Targeted inhibition of Wnt signaling with a bacterial toxin fragment suppresses breast cancer tumor-initiating/chemo-resistant cells

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ABSTRACT

BRCA1 germ-line mutations are a major cause of hereditary breast cancer and BRCA1-deficient breast cancer shares many characteristics as sporadic basal-like breast cancer (BLBC). Effective therapeutic targets for BRCA1-deficient BLBC remain lacking. By utilizing a BRCA1-deficient BLBC mouse model based on intraductal injection of *Krt8-Cre* adenovirus to inactivate *Brca1* and *Trp53* in luminal mammary epithelial cells, here we report that the Wnt receptor Frizzled 7 (FZD7) serves as a biomarker and therapeutic target in the resulting mammary tumor cells and is particularly enriched in cancer stem cells / tumor-initiating cells (CSCs/TICs). Inhibiting FZD7-mediated Wnt signaling using a nontoxic FZD-binding fragment of *C. difficile* toxin B ($TcdB^{FBD}$) attenuates growth of BRCA1-deficient tumor organoids and xenografted tumors, without damaging Wnt-sensitive tissues such as bones *in vivo*. Finally, FZD1/2/7-positive cells are enriched in chemotherapy-resistant cells in both BLBC and luminal breast tumors treated with cisplatin, and $TcdB^{FBD}$ synergizes strongly with cisplatin in inhibiting both tumor types. These findings demonstrate the therapeutic value for targeting FZD1/2/7 in treating breast cancers and establish $TcdB^{FBD}$ as a potential therapeutic agent targeting TICs and chemotherapy-resistant cancer cells.

INTRODUCTION

Breast cancers are heterogeneous and different subtypes require distinct treatments (1,2). Targeted therapy in breast cancer is most successful when subtype-specific key pathways that drive cancer cell growth are defined and serve as therapeutic targets. Among breast cancer subtypes, estrogen receptor $(ER)^+$ luminal breast cancers are treated by endocrine therapy (e.g., aromatase inhibitor, tamoxifen) that targets the ER signaling pathway, whereas breast cancers with HER2 overexpression can be targeted by Trastuzumab (Herceptin), a monoclonal antibody that blocks HER2 signaling. Basal-like breast cancer (BLBC) largely overlaps with triple-negative breast cancer (TNBC), which lacks ER and progesterone receptor (PR) expression and HER2 overexpression (3). BLBC/TNBCs lack clear driver mutations, as evident from recent sequencing studies (4). Thus, treatment of BLBC/TNBCs relies on standard chemotherapy, with the worst prognosis among all breast cancer subtypes (5). In addition, even among luminal breast cancers, those belonging to the luminal B subtype have high proliferation index and often do not respond to endocrine therapy well; thus, they are treated by chemotherapy as well (6). Chemotherapy can eliminate the bulk of cancer cells, but inevitably therapy-resistant cancer cells emerge, which typically possess stem cell-like properties (7,8). In order to eliminate these cells, it is important to define key programs that sustain their stemness so that therapeutic approaches can be designed to target them.

Wnt/β-catenin signaling plays key roles in stem cell self-renewal and injury repair (9). Mutations in components of the Wnt pathways are well-established as a dominant causal factor in colorectal cancer and subgroups of many other solid tumors (9,10). The therapeutic value of targeting Wnt signaling in these tumors are well established using numerous animal models and by a number of broad-spectrum pan-Wnt signaling inhibitors (9,10,14). Upregulation of Wnt signaling without any mutations in the pathways has also been broadly implicated in cancer development, epithelial–mesenchymal transition (EMT), metastasis, chemotherapy-resistance, and immune escape of a broad range of human cancers (10,11,12), possibly reflecting a universal requirement of Wnt signaling in maintaining the stemness of cancer cells. For instance, Wnt pathway activation has been previously reported to be enriched in BLBC and predicts poor outcome (13), and activation of Wnt signaling (without mutations in Wnt pathways) is observed in >50% of human breast cancer cases and is linked to reduced overall survival (10). These broad range of tumors potentially can benefit from Wnt signaling inhibition, but the therapeutic

efficacy of this approach remains to be established and the side effect associated with pan-Wnt inhibition also diminishes the therapeutic value particularly on cancers without Wnt signaling pathway mutations such as breast cancers (15,16).

Wnt and its receptors, the 7-pass transmembrane protein Frizzled (FZDs), are a large family (9,17). One way to minimize side effects is to target the specific Wnt-FZD pair(s) enriched in cancer cells. However, such a narrow spectrum inhibition has yet to be achieved *in vivo* due to high degrees of homology among Wnt-FZD members. Concerns on potential redundant expression of multiple Wnt-FZD pairs also raise the doubt on whether inhibiting a specific subgroup is sufficient to achieve any therapeutic effects *in vivo*.

There are ten FZD members in humans, divided into four subgroups (FZD1/2/7, 5/8, 3/6, 4/9/10) (17). They contain only one relatively small extracellular domain on their N-termini, designated cysteine-rich domain (CRD, ~ 120-150 residues), which serves as the binding site for Wnt (17). As CRDs are highly conserved across all mammals and different FZD members, they are not good immunogens to generate high-affinity antibodies, let alone neutralizing antibodies capable of blocking Wnt binding and distinguishing FZD members. The most advanced Wnt-signaling inhibition antibody, OMP-18R5 (developed by OncoMed Inc.), which was identified through *in vitro* phage-display approach, has been under clinical trial (14). Even though OMP-18R5 was originally developed to target only FZD7, it was found to bind five FZDs across two sub-groups (FZD1/2/7 and FZD5/8). Although OMP-18R5 showed anti-tumor efficacy in animal models, it exhibits adverse effects particularly loss of bone density in humans (10,18). The similar side effect was also observed with a Fc fusion protein containing FZD8-CRD (15,19), suggesting that bone density loss is a primary effect when both FZD1/2/7- and 5/8-mediated signaling pathways are blocked.

Here we have developed and evaluated a specific inhibitor targeting Wnt-FZD1/2/7 signaling utilizing a fragment of *C. difficile* toxin B, which uses FZD1/2/7 as its receptors (20,21). Taking advantage of this specific inhibitor and utilizing several breast cancer mouse models, we demonstrate that FZD1/2/7-mediated Wnt signaling plays a key role in mouse models of BLBC and human BLBC organoids, as well as in chemotherapy-resistant mammary tumor cells in both BLBC and luminal breast cancers. Inhibiting FZD1/2/7-mediated Wnt signaling suppresses tumor growth and cancer stem cell activities and synergizes with chemotherapy agent cisplatin, without side effects on bone density. These findings demonstrate

both the feasibility and therapeutic value for targeting a single subgroup of FZDs in treating a broad range of breast cancers.

RESULTS

FZD7 is the major FZD receptor expressed in BLBC

To identify the specific subgroups of FZDs expressed in BLBC, we examined expression of all 10 FZD members in human breast cancers and murine models. In publicly available human breast cancer expression data, *FZD5*, *6*, *7* and *9* are expressed at higher levels in BLBC cases than other subtypes (Supplementary Fig. S1). In a microarray dataset for various breast cancer mouse models (22), *Fzd7* and *Fzd6* were highly expressed in *C3(1)-Tag* and *Trp53*-null ER-negative mammary tumors (Supplementary Fig. S2), both of which represent mouse models for human BLBC/TNBC (23).

To further examine Wnt signaling in murine breast cancer models, we focused on two commonly used models, the BLBC model *C3(1)-Tag*, the luminal B breast cancer model *MMTV-PyMT* (23), as well as a novel BLBC mouse model we established recently with loss of p53 and BRCA1 (24). Our model is based on intraductal injection of a Cre-expressing adenovirus under the control of the keratin 8 (*Krt8*) promoter [*Ad-K8-Cre* (25)] to floxed *Trp53* and *Brca1* female mice carrying the conditional *Rosa26-LSL-EYFP* (*R26Y*) reporter ($Trp53^{L/L}$;$Brca1^{L/L}$;*R26Y*) (Supplementary Fig. S3A-B). This approach somatically inactivates *Brca1* and *Trp53* in luminal mammary epithelial cells (MECs), which are believed to serve as the cellular origin of *BRCA1*-associated BLBC (26-28), leading to development of mammary tumors that closely resemble the human BLBC subtype (24). Overall, these three models represent mammary tumor types that are not good candidates for hormone or anti-HER2 therapies but can be treated by standard chemotherapy.

