Sulfated glycosaminoglycans and low-density lipoprotein receptor contribute to Clostridium difficile toxin A entry into cells

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Clostridium difficile toxin A (TcdA) is a major exotoxin contributing to disruption of the colonic epithelium during C. difficile infection. TcdA contains a carbohydrate-binding combined repetitive oligopeptides (CROPs) domain that mediates its attachment to cell surfaces, but recent data suggest the existence of CROPs-independent receptors. Here, we carried out genome-wide clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated screens using a truncated TcdA lacking the CROPs, and identified sulfated glycosaminoglycans (sGAGs) and low-density lipoprotein receptor (LDLR) as host factors contributing to binding and entry of TcdA. TcdA recognizes the sulfation group in sGAGs. Blocking sulfation and glycosaminoglycan synthesis reduces TcdA binding and entry into cells. Binding of TcdA to the colonic epithelium can be reduced by surfen, a small molecule that masks sGAGs, by GM-1111, a sulfated heparan sulfate analogue, and by sulfated cyclodextrin, a sulfated small molecule. Cells lacking LDLR also showed reduced sensitivity to TcdA, although binding between LDLR and TcdA are not detected, suggesting that LDLR may facilitate endocytosis of TcdA. Finally, GM-1111 reduces TcdA-induced fluid accumulation and tissue damage in the colon in a mouse model in which TcdA is injected into the caecum. These data demonstrate in vivo and pathological relevance of TcdA-sGAGs interactions, and reveal a potential therapeutic approach of protecting colonic tissues by blocking these interactions.

The bacterium Clostridium difficile is a spore-forming opportunistic pathogen and one of the three ‘urgent threats’ classified by the Centers for Disease Control and Prevention of the United States. Disruption of gut flora by antibiotics allows C. difficile to colonize the colon, leading to diarrhoea and life-threatening pseudomembranous colitis. The occurrence of C. difficile infection is exacerbated by the emergence of hypervirulent and antibiotic-resistant strains. It is now the most common cause of antibiotic-associated diarrhoea and gastroenteritis-associated death in developed countries, accounting for around 500,000 cases and 29,000 deaths annually in the United States.

Two homologous exotoxins, C. difficile toxin A and B (TcdA and TcdB), which target and disrupt the colonic epithelium, are the major virulent factors of C. difficile. In addition, some hypervirulent strains also express a third toxin known as C. difficile transferase, which may suppress host eicosanoid responses. TcdA (~308 kDa) and TcdB (~270 kDa) consist of four functional domains: the N-terminal glucosyltransferase domain (GTD), a cysteine protease domain that mediates auto-cleavage and releases the GTD into the host cytosol, a central part containing both the transmembrane delivery domain and receptor-binding domain, and finally a C-terminal combined repetitive oligopeptides (CROPs) domain. The GTD glucosylates small GTPases of the Rho family, including Rho, Rac and CDC42, and inhibits their function, resulting in cytopathic cell rounding and ultimately cell death.

The CROPs domains of TcdA and TcdB bear similarity with carbohydrate-binding proteins and may mediate toxin attachment to cell surfaces through various carbohydrate moieties. Particularly, CROPs from TcdA was shown to bind the trisaccharide Galα1,3Galβ1,4GlcNAcβ. It has since been shown to also broadly recognize human I, Lewis X, and Lewis Y antigens, as well as glycosphingolipids, which all contain the Galβ1,4GlcNAc motif.

Recent studies have shown that truncating the CROPs only modestly reduces the potency of TcdA and TcdB on cultured cells, suggesting the existence of CROPs-independent receptors. Three candidate receptors have been reported for TcdB: chondroitin sulfate proteoglycan 4 (CSPG4), poliovirus receptor-like 3 (PVRL3) and the Wnt receptor frizzled proteins. Two proteins have been previously suggested as potential receptors for full-length TcdA: sucrase-isomaltase and glycoprotein 96 (Gp96). However, sucrase-isomaltase is not expressed in the colon epithelium and Gp96 resides mainly in the endoplasmic reticulum.

Here we used a truncated TcdA lacking the majority of the CROPs domain and carried out genome-wide clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated knockout (KO) screens, which identified sulfated glycosaminoglycans (sGAGs) and low-density lipoprotein...
receptor (LDLR) as CROPs-independent host factors mediating binding and entry of TcdA.

**Results**

**CRISPR screens identify host factors for TcdA.** To identify the CROPs-independent receptors involved in TcdA actions, we used a truncated TcdA (TcdA<sup>1–1874</sup>) lacking the majority of the CROPs domain (Supplementary Fig. 1a), which has previously been shown to retain high levels of toxicity to multiple cell lines<sup>17</sup>. We first validated the toxicity of TcdA<sup>1–1874</sup> on various human cell lines using the standard cytopathic cell-rounding assay, which measures the percentages of rounded cells after incubation with a series of concentrations of toxins for 24 h (Supplementary Fig. 1b,c). The toxin concentration that induces 50% of cells to become round is defined as CR<sub>50</sub>, and is used to compare the sensitivity of different cell lines to TcdA<sup>1–1874</sup>. HeLa cells are one of the most sensitive human cell lines to TcdA<sup>1–1874</sup>, and were selected to carry out genome-wide CRISPR–Cas9 mediated KO screens.

