LDLR, LRP1, and Megalin redundantly participate in the uptake of *Clostridium novyi* alpha-toxin

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*Clostridium novyi* alpha-toxin (Tcnα) is a potent exotoxin that induces severe symptoms including gas gangrene, myositis, necrotic hepatitis, and sepsis. Tcnα binds to sulfated glycosaminoglycans (sGAG) for cell-surface attachment and utilizes low-density lipoprotein receptor (LDLR) for rapid entry. However, it was also shown that Tcnα may use alternative entry receptors other than LDLR. Here, we define that LRP1 and Megalin can also facilitate the cellular entry of Tcnα by employing reconstitutive LDLR family proteins. LDLR, LRP1, and Megalin recognize Tcnα via their ligand-binding domains (also known as LDL receptor type A repeats). Notably, LDLR and LRP1 have contrasting expression levels in many different cells, thus the dominant entry receptor for Tcnα could be cell-type dependent. These findings together increase our knowledge of the Tcnα actions and further help to understand the pathogenesis of *C. novyi* infection-associated diseases.

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https://doi.org/10.1038/s42003-022-03873-0

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Clostridium novyi is an anaerobic, motile, and spore-forming bacterium that causes severe infectious diseases in humans and animals including gas gangrene, myositis, necrotic hepatitis, and sepsis1–3. C. novyi alpha-toxin (Tcnα) is a critical factor found in all pathogenic C. novyi strains, which are edematizing and lethal4. Tcnα belongs to a structurally related protein family called the large clostridium toxin (LCT) family. Members of the LCT family share similar domain arrangements as well as toxin action mechanisms2. All known LCTs (except for TpeL) consist of four functional domains, including a glucosyltransferase domain (GTD), an autocatalytic cysteine protease domain (APD), a delivery and receptor-binding domain (DRBD), and the combined repetitive oligopeptides (CROPs) domain5–7. Like other LCTs, Tcnα binds to the cell-surface receptors and enters cells via endocytosis. The low pH of endosomes induces structural changes in the toxin. The GTD is then delivered across endosomal membranes, released into the cytoplasm, and glucosylates small GTPases of the Rho and Ras family, leading to cytoskeleton disruption and eventual cell death8,9. Unlike other LCTs, Tcnα and TpeL are the only two LCTs that use UDP-N-acetylglucosamine (TpeL can also utilize UDP-glucose) to modify targeting small GTPases10–12.

Previously we reported that low-density lipoprotein receptor (LDLR) mediates the cellular entry of Tcnα. It was also suggested that LDLR family members other than LDLR may participate in the entry of Tcnα because LRPAP1 (also known as RAP), a general binder to LDLR family members, could functionally mediate the entry of Tcnα. Core members of the LDLR family, including LDLR, VLDLR, LRP1, Megalin (also known as LRP2 or gp330), ApoER2 (LRP8), LRP1B, and MEGF7, are well-known to mediate the endocytosis of a variety of ligands and maintain internal homeostasis13. These proteins have a large extracellular domain, a single transmembrane domain, and a relatively short cytoplasmic tail15. The extracellular domains of LDLR family proteins consist of several modular structures, including LDL receptor type A (LA) repeats, LDL receptor type B (LB) repeats (also known as epidermal growth factor precursor homology regions with β-propeller repeats), and an O-linked sugar domain16–18. Each LA module is about 40–60 residues long and displays a disulfide-bond stabilized charged surface16,17. The LA domains are commonly known as the ligand-binding regions recognizing various ligands such as ApoB, ApoE, LRPAP1, and Vesicular stomatitis virus (VSV)18–21. The bound ligands are commonly believed to be released in the low pH environment upon endocytosis22,23. The Asn-Pro-X-Tyr motif (NPxY; with x representing any amino acid) is found in several LDL receptor family members and can facilitate coated-pit-mediated endocytosis24.

