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(71) Applicant: **THE HOSPITAL FOR SICK CHILDREN** [CA/CA]; 555 University Avenue, Toronto, Ontario M5G 1X8 (CA).

(72) Inventors: **MELNYK, Roman A.**; 254 Douglas Avenue, Oakville, Ontario L6J 3S3 (CA). **AUGER, Anick**; 5190 Bl. St-Joseph, Lachine, Montreal, Québec H8T 1S2 (CA). **BEILHARTZ, Greg**; 3-374 Merton Street, Toronto, Ontario M4S 1B3 (CA). **MINASSIAN, Berge**; 11 Caravan Drive, Toronto, Ontario M3B 1M9 (CA). **SUGIMAN-MARANGOS, Seiji**; 1-197 Danforth Avenue, Toronto, Ontario M4K 1N2 (CA).

(74) Agents: **BOOCOCK, Graeme** et al.; Borden Ladner Gervais LLP, World Exchange Plaza, 100 Queen Street, Suite 1300, Ottawa, Ontario K1P 1J9 (CA).

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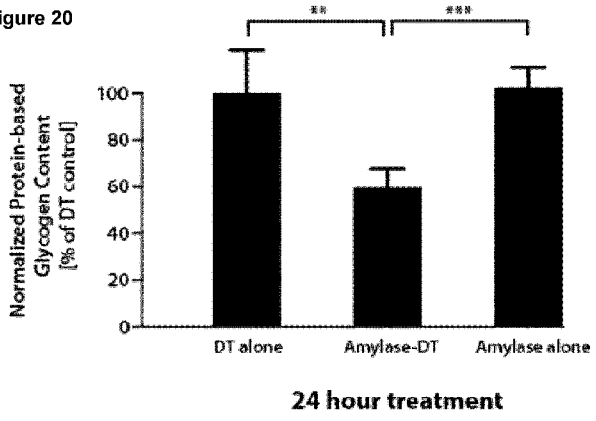
(54) Title: DELIVERY OF STRUCTURALLY DIVERSE POLYPEPTIDE CARGO INTO MAMMALIAN CELLS BY A BACTERIAL TOXIN

Figure 20



(57) Abstract: There is a need for delivery platforms with robust capacity that offer the possibility to deliver diverse protein-based therapeutics into specific cells. Described herein is a platform for delivering cargo polypeptides into cells, which is based on a recombinant molecule comprising: a cargo polypeptide, a diphtheria toxin enzymatic fragment (DTA), and a diphtheria toxin translocation fragment (DTB). The platform has been employed to deliver diverse cargo into cells, including those having low or high molecular weights. A hyper-stable cargo polypeptide has been delivered, as well as proteins of therapeutic significance {e.g, MecP2, SMN, FMRP, PNP, and alpha-amylase). The platform is also useful for delivering genome-modifying proteins, such as the CRISPR protein, Cas9. Associated nucleic acids, pharmaceutical compositions, methods, uses, and kits are also described, including those of therapeutic significance aimed at treating diseases or disorders caused by enzyme or protein deficiency.

DELIVERY OF STRUCTURALLY DIVERSE POLYPEPTIDE CARGO INTO MAMMALIAN CELLS BY A BACTERIAL TOXIN

FIELD

[0001] The present disclosure relates generally to a polypeptide delivery platform. More particularly, the present disclosure relates to a bacterial toxin-based platform for polypeptide delivery.

BACKGROUND

[0002] In contrast with small-molecule therapeutics and probes, which often readily penetrate biological membranes, larger macromolecules, such as peptides and proteins, are generally excluded from the cell interior. Given the vast array of applications for protein-based tools and therapeutics inside cells, there is great interest in developing safe and efficient protein delivery platforms that direct biologics into cells. To date, numerous approaches have been investigated to facilitate protein entry into the cytoplasm of cells, including cell-penetrating peptides, lipid-based molecules, nanoparticles, encapsulated protein containers, zinc-finger proteins, and super-charged green fluorescent proteins. Though each is capable of delivering protein cargo into cells to varying degrees, general mechanism-based limitations exist for these platforms. Cell-selectivity and/or efficiency-of-delivery remain particularly elusive features for most platforms owing to their shared nonspecific mode of interaction with membranes.

[0003] Platforms enabling targeted delivery of proteins into cells are needed to fully realize the potential of protein-based therapeutics with intracellular sites-of-action. As such, there remains a pressing need for delivery platforms with robust capacity that offer the possibility to deliver diverse protein-based therapeutics into specific cells.

SUMMARY

[0004] It is an object of the present disclosure to obviate or mitigate at least one disadvantage of previous approaches.

[0005] In one aspect, the present disclosure provides a recombinant molecule comprising a cargo polypeptide, a diphtheria toxin enzymatic fragment (DTA), and a diphtheria toxin translocation fragment (DTB). In one embodiment, the recombinant molecule has a general structure: x-C-y-DTA-DTB, wherein: x is a polypeptide or absent, C is the cargo polypeptide, and y is a polypeptide, a linker, or absent.

[0006] In another aspect, there is provided a nucleic acid encoding the above-described recombinant molecule.

[0007] In another aspect, there is provided a recombinant cell comprising at least one above-described nucleic acid.