HeLa cells stably expressing Cas9 were transduced with a lentiviral single guide RNA (sgRNA) library (GeCKO v.2) targeting 19,052 human genes<sup>24</sup>. The cells were subjected to three rounds of selection with TcdA<sup>1–1874</sup> sequentially at the indicated toxin concentrations. The genes identified after R3 were ranked and plotted. The y axis shows the number of unique sgRNAs for each gene. The x axis represents the number of sgRNA reads for each gene. The top-ranking genes are colour-coded and grouped on the basis of their functions. The dashed red line indicates the top-ranked hits. The NGS reads from R0 to R3 for the top-20 ranked (ordered by NGS reads) genes in R3 were colour-coded and plotted. The diameter of the circle represents the number of unique sgRNAs detected for the gene. All top-20 ranked genes were progressively enriched from R0 to R3.

**Fig. 1 | Genome-wide CRISPR-Cas9-mediated screen identifies host factors for TcdA.**

**a**, Schematic of the screening process using TcdA<sup>1–1874</sup> on HeLa cells. Round zero (R0) represents cells at the beginning of the screen. Rounds 1, 2 and 3 (R1, R2 and R3) represent surviving cells after exposure to TcdA<sup>1–1874</sup> sequentially at the indicated toxin concentrations. **b**, Genes identified after R3 were ranked and plotted. The y axis shows the number of unique sgRNAs for each gene. The x axis represents the number of sgRNA reads for each gene. The top-ranking genes are colour-coded and grouped on the basis of their functions. The dashed red line indicates the top-ranked hits. **c**, The NGS reads from R0 to R3 for the top-20 ranked (ordered by NGS reads) genes in R3 were colour-coded and plotted. The diameter of the circle represents the number of unique sgRNAs detected for the gene. All top-20 ranked genes were progressively enriched from R0 to R3.

The top-ranked gene encodes LDLR, a well-known receptor for low-density lipoproteins. Many other top-ranked genes encode key players in heparan sulfate biosynthesis and sulfation pathways<sup>25</sup>, including the glycosyltransferases exostosin-2 (EXT2) and exostosin-like 3 (EXTL3), the sulfotransferases heparan sulfate 6-O-sulfotransferase 1 (HS6ST1), N-deacetylase and N-sulfotransferase 1 (NDST1), and solute carrier family 35 member B2 (SLC35B2), which transports the activated form of sulfate into Golgi. Several other enzymes involved in glycosaminoglycan (GAG) synthesis were also identified (Supplementary Fig. 2a). Heparan sulfate is usually attached to core proteins as heparan sulfate proteoglycans (HSPGs). Both HSPGs and LDLR are widely expressed on the surface of various cells, and are therefore promising receptor candidates for TcdA.

Among the top-50 ranked genes, three (UGP2, PI4KB and ATP6V0D1) were also found in the top list of genes in our previous
whereas their sensitivities towards TcdB1–1830 remained the same as CROPs domain (Supplementary Fig. 1a). TcdA1–1874 and TcdA1–1832 CROPs to exclude any potential contribution from the residual KO cells to TcdA1-1832 was further confirmed SLC35B2 showed similar potency on HeLa cells in the cytopathic cell-rounding assays (Supplementary Fig. 1b).

ATP6V0D1 is a component of vacuolar-type H\(^+\)-ATPase for acidification of endosomes, which is an essential condition to trigger translocation of TcdA and TcdB25,26. PI4KB is a key player in phospholipid metabolism and signalling, and its role in toxin action remains to be established.

Other notable top hits include COG5, COG7, TMEM165 and RIC8A. COG5 and COG7 are members of the conserved oligomeric Golgi (COG) complex27. In fact, all eight COG members were identified in the final round of screening (Supplementary Fig. 2c). TMEM165 is a multi-pass transmembrane protein localized to the Golgi. Although the exact function of the COG complex and TMEM165 remains to be fully established, mutations in COG complex and TMEM165 both result in congenital disorders of glycosylation28,29, and affect multiple glycosylation pathways including biosynthesis of heparan sulfate30–32. RIC8A is a guanine nucleotide exchange factor and its role in TcdA action remains to be validated.

We also performed a parallel genome-wide CRISPR–Cas9-mediated KO screen using full-length TcdA on HeLa cells (Supplementary Fig. 2d). However, this screen only yielded UGP2 as the top hit. Two other hits, SGMS1 and ZNF283, were barely over our threshold. SGMS1 regulates lipid raft formation and may affect the endocytosis process. ZNF283 is a cytosolic protein, and its role in TcdA action remains to be validated. Lack of potential receptor candidates in the top hits suggests that full-length TcdA may utilize multiple receptors and entry pathways.

sGAGs contribute to cellular entry of TcdA1–1832. TcdA1–1874 still contains a short fragment of the CROPs domain. Therefore, we further generated a truncated TcdA (TcdA1–1874) that deletes the entire CROPs to exclude any potential contribution from the residual CROPs domain (Supplementary Fig. 1a). TcdA1–1874 and TcdA1–1832 showed similar potency on HeLa cells in the cytopathic cell-rounding assays (Supplementary Fig. 1b).