Although our previous study indicated that LDLR family members other than LDLR may serve as redundant endocytic receptor(s) for Tcnα, the receptor selectivity within the family remains unclear. Here, we examined the contribution of major LDLR family members in the cellular entry of Tcnα by ectopically expressing native or reconstituted proteins in the HeLa LDLR−/− cells. We reported that LRP1 and Megalin, but not other tested LDLR family members, could functionally mediate the entry of Tcnα. We also found that LDLR, LRP1, and Megalin have varying expression levels in different cell types, thus Tcnα may use different entry receptors to intoxicate various host cells.

**Results**

The LA repeats of LDLR are responsible for the uptake of Tcnα. The extracellular domain of LDLR consists of an LA domain, an LB domain, and an O-linked sugar region. To interrogate the regions in LDLR involving the uptake of Tcnα, we generated two Ldr truncates lacking either the LA repeats (LdrΔLA) or the LB repeats domain (LdrΔLB), as well as an Ldr with its NPxY motif deleted (Fig. 1a). The HeLa WT and LDLR−/− were exposed to different concentrations of Tcnα for 3 h.

**Fig. 1 The LA domain of LDLR is responsible for the uptake of Tcnα.** a Schematic drawing of Ldr, LdrΔLA, LdrΔLB and LdrΔNPxY. b The HeLa WT or LDLR−/− cells were transfected with mock, Ldr, LdrΔLA, LdrΔLB and LdrΔNPxY, followed by incubating with Tcnα for 3 h. The percentages of round-shaped cells are plotted on the chart. The blue dash line indicates 6 nM. Error bars (n = 6) indicate mean ± SD. c The indicated HeLa cells were incubated with Tcnα (6 nM, 3 h) and the images were captured. Red fluorescence (mCherry) marked transfected cells. The scale bar represents 50 μm. d The round-shaped cells among all mCherry-positive cells shown in c were quantified and plotted in a bar chart. Error bars (n = 6) indicate mean ± SD, *P < 0.05, ***P < 0.001, n.s. = not significant, two-sided Student’s t-test.
6 nM Tcna induced ~80% of the HeLa WT cells to become round in 3 h while the LDLR/−/− cells are generally normal (Fig. 1b). This assay condition was adopted for testing the sensitivity of other transfected HeLa cells unless otherwise stated. Ectopic expression of the full-length Ldlr and LdlrALB, but not LdlrALA, restored susceptibility of the LDLR/−/− cells to Tcna, suggesting the LA repeats are essential for mediating the entry of Tcna (Fig. 1b–d and Supplementary Fig. 1a). This data is in line with the previous finding that LRPAp1, which binds to the LA domain of LDLR21, can competitively protect cells from Tcna. Likewise, an Ldlr mutant with NPxy motif deleted could restore the susceptibility of LDLR/−/− cells but less efficiently (Fig. 1b–d). NPxy motif is responsible for the fast recycling of LDLR, which promotes the uptake of the toxin but is not necessary.

Reconstituted LRP1 and Megalin sensitize the HeLa LDLR/−/− cells to Tcna. All LDLR family core members contain at least one LA-repeats domain. Because LRPAp1 further protects the LDLR KO cells from Tcna, we postulate that the LA repeats from other LDLR family proteins may also recognize Tcna. HeLa LDLR/−/− cells are more resistant to Tcna compared to the WT cells and ectopic expression of a mouse Ldlr would restore their susceptibility. This cell system could be used for investigating other potential endocytic receptors of Tcna. LRP1, Megalin, and LRPlB are very large proteins (~600 kDa) that are hard to be expressed. The extracellular domains of both LRP1 and Megalin contain four canonical LA repeats domains, namely cluster I-IV, with clusters II and IV particularly important for ligand-binding25,26. Therefore, we fused the cluster II LA domains of LRP1, Megalin, and LRPlB to the C-terminal part (including the EGF-precursor domain, O-linked sugar domain, transmembrane region, and cytoplasmic domain) of Ldlr (LdlrC) and generated chimeric proteins, including LRP1CII-LdlrC, MegalinCII-LdlrC, and LRPlB-CII-LdlrC (Fig. 2a). To interrogate alternative entry receptor(s) of Tcna within the LDLR family, Ldlr, Vldlr, ApoER2, Lrp4, Lrp10, Lrp11, LRP1CII-LdlrC, MegalinCII-LdlrC, and LRPlB-CII-LdlrC were exogenously expressed in the HeLa LDLR/−/− cells by transient transfection (Supplementary Fig. 1b). The Tcna sensitivities of these transfected cells were measured by the cytotoxic cell rounding assay. Ectopic expression of Ldlr, LRP1CII-LdlrC, and MegalinCII-LdlrC, but not others, sensitized the HeLa LDLR/−/− cells to Tcna (Fig. 2b, c). We next switched the C-terminal part of LRP1CII-LdlrC to LRP1CII (Fig. 2a). As expected, this newly built LRP1CII-LRPlC also effectively mediates the entry of Tcna (Fig. 2b, c). These results suggest that the LA domains from LDLR, LRP1, and Megalin can selectively recognize Tcna.