[0008] In another aspect, there is provided a vector comprising at least one above-described nucleic acid.

[0009] In another aspect, there is provided a cell transformed with the above-described vector.

[0010] In another aspect, there is provided a pharmaceutical composition comprising the above-described recombinant molecule, and a pharmaceutically acceptable carrier.

[0011] In another aspect, there is provided a method of delivering a cargo polypeptide to a cell, comprising contacting the cell with the above-described recombinant molecule.

[0012] In another aspect, there is provided a method of delivery a cargo polypeptide to a cell of a subject, comprising contacting the cell with the above-described recombinant molecule.

[0013] In another aspect, there is provided a method of delivering a cargo polypeptide across the blood brain barrier, comprising administering to a subject the above-described recombinant molecule.

[0014] In another aspect, there is provided a method of increasing enzyme or protein activity in a cell, comprising contacting the cell with the above-described recombinant molecule.

[0015] In another aspect, there is provided a method of alleviating enzyme or protein deficiency in a cell, comprising contacting the cell with the above-described recombinant molecule.

[0016] In another aspect, there is provided a method of treating a disease or disorder caused by enzyme or protein deficiency in a subject, comprising administering to the subject the above-described recombinant molecule.

[0017] In another aspect, there is provided a method of manipulating the genome of a cell, comprising contacting the cell with the above-described recombinant molecule, wherein the cargo polypeptide comprises a genome-modifying protein.

[0018] In another aspect, there is provided a use of the above-described recombinant molecule for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide to a cell.

[0019] In another aspect, there is provided a use of the above-described recombinant molecule for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide to a cell of a subject.

[0020] In another aspect, there is provided a use of the above-described recombinant molecule for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide across the blood brain barrier.

[0021] In another aspect, there is provided a use of the above-described recombinant molecule for increasing, or for preparation of a medicament for increasing, enzyme or protein activity in a cell.

[0022] In another aspect, there is provided a use of the above-described recombinant molecule for alleviating, or for preparation of a medicament for alleviating, enzyme or protein deficiency in a cell.

[0023] In another aspect, there is provided a use of the above-described recombinant molecule for treating, or for preparation of a medicament for treating, a disease or disorder caused by enzyme or protein deficiency in a subject.

[0024] In another aspect, there is provided a use of the above-described recombinant molecule for manipulating the genome of a cell, wherein the cargo polypeptide comprises a genome-modifying protein.

[0025] In another aspect, there is provided a kit for delivering a cargo polypeptide to a cell comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[0026] In another aspect, there is provided a kit for delivering a cargo polypeptide to a cell of a subject, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[0027] In another aspect, there is provided a kit for delivering a cargo polypeptide across the blood brain barrier, comprising the above-described recombinant molecule, and instructions for administering the recombinant molecule to a subject.

[0028] In another aspect, there is provided a kit for increasing enzyme or protein activity in a cell, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[0029] In another aspect, there is provided a kit for alleviating enzyme or protein deficiency in a cell, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[0030] In another aspect, there is provided a kit for treating a disease or disorder caused by enzyme or protein deficiency in a subject, comprising the above-described

recombinant molecule, and instructions for administering the recombinant molecule to the subject.

[0031] In another aspect, there is provided a kit for manipulating the genome of cell, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule, wherein the cargo polypeptide comprises a genome-modifying protein.

[0032] Other aspects and features of the present disclosure will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] Embodiments of the present disclosure will now be described, by way of example only, with reference to the attached Figures.

[0034] **Figure 1** depicts representative structures of the three different passenger proteins: sumo protein; α-amylase; and eGFP. Arrows indicate the C-terminus of each protein.

[0035] **Figure 2** depicts a schematic of first generation chimeric fusions of different passenger proteins to the amino terminus of native diphtheria toxin (DT) via a flexible GSG linker.

[0036] **Figure 3** depicts dose titration curves of chimeric constructs on cells with wt-DT, Sumo-DT, Amylase-DT, and eGFP-DT.

[0037] **Figure 4** depicts data evaluating the effect of linker size between eGFP and dtA on cells.

[0038] **Figure 5** depicts the results of cell toxicity assays to measure the positional effects of dtA on inhibition of protein synthesis.

[0039] **Figure 6** depicts data in addition to Figure 5 to rule out the possibility that the amino terminal dtA fragment was affecting translocation.

[0040] **Figure 7** depicts data in addition to Figure 5 showing the same positional dependence for dtA when amylase is the passenger protein.

[0041] **Figure 8** depicts the results of cell toxicity assays indicating that passenger proteins reach the cytosol.

[0042] **Figure 9** depicts a time course of inhibition of protein synthesis of three constructs using 1nM of each toxin.

[0043] **Figure 10** depicts the results of differential scanning fluorimetry at various pH values for eGFP and mCherry.

[0044] **Figure 11** depicts pH-induced unfolding of dtA alone using differential scanning fluorimetry, with the transition midpoint of unfolding (Tm) shown across several pH values.

[0045] **Figure 12** depicts the results of cell toxicity assays indicating that mCherry is efficiently delivered into cells by DT.

[0046] **Figure 13** depicts the results of cell toxicity assays comparing delivery of dtA by dtB and TAT peptides.