Using TcdA1–1832, we first validated the role of EXT2 and EXT1, as they are specifically required for the elongation of the heparan sulfate chain, but not other types of GAGs. We generated EXT2 and EXT1 KO cells lines using the CRISPR–Cas9 system. Both cell lines showed a reduction of cell surface heparan sulfate levels compared with wild-type cells, as measured by flow cytometry analysis using a heparan sulfate antibody (Supplementary Fig. 3a). Both EXT2 and EXT1 KO cells showed a modest four-to-fivefold reduction in sensitivity to TcdA1–1832 compared with wild-type cells, whereas their sensitivities towards TcdB1–1830 remained the same as wild-type cells (Fig. 2a).

Several top-ranked genes identified in our screen, including SLC35B2, NDST, HS6ST, HS2ST and HS3ST, are involved in sulfation of GAGs25 (Supplementary Fig. 2a). To examine the role of sulfation, we generated three single clones of SLC35B2 KO HeLa cells using the CRISPR–Cas9 approach. Reduction of heparan sulfate in these cells was confirmed by flow cytometry analysis (Supplementary Fig. 3b). These cell lines all showed around tenfold reduction in sensitivity towards TcdA1–1832 compared with wild-type cells, whereas their sensitivities towards TcdB1–1832 were not changed (Fig. 2b). The reduced sensitivity of SLC35B2 KO cells to TcdA1–1832 was further confirmed by immunoblotting for RAC1 glucosylation (Supplementary Fig. 4a). Finally, SLC35B2 KO cells also showed approximately threefold reduction in sensitivity to full-length TcdA (Fig. 2c).

Characterizing the specificity of TcdA–sGAGs interactions. We next carried out competition assays to further validate the role of sGAGs. First, we used surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide), which is a small molecule that binds to and neutralizes negative charges on all sGAGs41. Pre-incubation of cells with surfen protected HeLa cells from TcdA1–1832 in a concentration-dependent manner, whereas it offered no protection from TcdB1–1830 (Fig. 2d and Supplementary Fig. 5a). Similar results were observed with Huh7 cells (Supplementary Fig. 5b).

To understand the selectivity of TcdA–GAG interactions, we carried out competition assays using a panel of GAGs including heparan sulfate, heparin, de-N-sulfated heparin, N-acetyl-de-O-sulfated heparin, chondroitin sulfate and dextran sulfate. Heparin is a highly sulfated variant of heparan sulfate and it is widely utilized as an anticoagulant. In addition, we also tested synthetic sulfated molecules GM-1111 and sulfated cyclodextrin. GM-1111 contains the same carbohydrate moieties and sulfation groups as heparan sulfate, but with distinct glycosidic bonds. It has been developed as a heparan sulfate mimic with reduced anticoagulation activities33. Sulfated cyclodextrin is a small molecule that is distinct from GAGs. Non-sulfated GAG hyaluronic acid and polysaccharide cellulose were also examined. These molecules are shown in Supplementary Fig. 6.

Pre-incubation of TcdA1–1832 with heparan sulfate, heparin, chondroitin sulfate, dextran sulfate, GM-1111 and sulfated cyclodextrin all reduced the level of cell rounding, whereas hyaluronic acid showed no effect (Fig. 2e). These results suggest that TcdA may not recognize heparan sulfate specifically, but rather interacts mainly with the sulfation group. Furthermore, the finding that de-N-sulfated heparin protected cells from TcdA1–1832, whereas N-acetyl-de-O-sulfated heparin did not offer any protection (Fig. 2e), suggests that TcdA preferentially recognizes O-sulfation.

To further characterize direct TcdA–sGAG interactions, we used bio-layer interferometry (BLI) assay by immobilizing biotinylated heparin onto the probe. Binding of TcdA to the immobilized heparin would result in a shift in the light interference pattern that can be monitored in real time. Biotinylated hyaluronate and cellulose were analysed in parallel as controls. Both full-length TcdA and TcdA1–1874 showed robust binding to biotin–heparin, but not to biotin–hyaluronate and biotin–cellulose (Fig. 2f and Supplementary Fig. 7a). TcdA–heparin interactions appear to be influenced by the ionic strength of the buffer: higher salt concentrations reduce heparin–TcdA interactions (Supplementary Fig. 7b). At 150 mM salt concentration, the apparent dissociation constants (K\(_d\)) for TcdA–heparin and TcdA1–1874–heparin are at similar levels (85.5 nM for TcdA1–1874 versus 23.2 nM for full-length TcdA; Supplementary Fig. 7c–e).

LDLR contributes to cellular entry of TcdA1–1832. To validate the role of LDLR, we generated LDLR KO HeLa cells using the CRISPR–Cas9 system. Three single-KO clones were established and the loss of LDLR expression was confirmed in the clones by immunoblot analysis (Fig. 3a). All three KO lines showed reduced sensitivity by about sevenfold to TcdA1–1832, whereas their sensitivity to TcdB1–1830 remained the same as that of wild-type cells (Fig. 3b). The reduced sensitivity of LDLR KO cells to TcdA1–1832 was also confirmed by immunoblot against RAC1 glucosylation (Supplementary Fig. 4b). LDLR\(^{-}\) cells also showed around threefold reduction in sensitivity to full-length TcdA, thus validating the role of LDLR in cytotoxic activity of full-length TcdA (Fig. 3c). The sensitivity to TcdA was restored when LDLR KO cells were transfected with mouse LDLr (Fig. 3d), which is not targeted by the sgRNA. Furthermore, Huh7 LDLR\(^{-}\) cells, which were previously generated and validated34, also showed reduced sensitivity to TcdA1–1832 compared with wild-type Huh7 cells (Supplementary Fig. 8).