We first analyzed activation of Wnt signaling in these tumors by assessing their levels of active β-catenin (i.e., non-phosphorylated β-Catenin). Both p53/BRCA1-deficient tumors and *C3(1)-Tag* Basal-like (BL) tumors exhibited higher levels of active β-catenin than that of *MMTV-PyMT* tumors, based on immunofluorescence (IF) staining and immunoblot analysis (Fig. 1A-C). Next, we measured expression levels of all *Fzd* genes in these mammary tumors by qRT-PCR and found that only *Fzd7* was expressed at higher levels in both p53/BRCA1-deficient and *C3(1)-Tag* tumors, but not in *MMTV-PyMT* tumors, than all other *Fzd* genes (Fig. 1D).

We then analyzed normal mammary gland tissues by fluorescence-activated cell sorting (FACS) with an APC-conjugated FZD7-specific antibody, which detected ~2.44% of cells as

FZD7-positive ($FZD7^+$) (Fig. 1E). Further analysis using CD24 and CD29 as markers revealed that $FZD7^+$ cells are largely considered basal MECs (~81.3%, Fig. 1E). Compared to the normal mammary tissues, *MMTV-PyMT* tumor tissues contains similar levels of $FZD7^+$ cells, whereas both *C3(1)-Tag* and p53/BRCA1-deficient BL tumors, particularly the latter, contained elevated levels of $FZD7^+$ cells (~6.05% and ~10.4%, respectively, Fig. 1F and Supplementary Fig. S3C). Together, these data suggest that $FZD7^+$ cells are enriched in BLBC tumor models.

$TcdB^{FBD}$ inhibits FZD1/2/7-mediated Wnt signaling in human breast cancer cell lines

To evaluate whether FZD7 may serve as a therapeutic target for treating BLBC, we took advantage of our recent finding that a bacterial toxin *C. difficile* toxin B (TcdB) recognizes FZD1, 2, and 7 as its high-affinity receptors and toxin binding inhibits FZD1/2/7-mediated Wnt signaling (20). Our previous studies have also defined an FZD-binding domain in TcdB (amino acid residues 1285-1804, designated as $TcdB^{FBD}$, Fig. 2A) (21). Both $TcdB^{FBD}$ and Wnt recognize the CRDs. FZD1, 2, and 7 form a sub-group with nearly identical CRDs within the FZD family. The co-crystal structure of $TcdB^{FBD}$ in complex with CRD of FZD2 (CRD2) has been solved, revealing that $TcdB^{FBD}$ effectively blocks Wnt signaling by targeting a region in CRD that is critical for docking of the palmitate in Wnt (21). All Wnts are modified by lipidation through the addition of a palmitoleic acid (PAM) to a conserved serine, which is essential for their secretion and binding to FZDs. Binding of $TcdB^{FBD}$ prevents docking of the Wnt PAM into a hydrophobic groove in CRDs. Key residues for $TcdB^{FBD}$ interactions are conserved in CRD1, 2, and 7, but varies in other FZD members, which are the reasons for the selective high-affinity binding of $TcdB^{FBD}$ to CRD1/2/7 (21,29).

We first tested the ability of $TcdB^{FBD}$ to inhibit Wnt signaling in a human TNBC cell line MDA-MB-231 using a well-established TOPFLASH/TK-Renilla dual luciferase reporter assay. It has been previously reported that FZD7 is upregulated in MDA-MB-231 (30), and qRT-PCR analysis confirmed that FZD1/2/7 are expressed at higher levels than other subgroups of FZDs in this cell line (Supplementary Fig. S4A). Wnt signaling in cells was stimulated using conditioned medium containing WNT3A. Nanomolar levels of $TcdB^{FBD}$ inhibited WNT3A-mediated signaling in a dose-dependent manner (Fig. 2B). This inhibitory effect sustained for over 72 h with a single exposure to $TcdB^{FBD}$ in the medium (Fig. 2C). As a control, a mutant form of TcdB ($TcdB^{mu}$) that could no longer bind FZD1/2/7, constructed by replacing the key CRD-binding

residues in TcdB^{FBD} with the corresponding residues in *C. difficile* toxin A that does not use FZDs as receptors (^{1595}VNFLQS changed to ^{1596}GFE, Fig. 2A) (21) (31), showed no inhibition of Wnt signaling at nanomolar concentrations (Fig. 2B-C). TcdB^{FBD} did not affect viability of MDA-MB-231 and a few other human cell lines (U2OS, 293T, and MCF7), confirming that this toxin fragment does not have general toxicity to cells (Supplementary Fig. S4B).

To further evaluate the ability of TcdB^{FBD} to inhibit Wnt signaling *in vivo*, we injected MDA-MB-231 cells with an integrated TOPFLASH reporter subcutaneously into immunodeficient athymic nude mice, which resulted in tumor growth. Recombinantly purified TcdB^{FBD} was subsequently injected intraperitoneally (*i.p.*) to these mice at a dose of 20 mg/kg once per day for 6 times. To imaging the Wnt signaling activity, D-luciferin was injected 5 min (*i.p.*) *in vivo* before tumors were dissected out, and their luminescence signals were then measured *ex vivo*. Tumors from TcdB^{FBD}-treated mice showed ~5-fold reduced signals compared with tumors from vehicle-treated control groups and TcdB^{mu}-treated groups (Fig. 2D).

We next evaluated the specificity of TcdB^{FBD} for targeting FZD1/2/7, but not other closely-related FZDs (e.g., FZD5). Two pancreatic cancer cell lines, PaTu8988s and HPAF-II, are known to express high levels of FZD5 and are sensitive to small molecule pan-Wnt signaling inhibitor, LGK974 (32), which targets the *O*-acyltransferase Porcupine required for palmitoylation of all Wnts (33). While LGK974 treatment (at a concentration of 100 nM) inhibited clonogenic growth as well as the sphere formation ability of these two cell lines, TcdB^{FBD} treatment at a similar concentration (150 nM) exhibited no growth inhibitory effect on them in both assays (Supplementary Fig. S4C-E).

TcdB^{FBD} inhibits growth of BL mammary tumors

To test whether TcdB^{FBD} could affect BL mammary tumors with FZD7 expression, we first treated tumor organoids derived from the above-described murine models with TcdB^{FBD} or TcdB^{mu}. Treatment with TcdB^{FBD}, but not TcdB^{mu}, reduced the size and number of organoids formed from the p53/BRCA1-deficient or *C3(1)-Tag* BL tumor cells; in contrast, TcdB^{FBD} treatment did not affect growth of the organoids formed from *MMTV-PyMT* luminal tumor organoids (Fig. 3A-C). Furthermore, TcdB^{FBD}-treated p53/BRCA1-deficient tumor organoids exhibited reduced expression of Wnt signaling-related genes (e.g., *Axin2*, *Rnf43*) and EMT-

related genes (e.g., *Vim*, *Zeb1*) compared with the control and $TcdB^{mu}$-treated organoids (Supplementary Fig. S5A).

Next, we utilized $TcdB^{FBD}$ to evaluate whether inhibiting FZD7-mediated Wnt signaling may offer any therapeutic benefits *in vivo*. Subcutaneous injection of p53/BRCA1-deficient tumor organoid cells (1×10^4 cells) into nude mice resulted in robust tumor growth. When tumors reached ~50 mm^3, $TcdB^{FBD}$ was injected *i.p.* into the recipient mice. $TcdB^{FBD}$ administration at a dose of 20 or 50 mg/kg with the intervals indicated in Fig. 3D, but not that of $TcdB^{mu}$, attenuated tumor growth, although the effect is modest (Fig. 3D). At molecular levels, genes related to Wnt signaling and EMT were downregulated in tumors from $TcdB^{FBD}$-treated mice, compared with those in vehicle or $TcdB^{mu}$-treated groups (Supplementary Fig. S5B). Consistently, expression of lymphocyte enhancer-binding factor 1 (LEF1), a representative Wnt signaling effector, is reduced at protein levels (Supplementary Fig. S5C).

To further evaluate whether $TcdB^{FBD}$ could attenuate growth of human BLBC/TNBC cells, we took advantage of a recently established biobank of over 100 primary and metastatic human breast cancer organoid lines (34). We chose two organoid lines from this biobank, 74T and 86T, which represent a luminal and a BL breast cancer line with low and high level of a BRCA1-deficiency signature (i.e., signature 3), respectively (34). The luminal organoid line 74T was insensitive to $TcdB^{FBD}$ treatment (Supplementary Fig. 6A). In contrast, growth of the BL line 86T organoids was attenuated by $TcdB^{FBD}$ (Supplementary Fig. 6B). Similar to the p53/BRCA1-deficient xenograft model, the inhibitory effect is rather modest. These results are consistent with the idea that Wnt signaling is not a driving force but rather one of the contributing factors in tumorigenesis of breast cancers. Nevertheless, these findings demonstrate that selective inhibition of FZD7-mediated signaling is sufficient to exhibit an inhibitory effect on growth of mouse and human BLBC cells.

