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Low-density lipoprotein receptor-related protein 1 is a CROPs-associated receptor for *Clostridioides infection* toxin B

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As the leading cause of worldwide hospital-acquired infection, *Clostridioides difficile* (*C. difficile*) infection has caused heavy economic and hospitalized burden, while its pathogenesis is not fully understood. Toxin B (TcdB) is one of the major virulent factors of *C. difficile*. Recently, CSPG4 and FZD2 were reported to be the receptors that mediate TcdB cellular entry. However, genetic ablation of genes encoding these receptors failed to completely block TcdB entry, implicating the existence of alternative receptor(s) for this toxin. Here, by employing the CRISPR-Cas9 screen in *CSPG4*-deficient HeLa cells, we identified LDL receptor-related protein-1 (LRP1) as a novel receptor for TcdB. Knockout of LRP1 in both *CSPG4*-deficient HeLa cells and colonic epithelium Caco2 cells conferred cells with increased TcdB resistance, while *LRP1* overexpression sensitized cells to TcdB at a low concentration. Co-immunoprecipitation assay showed that LRP1 interacts with full-length TcdB. Moreover, CROPs domain, which is dispensable for TcdB's interaction with CSPG4 and FZD2, is sufficient for binding to LRP1. As such, our study provided evidence for a novel mechanism of TcdB entry and suggested potential therapeutic targets for treating *C. difficile* infection.

***Clostridioides difficile*, low-density lipoprotein receptor-related protein 1, TcdB, toxin receptor, CRISPR screening**

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INTRODUCTION

Clostridioides difficile (formerly *Clostridium difficile*, or *C. difficile*) (Lawson et al., 2016; Oren and Rupnik, 2018) is a gram-positive, spore-forming pathogen that colonizes the mammalian intestinal tract. As one of the leading causes of healthcare-associated infections (HAIs), *C. difficile* infection (CDI) elicits a range of symptoms, including watery diarrhea, abdominal pain, fatal pseudomembranous colitis, toxic megacolon, and death (Martin et al., 2016; Rupnik et al., 2009). CDI has resulted in almost half a million infection cases and 29,000 deaths annually in the United States (Lessa et al., 2015). It has raised widespread public concerns as morbidity and mortality are increasing (Freeman et al., 2010; Leffler and Lamont, 2015; Lucado et al., 2012).

C. difficile exerts its toxicity through two homologous exotoxins, *C. difficile* toxin A (TcdA, 308 kD) and toxin B (TcdB, 270 kD) (Voth and Ballard, 2005), both of which belong to a family of structurally and functionally related bacterial toxins—large clostridial toxins (LCTs) (Just and Gerhard, 2004). LCTs are modular-structured and composed of four major domains, including an N-terminal glucosyltransferase domain (GTD), an autoprocessing domain, a pore-forming and delivery domain, and a C-terminal receptor-binding domain (RBD) (Just and Gerhard, 2004;

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Pruitt and Lacy, 2012; von Eichel-Streiber et al., 1996). The toxins enter cells in a clathrin-dependent manner (Papatheodorou et al., 2010). This endocytic process of TcdB has been proposed to be initiated by binding to the cellular receptor(s) through its receptor-binding domain, consisting of oligopeptide repeats (combined repetitive oligopeptides, CROPs) (Greco et al., 2006; Sauerborn et al., 1997; von Eichel-Streiber and Sauerborn, 1990). The receptor-bound toxins are internalized into early endosomes, and acidification of the endosome alters the toxins' conformations, followed by pore-forming and the release of GTD into the cytosol (Barth et al., 2001; Qa'Dan et al., 2000). The GTD catalyzes the transfer of a glucose moiety onto Rho family GTPase, such as Rho, Rac1, and Cdc42. This glucosylation process inactivates these enzymes and thereby causes the dysregulation of the cytoskeletons and the morphological changes of the cells (Jank and Aktories, 2008; Just et al., 1995).

As the premier process, receptor binding is pivotal for the cellular uptake of TcdA/B. Because the CROPs domain is homologous to the carbohydrate-binding domain and capable of binding to carbohydrate structures on the cell surface (Teneberg et al., 1996; Tucker and Wilkins, 1991; von Eichel-Streiber and Sauerborn, 1990), this domain is believed to mediate receptor binding. Indeed, recombinant fragments of CROPs and antibodies with epitopes in this region block endocytosis and cytotoxicity of toxins (Frey and Wilkins, 1992; Lyerly et al., 1986; Sauerborn et al., 1997). However, the discovery of a novel clostridial toxin lacking CROPs argues that CROPs domain is dispensable for endocytosis and cytotoxicity of the toxin (Amimoto et al., 2007). In addition, removal of CROPs compromises but does not abolish cellular uptake of TcdA, implicating an alternative receptor-binding domain other than CROPs (Olling et al., 2011). Based on these observations, a dual-receptor model for the endocytosis of clostridial toxins has been proposed: toxins enter cells by binding to cellular receptors through its CROPs and/or non-CROPs domain (Olling et al., 2011; Schorch et al., 2014).

Little was known about the cellular receptors for TcdB until recent years. Yuan et al. (2015) identified chondroitin sulfate proteoglycan 4 (CSPG4) as the first receptor for TcdB. The deficiency of *CSPG4* confers resistance to TcdB in both HeLa and HT29, a colonic epithelium cell line. Of note, CSPG4 binds to TcdB through the non-CROPs region of the receptor-binding domain, together with the first repeat of CROPs. Moreover, *CSPG4*-null cells are susceptible to the high concentration of TcdB, and *NG2* (a homolog of *CSPG4* in mice) knockout mice succumb to manifestation caused by TcdB, suggesting the existence of an alternative receptor(s) for TcdB. More recently, frizzled proteins FZD1/2/7 were found to mediate TcdB entry in a CROPs-independent fashion (Tao et al., 2016). These studies address the critical role of the non-CROPs receptor-binding domain in cellular uptake of TcdB. However, as removing the C-terminus of CROPs ($TcdB_{\Delta 1849\text{-}2366}$) almost abrogates the cytotoxicity of TcdB (Manse and Baldwin, 2015), the CROPs-associated TcdB receptor(s) remain to be identified to further clarify the endocytosis process of TcdB.

Here, leveraging a single guide RNA (sgRNA) library targeting 2,989 human membrane protein-associated coding genes, we performed a high-throughput CRISPR screen to explore alternative functional receptors for TcdB in *CSPG4*-deficient HeLa cells. We identified LRP1, a protein involved in endocytosis and intracellular signaling, as a receptor for TcdB. The C-terminus of CROPs ($TcdB_{1852\text{-}2366}$) is sufficient and responsible for the interaction of TcdB with LRP1. Our study unraveled a novel mechanism by which TcdB enters the cell and provided a potential therapeutic target for treating *C. difficile* infection.

