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STRUCTURAL SNAPSHOT

Structural insight into Wnt signaling inhibition by *Clostridium difficile* toxin B

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The incidence of *Clostridium difficile* infection (CDI) has increased significantly worldwide, causing substantial morbidity and mortality. One of the major virulence factor, TcdB, manages to enter the colonic epithelia via the human frizzled proteins (FZDs), which are physiological receptors for Wnt morphogens. Binding of TcdB to FZDs inhibits Wnt signaling, which may contribute to pathogenesis of CDI. Here, we review the structural mechanism by which TcdB exploits to recognize FZDs for cell entry and inhibiting Wnt signaling, which reveals new strategies to modulate Wnt signaling for therapeutic interventions.

Introduction

Clostridium difficile, or *C. diff* for short, is an opportunistic pathogen that can cause human diarrhea and pseudomembranous colitis [1]. It has been estimated that there were almost half a million cases of *C. difficile* infection (CDI) and approximately 29 000 associated deaths in the United States in 2011. Therefore, CDI is listed as an urgent threat by the Center for Disease Control and Prevention [2,3]. CDI is mainly caused by two *C. diff* virulence factors, toxin A (TcdA) and toxin B (TcdB). Both TcdA and TcdB are composed of various functional domains, including the glucosyltransferase domain (GTD), cysteine protease domain (CPD), delivery domain, and the combined repetitive oligopeptide domain (CROP) [4–6]. These toxins enter cells through receptor-mediated endocytosis. Once the GTD is delivered into the cytosol, it glucosylates small GTPases of the Rho family, including Rho, Rac, and CDC42 [7]. Glucosylation of Rho proteins inhibits their functions as molecular switches, leading to alterations in the actin cytoskeleton, resulting in cell-rounding and ultimately cell death [7–9]. The relative roles of these two toxins in the pathogenesis of CDI are not completely understood. But recent studies showed that TcdB is significantly more virulent than TcdA in animals [10–15], and TcdB alone is sufficient to elicit full spectrum of human diseases [10,16].

Host receptor recognition dictates the cell type tropism for a toxin and may determine the pathogenesis

Abbreviations
CDI, *Clostridium difficile* infection; CPD, cysteine protease domain; CRD, cysteine-rich domain; CROP, combined repetitive oligopeptide domain; CSPG4, chondroitin sulfate proteoglycan 4; FZD, frizzled; GTD, glucosyltransferase domain; PAM, palmitoleic acid; PVRL3, poliovirus receptor-like 3; TcdA, *C. diff* toxin A; TcdB, *C.* diff toxin B; $TcdB^{FBD}$, FZD-binding domain of TcdB.

process. Three receptors have been identified for TcdB: chondroitin sulfate proteoglycan 4 (CSPG4), Wnt receptor frizzled proteins (FZDs), and poliovirus receptor-like 3 (PVRL3), with FZDs thought to be the major receptors in the colonic epithelium [17–20]. FZDs are G protein-coupled receptor-like proteins, and the secreted Wnt glycoproteins initiate the canonical Wnt signaling by interacting with the extracellular cysteine-rich domain (CRD) of FZDs. The Wnt signaling pathway is crucial for development, homeostasis of multicellular organisms, stem cells functions, and many other processes [21]. TcdB binding to FZD inhibits Wnt signaling, which may contribute to the pathogenesis of CDI. In this review, we focus on the recent advances on understanding of the molecular mechanism by which TcdB recognizes FZDs for cell entry and inhibiting Wnt signaling, as well as their potential therapeutic implications.

