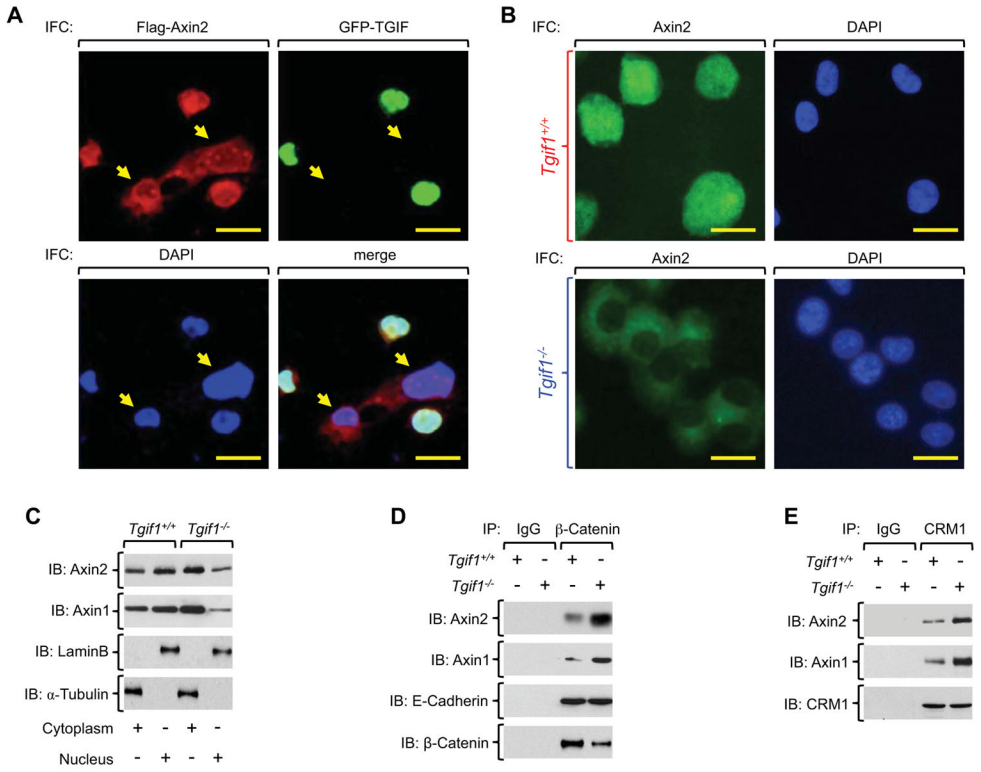

Figure 2. TGIF Interferes with the Nucleocytoplasmic Transit of Axin1/2

(**A**) COS-7 cells were transfected with Flag-Axin2 and GFP-TGIF and immunostained with anti-Flag and DAPI. Scale bars, 100 μM.

(**B**) Wild-type or *Tgif1*$^{-/-}$ MEFs were immunostained with anti-Axin2 and DAPI. Scale bars, 100 μM.

(**C**) Cytoplasmic or nuclear fractions from wild-type or *Tgif1*$^{-/-}$ MEFs were immunoblotted with anti-Axin1 or anti-Axin2. Purity of the nuclear and cytoplasmic fractions was verified by immunoblotting using anti-Lamin B or anti-α-Tubulin.

(**D, E**) Lysates from wild-type or *Tgif1*$^{-/-}$ MEFs were immunoprecipitated with anti-β-Catenin (D) or anti-CRM1 (E) and analyzed by immunoblotting with the indicated antibodies.

See also Figure S2.