We further carried out a competition assay using the soluble extracellular domain of LDLR (residues 22–788, LDLR22–788). Co-incubation of LDLR22–788 with TcdA1–1832 (200:1) reduced the percentage of rounded cells (Fig. 3e). LDLR belongs to a large family of proteins including VLDLR, LRPI, LRPIb, LRPI2 (also known as megalin), LRPS, LRPs and LRPS8 (also known as ApoER2), which...
Fig. 2 | sGAGs contribute to cellular entry of TcdA<sup>1–1832</sup>. a, The sensitivities of EXT2<sup>−/−</sup> and EXT3<sup>−/−</sup> HeLa cells to TcdA<sup>1–1832</sup> (left) and TcdB<sup>1–1830</sup> (middle) were quantified using the cytopathic cell-rounding assay. The percentage of rounded cells was quantified, plotted and fitted. The toxin concentration resulting in 50% cell rounding is defined as CR<sub>50</sub> and is used for comparisons by normalizing to the level of wild-type (WT) HeLa cells as normalized resistance (right; y axis, each data point is also shown as triangle in the bar graph). b, The sensitivities of three SLC35B2<sup>−/−</sup> HeLa cell lines to TcdA<sup>1–1832</sup> and TcdB<sup>1–1830</sup> were quantified using the cytopathic cell-rounding assay and normalized to the level of wild-type HeLa cells. Each data point is also shown as triangle in the bar graph (right). c, The sensitivities of wild-type and SLC35B2<sup>−/−</sup> (clone no. 5) HeLa cells to full-length TcdA were evaluated using the cytopathic cell-rounding assay. The percentage of rounded cells was quantified, plotted and fitted. d, Pre-incubation of surfen in the medium reduced the potency of TcdA<sup>1–1874</sup> but not TcdB<sup>1–1830</sup> on HeLa cells in a concentration-dependent manner, as measured by the cytopathic cell-rounding assay over time. e, Competition assay on HeLa cells by pre-incubating TcdA<sup>1–1874</sup> (2 nM) with the indicated GAGs, polysaccharides and synthetic sulfated molecules (all at 1 mg ml<sup>−1</sup>). The degree of protection from TcdA was evaluated by the cytopathic cell-rounding assay 4 h later (*<i>P</i> < 0.005; P > 0.05 is considered as non-significant (NS); two-sided Student’s t-test, n = 3). Each data point is also shown as a triangle in the bar graph. f, BLI assays showing that TcdA<sup>1–1874</sup> (1 µM) strongly bound to biotin–heparin but not to biotin–hyaluronate or biotin–cellulose. Experiments were repeated three times. In a–d, n = 6; data are mean ± s.d. The experiments were repeated three times independently with similar results.
share similar domains with LDLR and often act as redundant receptors for many LDLR ligands. Receptor associated protein (RAP) binds tightly to most LDLR family members and its binding inhibits binding of LDL and many other ligands. Adding RAP to the medium further reduced the sensitivity of LDLR KO cells to TcdA1–1832 (Fig. 4b and Supplementary Fig. 9). These results suggest that either that TcdA1–1832 binds to LDLR22–788–Fc in either BLI assays or an alternative dot blot assay (Supplementary Fig. 9). These results suggest that either that TcdA1–1874 binding to LDLR is weak or that their interactions may require additional cellular factors.

sGAGs are major cellular attachment factors for TcdA1–1874. To further understand the role of LDLR and sGAGs, we generated LDLR−/−; SLC35B2−/− double-KO cell lines by knocking out LDLR from HeLa SLC35B2−/− cells using the CRISPR–Cas9 approach. Two single-cell clones were established, and lack of LDLR expression was confirmed by immunoblot (Fig. 4a). However, these two double-KO cell lines did not further increase their resistance to TcdA1–1832 compared with LDLR and SLC35B2 single-KO cells (Fig. 4b and Supplementary Fig. 4c). Moreover, overexpression of exogenous mouse Ldr by transient transfection did not increase the sensitivity of SLC35B2−/− cells to TcdA1–1832 (Fig. 4c). These data suggest that LDLR and sGAGs are not redundant receptors, and that they could act cooperatively. We therefore examined binding of TcdA1–1874 to wild-type versus LDLR−/− and SLC35B2−/− HeLa cells, using TcdA1–1874 directly labelled with a fluorescent dye. As shown in Fig. 4d, LDLR−/− cells showed similar overall TcdA1–1874 binding as wild-type cells. By contrast, binding of TcdA1–1874 to SLC35B2−/− cells was diminished. These results suggest that sGAGs are the major attachment factor mediating binding of TcdA1–1874 on cell surfaces under our assay conditions.

sGAGs are attachment factors for TcdA1–1874 in the colonic epithelium. The colonic epithelium is the pathologically relevant target of TcdA. sGAGs are abundant both in the intestinal mucosa and on the basolateral side of the epithelium (43–46). To examine the contribution of sGAGs to TcdA binding to the colonic epithelium, we used a colon loop ligation assay (29). In brief, fluorescence-labelled TcdA1–1874 was injected into a ligated colon segment and incubated for 30 min. Colon tissues were then dissected and fixed. TcdA1–1874 showed strong binding to the apical side of the colonic epithelium and binding appears to extend into the lumen (Fig. 4e). Co-injecting surfen reduced binding of TcdA1–1874 (Fig. 4e). Similarly, heparin, GM-1111 and sulfated cyclodextrin all reduced binding of TcdA1–1874 whereas hyaluronic acid showed no effect (Fig. 4f). These results suggest that sGAGs are major attachment factors in the colonic epithelium for TcdA1–1874.