Structural basis for recognition of FZDs by TcdB

To better understand TcdB–FZD interactions, we biochemically mapped the FZD-binding domain in TcdB (residues 1285–1804, termed $TcdB^{FBD}$), which is located in the delivery domain. $TcdB^{FBD}$ could be readily expressed in *Escherichia coli* and is stable in solution. The crystal structure of $TcdB^{FBD}$ in complex with the CRD of human FZD2 (residues 24–156, referred to as CRD2) was determined at 2.5 Å resolution (Fig. 1A) [22]. Unexpectedly, the crystal structure revealed that an endogenous lipid, palmitoleic acid (PAM), is completely buried between CRD2 and $TcdB^{FBD}$ through extensive hydrophobic interactions with both proteins. In addition to PAM-mediated interactions, $TcdB^{FBD}$ also recognizes CRD2 through a network of hydrogen bonds and hydrophobic interactions that surround the PAM-binding groove in CRD2. Our extensive structure-based mutagenesis studies demonstrated that this endogenous PAM serves as a crucial co-receptor for $TcdB^{FBD}$ to strengthen its binding to CRD2.

Wnt proteins undergo extensive post-translational modifications, and all Wnt are modified by lipidation through the addition of a PAM to a conserved serine residue, which is essential for their secretion and function [23]. Remarkably, the endogenous PAM found in the $TcdB^{FBD}$–CRD2 complex is located in the same hydrophobic groove in CRD that accommodates the covalently linked PAM in Wnt [22,24]. Amino acid sequence analysis showed that CRD2 residues F76, P78, and L79 lining on one side of the PAM and residues M125, F128, and F130 on the other side are highly conserved across 10 human FZDs (Fig. 1B,C). Moreover, this pocket is always hydrophobic in all FZD CRDs (Fig. 1D–H), which is consistent with its physiological role as the binding pocket for the Wnt-PAM. However, subtle amino acid differences in this groove and the neighboring areas across different FZD members may lead to different affinities of CRDs toward endogenous lipids. The closely related FZD1, 2, and 7, the high affinity receptors for TcdB, have CRDs that bind endogenous lipids with sufficiently high affinities [18,22], while other CRDs likely have weaker affinities. For example, the purified recombinant CRD5 does not contain a PAM. Nevertheless, CRD5 was capable of binding a PAM when exogenous PAM was added to the solution, which subsequently improved its binding to $TcdB^{FBD}$ [22,25].

A novel mechanism for Wnt signaling inhibition

It is well established that Wnt engages CRD through two separate interfaces: one site is formed by docking the Wnt-PAM into its binding pocket in CRD (a.k.a. the palmitoylated 'thumb'), while the other site is mediated by protein–protein interactions at a distinct location (a.k.a. the 'index finger') (Fig. 2A) [24]. This clamp-like binding model is likely conserved for all Wnt–FZD pairs. The Wnt-PAM is clearly a hotspot that contributes considerably to the total binding energy and is essential for FZD binding.

Structural comparison between the $TcdB^{FBD}$–CRD2 complex and the Wnt8–CRD8 complex indicates that the CRD2-bound PAM almost completely overlaps with the Wnt-PAM (Fig. 2A) [24]. Therefore, the Wnt-PAM needs to displace the endogenous PAM bound on CRD2 during Wnt signaling activation. But, binding of $TcdB^{FBD}$ locks the endogenous PAM in position and prevents docking of the Wnt-PAM. It is worth noting that $TcdB^{FBD}$ engages CRD2 from the opposite side of the Wnt-binding interface and does not directly compete with Wnt for CRD binding (Fig. 2A). Therefore, preventing the binding of Wnt-PAM to CRD seems to play a central role in inhibiting Wnt binding and signaling by TcdB. These findings suggest that the Wnt-PAM could be Wnt's Achilles heel, and disrupting its binding to CRD may be sufficient to block Wnt binding to FZD (Fig. 2B).

While $TcdB^{FBD}$ prevents Wnt from binding to CRD, $TcdB^{FBD}$ is able to use the Wnt-PAM as a co-receptor to bind the Wnt–FZD complex (Fig. 2A). We found that Wnt could enhance binding of $TcdB^{FBD}$ to some CRDs (e.g., FZD4, FZD5, FZD8, and FZD9) that have weaker affinities for endogenous lipids [22]. This is advantageous for TcdB to recognize a broad range of FZDs despite their sequence variations.