Surface sGAG is essential for LDLR/LRP1/Megalin-mediated uptake of Tcna. Cell-surface sGAG can mediate the attachment of Tcna and Clostridiales difficile toxin A (TcdA) and allow them to be enriched on the cell surface13,27. To demonstrate the sGAG-binding potentials of other major LCTs, we performed the heparin-beads pulldown experiment with the purified LCT proteins. While Tcna strongly binds to the heparin beads, minimal bindings of TcsH and TpeL were observed, and no TcsL or TcdB binding was detected (Fig. 3a).

The previous study reported that direct interaction between LDLR and Tcna is weak. Using the biolayer interferometry (BLI) assay, we showed that both interactions between LdlrLA and Tcna and between LRPlCII and Tcna are weak (Supplementary Fig. 2a, b). Routine dot-blot assays showed no detectable signals for LRPlCII-Tcna binding (Supplementary Fig. 2c). However, if the dot-blot assays were performed by 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) cross-link28, obvious signals for LRPlCII/LDLRLA binding to membrane immobilized Tcna were detected (Supplementary Fig. 2d). These results suggest that the interactions between LRPlCII/LDLRLA and Tcna could be either weak or unstable. To investigate whether surface sGAG promote the LDLR/LRP1/Megalin-mediated cellular entry of Tcna, we employed HeLa SLC35B2−/− cells that lack sulfation in surface proteoglycans and are thus considered sGAG-negative27,29. HeLa SLC35B2−/− cells were transiently transfected with Ldlr, LRPlCII-LdlrC, or MegalinCII-LdlrC. Cells transfected with an empty vector served as the controls. These transfected cells were pre-incubated with 200 nM Tcna on ice for 30 min, washed with the fresh medium, and incubated at 37 °C for 3 h. Overexpression of Ldlr, LRPlCII-LdlrC, or MegalinCII-LdlrC failed to sensitize the SLC35B2−/− cells to Tcna (Fig. 3b, c). Together, these data demonstrated that cell-surface sGAG is essential for Ldlr-, LRP1-, and Megalin-mediated cellular entry of Tcna.

LRP1 versus LDLR in different cells. Although LRP1 and Megalin can mediate the cellular entry of Tcna, they were not found in the candidate list of our previous CRISPR screen for Tcna. Likewise, LRPlCII was demonstrated as an entry receptor for TcdA30 but it did not stand out from the previous genomewide screen27. We noticed that HeLa cells were employed in both genetic screens for Tcna and TcdA, as well as the following validation experiments. On the other hand, previously Schettendreier et al. used mouse embryonic fibroblasts (MEFs) for studying the role of LRPlCII in TcdA entry30. According to a public protein profiling database (http://www.proteinatlas.org), LDLR and LRP1 have contrasting mRNA expression profiles in many different cell lines, while Megalin is absent in most cell lines (Fig. 4a and Supplementary Fig. 3). Interestingly, HeLa cells express LDLR at a high mRNA level and LRPlCII at a low mRNA level (Fig. 4a), which may partly explain why LRPlCII did not stand out in the previous screens using HeLa cells.