Blocking sGAG–TcdA interactions reduces TcdA toxicity in the colon. We next examined the contribution of sGAGs-mediated binding in the context of full-length TcdA in vivo. Injecting fluorescence-labelled full-length TcdA into the ligated colon loop for 30 min resulted in robust binding to the apical side of the colonic epithelium (Fig. 5a). Co-injecting recombinantly produced CROPs fragment reduced binding of TcdA, consistent with the finding that the CROPs region mediates TcdA binding to cells (34). Co-injecting surfen with TcdA reduced binding of TcdA, confirming that sGAGs contribute to binding of full-length TcdA to the colonic epithelium (Fig. 5a). Similarly, co-injection with GM-1111 or sulfated cyclodextrin also reduced TcdA binding to the colonic epithelium (Fig. 5b). Interestingly, combining CROPs and surfen together largely ablated binding of TcdA to the colonic epithelium (Fig. 5a). Thus, both CROPs-mediated and sGAGs-mediated binding contribute to TcdA binding to the colonic epithelium.
Fig. 4 | sGAGs are major attachment factors for TcdA. a, The absence of LDLR expression in two SLC35B2−/−/LDLR−/− HeLa cell lines was confirmed by immunoblot analysis. The experiments were repeated three times independently with similar results. b, The sensitivities of two SLC35B2−/−/LDLR−/− HeLa cell lines and their parental cell line SLC35B2+/− (no. 5) to TcdA1–1837 were quantified using the cytopathic cell-rounding assay, and normalized to the level of wild-type HeLa cells. c, Ectopic expression of a mouse Ldlr did not restore TcdA1–1832 (2 nM, 4 h) entry into SLC35B2−/− cells under our assay conditions. Left, representative images; transfected cells are marked by GFP expression. Right, quantification of cell rounding.

To further examine the relevance of sGAG–TcdA interactions for TcdA-induced pathogenesis in vivo, we utilized a mouse caecum-injection model that was previously established to assess pathogenesis of TcdA and TcdB44. In brief, TcdA or TcdA premixed with inhibitors was injected into the caecum. Mice were allowed to recover for 6 h before euthanization. The caecum and the ascending colon were collected and weighed to assess the degree of fluid accumulation. The caecum tissue was also fixed and subjected to hematoxylin and eosin staining and histological score analysis based on four criteria (disruption of the epithelium, hemorrhagic congestion, mucosal edema and inflammatory cell infiltration) on a scale of 0–3 (normal, mild, moderate or severe). Injection of TcdA induced fluid accumulation in the colon tissues, severe mucosal edema, mild-to-moderate disruption of the epithelium, hemorrhagic congestion and inflammatory cell infiltration (Fig. 5c,d). Finding a suitable inhibitor for use in the caecum-injection model was challenging, as heparan sulfate and many sGAG mimics induced damage to colonic tissues after incubation for 6 h. After surveying many different sGAG mimics, we found that GM-1111, which was challenging, as heparan sulfate and many sGAG mimics induced damage to colonic tissues after incubation for 6 h. After surveying many different sGAG mimics, we found that GM-1111, which was...
specifically developed to reduce anticoagulation activity, can be used at the dose that reduces TcdA binding without itself inducing visible tissue damage. Co-injecting GM-1111 with TcdA significantly reduced fluid accumulation in the colon (caecal weight; Fig. 5c) and overall tissue damage as evidenced by histological scoring (Fig. 5d).

**Discussion**

The presence of numerous negatively charged sulfate groups in sGAGs provides an ideal multivalent landing pad for proteins and macromolecules through electrostatic interactions. These sulfate groups are known to interact with a large array of endogenous