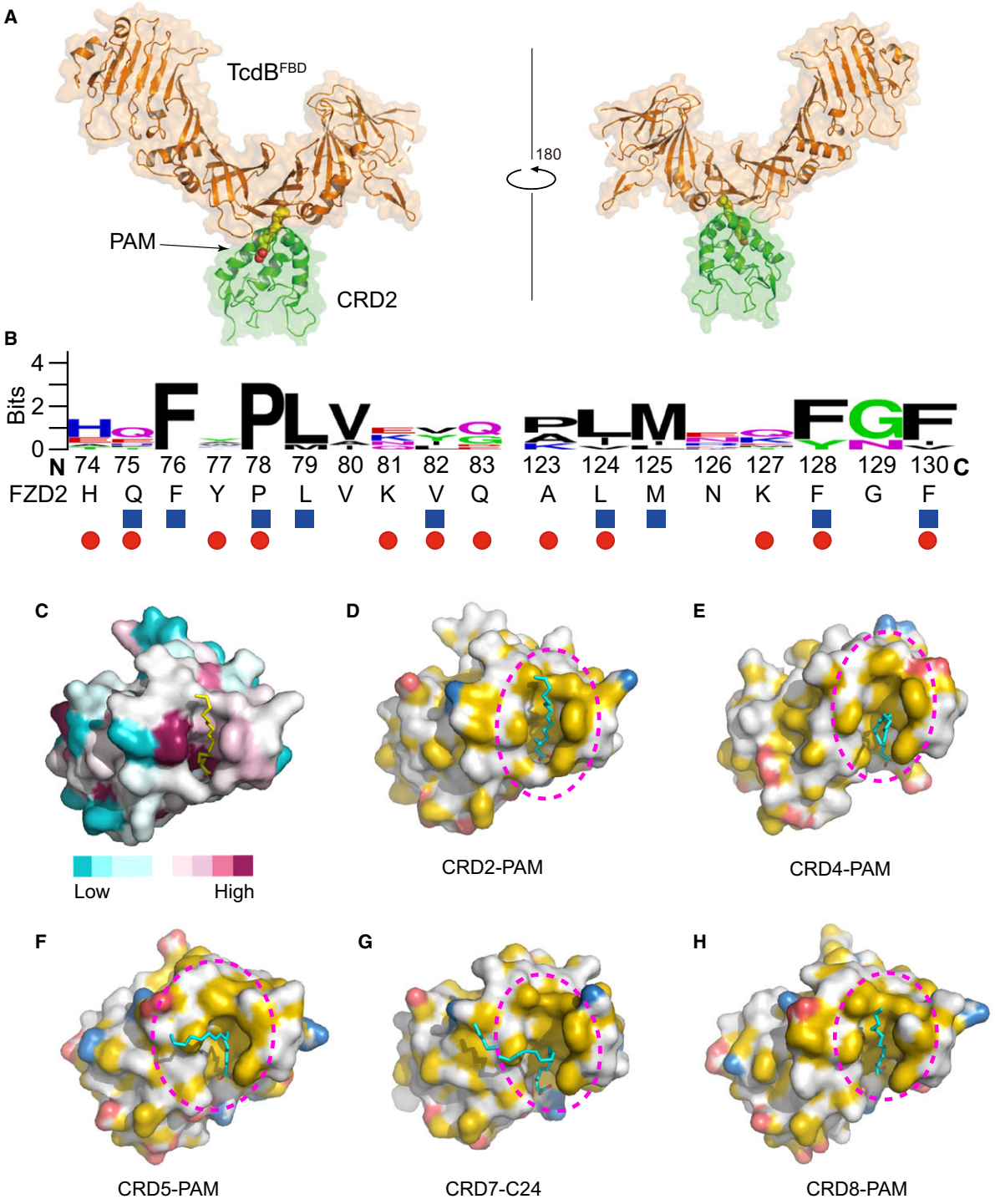

Fig. 1. TcdB^{FBD} uses an endogenous lipid as a co-receptor to recognize CRD2. (A) Overall structure of the TcdB^{FBD}–PAM–CRD2 complex. (B) Sequence conservation of key FZD residues that are involved in TcdB interaction (blue cubes) and/or PAM interaction (red ovals) among the 10 human FZDs [33]. (C) Molecular surface of CRD2, whereas the residues are colored according to their conservation across 10 human FZDs. (D–H) Hydrophobicity and charge distribution on CRD surfaces for representative CRD–lipid complexes [34]. The CRD-bound lipids are shown as sticks. Hydrocarbon groups without polar substitutions