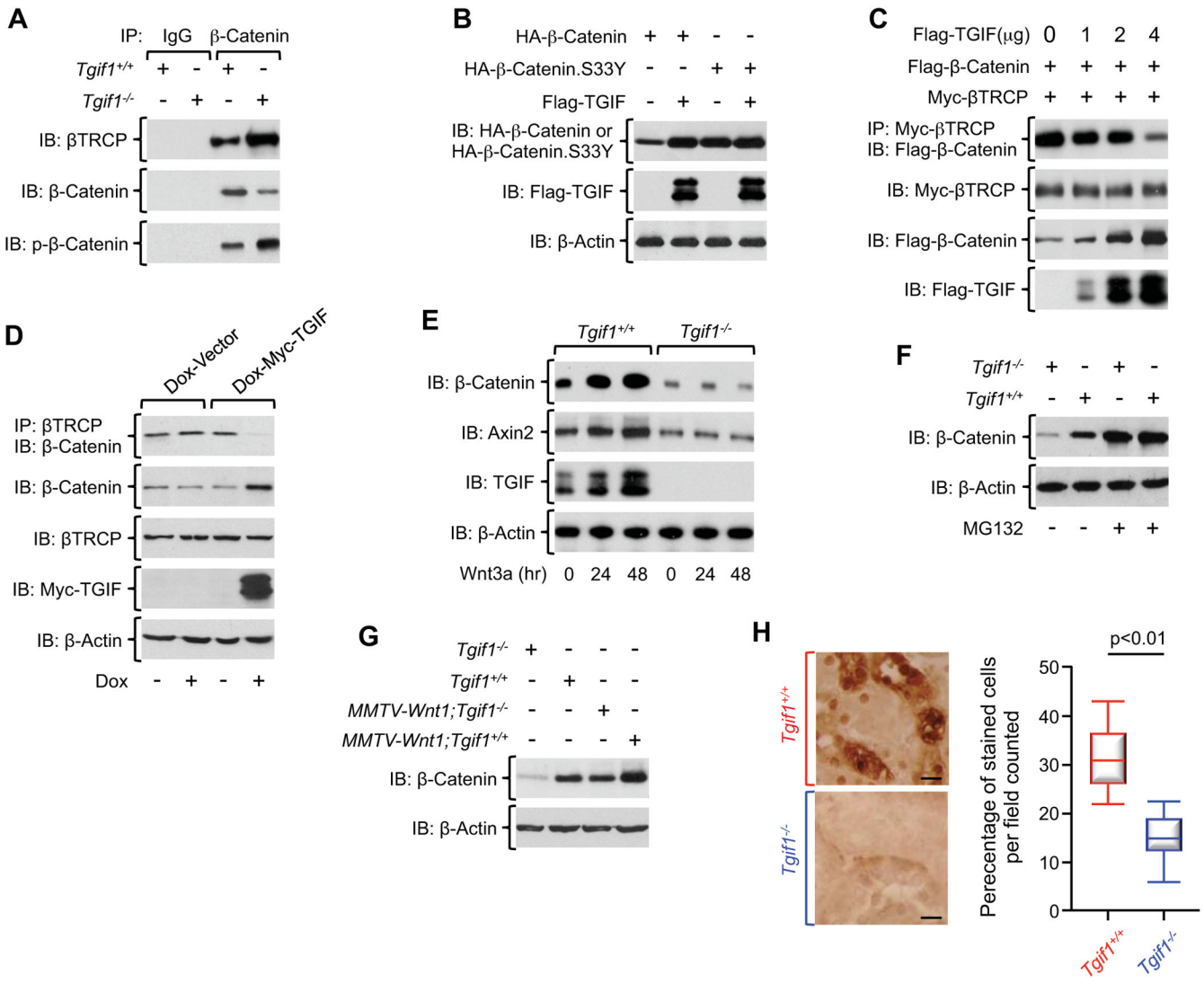

Figure 3. TGIF Interferes with β-Catenin Degradation

(**A**) Lysates from *Tgif1*$^{+/+}$ or *Tgif1*$^{-/-}$ MEFs were immunoprecipitated with IgG or anti-β-Catenin and analyzed by immunoblotting using the indicated antibodies.

(**B**) MCF7 cells were transfected with HA-β-Catenin or HA-β-Catenin.S33Y in the absence or presence of Flag-TGIF and cell lysates were immunoblotted with anti-HA or anti-Flag.

(**C**) HEK293T cells were transfected with Flag-β-Catenin together with Myc-βTRCP and increasing amounts of Flag-TGIF. Association of β-Catenin with βTRCP was analyzed by blotting anti-Myc immunoprecipitates with anti-Flag.

(**D**) MCF7-Dox-Myc-TGIF cells were cultured with or without doxycycline (Dox) for 48 hr. Association of β-Catenin with βTRCP was determined by blotting anti-βTRCP immunoprecipitates with anti-β-Catenin.

(**E**) *Tgif1*$^{+/+}$ or *Tgif1*$^{-/-}$ MEFs were cultured with control or Wnt3a CM for the indicated times and lysates were immunoblotted with anti-β-Catenin, anti-Axin2, or anti-TGIF.