**Fig. 5** | Blocking sGAG–TcdA interactions reduces TcdA toxicity in the colon. a, Co-injecting either surfen (50 µM) or TcdA CROPs (150 nM) with Alexa Fluor 555-labelled full-length TcdA (5 nM) partially reduces TcdA binding to the colonic epithelium. Co-injecting both surfen and TcdA CROPs with TcdA largely abolished toxin binding. Representative images (left), and quantification of binding (right; n = 3). b, Co-injection of GM-1111 or sulfated cyclodextrin with TcdA reduced TcdA binding to the colonic epithelium (n = 3). c, TcdA (4 µg), TcdA premixed with GM-1111 (0.5 mg ml⁻¹) or saline was injected into the caecum of mice. After 6 h, the caecum tissue was excised. Representative caecum tissues are shown, and the weight of each caecum was measured and plotted. Boxes represent mean ± s.e.m.; bars represent s.d.; two-sided Student’s t-test; n = 6 (saline), 11 (TcdA) or 10 (TcdA + GM-1111). d, Caecum tissues from c were sectioned and subjected to haematoxylin and eosiin staining. Representative images are shown and the histological scores were assessed on the basis of disruption of the epithelia, haemorrhagic congestion, mucosal oedema and inflammatory cell infiltration. Boxes represent mean ± s.e.m.; bars represent s.d.; Student’s t-test. In a and b, binding of TcdA was quantified using ImageJ; two-sided Student’s t-test with multiple comparisons. Data are mean ± s.d. Each data point is also shown as a triangle in the bar graph. Scale bars represent 200 µm in a and b, and 100 µm in d.
ligands, such as fibroblast growth factors, vascular endothelial growth factor, transforming growth factor β, chemokines and cytokines46,47. Unsurprisingly, these proteoglycans are also exploited by a long list of viral, bacterial and parasitic pathogens as attachment factors46,48. As TcdA is capable of binding to isolated sGAGs, it should be able to bind both to proteoglycans containing sGAGs as well as to free sGAGs on the cell surface and in the extracellular matrix. The exact binding sites for sGAGs in TcdA remain to be determined and it is possible that multiple positively charged surface regions of TcdA are involved.

LDLR belongs to a family of structurally related receptors, many of which act as redundant receptors for various ligands and viruses46,49. Interestingly, the LDLR family member LRP1 was previously established as the receptor for TpeL toxin49, which belongs to the same toxin family as TcdA but naturally lacks the CROPs domain. It is likely that LDLR family members other than LDLR can also contribute to TcdA1–1832 entry, as RAP further reduces the sensitivity of LDLR KO cells.

LDLR family receptors rapidly and constitutively recycle between cell membranes and endosomes. This provides an ideal mechanism by which to mediate endocytosis into cells. Indeed, LDLR has been exploited as a receptor for many viruses, such as vesicular stomatitis virus (VSV), hepatitis C virus and the minor group common cold virus36,38,48. Although it remains unknown whether TcdA is capable of binding to isolated sGAGs, it should to free sGAGs on the cell surface and in the extracellular matrix.

Genome-wide CRISPR–Cas9 screening with TcdA1–1832. The HeLa CRISPR genome-wide knockout library was generated as previously described31. In brief, the GEKO v2 library is composed of two sublibraries. Each sublibrary contains three unique sgRNA per gene and was independently prepared and screened. HeLa–Cas9 cells were transfected with sgRNA lentiviral library at a multiplicity of infection of 0.2. For each CRISPR sublibrary, 7.9 × 10⁶ cells were plated onto three 15-cm cell culture dishes to ensure sufficient sgRNA coverage, with each sgRNA being represented around 1,200 times. These cells were exposed to TcdA1–1832 for 48 h. Cells were then washed three times to remove loosely attached cells. The remaining cells were cultured with toxin-free medium to ~70% confluence and subjected to the next round of screening with higher concentrations of toxins. Three rounds of screenings were performed with TcdA1–1832 (40, 80 and 160 μM). Remaining cells from each round were collected and their genomic DNA was extracted using the Blood and Cell Culture DNA mini kit (Qiagen). DNA sequences containing the sgRNA sequences were amplified by PCR using primer lentiGP-1_F (AATGGCATATCATGTGTGTTCAATCTGAAAGTATTTCG) and lentiGP-3_R (ATGAAATCCTCGATATTGTCAGTAACGGTACAC). NGS (Illumina MiSeq) was performed by Genewiz.

Methods

Materials. HeLa (H1, CRL-1958), HT-29 (HTB-38), CHO-C6 and 293T (CRL-3216) cells were originally obtained from ATCC. They tested negative for mycoplasma contamination, but have not been authenticated. Huh7 and Huh7 LDLR−/− cells were provided by Y. Matsuura (Osaka University). The following mouse monoclonal antibodies were purchased from the indicated vendors: RAC1 (23A8, Abcam), non-glucosylated RAC1 (clone 102, BD Biosciences), β-actin (AC-15, Sigma) and heparan sulfate (F58-10E4, mouse IgM, Amsbio). Rabbit monoclonal IgG against LDLR (EP1553Y) was purchased from Abcam. Chicken polyclonal IgY (753A) against TcdA was purchased from List Biological Labs. Statistical analysis was performed using OriginPro 8 (v.8.0724, OriginLab) software.

Protein purification. Recombinant TcdA (from C. difficile strain VPI 10463), TcdAΔ1–1832, TcdAΔ1–1832 and CROPs (TcdAΔ1875–2710) were cloned into modified pWH1520 vector, and TcdBΔ1–1845 was cloned into pHis1522 vector, expressed in Bacillus megaterium and purified as His₆-tagged proteins. The expression plasmid pQTEV-LRPAP1 (31327) encoding RAP was obtained from Addgene and RAP was purified as a His₆-tagged protein. Genes encoding the ectodomains of human LDLR, LDLRΔ1–400, LDLRΔ401–1029 and LDLRΔ1–1029 were fused and cloned into pHis vec (provided by A. Jonathan (Harvard Medical School)). For the expression of Fc-tagged LDLRΔ21–294, HEK293T cells were transfected with Lipofectamine 3000 (Invitrogen). Transfected cells were grown for 5 h, and the culture medium was then replaced with serum-free medium for 4 d. LDLRΔ21–294-Fc in the culture medium was collected and purified.