$TcdB^{FBD}$ treatment does not affect the intestine and bones

We next analyzed whether inhibiting FZD7-mediated signaling by $TcdB^{FBD}$ at therapeutically effective doses is tolerated in Wnt sensitive tissues such as the intestine and bones. $TcdB^{FBD}$ was injected into mice at 20, 50, or 100 mg/kg twice a week for five weeks. These mice showed similar weight gains comparable with the control mice (Fig. 4A). To examine the potential impact on the intestine, we injected (*i.p.*) EdU, which incorporates into

replicating DNAs and marks proliferating cells, at the end of the fifth week. The intestine epithelium constantly turns over and newly generated cells are produced from stem cells located the bottom of the crypt region. Wnt signaling is a key pathway regulating intestinal stem cells and a reduction in EdU incorporation would reflect an inhibition on stem cell activity (35,36). The intestinal tissues were dissected out and EdU incorporation was measured. A shown in Fig. 4B, $TcdB^{FBD}$ treatment at 20 and 50 mg/kg levels did not reduce EdU levels in the intestinal tissues. There appears to be a slight reduction at 100 mg/kg, but it did not reach statistical significance.

Clinical trials with OMP-18R5, which blocks FZD1/2/7 and 5/8, revealed that bone density loss is the single most critical side effect in humans (15). Consistently, mice treated with pan-Wnt signaling inhibitors (Porcupine inhibitors LGK974 and ETC-1922159) exhibited loss of bone volume and density even at doses <10 mg/kg (37). We thus focused our analysis on bone mass and architecture utilizing micro-computed tomography (μCT) technology in mice treated with $TcdB^{FBD}$ at 20, 50, and 100 mg/kg doses for five weeks. No differences were observed in cancellous or cortical microstructure in the femur (Fig. 4C). Bone volume fraction (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th) in the distal femur metaphysis were not affected by $TcdB^{FBD}$, neither did cortical bone parameters, e.g., cortical thickness (Ct.Th) (Fig. 4D).

$TcdB^{FBD}$ inhibits $FZD7^{+}$ tumor-initiating cells *in vitro* and *in vivo*

FACS analysis demonstrate that ~90% of $TcdB^{FBD}$-bound cells from primary p53/BRCA1-deficient tumors were $FZD7^{+}$ (Supplementary Fig. S7A), confirming that $TcdB^{FBD}$ targeted $FZD7^{+}$ cells in tumor tissues. As Wnt signaling plays key roles in maintaining stem cell activities, we next examined whether $TcdB^{FBD}$ affected the activity of TICs in the p53/BRCA1-deficient tumor, utilizing the mammosphere/tumorsphere assay that enriches TICs by culturing cancer cells in suspension (38,39). A small percentage of these cells can survive and grow in suspension as spheroids, which often correlates with their ability to form tumors in mice and thus they are considered as TICs. Dissociated tumor cells from the p53/BRCA1-deficient model easily formed spheroids (i.e., tumorspheres) in suspension culture (Supplementary Fig. S7B). FACS analysis revealed that ~85.7% of cells forming tumorspheres are $FZD7^{+}$, confirming that most TICs express FZD7 (Supplementary Fig. S7C). $TcdB^{FBD}$ treatment greatly reduced sphere

formation of p53/BRCA1-deficient mammary tumor cells (Fig. 5A). After $TcdB^{FBD}$ treatment, the percentage of tumorsphere formation in the first passage was 1.2-fold reduced compared to that of control, increased to 1.7-fold reduction at the second passage, and became even more profound in the third passage (26.0-fold reduction) (Fig. 5B). This data indicate that $TcdB^{FBD}$ inhibited the self-renewal potential of p53/BRCA1-deficient tumor cells. To further determine the effect of $TcdB^{FBD}$, limiting dilution assay was employed by serial re-plating of p53/BRCA1-deficient tumor cells at various cell concentrations in the sphere culture. The frequency of sphere-forming cells, a surrogate for the frequency of TICs, was drastically reduced after $TcdB^{FBD}$ treatment, dropping from 0.42% (1/239) to 0.11% (1/895) compared to control (P = 0.0019) (Fig. 5C). These data demonstrate that $TcdB^{FBD}$ targeted $FZD7^{+}$ TICs and suppressed their capability to generate tumorsphere *in vitro*.

To further assess the effect of $TcdB^{FBD}$ on TICs *in vivo*, we performed the limiting dilution assay by transplanting ~10 – 1,000 p53/BRCA1-deficient tumor organoid cells to nude mice. FACS analysis confirmed that most of these tumor organoid cells were $FZD7^{+}$ (~81.7%, Supplementary Fig. S7D-E). $TcdB^{FBD}$ or $TcdB^{mu}$ treatment was given at 20 mg/kg twice a week starting from day 5 after inoculation until day 53, when most mice had reached their endpoint (Fig. 5D). Inoculating 1,000 organoid cells resulted in tumor formation in all mice within ~2 weeks. Reducing the number of organoid cells decreased the chances in tumor formation, and a repopulating frequency can be calculated from a serial dilution of organoid cells (Fig. 5E). $TcdB^{FBD}$ treatment reduced this frequency by 3.68-fold from 1/62 in control mice to 1/289.9, whereas $TcdB^{mu}$ treatment did not change the frequency (Fig. 5E). For instance, transplanting as few as 10 transplanted cells in the control or $TcdB^{mu}$-treated group formed tumors 2 out of 8 times, whereas no tumor was detected in mice treated with $TcdB^{FBD}$ (Fig. 5D-E). Furthermore, mice transplanted with 100 organoid cells and treated with $TcdB^{FBD}$ exhibited a significantly longer tumor-free period than those received vehicle or $TcdB^{mu}$ treatment (average tumor-free time 82.13 days vs. 44.37 days, P = 0.030) (Fig. 5F). Consistently, mice inoculated with 10,000, 1,000, or 100 p53/BRCA1-deficient tumor organoid cells and treated with $TcdB^{FBD}$ survived 1.2 days (P = 0.014), 14.84 days (P = 0.001), or 22.88 days (P = 0.017) longer than those treated with vehicle or $TcdB^{mu}$ (Fig. 5G). Collectively, these results suggest that the inhibition of $TcdB^{FBD}$ on tumor growth is mediated by its ability to target and suppress $FZD7^{+}$ TICs *in vivo*.

TcdB^{FBD} synergizes with cisplatin in treating both BL and luminal breast cancers

Even though TcdB^{FBD} exhibited efficacy in inhibiting growth of BLBC via targeting FZD7^{+} TICs, it is unlikely to eliminate tumors as a single agent. We thus examined its therapeutic value in combination with a standard chemotherapy drug (e.g., cisplatin). Xenograft experiments with the same initial number of tumor organoid cells from the two BLBC models (p53/BRCA1-deficient and *C3(1)-Tag*) were engrafted in nude mice. Mice were then treated with TcdB^{FBD} alone, cisplatin alone, or a combination of both agents following the schedule illustrated in Fig. 6A-B. TcdB^{FBD} alone reduced tumor growth in both models and its efficacy is similar to treatment with cisplatin alone (Fig. 6A-B). Combination treatment with both TcdB^{FBD} and cisplatin achieved the highest level of growth inhibition, close to complete suppression, than either agent alone on tumors derived from p53/BRCA1-deficient and *C3(1)-Tag* models, demonstrating a strong synergistic effect (Fig. 6A-B). To further confirm the synergistic effect, we also examined the sensitivity of tumor organoids of these two BL cancer models. A low concentration of cisplatin (0.2 μM) did not reduce the size or number of tumor organoids, whereas combining this level of cisplatin with TcdB^{FBD} reduced both size and number and organoids to a level lower than treatment with TcdB^{FBD} alone (Supplementary Fig. S8). These results suggest that targeting FZD7-mediated signaling may suppress the resistance to cisplatin, or cisplatin treatment may sensitize cells to FZD7-mediated inhibition by TcdB^{FBD}. It is possible that a subpopulation of FZD7^{+} TIC cells are intrinsically more resistant to chemotherapy, or FZD7 is upregulated in a subpopulation of cells that developed resistance. Indeed, FACS analysis of tumor cells revealed that the percentage of FZD7^{+} cells increased after treatment with cisplatin alone in both p53/BRCA1-deficient and *C3(1)-Tag* models, and co-administration of TcdB^{FBD} greatly reduced FZD7^{+} cells (Fig. 6C).