(**F**) *Tgif1*$^{+/+}$ or *Tgif1*$^{-/-}$ MEFs were treated with vehicle or MG132 for 12 hr and cell lysates were subject to immunoblotting using anti-β-Catenin.

(**G**) Expression of β-Catenin was analyzed using extracts from mouse mammary glands with the indicated genotypes.

(**H**) Analysis of active (non-phosphorylated) β-Catenin levels in mammary glands from *Tgif1*$^{+/+}$ or *Tgif1*$^{-/-}$ mice by immunohistochemistry. The percentage of stained cells in at least 20 independent fields is indicated. The line inside the box shows median percentage. The top and bottom of the box represent upper (third) and lower (first) quartile percentages, respectively. The lines above and below the box show maximum and minimum percentages, respectively. Scale bars, 50 μM.

See also Figure S3.

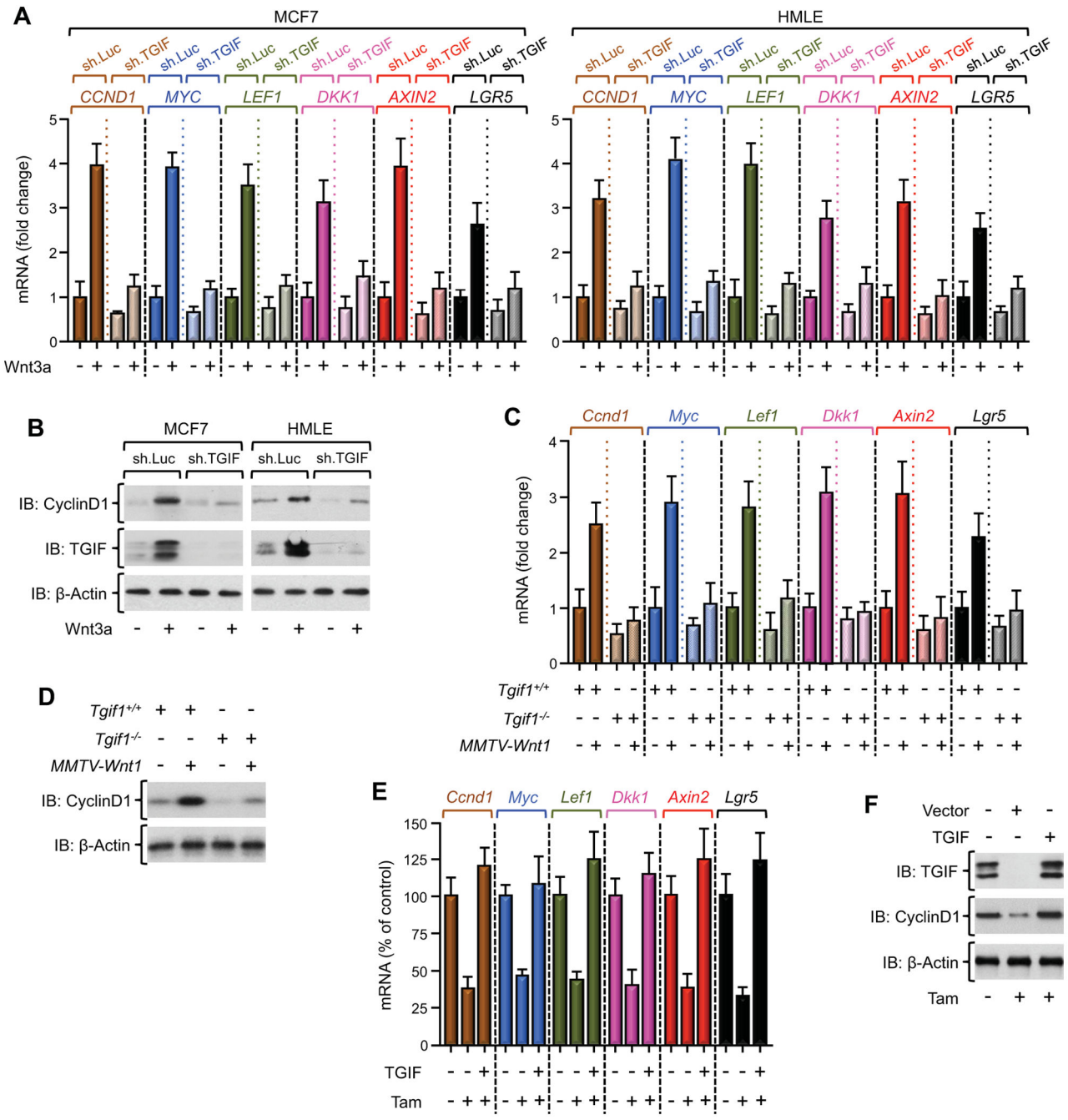

Figure 4. TGIF Promotes Wnt Signaling

(**A, B**) MCF7 or HMLE cells stably expressing control (sh.Luc) or TGIF (sh.TGIF) shRNAs were cultured with control or Wnt3a CM for 24 hr. Expression of Wnt target genes was analyzed by qRT-PCR (A) and the expression of CyclinD1 or TGIF was determined by immunoblotting (B).

(**C**, **D**) Expression of Wnt target genes in the mammary glands of mice with the indicated genotypes (8 in each group) was analyzed by qRT-PCR (C) and the expression of CyclinD1 was assessed by immunoblotting (D).