[0047] **Figure 14** depicts the results of cell toxicity assays demonstrating that enzymatically inactive DT competes with A-eGFP-a-B.

[0048] **Figure 15** depicts the results of cell toxicity assays examining the effect of a pore-formation formation/translocation-defective mutation (L350K).

[0049] **Figure 16** depicts further data indicating that the pore-formation/translocation mutant L350K is unable to enter cells and inhibit protein synthesis.

[0050] **Figure 17** depicts data obtained with the EnzChek™ Ultra Amylase Assay indicating that amylase fused to DT is folded and functional.

[0051] **Figure 18** depicts the experimental design for α-amylase-DT treatment of HEK 293 cells.

[0052] **Figure 19** depicts measurements of protein-based glycogen content in HEK cells after 24h or 48h treatment normalized on content in cells treated with either DT alone or amylase alone, respectively (n=1).

[0053] **Figure 20** depicts protein-based glycogen content in HEK cells after 24 h treatment with 1.0 uM DT, amylase-DT, or amylase alone.

[0054] **Figure 21** depicts results of protein toxicity studies cells indicating proof of delivery of MeCP2e1 into the cytosol of Vero cells.

[0055] **Figure 22** depicts results of cell toxicity assays indicating proof of delivery of MeCP2e1 into the cytosol of iPSC-derived neurons from Rett Syndrome patient fibroblasts.

[0056] **Figure 23** depicts results of cell toxicity assays indicating proof of delivery of SMN into the cytosol of Vero cells.

[0057] **Figure 24** depicts results of cell toxicity assays indicating proof of delivery of FMRP into the cytosol of Vero cells.

[0058] **Figure 25** depicts results of ^{3}H-leucine incorporation toxicity assays demonstrating delivery of PNP into the cytosol of Very cells.

[0059] **Figure 26** depicts results of ^{3}H-leucine incorporation toxicity assays demonstrating delivery of PNP into the cytosol of two-week old wild type (WT) neurons cells.

[0060] **Figure 27** depicts results of ^{3}H-leucine incorporation toxicity assays demonstrating delivery of Cas9 into the cytosol of Vero cells.

[0061] **Figure 28** depicts the results of NanoGlo assays demonstrating delivery of eGFP-CPD$_{Vc}$-DT into the cytosol of Vero-NlucP cells by fusion protein toxicity.

[0062] **Figure 29** depicts the results of cell viability assays to assess the effects of removing most of the DTA domain.

DETAILED DESCRIPTION

[0063] Generally, the present disclosure provides a platform for delivering cargo polypeptides into cells, which is based on a recombinant molecule comprising: a cargo polypeptide, a diphtheria toxin enzymatic fragment (DTA), and a diphtheria toxin translocation fragment (DTB). The platform may been employed to deliver diverse cargo into cells, including those having low or high molecular weights. Hyper-stable cargo polypeptide may be delivered, as well as proteins of therapeutic significance (e.g. MecP2, SMN, FMRP, PNP, and alpha-amylase). The platform may be useful in delivering genome-modifying proteins, such as the CRISPR protein, Cas9. Associated nucleic acids, pharmaceutical compositions, methods, uses, and kits are also described, including those which may be of therapeutic significance, e.g., for treating diseases or disorders caused by enzyme or protein deficiency.

[0064] Protein toxins with intracellular sites-of-action are promising systems to consider as delivery platforms as they have evolved elegant and sophisticated solutions to delivering proteins across membranes and into cells. Bacterial toxins are attractive systems to consider as templates for designing protein transduction systems as they naturally bind and enter specific cells with high efficiency.

[0065] Diphtheria toxin (DT) is among the smallest and best characterized toxin of the 'AB toxin' family. DT is a single chain 535-amino acid protein composed of an enzymatic A fragment (dtA) and a receptor-binding/translocation B fragment (dtB) linked through an intra-molecular disulfide bond with an intervening furin-like cleavage site. DT binds the heparin-binding epidermal growth factor-like growth factor (HB-EGF; also known as the diphtheria toxin receptor) on target cells via its C-terminal dtB domain triggering endocytosis into clathrin-coated vesicles, which are then converted into early endosomal vesicles. Upon exposure to low pH in the endosome, two hydrophobic α-

helical hairpins buried within the translocation domain of dtB unfurl and insert into the endosomal membrane, creating a transmembrane pore that facilitates translocation of the catalytic dtA domain into the cytosol. Once in the cytosol, the dtA domain catalyzes the transfer of the ADP-ribose moiety of NAD+ to eukaryotic elongation factor (eEF-2), which inhibits protein synthesis, and ultimately leads to cell death.

[0066] The properties of DT have previously been exploited to make therapeutic fusion proteins; however, in most cases, the receptor-binding region of the dtB-domain was replaced with alternate domains to direct the toxic dtA-fragment to kill specific cells bearing a particular receptor[1-3]. A fundamentally different concept is investigated herein, namely that of directing foreign proteins attached to the dtA-fragment into target cells using the receptor-binding and translocation properties of the native dtB-domain.