In addition to the two BL cancer models, we also tested the luminal tumor *MMTV-PyMT* model in xenograft models *in vivo,* which do not over-express FZD7^{+} and its organoids are resistant to TcdB^{FBD} *in vitro* (Figs. 1D, 3A). As expected, TcdB^{FBD} alone did not affect tumor growth in this model *in vivo* (Fig. 6D). Surprisingly, TcdB^{FBD} was able to synergize with cisplatin in this model as well and the combination greatly suppressed tumor growth than using cisplatin alone (Fig. 6D). Consistently, although neither a low concentration of cisplatin nor TcdB^{FBD} alone affected tumor organoids of *MMTV-PyMT* model, a combination of cisplatin and TcdB^{FBD} reduced the size and number of organoids (Fig. 6E-G). Thus, exposure to cisplatin may

have turned the TcdB^{FBD}-insensitive tumor into a sensitive one. These findings are consistent with our recent report that cisplatin treatment could lead to luminal to basal/mesenchymal cell fate changes, in part due to interstrand DNA crosslinks (24). Indeed, treatment with cisplatin increased expression of basal/mesenchymal genes, as well as *Fzd7* in *MMTV-PyMT* tumors, whereas these changes were reduced in the presence of TcdB^{FBD} (Fig. 6H), suggesting that cisplatin-induced cell fate change renders cells to become sensitive to the co-inhibition of FZD7-mediated Wnt signaling in luminal breast tumors.

DISCUSSION

Wnt signaling is a key pathway in maintaining the stemness of cells. Besides well-established cancer-causing mutations in Wnt pathways, recent studies have also suggested that Wnt signaling upregulation contributes to many aspects of cancer development and therapy resistance, including EMT, metastasis, and resistance to chemotherapy and immunotherapy, properties often attributed to CSCs/TICs. Thus, Wnt signaling represents a major therapeutic target in cancer treatment (15). Broadly inhibiting Wnt signaling inevitably results in side effects, diminishing the therapeutic value particularly for tumors that do not carry cancer-driving mutations in Wnt pathways. Inhibiting a subset of Wnt signaling may reduce the side effect and it needs to be done at the ligand-receptor level as this is the most diverged node in the pathway (17), but it has been difficult to develop inhibitors that can distinguish different FZD subfamily members. Furthermore, whether targeting only a subset of FZDs is going to be sufficient remains to be demonstrated *in vivo* due to concerns on potential signaling redundancy. Here we utilized the natural ability of a bacterial toxin to specifically recognize FZD1, 2, 7 subgroup and developed a therapeutic protein that can block FZD1/2/7-mediated Wnt signaling. We found that blocking FZD1/2/7-mediated Wnt signaling reduced growth of BLBC models *in vivo* and also synergized strongly with the traditional chemotherapy agent cisplatin in treating both BLBC and luminal breast tumor models. These findings demonstrate that inhibiting a single FZD subgroup, instead of pan-inhibition of Wnt signaling, is sufficient to exhibit a strong synergistic effect *in vivo* with the standard chemotherapy, and suggest a key role of FZD1/2/7-mediated Wnt signaling in the development of chemotherapy-resistance in breast cancers. Targeted inhibition of this pathway could be combined with chemotherapy to eliminate/prevent therapy-resistant stem cell-like cancer cells.

Chemotherapy remains to be the cornerstone of the first-line treatment for many tumors such as BLBC/TNBC that have no suitable targeted therapy. Conventional chemotherapy is effective in controlling primary tumors, but often led to a selection and/or an induction of CSCs/TICs that are more resistant to these agents, leading to eventual regeneration of chemo-resistant tumors and metastasis. Our findings that $TcdB^{FBD}$ synergizes strongly with cisplatin treatment add to the wealth of literatures suggesting that Wnt signaling play a key role in the development/selection of treatment-resistant cells and demonstrating that a combination of Wnt

signaling suppression with chemotherapy is a valid strategy to eliminate CSCs/TICs at once and/or prevent the emergence of therapy-induced CSCs/TICs.

Synergistic effect from Wnt signaling inhibition has also been reported between OMP-18R5 and the chemotherapy agent taxanes on a range of tumor models (14). Future studies can focus on testing whether $TcdB^{FBD}$ would also be effective in shrinking mammary tumors when combined with other types of chemotherapeutic agents (e.g., doxorubicin) and whether combined $TcdB^{FBD}$ and cisplatin treatment could be applied to other cancer types (e.g., serous ovarian cancer, oral squamous cell carcinoma). Upregulation of Wnt signaling has been associated with the resistance to many common chemotherapy agents as well as radiation therapy (12). The mechanistic link between Wnt signaling and therapy resistance remains to be fully established and is likely multifaceted (12). For instance, recent studies suggest that Wnt signaling enhances the DNA repair pathways in ovarian cancers (40), and it has been also proposed that therapy-induced senescence promotes cancer cell stemness via Wnt signaling (41). In our study, it is likely that cisplatin treatment leads to an epithelial cell fate change toward a mesenchymal state (24), which may represent a dedifferentiation process that involves Wnt signaling. Lastly, Wnt signaling has been shown to contribute to immune invasion as well as systemic inflammation in the tumor microenvironment that drives cancer metastasis (42-45). Whether $TcdB^{FBD}$ may provide a synergistic effect with immunotherapy such as PD-1/PD-L1 antibodies remains to be explored.

Intestines and bones are two major tissues sensitive to disruptions in Wnt signaling pathways and are often examined when testing Wnt signaling pathway inhibitors. The finding that $TcdB^{FBD}$ did not affect intestinal tissues is consistent with previous findings that the pan-Wnt inhibitors LGK974 did not cause any damage to the intestine until a dose much higher than the therapeutic dose (33). These findings are also supported by clinical observations that intestinal damage is not a major side effect in clinical trials of OMP-18R5 antibody. On the other hand, bone density loss is the single most important side effect observed from clinical trials of OMP-18R5. Our analysis showed that bones were not affected in mice by $TcdB^{FBD}$, which represents a major advantage over other existing pan-Wnt signaling inhibitors and neutralizing antibodies. This is possibly because both FZD7 and FZD5 are expressed in diaphyseal bone and potentially play redundant roles (46). FZD5-mediated Wnt signaling in bone tissues might be

sufficient to maintain bone density when FZD7 is selectively inhibited by $TcdB^{FBD}$, whereas the neutralizing antibody OMP-18R5 inhibits both FZD1/2/7 and FZD5/8 subfamilies.

The specificity of $TcdB^{FBD}$ for FZD1/2/7 allows us to establish this agent as an effective targeted therapy for FZD1/2/7^{+} TICs and cisplatin-resistant mammary tumor cells. The crystal structure of $TcdB^{FBD}$-CRD complex showed that the toxin targets the lipid docking site in CRD. The sequence variations surrounding this lipid docking site across different FZDs underlies the reason for the selectivity of TcdB toward FZD1/2/7 (21,29). These data suggest that this lipid docking site may serve as a promising therapeutic target for developing effective and selective Wnt signaling inhibitors. Although $TcdB^{FBD}$ is unlikely to be effective for blocking Wnt signaling in cancer cells with mutations in Wnt pathway components downstream of the receptor level (e.g., *CTNNB1*, *APC*), it should be able to inhibit Wnt signaling at the level of ligand/receptor interaction in cancer cells (breast cancer or other cancer types) that become dependent on Wnt ligands, in a way similar to Porcupine inhibitors (37), but is more selective based on utilization of specific FZD receptors (e.g., FZD7) and is less toxic to normal tissues (e.g., bone). These properties make this bacterial toxin-derived molecule a highly promising agent for targeting FZD1/2/7-mediated Wnt signaling *in vivo* for cancer treatment.

METHODS

Cloning, expression, and purification of recombinant proteins. The gene encoding $TcdB^{FBD}$ (residues 1285-1804) was cloned into pET28a vector, with an N-terminus HA tag. Mutated $TcdB^{FBD}$ variants ($TcdB^{mu}$) were generated by two-step PCR and verified by DNA sequencing. $TcdB^{FBD}$ and $TcdB^{mu}$ were expressed in *E. coli* strain BL21-Star (DE3) (Invitrogen). Bacteria were cultured at 37°C in LB medium containing kanamycin. The temperature was reduced to 16°C when OD600 reached ~0.8. Expression was induced with 1 mM IPTG (isopropyl-b-D-thiogalactopyranoside) and continued at 16°C overnight. Proteins were purified using Ni2+-NTA (nitrilotriacetic acid, Qiagen) affinity resins in a buffer containing 40 mM imidazole, 400 mM NaCl, and 50 mM Tris pH 8.0. The proteins were eluted with a high-imidazole buffer (300 mM imidazole, 400 mM NaCl, and 50 mM Tris, pH 8.0) and then dialyzed at 4°C against a buffer containing 150 mM NaCl and 20 mM HEPES, pH 7.5. Proteins were further purified by MonoQ ion-exchange (20 mM Tris, pH 8.5) and Superdex-200 size-exclusion chromatography (GE Healthcare, 20 mM Tris, pH 8.0, and 100 mM NaCl). Proteins were further incubated with Endotoxin Removal Resin (Thermo Scientific, # 88270) at 4 °C with gentle mixing for 1 h in columns, collected by centrifugation, further concentrated to ~10 mg/ml in PBS, and stocked in aliquots at -80°C.