(**E**, **F**) MWT23 cells stably expressing empty vector or TGIF were treated with vehicle or Tamoxifen (Tam) for 5 days, and expression of Wnt target genes was analyzed by qRT-PCR (E) and the expression of CyclinD1 or TGIF was determined by immunoblotting (F). Results (A, C, E) are expressed as mean ± SD of 3 independent samples.
See also Figure S4.

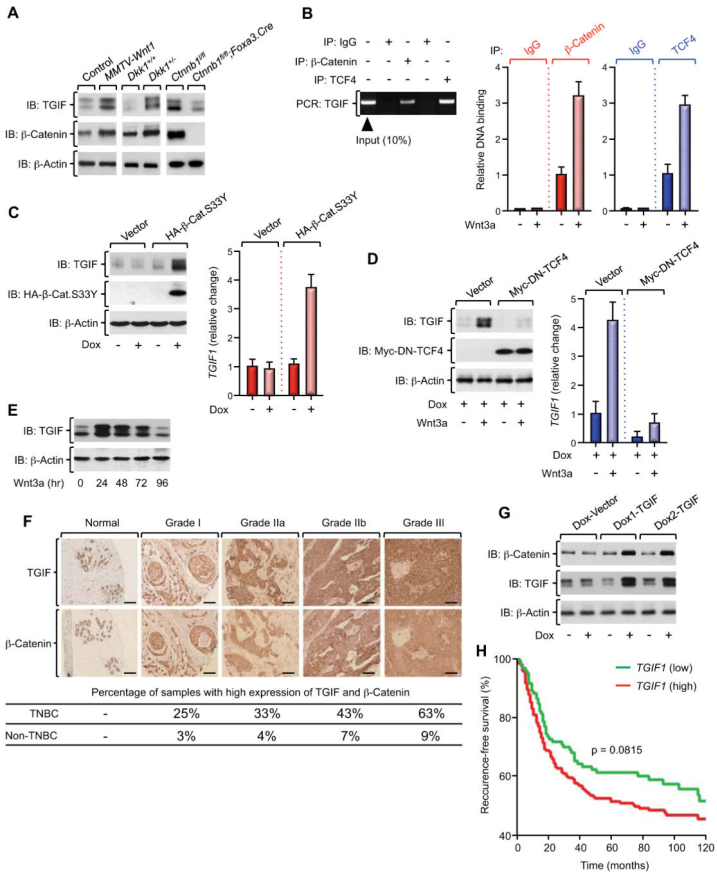

Figure 5. TGIF is a Wnt Target Gene

(**A**) Expression of TGIF or β-Catenin was assessed by immunoblotting using extracts from mammary glands of *MMTV-Wnt1* or *Dkk1* mice or liver from *Ctnnb1* conditional knockout.

(**B**) HMLE cells were left untreated or treated with control or Wnt3a CM for 24 hr and chromatin was immunoprecipitated with anti-β-Catenin, anti-TCF4, or IgG. Bound chromatin (in control cells) was amplified by PCR and analyzed by 2% agarose gel (left). Bound chromatin in control or Wnt3a-treated cells was analyzed by qRT-PCR.

(**C, D**) HMLE-Dox-HA-β-Catenin (C) or HMLE-Dox-Myc-DN-TCF4 (D) cells were treated with or without Dox for 48 hr before being cultured with or without Wnt3a CM for 16 hr. Expression of TGIF protein and mRNA was measured by immunoblotting and qRT-PCR, respectively.