[0067] The first piece of evidence that suggested that DT may have the capacity to function as a vehicle for cytosolic delivery of passenger proteins came from a seminal study by Madshus et al., showing that DT could deliver an extra dtA-domain, fused as an amino-terminal extension to the existing dtA, into the cytosol[4]. In subsequent studies, it was shown that short peptides and certain small protein cargo could also be co-delivered with dtA into cells[4-7]. A recurring – yet unexplained – observation in these studies was that passenger proteins appeared to decrease the efficiency of protein delivery to different extents when the activity of the associated dtA fragment was used to measure translocation. Also, because the passenger proteins used previously have been relatively small (i.e., < 20-kDa) and expected to be largely disordered prior to and during translocation[8], the extent to which DT could deliver proteins with properties more characteristic of typical proteins and would-be protein therapeutics is not known. Given the importance of cargo size, structure and stability in evaluating the suitability of DT as a universal protein delivery vector, an aim of this study was to resolve these questions using a number of model passenger proteins together with novel construct designs. The data presented here show that DT has great promise as an intracellular protein delivery platform, with some embodiments offering unique advantages of target cell specificity, translocation efficiency and passenger protein versatility.

[0068] The capacity of diphtheria toxin to function as an intracellular protein delivery vector is investigated. It is shown that diphtheria toxin can, in some embodiments, deliver an impressive array of passenger proteins spanning a range of sizes, structures and stabilities into cells in a manner that indicates that they are 'invisible' to the translocation machinery. Further, it is shown that α-amylase can be delivered into cells by a detoxified diphtheria toxin chimera, and that it digests intracellular glycogen in

live cells, providing evidence that delivered cargo can be folded, active and abundant. The efficiency and versatility of diphtheria toxin over existing systems open numerous possibilities for intracellular delivery of bioactive proteins.

[0069] Recombinant Molecules

[0070] In one aspect, there is provided a recombinant molecule comprising a cargo polypeptide, a diphtheria toxin enzymatic fragment (DTA), and a diphtheria toxin translocation fragment (DTB).

[0071] 'DTA', as used herein, refers to the diphtheria toxin enzymatic A fragment generally, while 'DTB' refers to the receptor-binding/translocation B fragment generally.

[0072] By 'fragment' is meant a sequence of amino acids that includes the relevant domain, or a subsequence thereof from which some or all of the relevant domain has been removed. Though terms "enzymatic fragment" or "receptor-binding/translocation" are used by convention, it will be understood that some such fragments are functional, while others may have reduced function or may not be functional. For example, in the case of DTA, a 'fragment' may encompass the entirety of SEQ ID NO: 1 (dtA) or SEQ ID NO: 2 (dta), but is also to be understood as encompassing subsequences thereof.

[0073] By 'domain' is meant a particular functional and/or structural unit of a protein, often responsible for a particular function or interaction that contributes to the overall role of a protein. Protein domains may be evolutionarily conserved.

[0074] Where 'dtA' is used, it refers to a catalytically active form of DTA, unless otherwise specified. Likewise, and 'dta' is used herein to refer to the catalytically inactive form, unless otherwise specified. 'dtB', as used herein, refers to functional DTB, unless otherwise specified.

[0075] By 'catalytically active' is meant that the DTA is enzymatically active, i.e. toxic to the relevant cells. By 'catalytically inactive' is meant that the DTA is enzymatically inactive, i.e. non-toxic to the relevant cells.

[0076] The recombinant molecule may be used with a cargo polypeptide of any size. The size can be less than 1 kDa, less than 2 kDa, less than 5 kDa, less than 10 kDa, or greater than 10 kDa. The recombinant molecule may be useful for delivering cargo polypeptides of relatively large size, for example, greater than 10kDa, 20 kDa, 30 kDa, 40 kDa, 50 kDA, 60 kDA, 70 kDa, 80 kDa, 90 kDa, 100 kDa, 110 kDa, 120 kDa, 130 kDa, 140 kDa, 150 kDa, or 160 kDa. For example, the cargo polypeptide may have a molecular weight of greater than 10 kDa. The cargo polypeptide may have a molecular weight greater than 20 kDa. The cargo polypeptide may have a molecular weight greater

than 30k Da. The cargo polypeptide may have a molecular weight greater than 50 kDa. The cargo polypeptide may also have a molecular weight of greater than 100 kDa. The cargo polypeptide may also have a molecular weight of greater than 150 kDa. The cargo polypeptide may be positioned at or upstream of the amino terminus of the diphtheria toxin enzymatic fragment.

[0077] The cargo polypeptide may be a modified sequence, e.g. containing chemically modified, mutated, or non-natural amino acids. For instance, the cargo polypeptides may be modified to increase stability as compared to, e.g., the unmodified or natural counterpart sequence.

[0078] In one embodiment, the recombinant molecule has a general structure: x-C-y-DTA-DTB, wherein: x is a polypeptide or absent, C is the cargo polypeptide, and y is a polypeptide, a linker, or absent. DTA can, for instance, be linked to the DTB by way of a disulphide linkage. This may be formed via a cysteine residue corresponding to the cysteine at position 186 of SEQ ID NOs: 1 or 2; and a cysteine residue corresponding to the cysteine at position 2 of SEQ ID NO: 3.