Cell lines and constructs. MB-MDA-231 (# HTB-26), MCF7 (# HTB-22), U2OS (# HTB-96), 293T (#CRL-3216), HPAF-II (#CRL-1997), L cells (#CRL-2648), and L/WNT3A (#CRL-2647) cells were originally obtained from ATCC. PaTu8988s was generously provided by Stephane Angers (University of Toronto, Canada). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 10,000 I.U./mL Penicillin, 50 µg/ml streptomycin (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37 °C. Stable Wnt-reporter cells (MB-MDA-231-TK/RL, U2OS-TK/RL, 293T-TK/RL cells) were generated by lentiviral transduction of MB-MDA-231, U2OS, 293T cells with constructs expressing Renilla and firefly luciferases (7xTcf-FFluc, # 24308; RLUC - IRES – FLUC, # 45642, Addgene), followed by selection with 3 µg/ml puromycin and 50 µg/ml geneticin.

Preparation of WNT3A-conditioned induction medium. WNT3A-conditioned induction medium was generated using L-WNT3A cells according to the manufacturer's protocol.

Conditioned medium from the corresponding L-cells was collected and used as a control. Briefly, the cells were grown in 10 mL of DMEM supplemented with 10% FBS for 4 days prior to collecting conditioned medium. To these cells, another 10 mL of fresh medium was added and cultured for 3 days to collect second batch of conditioned medium. The two batches of conditioned media were mixed at a 1:1 ratio and filtered using 0.22 μm filter and stored at 4°C until usage.

Wnt signaling assay. The TOPFLASH/TK-Renilla dual luciferase reporter assay (# E1910, Promega) was used to detect Wnt signaling activities. Briefly, Wnt signaling activates expression of TOPFLASH luciferase reporter (firefly luciferase), Renilla luciferase serves as an internal control. To obtain Wnt signaling inhibition dose response curve for $TcdB^{FBD}$ in Wnt reporter cells, MB-MDA-231- TK/RL, U2OS- TK/RL, 293T- TK/RL cells in 24-well plates were treated with a range of concentrations of $TcdB^{FBD}$ with 10 doses in 2-fold dilution series in WNT3A-conditional medium for 6 h. GraphPad Prism software was used for graphing and EC50 calculations.

Viability assay for cell lines. MB-MDA-231, MCF7, U2OS, 293T Cells were seeded at 3000-5000 cells per well in 96-well plates. Twenty-four h after seeding, cells were treated with $TcdB^{FBD}$ in triplicates, with 10 doses in 2-fold dilution for 72 h, the MTT solution was added to the culture medium (500 μg/ml). After incubation at 37 °C for 3 h, followed by the addition of 200 μl of dimethylsulfoxide (DMSO) to solubilize MTT. The absorbance at 562 nm was measured on a microplate reader. Normal cells without exposure to $TcdB^{FBD}$ were considered as 100% viable.

Colony formation assay. HPAF-II and PaTu8988s cells were seeded in 24-well plates at a density of 2000 cells per well, and cultured in the culture medium added with 100 nM LGK974 or 150 nM $TcdB^{FBD}$ or PBS vehicle control for 11 days and medium was refreshed every three days. Cells were washed by PBS, fixed by 4% paraformaldehyde for 15 min, stained with 0.5% crystal violet for 1 h, washed three times by ddH_2O and then photographed with a digital camera. The number of colonies was counted.

Mouse models. The *C3(1)-Tag* transgenic mice (FVB-Tg(C3-1-TAg)cJeg/JegJ , Stock No: 013591), *MMTV-PyMT* (FVB/N-Tg(MMTV-PyMT)634Mul/J, Stock No: 002374), *Trp53*^{L} (B6.129P2-*Trp53*^{tm1Brn}/J, Stock No: 008462), *Brca1*^{L} (STOCK *Brca1*^{tm1Aash}/J, Stock No: 017835), *R26Y* (B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J, Stock No: 006148) mice were obtained from The Jackson Laboratory. To target luminal MECs in *Trp53*$^{L/L}$*;Brca1*$^{L/L}$*;R26Y* adult female mice (2-3 months of age) were anaesthetized; and *Ad-K8-Cre* adenovirus (diluted in 0.1% Bromophenol blue in DMEM) was introduced into ducts of the fourth mammary gland via intraductal injection(47). Mice were then monitored via palpation or visual inspection weekly for tumor appearance. Once tumors were detected, animals were monitored three times a week for tumor development. For xenograft studies, six-eight weeks old female nude mice (Hsd: Athymic Nude-*Foxn1*^{nu}, 6903F) were purchased from Envigo RMS, Inc (Indianapolis, IN 46250 USA). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Brigham and Women's Hospital and Boston Children's Hospital where these mice were housed.

Tumor dissociation. Mammary tumors from the above mice were harvested, dissected and minced, and then incubated in digestion medium (2% Penicillin/Streptomycin, 0.1 mg/ml Gentamicin, 0.6% Nystatin, 2 mg/ml Collagenase A, 0.096 mg/ml Hyaluronidase in DMEM/F12) at 37℃ with shaking for 2 h. After digestion, the cells/tissues were treated sequentially with 0.25% trypsin/EDTA (37℃, 2 min), 5 mg/ml dispase with DNaseI (0.1 mg/ml, Sigma, St Louis, MO; 37℃, 5 min), cold red blood cell (RBC) lysis buffer (2–3 min). Between each treatment step, cells/tissues were washed with PBS. After treatment with the RBC lysis buffer, cells/tissues were filtered through 40 mm cell strainer to obtain single-cell suspension.

Organoid Culture. 2000 single cells digested from the corresponding primary tumors were seeded in 20 µl Matrigel in a 48-well-plate, cultured in 250 µl DMEM/F12 supplemented with 12 mM Hepes, 1% GlutaMAX, 1:50 B27, 0.21 µg/mL A83-01, 0.1 µg/ml EGF,10 µM Y-27632,100 ng/ml Noggin and 0.6 µg/ml R-spondin1. Established clonal organoids were trypsinized using TrypLE (Thermo Fisher Scientific). TcdB^{FBD} or TcdB^{mu} (150 nM) was added to the culture medium every day, PBS was used as a vehicle control.

Tumor implantation and evaluation. To establish xenograft tumors, a single p53/BRCA1 deficient tumor organoid cell suspension was harvested after trypsinization. The cells were resuspended in DMEM and Matrigel (V:V=1:1) and then were injected into the flank of nude mice. Tumors could be observed 5-7 days after organoid cell inoculation (1×10^4), tumor volumes were measured, and the mice were weighed twice weekly. Tumor volume was calculated using the formula: ½ (Length × Width^2). When tumors reach ~50 mm^3 (~9 days post inoculation of 1×10^4) mice were randomly divided into four groups (8 or 10 mice/group) and the mean tumor volumes of each group were similar. No mice were excluded during the treatment. Each group received one of the following treatments: PBS (150 μL/mice), endo-toxin free TcdB^{FBD} (20, 50, 100 mg/kg), or TcdB^{mu}, once a day on day 9,12, 14, 17, 20, and 23 post inoculation via intraperitoneal injection (*i.p.*). For combination treatment, Each group received one of the following treatments: PBS (150 μL/mice), cisplatin (5 mg/kg) alone or with endo-toxin free TcdB^{FBD} (20 mg/kg) once a day on the indicated day post inoculation via intraperitoneal injection (*i.p.*).

Bioluminescent assay. MDA-MB-231 cells that express integrated TOPFLASH were subcutaneously injected into immune-deficient athymic nude mice, when tumor length reached 10 mm, TcdB^{FBD} or TcdB^{mu} was injected at 20 mg/kg dose at indicated time. Five minutes before sacrifice, 100 mg/kg D-Luciferin was given (*i.p.*), tumors were dissected, put into 24-plate, and bioluminescent imaging were examined using a Xenogen IVIS-200 system (Xenogen). Images were analyzed by quantification of total photon flux of each tumor using Living Imaging Software. The p53/BRCA1-deficient tumor organoid cells were transduced by lentivirus with constructs 7xTcf-FFluc and subjected to the same analysis as described for MDA-MB-231 cells.