(**E**) MCF7 cells were left untreated or treated with Wnt3a for different times and expression of TGIF was assessed by immunoblotting.

(**F**) Expression of TGIF and β-Catenin in tissue microarrays including 173 human breast cancer samples was analyzed by immunohistochemistry. The percentages of samples with high expression of TGIF and β-Catenin in TNBC and non-TNBC subtypes are shown. Scale bars, 50 μM.

(**G**) MCF7 cells were stably transfected with doxycycline-inducible TGIF (two independent populations) or empty vector and treated with or without Dox for 48 hr. Expression of β-Catenin or TGIF was determined by immunoblotting.

(**H**) Kaplan-Meier graph representing the probability of cumulative recurrence-free survival in TNBC patients according to the TGIF expression, low (128) versus high (255).
Results in B (right), C (right), and D (right) are expressed as mean ± SD of 3 independent samples from representative experiments performed at least three times.
See also Figure S5.

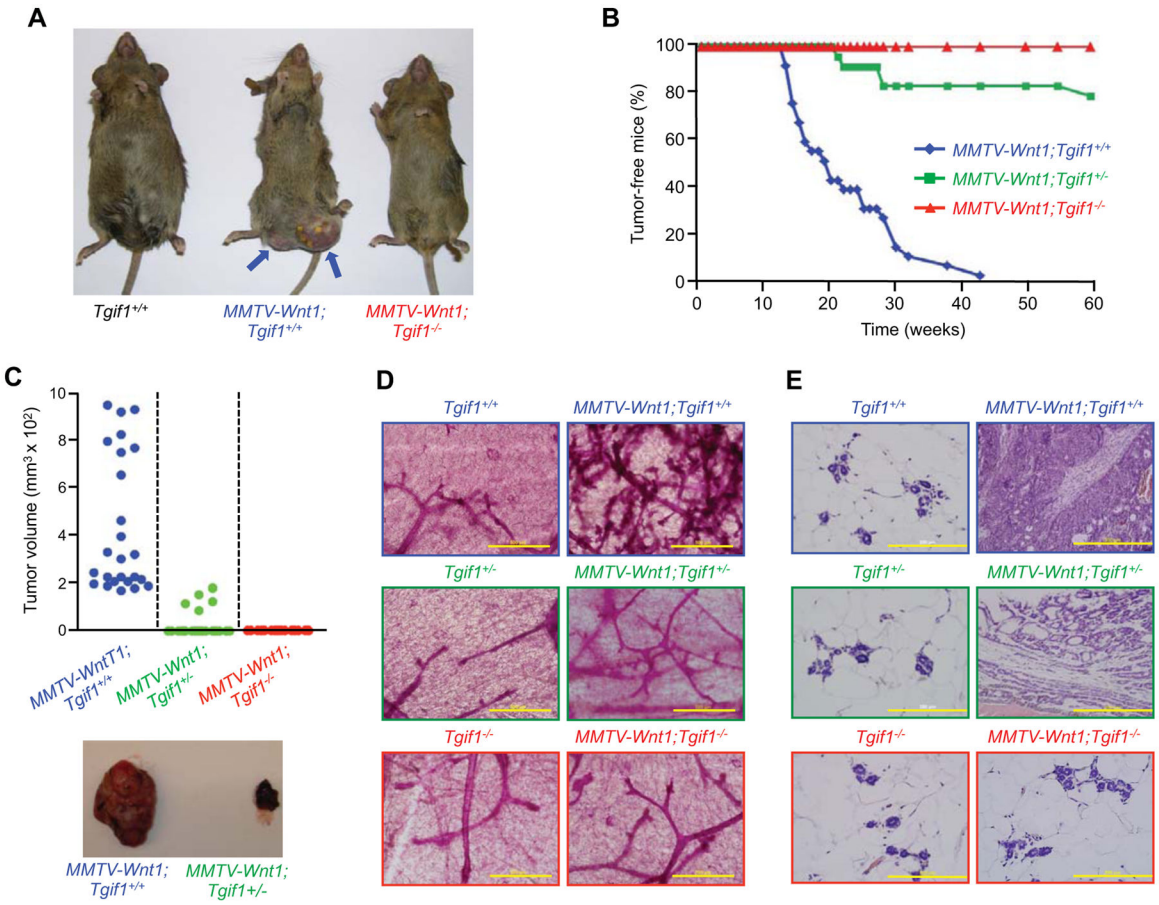

Figure 6. *Tgif1* deletion blocks Wnt1-Induced Mammary Tumors

(**A**) *Tgif1*$^{+/+}$, MMTV-Wnt1;*Tgif1*$^{+/+}$, or MMTV-Wnt1;*Tgif1*$^{-/-}$ mice were photographed three weeks after the tumors became palpable.