are shown in yellow; negatively charged oxygens of glutamate and aspartate are showed in red; nitrogens of positively charged functional groups of lysine and arginine are in blue; all remaining atoms including the polar backbone are showed in white.

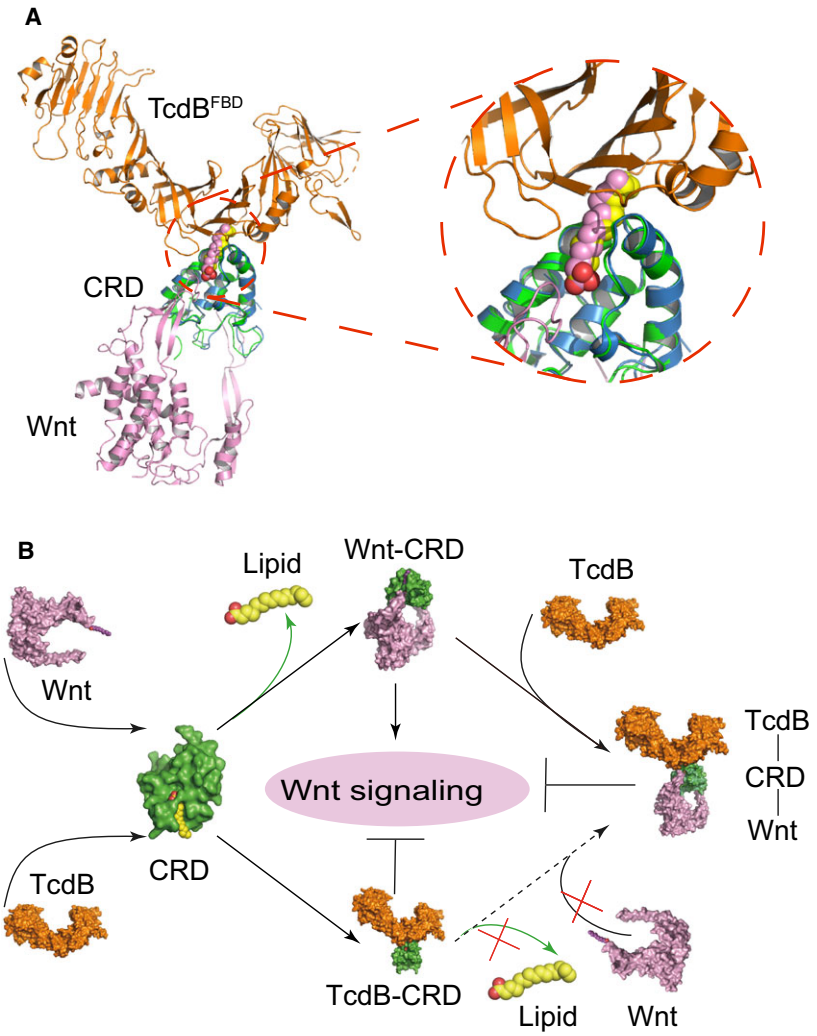

Fig. 2. A novel strategy to inhibit Wnt signaling by targeting the Wnt-PAM. (A) Superimposed structures of the $TcdB^{FBD}$–CRD2 complex (PDB: 6C0B) and the Wnt8–CRD8 complex (PDB: 4F0A) based on CRD. The yellow and pink sphere models represent the CRD2-bound PAM and the Wnt8-PAM, respectively. (B) A structural model for inhibition of Wnt signaling by TcdB.