RESULTS

CRISPR-Cas9 screen for novel TcdB receptors

Chondroitin sulfate proteoglycan 4 (CSPG4) has been previously demonstrated as a receptor for TcdB (Yuan et al., 2015). However, although genetic ablation of *CSPG4* causes cellular resistance to a low concentration of TcdB, increasing the dose of TcdB could still cause $CSPG4^{-/-}$ cell death. In addition, *NG2* (homolog of *CSPG4* in rodent) knockout mice remains vulnerable to TcdB (Yuan et al., 2015). These findings denoted the existence of receptors other than CSPG4. To explore alternative receptors for TcdB, we performed a CRISPR (clustered regularly interspaced short palindromic repeats) screen in the *CSPG4*-deficient HeLa cells (designated as HeLa_$CSPG4^{-/-}$, Figure 1A and Figure S1 in Supporting Information). The CRISPR screen employed a customized sgRNA library targeting 2,989 membrane protein-coding genes, and each of those was targeted by 10 sgRNAs. Two biological replicates were carried out. Before the screen, sgRNAs in two replicates were sequenced and analyzed to verify proper distributions and the consistency between two replicates (Figure S2 in Supporting Information). The cell library was exposed to TcdB, and cells vulnerable to TcdB were removed by mild pipetting. The resistant cells were then expanded for another round screen. For the first 5 rounds of screen, HeLa_$CSPG4^{-/-}$ was exposed to 5 ng mL^{-1} TcdB for 8 h. The survival cells were subjected to another 2 rounds of screen using 8 ng mL^{-1} TcdB. The screen was ceased after 7 rounds of screen, as ratio of resistant cells no longer increased (Figure 1B and C). The sgRNA sequence of survival cells was PCR amplified and subjected to next-generation sequencing (NGS).

sgRNA reads were analyzed with the MAGeCK method (Li et al., 2014) and ranked as genes according to the RRA

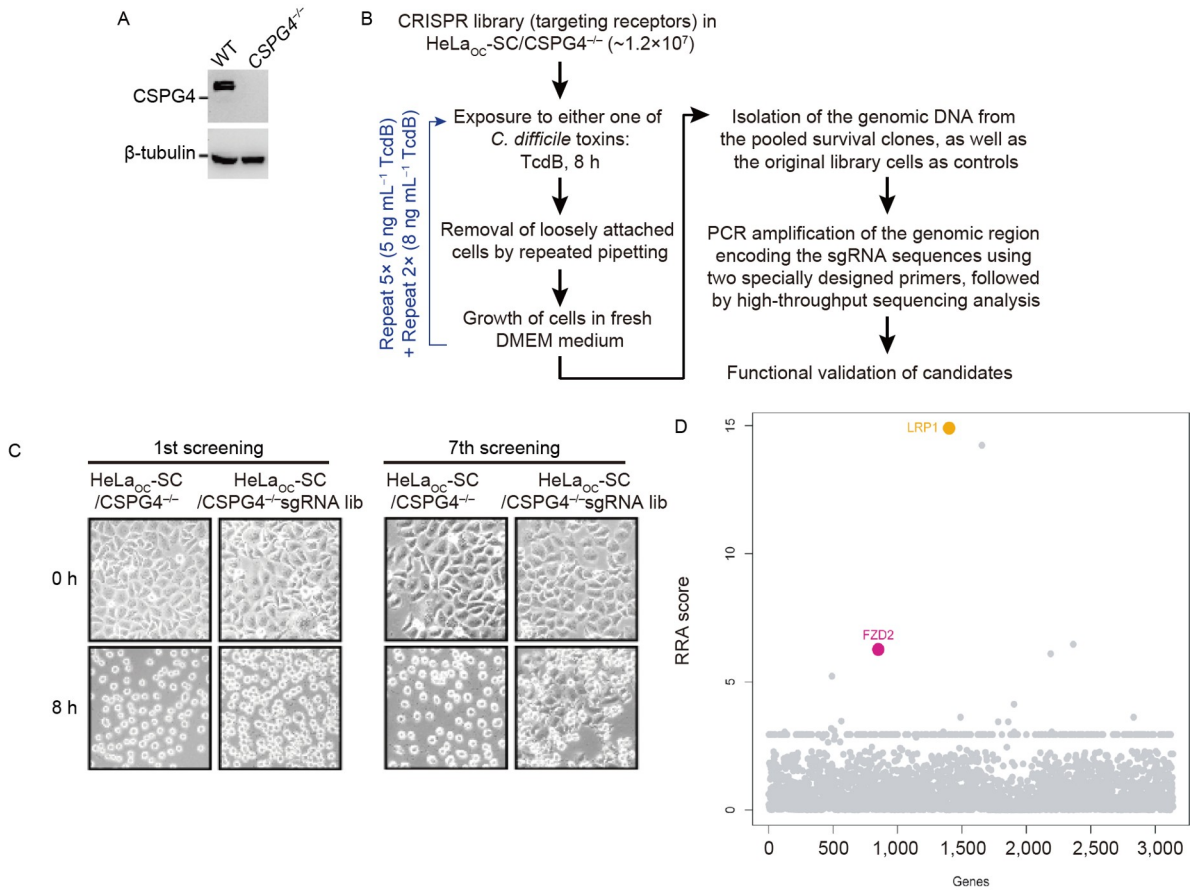

Figure 1 (Color online) CRISPR-Cas screening for the alternative receptor for TcdB. A, Immunoblot analysis of CSPG4 in HeLa and HeLa_*CSPG4*$^{-/-}$ cells. β-tubulin served as an internal control. B, The CRISPR-Cas9 screen was carried out by exposing to five rounds of 5 ng mL^{-1} of TcdB, followed by additional two rounds of the screen with 8 ng mL^{-1} of TcdB. The sgRNA sequence of survival cells was then analyzed by NGS. C, TcdB-resistant cells were enriched by exposure with TcdB. Both HeLa_*CSPG4*$^{-/-}$ cells and the majority of library cells were susceptible to TcdB treatment in the initial treatment with TcdB. After seven rounds of TcdB treatment, the majority of library cells were survival and resistant to TcdB, while HeLa_*CSPG4*$^{-/-}$ cells sustained susceptibility to TcdB. D, Ranking of sgRNAs of the TcdB-resistant cells. sgRNAs of survival cells were subjected to next-generation sequencing. Reads of sgRNAs were analyzed by MAgeCK method (Li et al., 2014) and ranked according to RRA score. The *x*-axis indicates genes, and the *y*-axis indicates the RRA score. Especially, LRP1 and FZD2 came out as candidates for TcdB receptors.