[0079] In one embodiment, y is an autoprocessing domain. Autoprocessing domains are those that effect their own cleavage. In one embodiment, an autoprocessing domain that cleaves at or near its own N-terminus, e.g. to "self clear" is desirable. Using an autoprocessing domain of this sort, cargo polypeptide may be released into the cytosol. The autoprocessing domain may comprise a cysteine protease domain (CPD). This protein family is well known. The CPD may be derived from a bacterium, such as *Vibrio cholerae* or *Clostridium difficile*. These cysteine protease domains may comprises an amino acid sequence as set forth in SEQ ID No: 20 or 21, respectively.

[0080] In one embodiment, y is a linker. The linker may be an amino acid linker. When placed between a cargo polypeptide and DTA or DTB, the linker may be of sufficient length so as not to inhibit (or reduce or minimize inhibit) DTA or DTB. The linker may comprise at least 1, 2, 3, or 4 amino acid residues. The linker may comprises, e.g. at least five amino acid residues. The amino acid linker may comprise $(G4S)_n$, wherein n is 1 or greater, for instance 1 to 3. In one embodiment, n is 3.

[0081] In one embodiment, x is absent.

[0082] DTB may comprise an amino acid sequence as set forth in SEQ ID No: 3.

[0083] DTA may be catalytically active (dtA) or catalytically inactive (dta). An example of a catalytically active DTA is one comprising an amino acid sequence as set forth in SEQ ID No: 1. An example of a catalytically inactive DTA is one bearing the mutations K51E and E148K, as numbered with respect to wild type sequence. For

instance, an inactive DTA may comprise an amino acid sequence as set forth in SEQ ID No: 2.

[0084] The cargo polypeptide may comprise any polypeptide for which cellular delivery is desired.

[0085] The cargo polypeptide may comprise an enzyme, or an active fragment thereof having substantially the same activity. By 'substantially the same activity' is meant that a core function of the enzyme is substantially unaltered in the fragment.

[0086] The cargo polypeptide may comprise a stably folded, or hyper stable polypeptide. By 'hyper stable' is meant a polypeptide that is not susceptible to unfolding. mCherry is one example of a stably folded protein. mCherry is not susceptible to unfolding at high temperatures, i.e. of 80 degrees Celsius. The cargo polypeptide may accordingly be a polypeptide that resists unfolding up to 60, 70, 80, 90, or 100 degrees Celsius. mCherry is also stable down to pH 4. The cargo polypeptide may according be a polypeptide that resists unfolding down to pH 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, or 1.0.

[0087] The cargo polypeptide may comprise a therapeutic protein. By 'therapeutic polypeptide' is meant any protein, the cellular delivery of which could be used for a therapeutic purpose. It is well known, for example, that many human diseases or disorders are caused by or characterized by protein deficiency. Therapeutic proteins encompass proteins, the delivery of which could ameliorate or correct such a deficiency. A therapeutic protein may act to replace a protein that is deficient in the disease or disorder. A therapeutic protein may be the protein that is deficient in the disease or disorder. However, a therapeutic protein need not necessarily be identical to the protein that is deficient in the disease or disorder. For instance, a therapeutic protein may be an active fragment or modified form of a deficient protein. A therapeutic protein may also partially or fully functionally compensate for the protein deficiency underlying the disease or disorder. A therapeutic protein may also ameliorate or correct downstream or secondary effects of the cellular deficiency in a particular protein. As an example, while Lafora disease is caused e.g. my mutations in EPM2A or NHLRC1 (EPM2B), it is envisaged that delivery of an amylase, such as an alpha-amylase, as a therapeutic protein could help to reduce or clear Lafora bodies. The cargo polypeptide may comprise MecP2 (e.g. SEQ ID No: 16 or 17), SMN (e.g. SEQ ID No: 19), FMRP (e.g. SEQ ID No: 18), PNP (e.g. SEQ ID No: 24), or alpha-amylase (e.g. SEQ ID No: 15).

[0088] The cargo polypeptide comprises a genome-modifying protein. The genome-modifying protein comprises a zinc finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), or a CRISPR (clustered regularly interspaced

short palindromic repeat) protein. The CRISPR protein may be Cas9 (e.g. SEQ ID No: 22).The cargo polypeptide may comprise a complex of the genome-modifying protein and a nucleic acid, such as a guide nucleic acid. For instance, Cas9 may be complexed with a nucleic acid (such as a guide RNA), such as crRNA, trRNA, and/or sgRNA.

[0089] The amino acid sequences referred to herein encompass sequence differences compared to the references sequences (such as those set forth in Table 1, below). These may be variants, mutations, insertions, or deletions. In some applications, it may be important to ensure that the primary function of the protein is not substantially altered or abrogated, but this can be readily tested, e.g. using assays described herein. The amino acid sequences described herein may comprise a sequence of 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater identity to the references sequences. The amino acid sequences may encompass conservative amino substitutions. Conservative amino acid substitutions which are known in the art are as follows with conservative substitutable candidate amino acids showing in parentheses: Ala (Gly, Ser); Arg (Gly, Gln); Asn (Gln; His); Asp (Glu); Cys (Ser); Gln (Asn, Lys); Glu (Asp); Gly (Ala, Pro); His (Asn; Gln); Ile (Leu; Val); Leu (Ile; Val); Lys (Arg; Gln); Met (Leu, Ile); Phe (Met, Leu, Tyr); Ser (Thr; Gly); Thr (Ser; Val); Trp (Tyr); Tyr (Trp; Phe); Val (Ile; Leu). Some so-called 'functional' variants, mutations, insertions, or deletions encompass sequences in which the function is substantially the same as that of the reference sequence, e.g. from which it is derived. This can be readily tested using assays similar to those described herein.