The structural flexibility of the lipid bound in the hydrophobic groove in FZD CRD

In addition to the Wnt8–CRD8 complex, several structures of CRDs in complex with an endogenous lipid or an exogenous lipid added during crystallization have been reported, such as CRD2 in complex with PAM (PDB: 6C0B), CRD4 in complex with PAM (PDB: 5UWG), CRD5 in complex with PAM (PDB: 5URY), and CRD7 in complex with a C24 fatty acid (PDB: 5URV) [22,24–26]. Structural superposition of these CRD–lipid complexes reveals a common lipid-binding groove (Fig. 3A), but the relative positions and conformations of the bound lipids are different (Fig. 3B–F).

As the Wnt-PAM binds in this CRD pocket under physiological conditions, we used the Wnt-PAM as a bench mark for structural comparison and assigned the location of its carboxylic group in the Wnt8–CRD8 complex as state 0 (Fig. 3B). The CRD2-bound endogenous PAM also adopts a state 0 configuration, which puts its head group within proximity of the consensus acylation site in Wnt, therefore faithfully mimicking the Wnt-PAM [22]. This is consistent with the observation that TcdB could use the Wnt-PAM as a co-receptor for binding [22]. Interestingly, the carboxylic group of the lipids in the structures of the CRD4–PAM and the CRD7–C24 complexes shifts up toward Wnt by 2 and 3 carbon lengths, respectively (Fig. 3C,F). The structure of the CRD5–PAM complex revealed two different lipid configurations: PAM could shift away from Wnt (state -2) or adopt state 0 (Fig. 3D,E). Therefore, the exogenous lipids seem to have some freedom to slide in this hydrophobic groove in CRDs. In contrast, the Wnt-PAM is covalently attached to a conserved Ser residue (e.g., Ser187 in *xenopus* Wnt8) and the position of its carboxylic group