(robust rank aggregation) score (Figure 1D). As a result of the stringent screening process of up to seven rounds, limited genes stood out from the screen. Among those, low-density lipoprotein-related protein 1 (LRP1)-coding gene was top-ranked with seven individual sgRNAs enriched. LRP1 is a widely expressed receptor that plays critical roles in diverse biological processes, including endocytosis (Herz and Strickland, 2001; Lillis et al., 2008). It has numerous ligands, including bacterial toxins *Pseudomonas* Exotoxin A and TpeL (Kounnas et al., 1992; Schorch et al., 2014). Of note, frizzled class receptor 2 (FZD2) came out as one of the top hits in our screen, which has recently been identified as a functional receptor for TcdB (Tao et al., 2016).

Depletion of LRP1 prevents the uptake of TcdB in HeLa cells

We next focused on LRP1. To validate LRP1's function, we disrupted the *LRP1* gene in HeLa_*CSPG4*$^{-/-}$ cells using TALENs, thereby generating a cell line deficient in both *CSPG4* and *LRP1* (designated as HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$) (Figure 2A and Figure S3 in Supporting Information). Given that TcdB treatment could turn cell morphology from spindle to round shape, cell rounding assay was employed to assess the cellular susceptibility to TcdB. Both HeLa_*CSPG4*$^{-/-}$ and HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$ cells were challenged with serially diluted TcdB. By analyzing cell rounding, dosage-response curves were depicted, and the median lethal concentration (LC_{50}) was calculated (Figure 2B). Lack of *LRP1* remarkably increased cell resistance to TcdB, as LC_{50} of HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$ cells was 3.5-fold higher than that of HeLa_*CSPG4*$^{-/-}$ cells. After exposing to 6.4 ng mL^{-1} TcdB for 5 h, the majority of the HeLa_*CSPG4*$^{-/-}$ cells turned round, while HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$ cells remained spindle shape (Figure 2C). Rac1 glucosylation assay was often used to assess intoxication caused by TcdB because the total Rac1

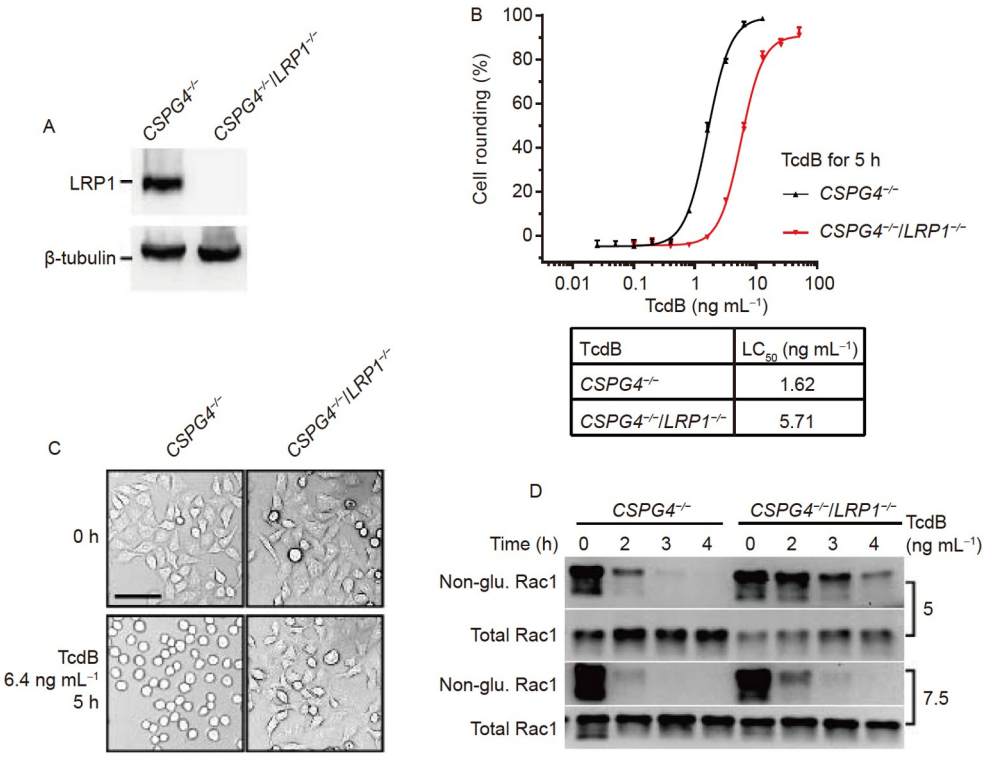

Figure 2 (Color online) *LRP1*-deficiency conferred HeLa cells with resistance to TcdB. A, Immunoblot analysis of LRP1 in HeLa_*CSPG4*$^{-/-}$ and HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$ cells. Antibody against LRP1's β-subunit (~85 kD) was used to detect the expression of LRP1. β-tubulin served as an internal control. B, Cell rounding assay was conducted to assess resistance to TcdB. Both HeLa_*CSPG4*$^{-/-}$ cells and HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$ cells were exposed to TcdB of indicated concentrations for 5 h. Cell morphologies were recorded and analyzed using Opera Phenix high content screening system (Perkin Elmer). The ratio of rounding cells was plotted by Prism6. The dose-response curves were analyzed using nonlinear regression. These data were represented on a semi-logarithmic plot, where the concentration of TcdB is plotted on the *x*-axis in a log form, and the ratio of cell rounding is plotted on the *y*-axis using a linear scale. Comparing with HeLa_*CSPG4*$^{-/-}$ cells, deletion of LRP1 further elevated resistance to TcdB, that LC_{50} increased from 1.62 to 5.71 ng mL^{-1}. The data are mean±SD, *n*=3. C, Microscopic images of cells from (B) under the condition as indicated. Images were taken using Opera Phenix high content screening system (Perkin Elmer). Scale bar=100 μm. D, Glucosylation of Rac1 was examined by immunoblot analysis. The whole-cell lysate was harvested at indicated timepoints following treatment with 5 or 7.5 ng mL^{-1} TcdB. Both total Rac1 and non-glucosylated Rac1 (non-glu. Rac1) were detected with the specific antibody. Lack of LRP1 significantly decelerated glucosylation of Rac1 caused by TcdB exposure.

level remains unchanged after the exposure of TcdB, while the glucosylated Rac1 accumulates with the prolonged exposure time, resulting in the concomitant decrease of the non-glucosylated Rac1 (Giesemann et al., 2008; Huelsenbeck et al., 2007; Just and Gerhard, 2004). Hence, we examined both total Rac1 and non-glucosylated Rac1 by immunoblot at different timepoints after TcdB treatment. In HeLa_*CSPG4*$^{-/-}$ cells, depletion of *LRP1* significantly decelerated Rac1 glucosylation when exposed to either 5 ng mL^{-1} or 7.5 ng mL^{-1} TcdB (Figure 2D). Altogether, our results confirmed LRP1's role in mediating endocytosis and cytotoxicity of TcdB.