[0090] The amino acid sequences referred to herein, in particular the DT sequences may be modified for some applications. It may be desirable, for instance, to reduce the antigenicity of the fusion protein or the DT domains. They may be accomplished in a number of ways. For example, an amino acid sequence could be PEGylated. The amino acid sequence may also be mutated, e.g. to reduce antigenicity, for example by removing B- and/or T-cell epitopes. Humanization is one example mode of sequence modification.

[0091] In one embodiment, the cargo comprises an ubiquitin or a variant thereof. In one embodiment, the cargo comprises ubiquitin.

[0092] As mentioned previously, a 'variant' may encompass sequences that encompasses sequence differences with respect to a reference sequence, e.g. which may have 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater identity to the references sequence. A 'variant' may also encompass amino acid substitutions, such as aforementioned conservative amino

substitutions. Variants may also encompass sequence changes aimed at humanizing and/or reducing antigencitiy.

[0093] In one embodiment, the cargo polypeptide comprises a therapeutic polypeptide. By 'therapeutic peptide' is meant any amino acid sequence that is delivered for a therapeutic purpose, e.g. to treat, prevent, or ameliorate a disease or pathological state.

[0094] In one embodiment, y comprises a ligation site. By 'ligation site' is meant the product of a ligation reaction. This could encompass, e.g., a particular sequence or a chemical structures that is the product of a ligation reaction. In one embodiment, the ligation site is a sortase ligation site.

[0095] In some embodiments, it may be advantageous to reduce the size of the recombinant molecule, i.e. to provide a smaller construct or lower antigenicity.

[0096] The DTA may be a subsequence of dtA or dta in some embodiments. In one embodiment, the DTA is a C-terminal fragment comprising a cysteine corresponding to the cysteine at position 186 of SEQ ID NO: 1. By 'corresponding to' is meant a position at the equivalent or cognate position when, e.g., two sequences are compared or aligned.

[0097] In one embodiment, the C-terminal fragment comprises a polypeptide having a sequence CAGNRVRRSVGSSL (SEQ ID NO: 26). In one embodiment, the C-terminal fragment consists of a polypeptide having a sequence CAGNRVRRSVGSSL (SEQ ID NO: 26). However, in some embodiments, DTA may be a different C-terminal fragment longer than SEQ ID NO: 26 but shorter than SEQ ID NOs: 1 or 2.

[0098] Nucleic Acids, Vectors, and Cells

[0099] In one aspect, there is provided a nucleic acid encoding the above-described recombinant molecule. It will be appreciated that DTA and DTB, being separate polypeptides in the wild type diphtheria toxic linked by a disulphide bridge, may be separately encoded. Accordingly, in the nucleic acid, the DTA and DTB may be separately encoded. Separate nucleic acids encoding each of DTA and DTB may also be provided.

[00100] A skilled person would readily appreciate there are many ways to encode the above-described recombinant molecule (e.g. due to degeneracy of the genetic code), all of which are encompassed. Deletions, insertions, and substitutions may also be permitted if protein function remains substantially intact. For instance, nucleic acids may have 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater identity to wild-type or references sequences may be encompassed. The

above-noted nucleic acids could also be codon optimized depending on the organism or expression system in which it is intended to be expressed.

[00101] In one aspect, there is provided a recombinant cell comprising the above-described nucleic acid.

[00102] In one aspect, there is provided a vector comprising the above-described nucleic acid. Vectors suitable for propagated nucleic acid in bacterial and/or eukaryotic cells are well known in the art.

[00103] In one aspect, there is provided a cell transformed with the above-described vector. Transformation methods for obtaining such cells are well known.

[00104] Pharmaceutical Compositions and Dosage Forms

[00105] In one aspect, there is provided a pharmaceutical composition comprising the above-described recombinant molecule and a pharmaceutically acceptable carrier. In some applications, a recombinant molecule comprising non-toxic, catalytically inactive DTA (dta) may be preferred. For example, a DTA having K51E and E148K mutations may be useful in such applications. A skilled person could generate and test other mutations, e.g. using cellular assays such as those described herein, to determine which have desirable properties in this regard. The DTA may comprise a sequence as set forth in SEQ ID No: 2. The DTA may comprise variants or modification of this sequence, such as those discussed above.

[00106] For some therapeutic applications, it may be desirable to reduce the antigenicity of the fusion protein or the DT domains. They may be accomplished in a number of ways. For example, an amino acid sequence could be PEGylated. The amino acid sequence may also be mutated, e.g. to reduce antigenicity, for example by removing B- and/or T-cell epitopes. Humanization is one example mode of sequence modification.