FACS analysis. In brief, cells were collected with 1 mM EDTA in PBS and subsequently resuspended with 4% BSA. Cells were incubated with either the 10E4 monoclonal antibody against heparan sulfate (1:400), or mouse IgM (1:200; ab18401, Abcam) for 1 h on ice. Cells were washed twice with PBS and incubated with goat anti-mouse IgG/IgM Alexa488 (11:1000; A16080, Molecular Probes) for 1 h on ice, washed twice, and followed by single-cell sorting using a FACs MoFlo Astrios EQ cell sorter (Beckman Coulter). Data were analysed using Flowjo software (Flowjo).

Cytopathic cell-rounding assay. The cytopathic effect of TcdA and TcdB was analysed using the standard cell-rounding assay. In brief, cells were exposed to TcdA, TcdAΔ1–1832 or TcdBΔ1–1845 for 24 h, and phase-contrast images of cells were recorded (Olympus IX51, 10–20 objectives). A zone of 300 x 300 μm² was measured including either the 10E4 monoclonal antibody against heparan sulfate (1:400), or mouse IgM (1:200; ab18401, Abcam) for 1 h on ice. Cells were washed twice with PBS and incubated with goat anti-mouse IgG/IgM Alexa488 (11:1000; A16080, Molecular Probes) for 1 h on ice, washed twice, and followed by single-cell sorting using a FACs MoFlo Astrios EQ cell sorter (Beckman Coulter). Data were analysed using Flowjo software (Flowjo).

Competition assays with RAP or surfen. The cells were pre-incubated with RAP or surfen in the medium at indicated concentrations at 37 °C for 20 min.
The medium was then supplemented with 2 mM TcdA-1486 and cells were incubated further at 37°C and the percentage of rounded cells over time was recorded and analysed.

Dot blot assay. The indicated amounts of RAP, TcdA-1486, and TcdB-1486 were spotted onto a nitrocellulose membrane and allowed to dry completely in air. The membrane was then blocked with 5% skimmed milk for 1 h at room temperature followed by overnight incubation with LDLR-1254–156.4 Ec at 4°C. The bound LDLR-1254–156.4 Ec was detected with a monoclonal antibody against human Fc fragment. The experiments were repeated in triplicate.

Surface binding of TcdA1–1874 on HeLa cells. TcdA and TcdA1–1874 were labelled using an Alexa 555 antibody labelling kit (A20187, Thermofisher Scientific) following the manufacturer's instruction. Wild-type, SLCC5B2−/− or LDLR−/− HeLa H1-Cas9 cells were incubated with 3 nM Alexa 555-labelled TcdA-1486 or TcdA1–1874 in PBS for 30 min on ice. Cells were washed three times with ice-cold PBS and fixed with 4% paraformaldehyde. Cell nuclei were labelled with Hoechst dye. Confocal images were captured with the Ultraview Vx Spinning Disk Confocal System.

BLI assay. The binding affinities between TcdA-1486 and heparin were measured by BLI assay using the Blitz system (ForteBio). In brief, biotinylated heparin (4 μg ml−1) was quantified using ImageJ software. The binding signal intensity was averaged and binding affinities (Kd) were calculated using the Blitz system software (Fortebio). The experiments were repeated in triplicate.

Colon loop ligation assay. All animal studies were conducted in accordance with ethical regulations under protocols approved by the Institute Animal Care and Use Committee (IACUC) at Boston Children’s Hospital (no. 3028). Statistical consideration was not used to determine the sample size of mice. Animals were distributed to each experimental group randomly. Experiments and data analysis were carried out without blinding. Colonoids from adult CD1 mice (6–8 weeks, both male and female, from Envigo) were dissected out and incubated in PBS on ice. A ~2 cm loop in the ascending colon was sealed with silk ligatures. One hundred microlitres of Alexa555-labelled TcdA-1486 or TcdA (5 nM each) in PBS was injected through an intravenous catheter into the sealed colon segment with or without TcdA1–1874 (150 nM) and/or surfen (5 μM), GM-1111 (1 μg ml−1), sulfated and cycloedextrin (1 mg ml−1). The colon segments were incubated on ice for 30 min, then cut open, washed with PBS, fixed with paraformaldehyde and subjected to cryosectioning to sections 10 μm thick. Confocal images were captured with the Ultraview Vx Spinning Disk Confocal System. Toxin binding was quantified using ImageJ software. The binding signal intensity was averaged on the length of the epithelium. Three images were analysed and the P value was calculated by Student's t-test.

Caecum-injection assay. Mice (CD1, 6–8 weeks of age, male and female, Envigo) were anaesthetised with 3% isoflurane after overnight fasting. A midline laparotomy was performed. TcdA (4 μg in 100 μl saline), TcdA premixed with GM-1111 (4 μg TcdA + 0.5 mg ml−1 GM-1111), or saline was injected into the caecum through the ileocaecal junction. The gut was then returned to the abdomen. The incision was closed using 4–0 chromic catgut and mice were allowed to recover. After 6 h, mice were euthanased and the caecum plus the ascending colon (~1.5 cm) was excised and weighed. The caecal tissue was fixed with 10% phosphate buffer formalin and embedded in paraffin. Tissue sections were subjected to haematoxylin and eosin staining for histological score analysis based on four criteria (disruption of the epithelium, haemorrhagic congestion, mucosal oedema and inflammatory cell infiltration) on a scale of 0–3 (normal, mild, moderate or severe).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding authors upon request.