Considering that FZD2 has also been identified in our study, which was confirmed by a previous report (Tao et al., 2016), and that members of LDL receptor family and frizzled family are closely associated in Wnt signaling transduction (He et al., 2004; Huang and Klein, 2004; Qian et al., 2014; Wehrli et al., 2000), we next interrogated and compared their roles in the absence of CSPG4. To address this, HeLa_*CSPG4*$^{-/-}$/*FZD2*$^{-/-}$ cells and HeLa_*CSPG4*$^{-/-}$/*FZD2*$^{-/-}$/*LRP1*$^{-/-}$ cells were generated. As shown in the cell rounding assay, *CSPG4*$^{-/-}$/*FZD2*$^{-/-}$ is more resistant to TcdB than *CSPG4*$^{-/-}$/*LRP1*$^{-/-}$, which implies FZD2's activity in TcdB entry is more potent. Moreover, triple knockout of these genes further impeded TcdB-mediated intoxication of cells and increased cell resistance to TcdB to ~1.5-fold in terms of LC_{50}, comparing with HeLa_*CSPG4*$^{-/-}$/*FZD2*$^{-/-}$ cells (Figure S4 in Supporting Information). This result indicated that both LRP1 and FZD2 play important but non-redundant roles in TcdB endocytosis in HeLa cells.

Overexpression of LRP1 sensitizes cells to TcdB

We then asked whether LRP1 overexpression could promote TcdB cellular endocytosis. LRP1 is a huge protein of ~600 kD, and its coding sequence is approximately 14 kb; thus, it is challenging to clone and express this gene in full-length. To solve this issue, we generated a stable cell line overexpressing LRP1 by inserting a bi-directional cytomegalovirus (CMV) promoter in the promoter region of native

LRP1. This insertion was achieved via an NHEJ-mediated (Men et al., 2017) targeted integration approach using the CRISPR system (Figure 3A). By fluorescence-activated cell sorter (FACS) sorting and immunoblotting analysis, a single cell clone (designated as HeLa_*LRP1*-OE) with relatively high LRP1 expression was generated (Figure 3B).

In cell rounding assays, the upregulation of LRP1 dramatically increased the susceptibility of HeLa cells to TcdB. The LC_{50} slumped from 0.367 ng mL^{-1} in HeLa cells to 0.075 ng mL^{-1} in HeLa_*LRP1*-OE cells (Figure 3C). Most HeLa_*LRP1*-OE cells turned round after challenged by 0.1 ng mL^{-1} TcdB for 5 h, while the majority of HeLa cells maintained normal morphology (Figure 3D). In addition, Rac1 glucosylation was highly accelerated in HeLa_*LRP1*-OE cells (Figure 3E). Combined with observations in *LRP1*$^{-/-}$ cells, we conclude that LRP1 is critical for TcdB's entry in HeLa cells.

LRP1 involves in the endocytosis of TcdB in the colonic cell line

As the colon is one of the primary targets for *C. difficile*, we conducted experiments using a more physiologically relevant cell line—Caco2 (Di Bella et al., 2016; Thelestam and Chaves-Olarte, 2000). Caco2 is a commonly used colonic epithelium that is susceptible to TcdB (Chumbler et al., 2016; Feltis et al., 1999). Importantly, Caco2 has been shown to be *CSPG4*-deficient (Schöttelndreier et al., 2018; Tao et al., 2016), making it convenient to study the role of LRP1 in the absence of CSPG4. The *LRP1*-deficient Caco2 cell line was subsequently generated using lentiviral delivered Cas9 and its corresponding sgRNA (Figure 4A).

We then measured TcdB-mediated toxicity in these colonic cell lines. Both Caco2 and Caco2_*LRP1*$^{-/-}$ cells were exposed to serially diluted TcdB for 12 h, followed by the Rac1 glucosylation assay. We found that knockout of *LRP1* decreased TcdB-induced Rac1 glucosylation at the concentration of 6.25–50 ng mL^{-1} (Figure 4B and C). The difference in Rac1 glucosylation between two cell lines was marginal when the TcdB concentration was below 6.25 ng mL^{-1}. This is likely because lower levels of TcdB are not sufficient to induce detectable changes of Rac1 glucosylation in Caco2 cells. Consistently, the dose-response curves of Rac1 glucosylation also revealed that *LRP1*$^{-/-}$ cells appeared more resistant to TcdB than Caco2 cells (Figure 4D).

LRP1 interacts with TcdB via CROPs domain

We then examined if LRP1 physically interacts with TcdB. Because LRP1 is too large to be expressed and purified, we enriched LRP1 protein from the HeLa_*LRP1*-OE cells using an anti-LRP1 antibody immobilized on Protein A/G sepharose. Meanwhile, isotype IgG was used as a negative control, and CSPG4-Myc protein enriched by Myc antibody-immobilized sepharose was used as a positive control. On mixing of the indicated protein-bound sepharose with the purified TcdB holotoxin, control IgG failed to precipitate TcdB, while both LRP1 and CSPG4 co-precipitated with substantial TcdB holotoxin, indicating that LRP1 interacts with TcdB (Figure 5B).

We next set out to determine the exact region of TcdB that mediates LRP1 binding. As previously reported, LRP1 is not involved in the cellular uptake of CROPs-deficient TcdA and TcdB (Schorch et al., 2014). We thus speculated that TcdB interacts with LRP1 through CROPs domain. To this end, we purified GST-tagged truncated TcdB CROPs (GST-$TcdB_{1852\text{-}2366}$) and examined their interaction with LRP1 (Figure 5A and C). As expected, LRP1 was capable of co-precipitating with GST-$TcdB_{1852\text{-}2366}$, while CSPG4, which interacts with TcdB independent of its CROPs (Yuan et al., 2015), failed to do so (Figure 5C). These data suggested that LRP1 mediates TcdB endocytosis by interacting with its CROPs domain.