[00107] Pharmaceutically acceptable carriers include solvents, diluents, liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, or lubricants. Carriers may be selected to prolong dwell time for sustained release appropriate to the selected route of administration. Exemplary carriers include sugars such as glucose and sucrose, starches such as corn starch and potato starch, fibers such as cellulose and its derivatives, sodium carboxymethyl cellulose, ethyl cellulose, cellulose acetate, powdered tragacanth, malt, gelatin, talc, cocoa butter, suppository waxes, oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; glycols such as propylene glycol, esters such as ethyl oleate and ethyl laurate, agar, buffering agents such as magnesium hydroxide and aluminum hydroxide, alginic acid, pyrogen-free water, isotonic

saline, Ringer's solution, ethyl alcohol, phosphate buffer solutions, non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, coloring agents, releasing agents, coating agents, sweeteners, flavors, perfuming agents, preservatives, and antioxidants.

[00108] Compositions can be administered to subjects through any acceptable route, such as topically (as by powders, ointments, or drops), orally, rectally, mucosally, sublingually, parenterally, intracisternally, intravaginally, intraperitoneally, bucally, ocularly, or intranasally.

[00109] Liquid dosage forms for oral administration may include emulsions, microemulsions, solutions, suspensions, syrups and elixirs. Liquid dosage forms may contain inert diluents such as water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils such as cottonseed, groundnut, corn, germ, olive, castor, and sesame oils, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

[00110] Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, or patches. The active agent is admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required.

[00111] Injectable preparations, such as sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. The injectable formulations can be sterilized prior to addition of spores, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which

can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[00112] It is often desirable to slow the absorption of the agent from subcutaneous or intramuscular injection. Delayed absorption of a parenterally administered active agent may be accomplished by dissolving or suspending the agent in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the agent in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of active agent to polymer and the nature of the particular polymer employed, the rate of active agent release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the agent in liposomes or microemulsions which are compatible with body tissues.

[00113] Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the active agent(s) of this invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active agent(s).

[00114] Solid dosage forms for oral, mucosal or sublingual administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active agent is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate, fillers or extenders such as starches, sucrose, glucose, mannitol, and silicic acid, binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, humectants such as glycerol, disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, solution retarding agents such as paraffin, absorption accelerators such as quaternary ammonium compounds, wetting agents such as, for example, cetyl alcohol and glycerol monostearate, absorbents such as kaolin and bentonite clay, and lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof.

[00115] Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the

pharmaceutical formulating art. In such solid dosage forms the active agent(s) may be admixed with at least one inert diluent such as sucrose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, such as tableting lubricants and other tableting aids such a magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents. They may optionally contain opacifying agents and can also be of a composition that they release the active agent(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

[00116] The therapeutically effective amount may be determined on an individual basis or on the basis of the established amount necessary. The dosage for an individual subject is chosen in view of the subject to be treated. Dosage and administration may be adjusted to provide sufficient levels of the active agent(s) or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, contact with infectious agent in the past, potential future contact; age, weight, gender of the subject, diet, time and frequency of administration, drug combinations, reaction sensitivities, and tolerance/response to therapy. Sustained release compositions might be administered less frequently than fast-acting compositions.

[00117] Methods

[00118] In one aspect, there is provided a method of delivering a cargo polypeptide to a cell, comprising contacting the cell with the above-described recombinant molecule.

[00119] In one aspect, there is provided a method of delivery a cargo polypeptide to a cell of a subject, comprising contacting the cell with the above-described recombinant molecule.

[00120] In one aspect, there is provided a method of delivering a cargo polypeptide across the blood brain barrier, comprising administering to a subject the above-described recombinant molecule.

[00121] In one aspect, there is provided a method of increasing enzyme or protein activity in a cell, comprising contacting the cell with the above-described recombinant molecule.

[00122] In one aspect, there is provided a method of alleviating enzyme or protein deficiency in a cell, comprising contacting the above-described recombinant molecule. In one embodiment, the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity. In another embodiment, the cargo polypeptide compensates for the enzyme or protein deficiency.

[00123] By 'compensate', as used herein, is meant that the cargo polypeptide corrects or at least partially ameliorates the protein or enzyme deficiency, an aspect of the deficient protein or enzyme's function, or one or more of its downstream or secondary cellular effects or consequences.

[00124] In one aspect, there is provided a method of treating a disease or disorder caused by enzyme or protein deficiency in a subject, comprising administering to the subject the above-described recombinant molecule. In one embodiment, the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity. In another embodiment, the cargo polypeptide compensates for the enzyme or protein deficiency. The disease or disorder may be Rett syndrome, and the cargo polypeptide may comprise MecP2 (e.g. SEQ ID No: 16 or 17). The disease or disorder may be Spinal Muscular Atrophy syndrome, and the cargo polypeptide may comprise SMN (e.g. SEQ ID No: 19). The disease or disorder may be Fragile X syndrome, and the cargo polypeptide may comprise FMRP (e.g. SEQ ID No: 18). The disease or disorder may be PNP-deficiency, and the cargo polypeptide may comprise PNP (e.g. SEQ ID No: 24). The disease or disorder may be Lafora Disease, and the cargo polypeptide may comprise alpha-amylase.

[00125] In the methods described herein, the cargo polypeptide may have a molecular weight of less than 10 kDa, greater than 10 kDa, greater than 20 kDa, greater than 30 kDa, greater than 50 kDa, greater than 100 kDa, or greater than 150 kDa.