Both LDLR and LRPlCII participate in the Tcna entry in U-87 MG cells. We next performed immunoblot analysis to validate the protein levels in some commonly used cell lines including MCF-7, HeLa, HepG2, MEFs, BJ, and U-87 MG. HeLa cells express a minimal amount of LRPlCII, which is consistent with the mRNA data (Fig. 4a, b). Both MEFs and U-87 MG cells express considerable amounts of LDLR and LRPlCII (Fig. 4b). MEFs were previously used to study the role of LRPlCII in mediating the entry of TpeL and TcdA but these are mouse cells30,33. Therefore, we chose U-87 MG, a human glioma cell line that expresses both LDLR and LRPlCII, to generate LDLR and LRPlCII knockout cells using the CRISPR/Cas9 approach (Fig. 4c). In this cell line, knocking-out LRPlCII does not affect the expression level of LDLR and vice versa (Fig. 4c). We observed that Tcna is equally bound to the U-87 MG WT, LDLR−/−, and LRPlCII−/− cells in the binding assay (Fig. 4d), which is consistent with the view that LDLR and LRPlCII are not dominant attachment factors for Tcna.

We next assessed the colocalization of the endocytosed Tcna and LDLR/LRPlCII using the toxin internalization assay, followed by confocal fluorescence analysis. In the HeLa cells, knocking out LDLR largely reduced the internalization of Tcna, indicating that LDLR is a dominant entry receptor for Tcna in these cells (Supplementary Fig. 4). In contrast, a considerable amount of internalized Tcna was observed in the U-87 MG LDLR−/− cells using the internalization assay (Fig. 4e). Moreover, the internalized Tcna better colocalized with LDLR in the U-87 MG LRPlCII−/− cells when compared to the WT cells (Fig. 4e, f).

Finally, we investigated the roles of LDLR- and LRPlCII-mediated Tcna entry and intoxication in the U-87 MG cells. We found that both U-87 MG LDLR−/− and LRPlCII−/− cells were more resistant to...
when compared to the WT cells (Fig. 5a). To quantitatively determine the increased resistance, we defined the toxin concentration that results in 50% cell rounding as CR50. The CR50 for Tcnα in the U-87 MG WT is about 15.8 pM. The LDLR−/− cells showed ~36-fold increased resistance while the LRP1−/− cells showed ~18-fold increased resistance, compared to the WT cells (Fig. 5b). While the sensitivity of the LDLR−/− cells to Tcnα can be restored by the transient transfection of Ldlr, we further showed that ectopic expressing LRP1CII-LdlrC restored the sensitivity of the U-87 MG LRP1−/− cells (Fig. 5c, d). These data together suggest that both LDLR and LRP1 functionally mediate the endocytosis of Tcnα and are redundant receptors for Tcnα in cells such as U-87 MG.

Discussion

Tcnα is the most important virulence factor responsible for human and animal diseases associated with C. novyi infection. Our previous study demonstrated that sGAG and LDLR synergistically mediate the cellular entry of Tcnα13. It was also shown that other LDLR family proteins may be redundant entry receptors for Tcnα, but the receptor specificity within the LDLR family remains unclear. However, some LDLR family proteins, such as LRP1, Megalin, and LRP1B, have very high molecular weights that are hard to be studied directly. Here, we used reconstitutive proteins to investigate the roles of LDLR family members in the cellular uptake of Tcnα. Although the truncated/chimeric proteins may not completely represent the biological properties of native proteins, they act as powerful tools to study the ligand-binding properties of LDLR family proteins. For example, Ganaie et al. recently used various chimeric LRP1 to investigate the cellular entry of the Rift Valley fever virus34. By employing the reconstitutive LDLR family proteins, we successfully defined that LDLR, LRP1, and Megalin serve as redundant entry receptors for Tcnα and their LA domains are responsible for toxin recognition.