Then, we sought to identify the region of LRP1 critical for TcdB binding. The extracellular domain of LRP1 contains several cysteine-rich complement-type repeats (CRs), forming four canonical ligand-binding domains, namely cluster I-IV (Herz and Strickland, 2001; Horn et al., 1997; Lillis et al., 2008; Neels et al., 1999). LRP1 has a number of different ligands, the majority of which bind to cluster II and cluster IV (Herz and Strickland, 2001; Neels et al., 1999; Willnow et al., 1994). In particular, cluster IV of LRP1 has been demonstrated to mediate LRP1's interaction with another clostridial toxin, TpeL (Schorch et al., 2014). To determine the exact TcdB-binding domain of LRP1, we engaged LRP1 mini-receptors (Bu and Rennke, 1996; Jen et al., 2010; Li et al., 2000) that encompass signal peptide, either of ligand-binding clusters as indicated, immediate extracellular domain after the last CR repeat, transmembrane domain, and the cytosolic domain. Surprisingly, neither ectopically expressed LRP1 mini-receptors could rescue the lost function of LRP1 in HeLa_*CSPG4*$^{-/-}$/*LRP1*$^{-/-}$ cells (Figure S5A in Supporting Information). Consistently, neither of the four canonical ligand-binding clusters could solely pull down TcdB (Figure S5B in Supporting Information). It is possible that LRP1-mediated TcdB entry is dependent on the cooperation of multiple ligand-binding domains, or the region except for ligand-binding domains.

DISCUSSION

We identified LRP1 as a novel receptor for TcdB by performing a CRISPR-Cas9 screen in *CSPG4*-deficient HeLa cells. Depletion of LRP1 in HeLa_*CSPG4*$^{-/-}$ cells increased its resistance to TcdB, whereas the upregulation of LRP1 has

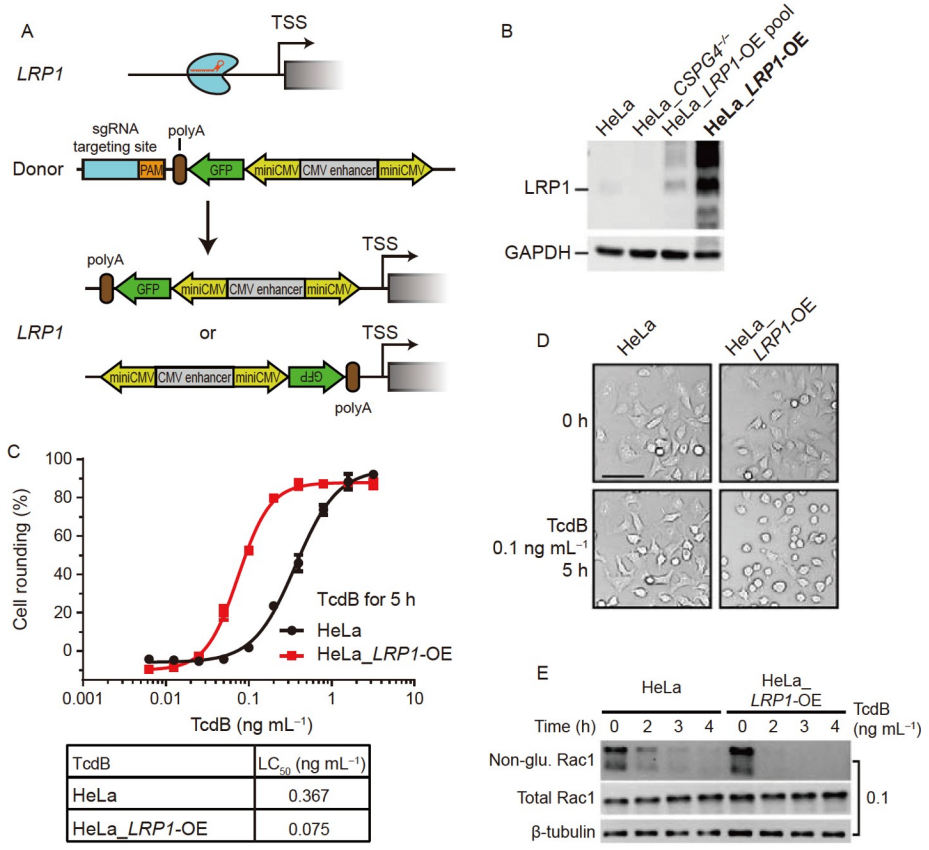

Figure 3 (Color online) Overexpression of *LRP1* facilitated cellular uptake of TcdB. A, Schematic of generation of stable cell lines overexpressing endogenous *LRP1* via CRISPR/Cas9 system. The linear donor consisted of the same sgRNA targeting site as the one in *LRP1*'s promoter region (upstream of the transcription start site, TSS), NGG PAM, polyA, inverted EGFP coding sequence and bi-directional CMV promoter. The artificial donor was integrated in the genome at the cleavage site, following Cas9 induced DSB in genome loci and the linear donor. Insertion of donors leads to both forward and backward insertion, both of which could enable the bi-directional CMV to upregulate expression of LRP1 while expressing EGFP as a selection marker. B, LRP1 overexpression stable single clone was validated by immunoblot. Comparing with HeLa cells, the expression of LRP1 in HeLa_*LRP1*-OE was extremely upregulated. GAPDH served as an internal control. C and D, Resistance against TcdB was measured by cell rounding assay. The dose-response curves (C) and relative microscopic images (D) as indicated illustrated that overexpression of LRP1 significantly diminished resistance to TcdB in HeLa cells. The data are mean±SD, n=3. Scale bar=100 μm. E, Overexpression of LRP1 promoted the intoxication of TcdB in HeLa cells, as implicated by glucosylation of Rac1 induced by 0.1 ng mL^{-1} TcdB.

the reverse effects. In addition, LRP1 is involved in cellular uptake of TcdB in the colonic epithelium Caco2 cells. We demonstrated that TcdB interacts with LRP1 through its C-terminus of CROPs domain (TcdB$_{1852\text{-}2366}$). This region is crucial for the full potency of TcdB, as exemplified by the fact that the deletion of TcdB$_{1849\text{-}2366}$ markedly attenuates TcdB's cytotoxicity to both Vero and CHO cells (Manse and Baldwin, 2015). However, how the C-terminal region of CROPs domain dominates TcdB cytotoxicity remains unresolved. Here, we propose LRP1 as a CROPs-associated receptor for TcdB, thus providing a novel model by which TcdB enters the cell.

LRP1 is a multifunctional receptor with numerous ligands. It has been opted as receptors for multiple bacterial toxins such as Pseudomonas Exotoxin A and TpeL (Kounnas et al., 1992; Schorch et al., 2014). Of note, TpeL is a CROPs-deficient clostridial toxin (Amimoto et al., 2007), which utilizes LRP1 to enter cells by binding to LRP1 cluster IV (Schorch et al., 2014). However, LRP1 cluster IV, the canonical ligand-binding domain of LRP1, is insufficient to mediate binding with TcdB. This could be partially explained by our observation that LRP1 interacts with the C-terminus of TcdB's CROPs domain (TcdB$_{1852\text{-}2366}$), instead of the non-CROPs region TcdB$_{1349\text{-}1811}$, homologous to LRP1-binding region of TpeL (TpeL$_{1335\text{-}1779}$). This indicates that LRP1 is a versatile receptor for clostridial toxins and facilitates endocytosis of clostridial toxins in either CROPs-dependent or -independent manner by different mechanisms.