(**B, C**) MMTV-Wnt1 mice with *Tgif1*$^{+/+}$, *Tgif1*$^{+/-}$ or *Tgif1*$^{-/-}$ genotypes were palpated twice weekly from puberty onwards for 60 weeks and dates of tumor incidence were recorded (B). For comparison, tumor volumes at sacrifice were shown (C).

(**D**) Whole-mount staining of mammary glands from 4-month-old virgin mice with the indicated genotypes.

(**E**) H&E staining of mammary glands from mice with the indicated genotypes three weeks after tumor formation. Scale bars, 500 μM for D and 200 μM for E.

See also Figure S6.

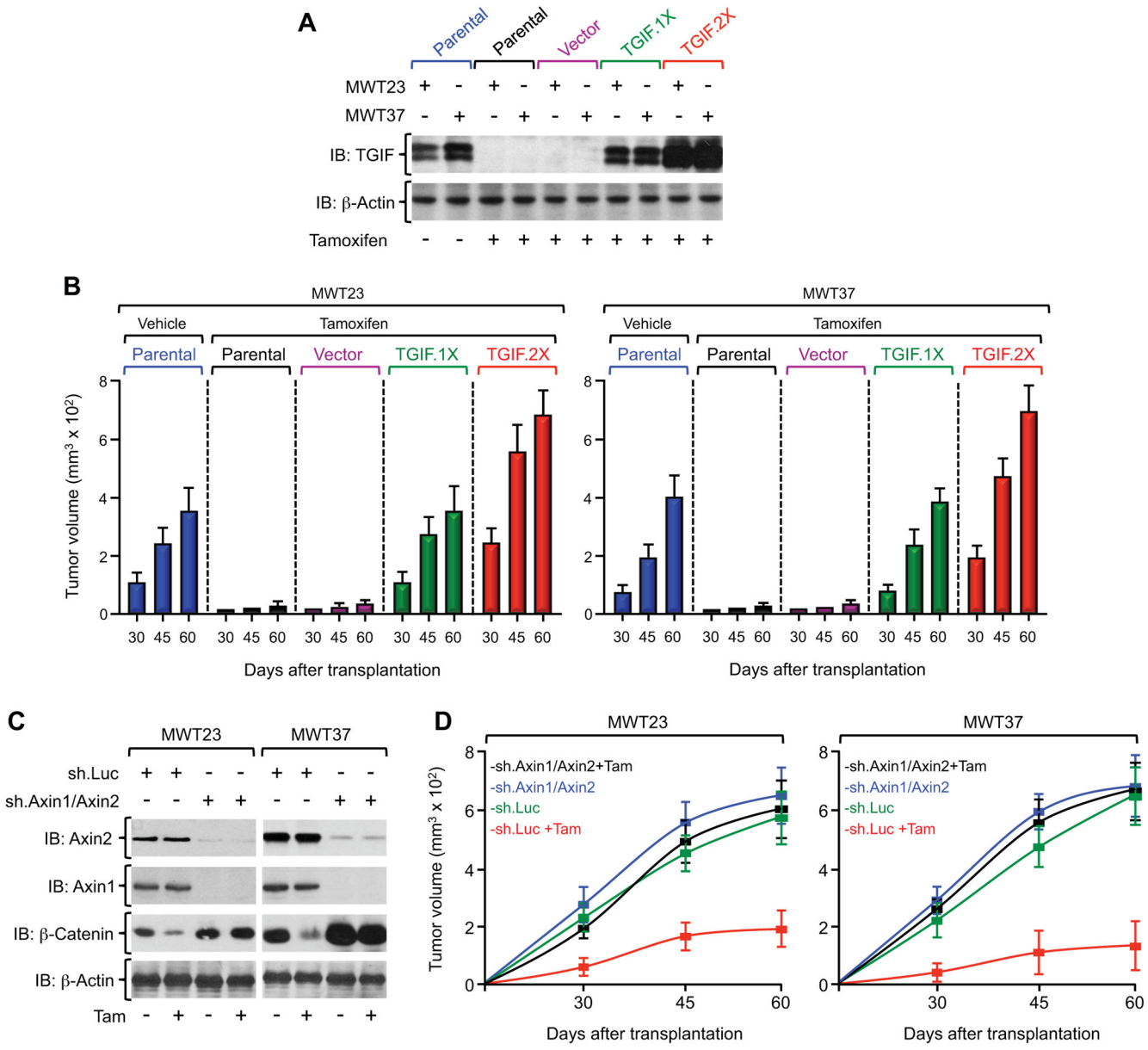

Figure 7. TGIF promotes Mammary Tumors by a Mechanism Dependent on its Ability to Antagonize Axin1/2

(**A**) Flip-In MWT23 or MWT37 cells carrying one or two copies of TGIF were treated with vehicle or Tam for 5 days. Expression of TGIF was assessed by immunoblotting.

(**B**) Cells (as in A, 10^5) were implanted into mammary fat pad of FVB mice and tumor volumes were measured at the indicated times.