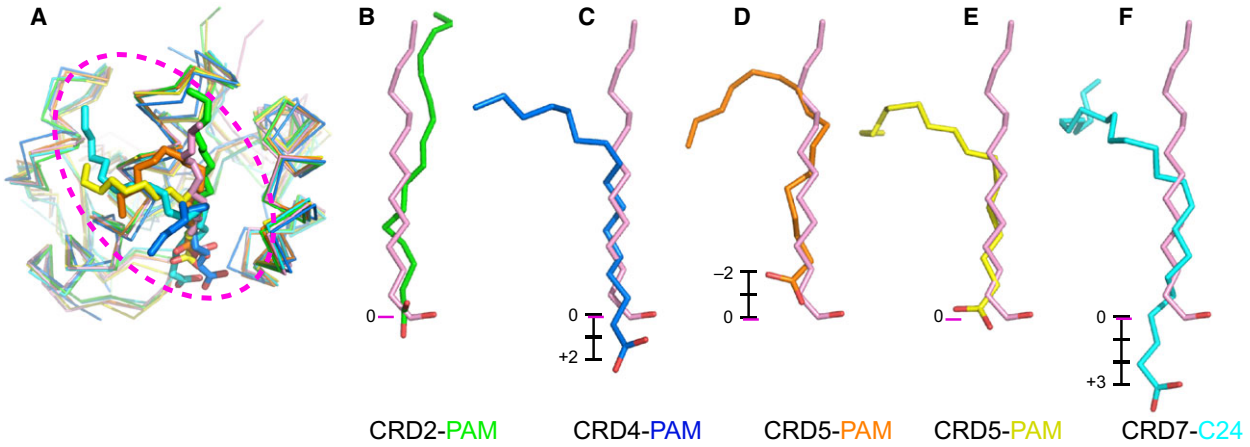

Fig. 3. Structural flexibility of the CRD-bound lipids. (A) Structural superposition of five CRD–lipid complexes with the Wnt8-bound CRD8 (pink, PDB: 4F0A): CRD2–PAM (green, PDB: 6C0B); CRD4–PAM (blue, PDB: 5UWG); CRD5–PAM (chain A: orange; chain B: yellow, PDB: 5URY); and CRD7–C24 (cyan, PDB: 5URV). (B–F) The lipid molecules in these complexes are individually compared with the Wnt8–PAM.

is constrained. Furthermore, the Wnt-PAM binding to CRD is facilitated by the protein moiety of Wnt [24]. Therefore, the flexibility of the bound lipid to slide in this CRD pocket may provide the structural basis for replacing this lipid by the Wnt-PAM during Wnt activation.

The Wnt lipid may mediate FZD dimerization for signaling

It is worth noting that the lipids found in the structures of CRD4 (PAM), CRD5 (PAM), and CRD7 (C24) adopt curved conformations (Fig. 3C–F), with their protruding methyl tail bound in the equivalent lipid-binding groove in a neighboring CRD molecule [25,26]. As a result, a single unsaturated fatty acid binds in a contiguous U-shaped hydrophobic groove formed between a CRD homodimer: the carboxylic acid end of the lipid is located on one CRD, while the methyl tail of the lipid is bound on the other CRD. The kinked unsaturated bond in the lipid is situated at the bottom of the U-shape crossing the CRD–CRD interface. It is suggested that the curved lipids stabilize the CRD dimerization and contribute to activate Wnt signaling. But its physiological relevance remains to be fully established.

Several distinct dimer configurations of CRD have been observed, which lead to different curvature of the contiguous U-shaped lipid-binding groove (Fig. 4A–D), whereas the angle of the kink in the CRD-bound lipid seems to vary accordingly (Fig. 3C–F). A recent study reported a homodimeric peptide (dFz21) that binds to CRD7 at a site close to the CRD dimer interface (Fig. 4D) [27]. This peptide forces the CRD7 dimer to open up relative to each other, which makes the U-shaped lipid-binding groove become more extended (Fig. 4D). It is believed that this unique open-configuration of a CRD7 dimer bound by dFz21 is incompetent to form the functional Wnt–FZD–LRP complex for signaling. The physiological contribution of FZD CRD dimerization to activating Wnt signaling *in vivo* warrants further studies.

Interestingly, the CRD8-bound Wnt-PAM adopts an extended conformation (Figs 1H and 2A), while CRD8 exists as a monomer in the crystal structure of the Wnt8–CRD8 complex [24]. We suspect that this is likely an intermediate conformation 'frozen' by the crystal packing effect, which preferentially crystallized CRD8 in the monomeric form. The authors acknowledged that higher order species of the Wnt8–CRD8 complex were observed in solution. Indeed, the tail of the Wnt-PAM acyl chain in the extended conformation is bound by a neighboring Wnt8 molecule due to crystal packing, which also blocks the formation of a potential CRD8 dimer [24]. In the $TcdB^{FBD}$–CRD2 complex, the endogenous PAM is completely buried between the two proteins, and the structurally flexible long acyl chain of PAM is 'forced' by $TcdB^{FBD}$ to adopt an extended conformation. At the same time, $TcdB^{FBD}$ also sterically blocks the formation of a CRD dimer (Fig. 4E). Taken together, TcdB renders the Wnt–FZD complex incompetent for proper downstream signaling (Fig. 2B).