LDLR family receptors rapidly and constitutively recycle between cell membranes and endosomes, which provides an ideal route for mediating the endocytosis of target cargoes into cells. Several LDLR family core members commonly share their ability to bind a variety of ligands from endogenous lipoproteins to pathogenic viruses and bacterial toxins, such as LRPAP1, ApoE, TcdA, and vesicular stomatitis virus21,27,30,35,36. The LA repeats of the LDLR family core members are closely related modules that are responsible for the binding of most ligands14. We also defined

Fig. 2 LA domains of LRP1 and Megalin recognize Tcnα. a Schematic drawing of chimeric receptor proteins including VLdlrLA-LdlrC, LRP1CII-LdlrC, MegalinCII-LdlrC, LRP1BCII-LdlrC, and LRP1CII-LRP1C. b The HeLa LDLR−/− were transfected with mock, Ldlr, Vldlr, ApoER2, Lrp4, Lrp10, Lrp11, LRP1CII-LdlrC, MegalinCII-LdlrC, LRP1BCII-LdlrC, VldlrLA-LdlrC, and LRP1CII-LRP1C, followed by the incubation with Tcnα (6 nM, 3 h). Red fluorescence (mCherry) marked transfected cells. Representative images are shown. The scale bar represents 50 μm. c The round-shaped cells among all mCherry-positive cells shown in b were quantified and plotted in a bar chart. Error bars (n = 6) indicate mean ± SD, ***P < 0.001 versus mock, two-sided Student’s t-test.
that the LA domains of LDLR, LRP1, and Megalin are capable of recognizing Tcnα and mediating its entry. Owing to the similarity of these LA repeats, we propose that they may interact with Tcnα in a similar mode of action. However, other LDLR family members, such as Vldlr and ApoER2, failed to recognize Tcnα, indicating that the interactions between Tcnα and LDLR family proteins are somehow selective.

LDLR, LRP1, and Megalin can functionally mediate the cellular entry of Tcnα. According to the public datasets, both LDLR and LRP1 are widely distributed in various tissues including the liver and muscles, which are common targets for Tcnα. Megalin is expressed in limited organs like the brain and endocrine tissue, and muscles, which are common targets for Tcnα.

As the host receptors are demonstrated as keys to determining the pathology for LCTs,45–50 this study may further help to understand the pathogenesis of C. novyi infection-associated diseases.

Methods

Materials. HeLa (H1, CRL-1958) and MCF-7 (HTB-22) cells were originally obtained from ATCC. MEFs (CTCC-003-0036), BJ (CTCC-400-0144), and U-87 MG (CTCC-ZYHC-0434) cells were purchased from Chinese Tissue Culture Collections (CTCC). Expi293F cells (A14527) were purchased from ThermoFisher Scientific. They were tested negative for mycoplasma contamination. HeLa cells were authenticated via STR profiling (Shanghai Biowing Biotechnology Co. LTD, Shanghai, China). Hela LDLR−/− and SLC35B2−/− cells were previously generated laboratory stocks.27,31 All cell lines were cultured in DMEM media plus 10% fetal bovine serum (FBS) and 100 U penicillin/0.1 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37 °C.