TcdB^{FBD} *in vivo* toxicity assay or EdU staining. Six weeks old female nude mice were injected (*i.p.*) with the TcdB^{FBD} (20, 50, or 100 mg/kg) or TcdB^{mu} (20 mg/kg) twice a week for five weeks. Mice were injected intraperitoneally (*i.p.*) once with EdU at the dose of 100 mg/kg body weight 12 h before euthanization. Intestine tissues were cleaned with cold PBS, fixed in 4% formaldehyde, and embedded in paraffin. Four-μm-thick sections were prepared, and the intestine crypt proliferation was conducted using Click-iT® EdU Alexa Fluor® 594 Imaging Kit (C10339, Invitrogen) according to manufacturer's introduction. Briefly, the sections were

541 washed twice with 3% BSA in PBS and permeabilized in 0.5% Triton® X-100, then incubated
542 with a Click-iT™ reaction cocktail, followed by incubation in 5 μg/mL Hoechst 33342 according
543 to the manufacturer's protocol.
544
545 **Micro-computed tomography (μCT).** The right femur was extracted at euthanization and fixed
546 in 4% paraformaldehyde for 2 days, then transferred into 70% ethanol. A 2.6-mm span of the
547 distal femoral metaphysis was scanned on a desktop μCT (μCT-35; Scanco Medical AG) at 10-
548 μm resolution using 50-kV peak tube potential and 151-ms integration time to
549 measure cancellous three-dimensional morphometric properties as previously described(48).
550 Standard trabecular bone parameters (BV/TV, Tb.N, Tb.Th) were calculated from each
551 reconstructed stack through the metaphysis. Cortical thickness (Ct. Th) and area (Ct.Ar) were
552 obtained from 20 slices reconstructed through the midshaft femur.
553
554 **Immunofluorescence staining.** Immunofluorescence (IF) staining was performed on tissue
555 sections that were fixed in 10% formalin (Fisher Scientific, Hampton, NH) and embedded in
556 paraffin. Antigen retrieval (Citrate buffer pH 6.0, 20 min boil in microwave oven) was
557 performed before blocking and endogenous peroxidase activity was quenched on the slides
558 intended for IF by incubation in 0.3% H_2O_2. Antibodies included: LEF1 (clone C12A5, #2230;
559 Cell Signaling;1:100), active β-catenin (clone D13A1, #8814; Cell Signaling;1:100).
560
561 **Flow cytometry.** Flow cytometric (FACS) analysis was performed after single cells were
562 obtained using an Accuri C6 analyzer (BD Biosciences, San Jose, CA) and analyzed with CFlow
563 software (BD Biosciences). The following antibodies were utilized: CD24 (clone M1/69, 564237;
564 BD Biosciences; 1:100), CD29 (clone eBioHMb1-1, 12-0291-82; 1:250), FITC-TcdB^{FBD} (0.1
565 mg/ml), FZD7 (Clone 151143, FAB1981A; RD system; 1:100), CD31 (clone 390, 13-0311-85;
566 eBioscience 1:100), CD45 (clone 30-F11, 13-0451-82; eBioscience; 1:100) and TER119 (clone
567 Ter-119, 13-5921-85; eBioscience; 1:100).
568
569 **Tumorsphere formation and *ex vivo* tumorigenicity assays**. Single cells obtained from cell
570 lines or tumor tissues were resuspended and plated into round-bottom 96-well ultralow
571 attachment plates (Corning) at a density of 1, 5, 10, 100, and 200 cells per well, or at 5000 cells

per well in 24-well flat-bottom ultralow attachment plates in the sphere culture medium (DMEM/F12 media supplemented with B27 (100 units/mL), Insulin (10 µg/mL), EGF (20 ng/mL) and bFGF (20 ng/mL) with 150 nM TcdB$^{\mathbf{FBD}}$ or TcdB$^{\mathbf{mu}}$, PBS served as control. The frequency of TICs was calculated using the ELDA website (http://bioinf.wehi.edu.au/software/elda/index.html). The tumorsphere formation frequency in 24-well plates was calculated according to the formula F= Numbers of forming tumorspheres / Number of single cells plated. For secondary and tertiary tumorsphere formation, single cell suspensions prepared from the previous generation of tumorspheres were re-plated under the same conditions as the first generation.

***In vivo* tumorigenicity assay.** p53/BRCA1-deficient tumor organoid cells (10, 100, or 1000 cells) were injected subcutaneously into nude mice in serum-free DMEM/Matrigel. The growth of tumor was evaluated daily over a 3-month period. The animal ethics endpoint was tumor reaching a size of 10 mm. Tumor volume was monitored and calculated as described above. The TIC frequency was derived as described above. For the Kaplan-Meier tumor free survival curves, mice were considered tumor free until tumors were visible or palpable. For the Kaplan-Meier survival curves, mice were considered alive until tumors reach ethics endpoint a size of 10 mm.

Reverse transcription and quantitative real-time PCR. Total RNAs from tumors were purified by either Trizol or the Allprep DNA/RNA mini kit (Qiagen). cDNA was generated with iScript (Bio-Rad, Berkeley, CA) according to the manufacturer's protocol. Quantitative RT–PCR (qRT–PCR, for RNA) and PCR (for genomic DNA) were performed using FastStart SYBR Green Master (Roche, Indianapolis, IN). PCR primers are listed below:
FZD1:forward:5′-GAGTTCTGGACCAGTAATCCGC-3′;reverse:5′-ATGAGCCCGTAAACCTTGGTG-3′; *FZD2*: forward: 5′-CTTCTCGCAAGAGGAGACTCG-3′; reverse: 5′-GTGGTGACCGTGAAGAAAGTG-3′; *FZD3*: forward: 5′-ATGGCTGTGAGCTGGATTGTC-3′; reverse: 5′-GGCACATCCTCAAGGTTATAGGT-3′; *FZD4*: forward: 5′-AACCTCGGCTACAACGTGAC-3′; reverse: 5′-GGCACATAAACCGAACAAAGGAA-3′; *FZD5*: forward: 5′-GGTGTGCCAGGAAATCACG-3′; reverse: 5′-CACAAGCGGCCAGAATTGG-3′; *FZD6*: forward: 5′-TCTGCCCCTCGTAAGAGGAC-3′; reverse: 5′-

GGGAAGAACGTCATGTTGTAAGT-3′; *FZD7*: forward: 5′-GCCACACGAACCAAGAGGAC-3′; reverse: 5′-CGGGTGCGTACATAGAGCATAA-3′; *FZD8*: forward: 5′-GGGTTACCTGTTGGAAGTGAC-3′; reverse: 5′-GGCACCGTGATCTCTTGGC-3′; *FZD9*: forward: 5′-CGCACGCACTCTGTATGGAG-3′; reverse: 5′-GCCGAGACCAGAACACCTC-3′; *FZD10*: forward: 5′-CATGCCCAACCTGATGGGTC-3′; reverse: 5′-GCCACCTGAATTTGAACTGCTC-3′; *Gapdh*: forward: 5′-GGTGAAGGTCGGTGTGAACG-3′; reverse: 5′-CTCGCTCCTGGAAGATGGTG-3′; *Axin2*: forward: 5′-ATGAGTAGCGCCGTGTTAGTG-3′; reverse: 5′- GGGCATAGGTTTGGTGGACT -3′; *Vim*: forward: 5′-CGGCTGCGAGAGAAATTGC-3′; reverse: 5′-CCACTTTCCGTTCAAGGTCAAG-3′; *Zeb1*: forward: 5′-GCTGGCAAGACAACGTGAAAG-3′; reverse: 5′-GCCTCAGGATAAATGACGGC-3′; *Rnf43*: forward: 5′-CACGAGTTTCATCGAACGTGT-3′; reverse: 5′-CTGGCGAATGAGGTGGAGT-3′.

Viability assay for human breast cancer organoids. Organoid lines derived from breast cancer patients are identified, as previously reported(34). 74T was a luminal organoid line and 86T was a basal-like line. The organoid was cultured in 250 μl DMEM/F12 supplemented with 10 mM Hepes, 50 μg/ml Primocin, 1% GlutaMAX, 1:50 B27, 1mM N-Acetylcysteine, 50 ng/ml EGF, 20 ng/ml FGF 2, 10 μM Y-27632, 100 ng/ml Noggin. $TcdB^{FBD}$ or $TcdB^{mu}$ (150, 300, 500, 1000 nM) was added to the culture medium every day for 10 days, PBS was used as a vehicle control. The viability of organoids was assessed using the CellTiter-Glo luminescent cell viability assay (# G7570, Promega) according to the manufacturer's protocol.