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Author contributions
L.T. and M.D. initiated and designed the project. L.T. and S.T. carried out the CRISPR–Cas9 screen. L.T., S.T. and J.Z. carried out colon loop ligation assays. S.T. and J.Z. carried out caecum-injection assays. Z.L., L.R.-M. and S.P.J.W. generated heparan sulfate-deficient cells, analysed cell surface heparan sulfate levels and provided related reagents. S.M. purified LDLR–Fc. R.G. provided TcdA and performed the experiment on CHO cells. D.T.B. and S.O. provided key reagents and advice. L.T. and M.D. wrote the manuscript with input from all co-authors.

Competing interests
The authors declare no competing interests.

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| ☑   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
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Our web collection on statistics for biologists may be useful.

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Data collection

ImageJ, Origin Lab, Excel, FlowJo software (FlowJo Inc, Ashland, OR). (all commercially available).

Data analysis

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| Sample size | Sample size was determined based on authors' past experience and what is commonly accepted sample size number in the literature. Each sample size was selected so that a reasonable scientist would conclude that the size is sufficient to draw a statistical conclusion. At least two biological replicates were carried out. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data exclusions. |
| Replication | All experiments were replicated at least twice. All attempts at replication were successful. |
| Randomization | Samples were allocated into experimental groups randomly. |
| Blinding | Blinding was not performed, as virtually all the data are quantitative and not easily subject to operator bias. |

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| Study description | Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study). |
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| Sampling strategy | Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed. |
| Data collection | Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection. |
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Describe the research sample (e.g. a group of tagged Passer domesticus, all Stenocerus thurberi within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.

Sampling strategy

Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.

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Describe the data collection procedure, including who recorded the data and how.

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Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.

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Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.

Did the study involve field work?  Yes  No

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Field conditions

Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).

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State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).

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Describe any disturbance caused by the study and how it was minimized.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a  Involved in the study

- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

n/a  Involved in the study

- ChiP-seq
- Flow cytometry
- MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique materials are readily available from the authors or from standard commercial sources (noted in the method section).

Antibodies

Antibodies used

The following mouse monoclonal antibodies were purchased from the indicated vendors: RAC1 (23A8, Abcam), non-glycosylated RAC1 (Clone 102, BD Biosciences), β-actin (AC-15, Sigma) and HS (FS8-10E4, mouse IgM, Ambion). Rabbit monoclonal IgG against LDLR (EP1553Y) was purchased from Abcam. Chicken polyclonal IgY (#753A) against TcdA was purchased from List Biological Labs.
Validation
- Each primary antibody was validated using WB, IHC, IFC, or flow cytometry according to Manufacturer’s instructions.

### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | HeLa (H1, #CRL-1958), HT-29 (#HTB-38), CHO-C6, and 293T (#CRL-3216) cells were originally obtained from ATCC. |
|---------------------|------------------------------------------------------------------------------------------------------------------|
| **Authentication**  | All cell lines are from laboratory-derived frozen ampoules produced from ATCC vials.                               |
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| Laboratory animals | Adult CD-1 mice (6-8 weeks, both male and female, from Envigo, NJ). |
|--------------------|-------------------------------------------------------------------|
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- Provide a list of all files available in the database submission.

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Methodology

Replicates
Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth
Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies
Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters
Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

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Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software
Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

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☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑ All plots are contour plots with outliers or pseudocolor plots.
☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Cells were harvested and stained with primary antibody and single cells were sorted using a FACS.

Instrument
FACS MoFlo Astrios EQ (cell sorter-Beckman Coulter)

Software
FlowJo software (FlowJo Inc, Ashland, OR)

Cell population abundance
Single cell clones were cultured and confirmed by heparan sulfate staining and analyzed using FlowJo software

Gating strategy
Forward and side scatter gating. The major density of events is captured by this gate. The events with very low FSC and SSC, as well as those with low FSC and high SSC are eliminated. These events represent debris, cell fragments and pyknotic cells.

☑ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type
Indicate task or resting state; event-related or block design.

Design specifications
Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures
State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)
Specify: functional, structural, diffusion, perfusion.

Field strength
Specify in Tesla

Sequence & imaging parameters
Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

Area of acquisition
State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.
| Preprocessing |  |
| --- | --- |
| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
| Normalization | If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

| Statistical modeling & inference |  |
| --- | --- |
| Model type and settings | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
| Effect(s) tested | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Specify type of analysis: | Whole brain | ROI-based | Both |
| Statistic type for inference | See Eklund et al. 2016 | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

| Models & analysis |  |
| --- | --- |
| n/a | Involved in the study |
| Functional and/or effective connectivity |  |
| Graph analysis |  |
| Multivariate modeling or predictive analysis |  |
| Functional and/or effective connectivity | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
| Graph analysis | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
| Multivariate modeling and predictive analysis | Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics. |