LRP1 belongs to the LDL receptor family that consists of receptors traffic between the membrane and endocytic compartments (Go and Mani, 2012; Li et al., 2001). Thus, LRP1 may function as an endocytic receptor for TcdB and contribute to its internalization. Indeed, rather than known TcdB receptors CSPG4, FZD2/7, and PVRL3, LRP1 is efficiently endocytosed in both fibroblasts and Caco2 cells (Schöttelndreier et al., 2018). Apart from LRP1, another

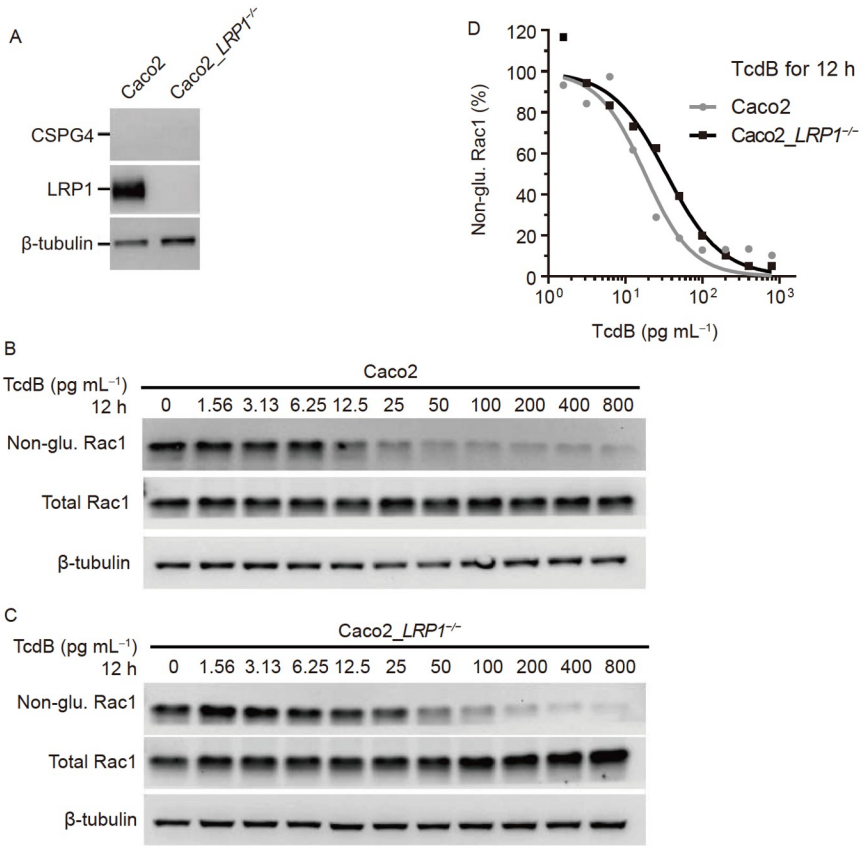

Figure 4 LRP1 is involved in the endocytosis of TcdB in human colonic cells. A, Immunoblot of CSPG4 and LRP1 in Caco2 cells and Caco2_*LRP1*$^{-/-}$ cells, respectively. β-tubulin served as an internal control. B and C, Glucosylation of Rac1 in Caco2 cells (B) and Caco2_*LRP1*$^{-/-}$ cells (C) were examined by immunoblot, after cells subjected to TcdB of an increasing serial dosage for 12 h. D, The dose-response curves of Rac1 glucosylation caused by TcdB. Bands of total and non-glucosylated Rac1 from (B) and (C) were subjected to relative quantification analysis using ImageLab (Bio-Rad). Ratios of non-glucosylated Rac1 were calculated and plotted in GraphPad Prism6. Nonlinear regression was exploited to generate dose-response curves. (Grey line indicates Caco2 cells; black line indicates Caco2_*LRP1*$^{-/-}$ cells).

member of LDL receptor family, LDLR has been recently established as a receptor for TcdA through CRISPR-Cas9 screening using CROPs-deficient TcdA (TcdA$_{1\text{-}1874}$). LDLR exerts its role through coordinating with sulfated glycosaminoglycans (sGAGs) and facilitating endocytosis of cGAGs-bound TcdA. We speculate that TcdA and TcdB employ entirely distinct endocytic apparatus although they are structurally related: TcdA initiates attachment to the cell surface by binding to sGAGs through its non-CROPs RBD, followed by being presented to LDLR and spontaneously internalized, while TcdB engages CSPG4, FZD1/2/7, and LRP1 through non-CROPs RBD and CROPs, respectively, and is then internalized. The mechanism of synergistic action between receptors of TcdB needs to be further clarified.

LRP1 is a widely expressed receptor with various ligands, like extracellular matrix proteins, growth factors, protease, and toxins (Lillis et al., 2008). LRP1 is involved in the homeostasis of many secreted proteins and the integrity of the extracellular matrix, as it participates in clearing of proteases, like metalloproteinases (Van Gool et al., 2015). Abnormal expression of LRP1 strongly correlated with poor clinical outcomes of colon cancer (Boulagnon-Rombi et al., 2018). CSPG4 binds to extracellular matrix proteins, growth factors, integrins, and lectins (Iida et al., 2007; Ilieva et al., 2017; Timpl et al., 2000). It also facilitates tissue development or homeostasis by engaging galectin-3 and integrin (Fukushi et al., 2004). FZD2 is involved in the Wnt signaling pathway, which is pivotal for the self-renewal of colonic epithelium and the maintenance of colonic stem cells (Crosnier et al., 2006; Gregorieff and Clevers, 2005). Both TcdA and TcdB have been suggested to attenuate Wnt signaling in cells (Bezerra Lima et al., 2014; Tao et al., 2016). Thus, TcdB may cause pathological outcomes by hijacking these important receptors and disrupting their normal function.