Immunoblot. Tumor tissues were lysed by RIPA buffer (50 mM Tris, 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Roche). Total proteins were loaded on SDS-PAGE and transferred to PVDF membrane. After blocking, proteins were detected with a 1:1000 dilution of primary antibody active β-catenin (clone D13A1, #8814; Cell Signaling;1:1000) using the enhanced chemiluminescence (ECL) method (Pierce).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA). An unpaired t test was used for comparisons between two experimental groups, and ANOVA was used for comparisons of more than two groups. Unless otherwise indicated, all results were averaged from biological triplicates and values are reported as means ± s.e.m. $P < 0.05$ was considered statistically significant.

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Authors' Contributions

Conception and design: A.H., D.X., M.D., R.J., and Z.L.

Development of methodology: A.H., D.X, Z.L.

Acquisition of data: A.H., D.X., O.K., H.C., D.J.H., A.G.R., R.T.B.,

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.S., X.H., L.S., K.Z., D.X., Y.H., Z.S.

Writing, review, and/or revision of the manuscript: A.H., D.X., M.D., Z.L.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.C., R.J., R.S., X.H.

Study supervision: M.D., Z.L.

Conflicts of Interest

A patent application on utilizing TcdB fragments for inhibiting Wnt signaling has been filed by Boston Children's Hospital, with M.D., R.J., L.T., A.H., and P.C. as inventors.

Data Availability Statement

All data is available in the main text or supplementary materials. All materials created in this study are available with material transfer agreements approved by Boston Children's Hospital to any researcher for purposes of reproducing or extending the analysis.

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Figure legends

Fig.1 | Wnt/FZD7 signaling is active in basal-like mammary tumors. (A) Representative immunostaining images detecting the levels of active β-catenin (non-phosphorylated form) in tumor tissues from p53/BRCA1-deficient, *MMTV-PyMT* and *C3(1)-Tag* breast cancer mouse models**.** DAPI staining marks the cell nucleus. Scale bar = 50 μm. **(B)** Quantitation of active β-catenin positive cells in (A). **(C)** Immunoblot analysis of active β-catenin expression in mammary tumors from models described in (A-B). Actin serves as a loading control. **(D)** Quantitative real-time (qRT)–PCR analysis of expression of *Fzd* family genes in p53/BRCA1-deficient (n=3), *C3(1)-Tag* (n=3) and *MMTV-PyMT* (n=2) tumors; error bars indicate mean ± SEM. **(E)** Upper panel: FACS analysis using a FZD7-specific antibody identified ~2.44% cells as $FZD7^+$ in normal mammary epithelial cells (MECs). Lower panel: $FZD7^+$ cells were isolated and subjected to further FACS analysis using antibodies against CD24 and CD29 to differentiate basal versus luminal EMCs, revealing that ~81.3% of $FZD7^+$ cells are basal EMCs. **(F)** The percentages of $FZD7^+$ cells in p53/BRCA1-deficient, *C3(1)-Tag* and *MMTV-PyMT* tumors, as well as in normal mouse mammary glands, were analyzed by FACS. The $FZD7^+$ peaks are marked and compared in the upper panel and quantified in the lower panel (The *P* values for p53/BRCA1-deficient, *C3(1)-Tag* and *MMTV-PyMT* models were <0.0001, 0.8556, 0.0178 respectively compared to normal mouse mammary glands). The representative FACS plots are shown in Supplementary Fig. 3.

Fig.2 | $TcdB^{FBD}$ inhibits FZD1/2/7-mediated Wnt signaling. (A) Schematic diagrams showing the domain structures of TcdB, and the two short fragments derived from TcdB ($TcdB^{FBD}$ and $TcdB^{mu}$) used in this study. GTD: glucosyltransferase domain; CPD: cysteine protease domain; Delivery/RBD: membrane translocation and receptor-binding domain; CROPs: combined repetitive oligopeptides domain. The structural model of $TcdB^{FBD}$-CRD7 complex shown is modeled based on the crystal structure of $TcdB^{FBD}$-CRD2 (PDB code: 6C0B) and CRD7 (PDB code:5T44). $TcdB^{FBD}$, CRD2, and CRD7 are colored pink, green, and blue, respectively. CRD: cysteine-rich domain. **(B)** $TcdB^{FBD}$ blocked WNT3A-mediated signaling in MDA-MB-231 cells in a dose-dependent manner, whereas $TcdB^{mu}$ showed no inhibition at nanomolar concentrations. Wnt signaling activity was analyzed using the TOPFLASH/TK-Renilla (TK/RL) dual luciferase

reporter assay (error bars indicate mean ± SEM, three independent experiments). **(C)** Wnt signaling activity in MDA-MB-231 cells was monitored using TK/RL assays over 5 days after induction by WNT3A conditioned medium with the indicated concentrations of $TcdB^{FBD}$ or $TcdB^{mu}$. Error bars indicate mean ± s.e.m, three independent experiments. **(D)** Nude mice were subcutaneously transplanted with TK/RL-transduced MDA-MB-231 cells and then treated at the indicated time point with $TcdB^{FBD}$ or $TcdB^{mu}$ (20 mg/kg of body weight) by intraperitoneal (*i.p.*) injection. D-Luciferin was injected 5 min before tumor tissues were isolated and the luciferase activity in tumor tissues was then measured *ex vivo* and quantified (error bars indicate mean ± SEM, n= 4-5 tumors).

Fig.3 | $TcdB^{FBD}$ inhibits growth of BL mammary tumors. (A) ~2000 cells digested from the indicated primary tumors were seeded in 20 μl Matrigel in a 48-well-plate. $TcdB^{FBD}$ or $TcdB^{mu}$ (150 nM) was added to the culture medium once per day. PBS was used as a vehicle control. Representative images of tumor organoids at day 5 in culture are shown. Scale bar = 100 μm. **(B)** Quantitation of organoids sizes at day 5 in culture for the indicated groups in (A). **(C)** Quantitation of organoid numbers at day 5 in culture. **(D)** Mice were subcutaneously transplanted with p53/BRCA1-deficient mammary tumor organoid cells (10,000 cells per mice) and treated for the indicated period when tumor volumes reached 50 mm^3 (left panel). The averaged tumor volumes over time were measured and plotted (middle panel). Tumor tissues were harvested at day 24 and weighted (right panel). Error bars represent SEM of 8-10 independently injected mice.

Fig.4 | $TcdB^{FBD}$ treatment *in vivo* does not affect the intestine and bones. (A) Six weeks old female nude mice (Hsd: Athymic Nude-*Foxn1*^{nu}) were injected (*i.p.*) with the $TcdB^{FBD}$ (20, 50, or 100 mg/kg) or $TcdB^{mu}$ (20 mg/kg) twice a week for five weeks. Their body weight gains were monitored and plotted. Error bars indicate mean ± SEM, n = 8-10 mice. **(B)** Mice were treated with $TcdB^{FBD}$ as described in panel A. EdU was injected (*i.p.*, 100 mg/kg) 12 h before euthanization. Intestine tissues were harvested, fixed, and analyzed. The representative images were shown in the left panel and quantification of the percentage of EdU-positive cells per crypt was plotted in the right panel. Scale bar = 200 μm. P=0.28**. (C)** Mice were treated with $TcdB^{FBD}$ as described in panel A and their right femur bones were extracted, fixed, and subjected to micro-computed tomography (μCT) analysis. Representative μCT reconstructions of the

midshaft femur cortical bone (upper row), distal femur metaphyseal bone (middle row), and entire distal femur with the ventral half of the femur digitally removed to reveal the cancellous compartment (lower row) are shown. N = 6 /dose group. **(D)** Quantification of the μCT analysis described in panel C for trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and cortical area (Ct.Ar), *P*=0.67, 0.63. 0.68 ,0.67, respectively.