The following antibodies, reagents, and recombinant proteins were purchased from the indicated vendors: Alexa Fluor 488 goat anti-rabbit IgG (ab150077, 1:1000, Abcam), rabbit polyclonal IgG against β-Actin (ab227387, 1:5000, Abcam), rabbit monoclonal IgG against LDLR (ab52818 for western blot, 1:500; ab30532 for immunofluorescence, Abcam), rabbit monoclonal IgG against LRP1 (ab92544, 1:20000 for western blot and 1:200 for immunofluorescence, Abcam), HRP-conjugated goat anti-human IgG-Fc antibody (SAA001, 1:3000, Sino Biological), Hoechst 33258 staining solution (E607301, BBI), NHS-Rhodamine fluorescent labeling kit (#46406, Thermo Fisher Scientific), recombinant human LRP1 Cluster II Fc chimera (R&D Systems, 2368-L2), Precast PAGE Gel (abs9309, Abcam), Polyethyleneimine Linear (PEI) MW25000 (40816ES03, YEASEN), and Heparin-Sepharose (ab92544, 1:200 for immunofluorescence, Abcam), rabbit monoclonal IgG against LRP1 (ab92544, 1:20000 for western blot and 1:200 for immunofluorescence, Abcam), HRP-conjugated goat anti-human IgG-Fc antibody (SAA001, 1:3000, Sino Biological), Hoechst 33258 staining solution (E607301, BBI), NHS-Rhodamine fluorescent labeling kit (#46406, Thermo Fisher Scientific), recombinant human LRP1 Cluster II Fc chimera (R&D Systems, 2368-L2), Precast PAGE Gel (abs9309, Abcam), Polyethyleneimine Linear (PEI) (MW25000 (40816ES03, YEASEN), and Heparin-Sepharose (Abcam, ab193268).

Genes and cloning. The DNA fragments encoding LRP1ΔC, MegalinΔC, LRP1BCII, and LRP1ΔC were synthesized by a commercial vendor (Genscript, Nanjing). The DNA fragments encoding Ldlr, LdlrΔLA, LdlrΔLB, Vldlr, Lrp4, Lrp10, Lrp11, and ApoER2 were PCR amplified from DharmaconTM cDNA/ORF Library and cloned...
Fig. 4 LDLR versus LRP1 in various cells. a The mRNA levels of LDLR, LRP1, and Megalin in MCF-7, THP-1, PC-3, A549, HeLa, HepG2, BJ, U-87 MG, and Caco-2 cells are shown. Data were obtained from a public database (http://www.proteinatlas.org). b The protein levels of LDLR and LRP1 in the HeLa, MCF, MEF, BJ, and U-87 MG cells were measured by immunoblot analysis. c The depletion of LDLR and LRP1 in the U-87 MG LDLR<sup>−/−</sup> and LRP1<sup>−/−</sup> cells showed by immunoblot analysis. Actin served as a loading control. The experiments in b, c have been repeated independently twice with similar results. d Immunofluorescence analysis shows that Alexa Fluor 555-labeled Tcnα (50 nM) is robustly bound to the U-87 MG WT, LDLR<sup>−/−</sup>, and LRP1<sup>−/−</sup> cells. Cell nuclei were stained with Hoechst dye. Representative images are shown. The scale bar represents 50 μm. e Immunofluorescent staining shows cellular localization of LDLR and endocytosed Tcnα in the U-87 MG WT, LDLR<sup>−/−</sup>, and LRP1<sup>−/−</sup> cells. Cell nuclei were stained with Hoechst. Representative images are shown. Scale bars represent 10 μm. f Colocalization of LDLR and endocytosed Tcnα in the U-87 MG WT, LDLR<sup>−/−</sup>, and LRP1<sup>−/−</sup> cells were analyzed by software ImageJ ver1.53. The percentage of the Tcnα signals that overlapped with LDLR in each cell was calculated and plotted as an open circle. Error bars (n = 10) indicate mean ± SD, ***P < 0.001 versus WT, two-sided Student’s t-test.

Expression and purification of recombinant Proteins. Recombinant Tcnα, TcdB, TpeI, TcSI, and TcSH were expressed in Bacillus subtilis SL401 and purified as Histagged proteins<sup>52</sup>. In brief, B. subtilis cells were cultured at 37 °C till OD<sub>600</sub> reached 0.6 and then induced with 1 mM isopropyl β-D-thiogalactoside at 25 °C for 20 h. The recombinant LDLR<sub>A</sub>-Fc with His-tag at C-terminus was expressed in E. coli. The supernatant was collected 4 days post-transfection and applied to purification. All above recombinant proteins were purified by Ni-affinity chromatography and size-exclusion chromatography (GE Healthcare).