(**C**) MWT23 cells stably expressing the indicated shRNAs were treated with vehicle or Tam for 5 days. Expression of Axin1, Axin2, or β-Catenin was assessed by immunoblotting.

(**D**) Cell (as in C, 5×10^5) were transplanted into mammary fat pad of FVB mice and tumor volumes were measured at the indicated times.

Results (B, D) are expressed as mean ± SD of measurements obtained with 6 animals in each group.

See also Figure S8.

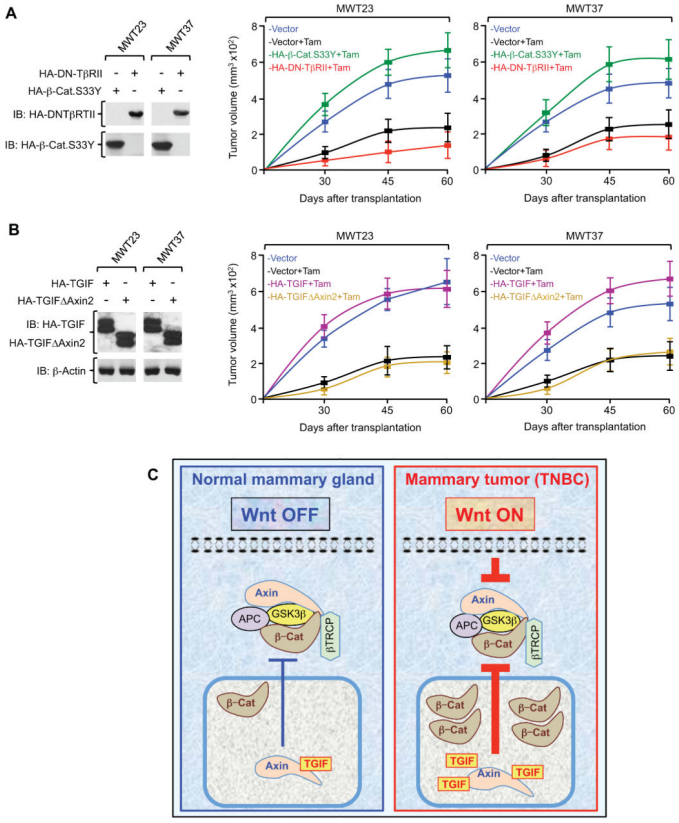

Figure 8. TGIF Promotes Wnt1-Induced Mammary Tumor Formation by a TGF-β-Independent Mechanism

(**A, B**) MWT23 or MWT37 cells stably expressing empty vector, HA-DN-TβRII, or HA-β-Catenin.S33Y (A) or empty vector, HA-TGIF, or HA-TGIFΔAxin2 (B) were treated with vehicle or Tam for 5 days. Expression of transfected proteins was determined by immunoblotting (left). Cells (5×10^5) were transplanted into mammary fat pad of FVB mice and tumor volumes were measured at the indicated times (right).

(**C**) A model depicting the link of TGIF to the Wnt signaling network.

Results (A, B) are expressed as mean ± SD of measurements obtained with 6 animals in each group.

See also Figure S8.