Fig.5 | TcdB^{FBD} inhibits tumorigenic potential of BL mammary tumor cells. **(A)** Cells dissociated from p53/BRCA1-deficient mammary tumor tissues were resuspended and plated into round-bottom 96-well ultralow attachment plates in the sphere culture medium containing with 150 nM TcdB^{FBD}, TcdB^{mu}, or PBS (control). Representative images of tumorspheres formed at day 5 and day 15 in culture are shown. Scale bar = 200 μm. **(B)** The percent of tumorspheres were quantified following three passages of p53/BRCA1-deficient mammary tumor cells (5,000 cells/well for primary culture, and 2,000 cells/well for secondary and tertiary culture) with 150 nM TcdB^{FBD} or TcdB^{mu} or PBS in the sphere culture medium. **(C)** Analysis of the sphere-forming capabilities of p53/BRCA1-deficient mammary tumor cells using the limiting dilution assay. Cells were seeded in the presence of 150 nM TcdB^{FBD} or TcdB^{mu} or PBS vehicle. Sphere formation was counted 10-15 days post-seeding. The frequency of sphere-forming cells (TICs) was calculated using the ELDA website (http://bioinf.wehi.edu.au/software/elda/index.html): Control = 1/239 (lower 449, upper 128); TcdB^{FBD} =1 / 895 (lower 1,594, upper 502), *P*= 0.00192, compared with control, TcdB^{mu} =1 / 318 (lower 579, upper 175). **(D)** Tumor organoid cells (10, 100, or 1000 cells) derived from p53/BRCA1-deficient tumors were injected subcutaneously into nude mice. The growth of tumor was evaluated daily for 3-month. Once one tumor grows out in each group (10, 100, or 1000 cells), the mice was divided into three groups, received one of the following treatments twice a week: PBS (150 μL/mice), endo-toxin free TcdB^{FBD} (20 mg/kg), or TcdB^{mu} (20 mg/kg). Values in brackets indicate the number of organoid cells, the number of tumors obtained versus the total injected mice number. **(E)** Analysis of the tumorigenesis of p53/BRCA1-deficient mammary tumor organoid cells using the limiting dilution assay as described in (D). Frequency of TICs: Control = 1/62 (lower 134, upper 28.8); TcdB^{FBD} =1/289.9 (lower 701, upper 120.1), *P* = 0.0013, compared with control, TcdB^{mu} =1/73.1 (lower 160, upper 33.4). **(F)** Tumor latency plotted as percentage of tumor-free mice implanted with 100 p53/BRCA1-deficient mammary tumor

organoid cells following the indicated treatment. **(G)** Kaplan-Meier survival curves of nude mice bearing xenograft tumors treated as indicated.

Fig. 6 | TcdB^{FBD} inhibits cisplatin-resistant mammary tumor cells. **(A-B)** Tumor organoid cells from p53/BRAC1-deficient tumor model (A) or *C3(1)-Tag* model (B) were injected into nude mice, and mice were then treated with TcdB^{FBD} alone, cisplatin alone, or a combination of both TcdB^{FBD} and cisplatin as indicated. Tumor volume were recorded and plotted over time. Error bars indicate mean ± SEM, n= 8 mice. **(C)** FACS analysis of FZD7^{+} cells in the indicated tumors from p53/BRCA1-deficient (n=5) and *C3(1)-Tag* (n=4) models. Error bars indicate mean ± SEM. **(D)** Tumor organoid cells from *MMTV-PyMT* model were injected into nude mice, and mice were then treated with TcdB^{FBD} alone, cisplatin alone, or a combination of both TcdB^{FBD} and cisplatin as indicated. Tumor volume were recorded and plotted over time. Error bars indicate mean ± SEM, n= 6-8 mice. **(E)** Representative images of *MMTV-PyMT* mammary tumor organoids treated with TcdB^{FBD} alone (150 nM), cisplatin alone (0.2 μM), or a combination of cisplatin and TcdB^{FBD}. Scale bar = 100 μm. **(F)** Quantitation of organoid sizes for indicated groups described in (E). **(G)** Quantitation of organoid numbers for indicated groups described in (E). **(H)** qRT-PCR analysis of EMT-related genes (*Vim, Zeb1, Tcf4*) and *Fzd7* genes in tumor cells from *MMTV-PyMT* model after treatment with TcdB^{FBD}, cisplatin, or a combination of both (P<0.01).

Supplementary Figure Legends:

Supplementary Fig. S1 | Expression levels of *FZDs* in human breast cancers. Expression levels of *FZDs* in different subtypes of human breast cancer based on bc-GenExMiner online tool.

Supplementary Fig. S2 | Expression levels of *FZDs* in murine breast cancer models. High expression levels of *Fzd7* and *Fzd6* in mouse models for human BLBC/TNBC; data was based on GEO accession # GSE25488 (in heatmap, red to blue represents highest to lowest expression levels). The first five from the left side (marked as mammary tumor tissue rep1-5) represent the *C3(1)-Tag* model.

Supplementary Fig. S3 | The percentage of FZD7^{+} cells are assessed in three breast cancer models. (A) Schematic diagram showing the generation of a p53/BRAC1-deficient breast cancer model by intraductal injection of *Ad-K8-Cre* into *Trp53$^{L/L}$;Brca1$^{L/L}$;R26Y* female mice. **(B)** Kaplan-Meier tumor-free results showing that *Trp53$^{L/L}$;Brca1$^{L/L}$;R26Y* female mice (n=17) injected with *Ad-K8-Cre* developed malignant mammary tumors over time after *Ad-K8-Cre* injection. **(C)** Representative FACS plots of FZD7^{+} cells from normal mammary glands, p53/BRCA1-deficient, *MMTV-PyMT*, and *C3(1)-Tag* tumors.

Supplementary Fig. S4 | TcdB^{FBD} inhibits Wnt signaling in human breast cancer cell lines. (A) Left panel: Phylogenetic analysis of human FZD proteins (15); Right panel: qRT–PCR analysis of *Fzds* in MDA-MB-231 cells. **(B)** Cell viability measured by the MTT assay showed that TcdB^{FBD} did not exhibit cytotoxicity to the indicated human cell lines (error bars indicate mean ± SEM, three independent experiments). **(C)** Representative images of clonogenic growth of PaTu8988s and HPAF-II cells cultured in the presence of TcdB^{FBD} (150 nM) or LGK974 (100 nM). **(D)** Representative images of sphere formation assay in PaTu8988s and HPAF-II cells cultured in the presence of TcdB^{FBD} (150 nM) or LGK974 (100 nM). **(E)** Quantitation of colony numbers and sphere formation from (C-D), error bars indicate mean ± SEM, n= 4.

Supplementary Fig. S5 | TcdB^{FBD} inhibits Wnt signaling in p53/BRCA1-deficient mammary tumor cells. (A) Experiments were carried out as described in Fig. 3A. qRT-PCR analysis showing downregulation of Wnt signaling-related genes (*Axin2* and *Rnf43)* and EMT-associated genes (*Vim* and *Zeb1*) in tumor organoids upon TcdB^{FBD} treatment. **(B)** Experiments were carried out as described in Fig. 3D. qRT-PCR analysis showing downregulation of Wnt-associated genes (*Axin2* and *Rnf43*) and EMT-associated genes (*Vim* and *Zeb1*) in TcdB^{FBD}-treated xenograft tumors. **(C)** Experiments were carried out as described in Fig. 3D. Representative immunostaining images of LEF1 expression (green) in different treatment groups are shown (Control, TcdB^{FBD} or TcdB^{mu}). Scale bar = 50 μm.

Supplementary Fig. S6 | TcdB^{FBD} inhibits growth of human TNBC organoids. The viability of two lines of human breast cancer organoids, a luminal tumor line 74T (panel A) and a BL tumor line 86T (panel B), were exposed to the indicated concentrations of TcdB^{FBD} or TcdB^{mu}. Cell viability was assessed using the CellTiter-Glo luminescent assay.

Supplementary Fig. S7 | TICs in p53/BRCA1-deficient mouse mammary tumors are largely FZD7^{+}. (A) FACS analysis using FITC-TcdB^{FBD} and anti-FZD7 antibody showed that primary p53/BRCA1-deficient tumors cells targeted by TcdB^{FBD} are largely FZD7^{+}. (**B**) Representative images showing the tumorspheres formed from p53/BRCA1-deficient tumor cells. **(C)** Tumorsphere cells cultured from p53/BRCA1-deficient tumors were examined by FACS analysis using an anti-FZD7 antibody. **(D)** Representative images showing the tumor organoid formed from p53/BRCA1-deficient tumor cells. **(E)** FACS analysis of FZD7^{+} cells in the tumor organoids described in (D) showing that most organoid cells express FZD7.

Supplementary Fig. S8 | TcdB^{FBD} and cisplatin showed synergistic effect in inhibiting BL mammary tumor organoids. (A) Representative images of p53/BRCA1-deficient mouse mammary tumor organoids treated with TcdB^{FBD} alone (150 nM), cisplatin alone (0.2 μM), or a combination of both. **(B)** Quantitation of organoids sizes for the indicated treatment groups in (A). **(C)** Quantitation of organoid numbers for the indicated groups in (A). **(D)** Representative pictures of *C3(1)-Tag* mouse mammary tumor organoids treated with TcdB^{FBD} alone, cisplatin alone, or a combination of both. **(E)** Quantitation of organoids sizes for the indicated treatment

groups in (D). **(F)** Quantitation of organoid numbers for the indicated groups in (D). Scale bar = 100 μm.