Gene knockout in U-87 MG cell line. To generate U-87 MG LDLR<sup>−/−</sup> cell line, the following two sgRNA sequences were cloned into LentivGuide-puro-mKate2 vectors (Addgene #52963) to target LDLR genes: 5′-CCAGCTGGACCCCCCAGCGGA-3′. To generate U-87 MG LRP1<sup>−/−</sup> cell line, the following two sgRNA sequences were cloned into LentivGuide-puro-mKate2 vectors to achieve fragment knockout: 5′-CTGCCCGAGCCGATCGTGGCAGG-3′ and 5′-TGGCAGTACGACACAGCAGTTG-3′. Lentiviruses were generated by transfecting 293T cells with sgRNA plasmid, psiPAX2, and pMD2g. U-87 MG Cas9 cells were transduced with lentiviruses that express the sgRNAs. Mixed populations of infected cells were selected with puromycin (5 μg/ml). The KO efficiency of all mixed populations of KO cells was validated by immunoblot analysis.

Cytopathic cell rounding assay. HeLa and U-87 MG cells were transiently transfected using Polyjet following a manufacturer’s instruction. Thirty-six hours post-transfection, the transfected cells were trypsin-digested and plated to the new 24-well plates. Cells were allowed to grow for additional 12 h and then applied to toxin treatment. The transfected HeLa LDLR<sup>−/−</sup> cells were exposed to a series of diluted Tcnα at 37 °C for 3 h. The transfected HeLa LSLC5B2<sup>−/−</sup> cells were first incubated with 200 nM Tcnα on ice, changed with the fresh medium, and then incubated at 37 °C for 3 h. The U-87 MG cells were exposed to a series of diluted Tcnα at 37 °C for 20 h. The phase-contrast images of cells were then taken (Olympus IX73, 10× objectives). A zone of 200 × 200 μm was selected randomly, which contains 20–50 cells. Round-shaped and normal-shaped cells were counted manually. The percentage of round-shaped cells was analyzed using OriginPro (OriginLab, v8.5). All experiments were performed in three independent biological replicates. Statistical analysis was performed using OriginPro (OriginLab, v8.5).

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Fig. 5 The U-87 MG LDLR−/− and LRP1−/− cells are more resistant to Tcnα. a U-87 MG WT, LDLR−/−, and LRP1−/− cells were incubated with 30 pM Tcnα at 37 °C for 20 h. Cytopathic effect was observed in the U-87 MG WT cells but not the LDLR−/− and LRP1−/− cells using microscopic analysis for cell morphology. The scale bar represents 50 μm. b The sensitivities of the U-87 MG WT, LDLR−/−, and LRP1−/− cells to Tcnα were quantified using the cytopathic cell rounding assay. The percentage of round-shaped cells was measured and plotted on the chart. Error bars (n = 6) indicate mean ± SD. The CR50 for Tcnα in the U-87 MG WT, LDLR−/−, and LRP1−/− cells were calculated and listed. c The indicated U-87 MG cells were transfected with mock, LDLr, or LRP1c-LdlrC, followed by incubation with Tcnα for 20 h. The percentages of round-shaped cells are plotted on the chart. Error bars (n = 6) indicate mean ± SD. d The indicated U-87 MG cells were incubated with Tcnα (30 pM, 20 h) and the images were captured. Representative images are shown. Red fluorescence (mCherry) marked transfected cells. The scale bar represents 50 μm.

Heparin-Sepharose pulldown assay. Tcnα, Tcα, Tcβ, Tcγ, and Tcδ were diluted into a final concentration of 0.5 μg/μL. Then 20 μL of toxin protein was incubated with 20 μL of Heparin-Sepharose (Abcam, ab193268) for 1 h at 4 °C. The Heparin-Sepharose beads were washed three times with PBS and collected as samples. All the samples were analyzed via SDS-PAGE analysis.