The expression profile of LRP1 and other receptors for TcdB are highly distinct in different cell types. For instance, both LRP1 and FZD2 are expressed in HeLa, Caco2, and HT-29 cells, while the expression of CSPG4 could not be detected in Caco2 cells (Jiang and Lönnerdal, 2017; Tao et al., 2016; Yuan et al., 2015). Regarding *in vivo* expression, LRP1 and FZD2/7 are expressed in colonic epithelium, while

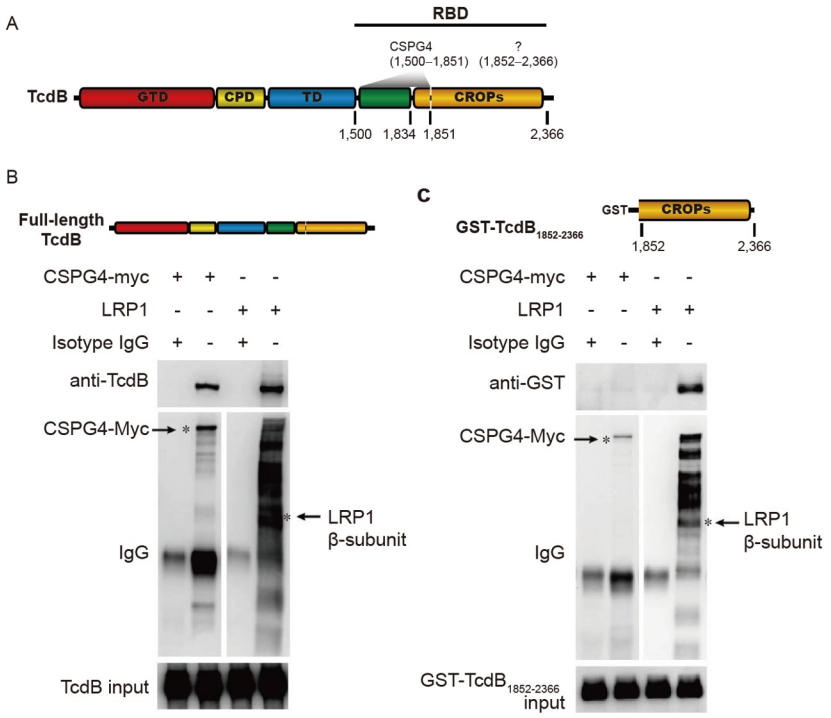

Figure 5 (Color online) TcdB interacted with LRP1 through the C-terminal of CROPs. A, Schematic of TcdB and its functional domains. GTD, glucosyltransferase domain; CPD, cysteine protease domain; TD, translocation domain; RBD, receptor-binding domain. The C-terminal receptor-binding domain ($TcdB_{1500-2366}$) includes both CROPs and non-CROPs regions. Amino acid residues 1,500–1,851 has been indicated to interact with CSPG4, while receptors interact with C-terminal of CROPs ($TcdB_{1852-2366}$) remain unknown. B and C, Purified TcdB holotoxin (B) and GST-tagged C-terminal of CROPs (GST-$TcdB_{1852-2366}$) (C) were incubated with endogenous LRP1 immobilized on Protein A/G sepharose by anti-LRP1 antibody. Isotype IgG served as an internal control for antibodies. Both TcdB and GST-$TcdB_{1852-2366}$ were co-immunoprecipitated with endogenous LRP1. TcdB holotoxin, but not GST-$TcdB_{1852-2366}$, interacted with myc-tagged CSPG4. The asterisks indicate CSPG4-myc or LRP1 β-subunits, respectively.

CSPG4 is mainly found in the multi-nucleated intestinal sub-epithelial myofibroblasts (ISEMFs) (Boulagnon-Rombi et al., 2018; Tao et al., 2016). Moreover, only LRP1 and FZD7 are detected in human intestinal organoids. Taken together, we favor a multi-receptor model for TcdB (Figure 6). TcdB enters cells by engaging LRP1, CSPG4, and FZD1/2/7 through the distinct binding sites in different cell types. TcdB may invade the intestinal tract by binding LRP1 and FZD1/2/7 that are expressed in colonic epithelium, through the regions spanning residues $TcdB_{1852-2366}$ and $TcdB_{1285-1804}$, respectively, and further disrupt sub-epithelial cells through CSPG4-mediated endocytosis. As HeLa_$CSPG4^{-/-}$/$FZD2^{-/-}$/$LRP1^{-/-}$ cells are still susceptible to the high concentration of TcdB, we speculate that TcdB may exploit yet unknown endocytic receptors or other receptor-independent endocytic pathways to enter the cells. The full mechanism of TcdB cellular entry still needs further investigation to clarify.

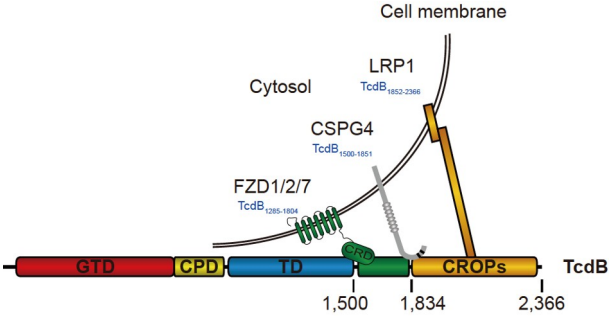

Figure 6 (Color online) Multiple-receptor model for endocytosis of TcdB. TcdB enters cells by hijacking three types of host receptors. TcdB binds to the cysteine-rich domain (CRD) of frizzled proteins (FZD1/2/7) in a CROPs-independent manner through amino acid residues 1,285–1,804, which is adjacent to the N-terminus of CROPs domain. Non-CROPs region of the receptor-binding domain and first repeat of CROPs domain ($TcdB_{1500-1852}$) constitute CSPG4-binding site for TcdB. As a CROPs-dependent receptor for TcdB, LRP1 interacts with solely the C-terminal of the TcdB CROPs domain ($TcdB_{1852-2366}$).

MATERIALS AND METHODS

Cell lines

HeLa cells, stably expressing SpCas9 were from the previous study (Zhou et al., 2014). Cas9-expressing HeLa cells, HEK293T, Caco2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) with 10% (vol/vol) FBS and penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1}). All cells were incubated with 5% CO_2 at 37°C.

Antibody and proteins

The primary antibodies used in this study were rabbit

monoclonal antibody against human LRP1 (Abcam, USA), rabbit monoclonal antibody against human CSPG4 (Abcam); mouse monoclonal antibodies against TcdB (GeneTex, USA), non-glucosylated Rac1 (BD, USA), Rac1 (Millipore, USA), and mouse monoclonal antibody against c-myc (CWBIO, Beijing, China). The isotype IgG controls were normal rabbit IgG (Cell Signaling Technology, USA) .