Future prospects

Abnormal activation of Wnt signaling is tumorigenesis and is frequently associated with cancers, such as

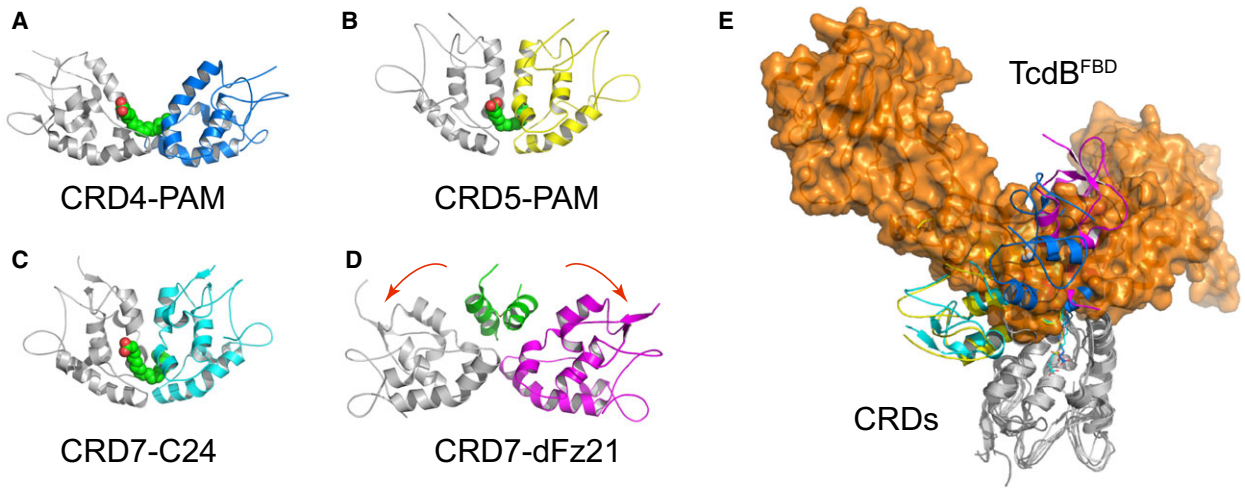

Fig. 4. CRD homodimers could adopt various configurations. (A) CRD4–PAM complex; (B) CRD5–PAM complex; (C) CRD7–C24 complex; (D) CRD7–dFz-21 complex (PDB: 5WBS). The lipids are shown as green sphere models and peptide dFz-21 in a green cartoon model. (E) The CRD dimers shown in panels A–D are superimposed to the $TcdB^{FBD}$-bound CRD2 based on the CRD molecule colored in gray. In each case, the other CRD molecule in a dimer clashes with $TcdB^{FBD}$.

breast, colorectal, and pancreatic cancers [21]. Upregulation of Wnt signaling may also play a critical role in epithelial–mesenchymal transition and cancer metastasis [28–31]. Furthermore, cancer cells that are exiting chemotherapy-induced senescence were shown to be associated with high levels of Wnt signaling activity [32]. Therefore, inhibiting Wnt signaling represents an important route to target these hard-to-treat cancer cells. The unique mechanism exploited by TcdB to target Wnt-PAM and block Wnt binding to FZD reveals a new strategy to develop Wnt antagonists for basic research and therapeutic application. For example, we have begun to test the hypothesis that the nontoxic $TcdB^{FBD}$ or its modified variants may be able to inhibit cancers that are associated with abnormal activation of Wnt signaling. Alternatively, it would be exciting to develop novel antibodies or small molecules as Wnt antagonists that prevent the binding of Wnt-PAM to CRD in a way similar to what $TcdB^{FBD}$ does. At the same time, the utilization of PAM as a co-receptor by TcdB also exposes its vulnerability, which may help to develop antitoxins to fight CDI. So, once again, pathogens have served us well as key scientific tools, which not only help us better understand the complicated biological processes but also open a new door to develop novel therapeutic agents.

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Author contributions

PC prepared the figures and wrote the paper with inputs from LT, ZL, MD, and RJ.

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