Immunoblot analysis. Cells were scraped and washed three times with PBS. Cell pellets were lysed with RIPA buffer (50 mM Tris, pH 7.5, 1% NP-40, 150 mM NaCl, 0.5% sodium deoxycholate, 1% SDS, protease inhibitor cocktail) on ice for 30 min. The protein amounts in cell lysates were determined by BCA assay (Beyotime, P0011). The cell lysates were heated for 5 min at 95 °C, analyzed by SDS-PAGE, and transferred onto a nitrocellulose membrane (GE Healthcare, 10600002). The membrane was blocked with TBS-T buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% skim milk at room temperature for 1 h. The membrane was then incubated with the primary antibodies for 2 h, washed, and incubated with secondary antibodies for 1 h at room temperature. Signals were detected using the enhanced chemiluminescence method (Thermo Fisher Scientific, 34080) with GE imaging system AL600RGB.

BLI assay. BLI assay was performed with an Octet RED96e system and the data were analyzed with Octet Data Analysis software (Version:12.0.1.2, ForteBio, Fremont, CA, U.S.). In brief, 200 nM Fc-tagged proteins were immobilized onto capture biosensors (AHC biosensor, ForteBio) and balanced with binding buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). The biosensors were then exposed to capture biosensors (AHC biosensor, ForteBio) and balanced with binding buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). The biosensors were then exposed to the indicated concentrations of Tcnα or RAP, followed by dissociation in the binding buffer.

Dot-blot assay. LRPA1 and Tcnα of indicated amounts were spotted onto a nitrocellulose membrane and allowed to dry completely in the air. The membrane was blocked with 5% skim milk for 1 h at room temperature, followed by incubating with LRPA1-Fc/LDLRα-Fc/IgG Fc at room temperature for 4 h. The bound LRPA1-Fc/LDLRα-Fc was detected with a monoclonal antibody against human IgG Fc. For membrane EDC cross-link, after the LRPA1-Fc/LDLRα-Fc/IgG Fc incubation, the blots were further incubated with 5 mM EDC at room temperature for 1 h.

Cell-surface toxin-binding assay. Tcnα was labeled using an NHS-Rhodamine fluorescent labeling kit (#46406, Thermo Fisher Scientific) following the manufacturer’s instructions. U-87 MG WT, LDLR−/−, and LRP1−/− cells were incubated with 50 nM Rhodamine-labeled Tcnα in PBS for 30 min on ice. Cells were washed five times with ice-cold PBS and fixed with 4% paraformaldehyde (PFA) and the cell nuclei were labeled with Hoechst. Confocal images were captured with the Zeiss LSM 880 NLO with AiryScan System.

Toxin internalization assay. Tcnα were labeled using an NHS-Rhodamine fluorescent labeling kit (#46406, Thermo Fisher Scientific) following the manufacturer’s instructions. HeLa or U-87 MG cells were incubated with 400 nM Rhodamine-labeled Tcnα in PBS for 30 min on ice. Cells were washed five times with ice-cold PBS and subjected to immunofluorescence analysis.

Immunofluorescence analysis. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 5% BSA, and then incubated with the LDLR antibody (ab30532, Abcam) overnight at 4 °C. The cells were then washed, incubated with the secondary antibody (goat anti-rabbit IgG Alexa488) for 1 h at room temperature, and stained with Hoechst for cell nuclei. Confocal images were captured with the Zeiss LSM 880 NLO with AiryScan System. Colocalization of Tcnα and LDLR was analyzed by the software ImageJ ver.1.52.

Statistics and reproducibility. Data are presented as mean ± standard deviation (SD). The number of the sample size (n) and statistical hypothesis testing method (two-sided Student’s t-test) are described in the legends of the corresponding figures. Statistical analyses of data were performed with GraphPad Prism v9.3 or OriginPro v8.5. **P < 0.05, ***P < 0.01, ****P < 0.001, n.s. = not significant. For western blot analysis, the experiments have been repeated independently at least twice with similar results.

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

Data availability. The source data behind the graphs and charts in the paper are provided as Supplementary Data. Uncropped blots are available in Supplementary Information.
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