Gene knockout

To avoid unexpected screening results caused by the presence of CSPG4 or to validate *LRP1*'s role in an independent way, we used an orthogonal gene-editing system, TALEN. In terms of CSPG4, the same pair of TALENs as used in the previous study (Yuan et al., 2015) was employed to generate CSPG4-deficient cells. The targeting sequences are 5′-CTGGCCAACATAGTC-3′ and 5′-TCCAGCCCCCGGCCT-3′, targeting the second exon of CSPG4. The method for assembly of TALENs was described previously. In respect to LRP1, the TALENs targeting sequences are 5′-GACTTGCAGCCCCAAG-3′ and 5′-ATACAGGTTATTTGA-3′. Both of the TALEN expressing plasmids were transiently transfected into designated cells concomitantly. The gene knockout was validated by both Sanger sequencing and immunoblots.

As for knockout of *LRP1* in Caco2, an all-in-one sgRNA-expressing vector lentiCRISPR v2 (Addgene, USA) was employed, which expresses sgRNA and Cas9 protein concomitantly. sgRNA targeting *LRP1*, targeting sequence of which is 5′-CTGCTGCCCCTGCTCTCAGCTC-3′, was cloned into the vector as described (Sanjana et al., 2014). The $sgRNA^{LRP1}$ lentivirus was generated using a second-generation package system. Caco2 cells were infected by $sgRNA^{LRP1}$ lentivirus, with 8 μg mL^{-1} polybrene to facilitate the lentiviral infection. Single clones were selected and confirmed by immunoblot.

CRISPR-Cas9 screening with TcdB

The sgRNA library consists of sgRNAs targeting 2,989 membrane-associated protein-coding genes with 10 sgRNAs for each gene. The cellular sgRNA library was established as described previously (Zhou et al., 2014). Briefly, oligonucleotides encoding sgRNAs were synthesized as a mixed pool. Subsequently, sgRNAs were amplified and cloned into the lentiviral backbone encompassing the sgRNA scaffold and GFP selection marker. The lentiviral sgRNA library was generated with plasmids expressing sgRNA library and second lentiviral packaging plasmids. 1×10^8 HeLa_*CSPG4*$^{-/-}$ cells were infected with sgRNA library lentivirus using MOI=0.3. Cells were obtained by cell sorting of GFP-positive cells and followed by 14-day culture. The final cell library was aliquot and subjected to cryopreservation, NGS as a reference, and screening with TcdB. Cells that survived after TcdB screening were collected and subjected to NGS sequencing and analysis to enrich sgRNA.

Recombinant full-length and GST-tagged $TcdB_{1852\text{-}2366}$

Recombinant full-length TcdB was expressed by *Bacillus megaterium* and purified as described previously (Yang et al., 2008). pHIS1522-TcdB plasmid extracted from the TcdB-expressing *B. megaterium* strain and served as the PCR template for truncated TcdB. $TcdB_{1852\text{-}2366}$ was amplified with primers as indicated and ligated into pGEX-4T-1 between BamHI and EagI. The ligation product was transformed into BL21 (TransGen Biotech, Beijing, China) competent cells. Several clones were picked and validated by Sanger sequencing. Correct clones were subjected to pilot expression, under 1 mmol L^{-1} IPTG. The cell culture was lysed by Western blot lysis buffer (50 mmol L^{-1} Tris at pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 137 mmol L^{-1} sodium chloride, 1 mmol L^{-1} sodium orthovanadate) by boiling at 98°C for 10 min. Clone with the highest expression level of GST-$TcdB_{1852\text{-}2366}$ was chosen for the final expressing clone. 500 mL cell culture induced by 1 mmol L^{-1} IPTG was subjected to purification with Glutathione Sepharose 4 Fast Flow (GE Healthcare, USA) as manufacturer's instructions. TcdB 1852-F BamHI: 5′-CGCGGATCCTTGATAACTGGATTTGTGACTGTA-3′; TcdB 2366-R EagI: 5′-TATCGGCCGCTATTCACTAATCACTAATTGAGC-3′.

Construction of HeLa_LRP1-OE cells

A bi-directional CMV promoter was inserted upstream of the endogenous *LRP1*-coding sequence by gene editing using Cas9 to generate *LRP1*-overexpressing cells. The sgRNA targeting 5′ untranslated region (UTR) of *LRP1* (5′-CACTTCAGTCCGGGGAACAG-3′) was cloned as described previously (Zhou et al., 2014). Donor DNA, which consisted of GFP, sgRNA targeting sequence and bi-directional CMV promoter, was cloned into pGL3 basic backbone between KpnI and NheI. The donor plasmid was verified by Sanger sequencing. sgRNA-expressing plasmid and donor plasmid were co-transfected into Cas9-expressing HeLa cells with the ratio of 1:1. Sixteen days after transfection, GFP-positive cells were harvested by FACS. At this time, only cells with integrated donor DNA expressed GFP, as episomal donor plasmid were degraded. The HeLa_*LRP1*-OE pool was validated by immunoblot. Several single clones were derived from HeLa_*LRP1*-OE pool, and the one with the highest expression level of LRP1 (designed as HeLa_*LRP1*-OE) was used for subsequent assays.

Co-immunoprecipitation

HeLa_*LRP1*-OE cells were seeded or a plasmid expressing CSPG4-myc was transfected into HEK293T cell. Twenty-four hours later, cells expressing either LRP1 or CSPG4-myc were scrapped and washed with cold PBS for twice. Re-suspended and lysed about 1×10^7 transfected cells in 1 mL Co-IP lysis buffer (20 mmol L^{-1} Tris-HCl, pH 7.5, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 1% Triton X-100, EDTA-free Protease Inhibitor Cocktail, Roche, Switzerland), at 4°C for 1 h. Centrifuged at the highest speed and remove supernatant to new 1.5 mL EP tubes. Saved 20 μL supernatant as LRP1 or CSPG4-myc input sample. 3 μg of either anti-LRP1 antibody or anti-myc antibody was added to the corresponding supernatant and gently mixed at 4°C overnight. Isotype IgG served as control. Aliquoted 30 μL Protein G Sepharose 4 Fast Flow resin (GE Healthcare) was added in each sample. Centrifuged at 4°C and removed the storage buffer, washed with cold Co-IP lysis buffer twice. Resuspended Protein G sepharose with the overnight incubation product and mix at 4°C for 1 h. Wash the resin with Co-IP wash buffer (20 mmol L^{-1} Tris-HCl, pH 7.5, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 0.1% Triton X-100, EDTA-free Protease Inhibitor Cocktail, Roche) for twice. Resuspended the resin with 500 μL Co-IP lysis buffer containing 5 μg TcdB and gently mixed at 4°C overnight. Saved 20 μL supernatant as TcdB input sample. Wash the resin with Co-IP wash buffer for twice. Add 30 μL Western blot lysis buffer and 30 μL 2× Laemmli Sample Buffer (Bio-Rad, USA) to the resin, boiled at 98°C for 10 min. Load samples into SDS-PAGE gel and detected proteins with indicated antibodies.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

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