[00126] In one aspect, there is provided a method of manipulating the genome of a cell, comprising contacting the cell with the above-described recombinant molecule, wherein the cargo polypeptide comprises a genome-modifying protein. Genome-modifying proteins for genetic engineering are widely known. The genome-modifying protein may be, for example, a zinc finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), or a CRISPR clustered regularly interspaced short palindromic repeat) protein. For example, the CRISPR protein may be Cas9 (e.g. SEQ ID No: 22). In some embodiments, these nucleic acids, such as guide RNAs, may be separately delivered to cells. In others, a pre-complex of protein and nucleic acid may be formed for delivery into a cell.

[00127] For applications of the above methods involving subjects or therapy, a recombinant molecule comprising non-toxic, catalytically inactive DTA (dta) may be preferred. For example, a DTA having K51E and E148K mutations may be useful in such applications. A skilled person could generate and test other mutations, e.g. using cellular assays such as those described herein, to determine which have desirable properties in

this regard. The DTA may comprise a sequence as set forth in SEQ ID No: 2. The DTA may comprise variants or modification of this sequence, such as those discussed above.

[00128] For some therapeutic applications, it may be desirable to reduce the antigenicity of the fusion protein or the DT domains. They may be accomplished in a number of ways. For example, an amino acid sequence could be PEGylated. The amino acid sequence may also be mutated, e.g. to reduce antigenicity, for example by removing B- and/or T-cell epitopes. Humanization is one example mode of sequence modification.

[00129] Uses

[00130] In one aspect, there is provided a use of the above-described recombinant molecule for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide to a cell.

[00131] In one aspect, there is provided a use of the above-described recombinant molecule, or for preparation of a medicament for delivery, of the cargo polypeptide to a cell of a subject

[00132] In one aspect, there is provided a use of the above-described recombinant molecule for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide across the blood brain barrier.

[00133] In one aspect, there is provided a use of t the above-described recombinant molecule for increasing, or for preparation of a medicament for increasing, enzyme or protein activity in a cell.

[00134] In one aspect, there is provided a use of the above-described recombinant molecule for alleviating, or for preparation of a medicament for alleviating, enzyme or protein deficiency in a cell. In one embodiment, the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity. In another embodiment, the cargo polypeptide compensates for the enzyme or protein deficiency.

[00135] In one aspect, there is provided a use of the above-described recombinant molecule, or for preparation of a medicament for treating, a disease or disorder caused by enzyme or protein deficiency in a subject. In one embodiment, the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity. In another embodiment, the cargo polypeptide compensates for the enzyme or protein deficiency. The disease or disorder may be Rett syndrome, and the cargo polypeptide may comprise MecP2 (e.g. SEQ ID No: 16 or 17). The disease or disorder may be Spinal Muscular Atrophy syndrome, and the cargo polypeptide may comprise SMN (e.g. SEQ ID No: 19). The disease or disorder may be Fragile X

syndrome, and the cargo polypeptide may comprise FMRP (e.g. SEQ ID No: 18). The disease or disorder may be PNP-deficiency, and the cargo polypeptide may comprise PNP (e.g. SEQ ID No: 24). The disease or disorder may be Lafora Disease, and the cargo polypeptide may comprise alpha-amylase (e.g. SEQ ID No: 15).

[00136] In the uses described herein, the cargo polypeptide may have a molecular weight of less than 10 kDa, greater than 10 kDa, greater than 20 kDa, greater than 30 kDa, greater than 50 kDa, greater than 100 kDa, or greater than 150 kDa.

[00137] In one aspect, there is provided a use of the above-described recombinant molecule for manipulating the genome of a cell, wherein the cargo polypeptide comprises a genome-modifying protein. Genome-modifying proteins for genetic engineering are widely known. The genome-modifying protein may be, for example, a zinc finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), or a CRISPR clustered regularly interspaced short palindromic repeat) protein. For example, the CRISPR protein may be Cas9 (e.g. SEQ ID No: 22). In some embodiments, these nucleic acids, such as guide RNAs, may be separately delivered to cells. In others, a pre-complex of protein and nucleic acid may be formed for delivery into a cell.

[00138] For applications of the above uses involving subjects or therapy, a recombinant molecule comprising non-toxic, catalytically inactive DTA (dta) may be preferred. For example, a DTA having K51E and E148K mutations may be useful in such applications. A skilled person could generate and test other mutations, e.g. using cellular assays such as those described herein, to determine which have desirable properties in this regard. The DTA may comprise a sequence as set forth in SEQ ID No: 2. The DTA may comprise variants or modification of this sequence, such as those discussed above.

[00139] For some therapeutic applications, it may be desirable to reduce the antigenicity of the fusion protein or the DT domains. They may be accomplished in a number of ways. For example, an amino acid sequence could be PEGylated. The amino acid sequence may also be mutated, e.g. to reduce antigenicity, for example by removing B- and/or T-cell epitopes. Humanization is one example mode of sequence modification.

[00140] Kits

[00141] In one aspect, there is provided a kit for delivering a cargo polypeptide to a cell comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[00142] In one aspect, there is provided a kit for delivering a cargo polypeptide to a cell of a subject, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[00143] In one aspect, there is provided a kit for delivering a cargo polypeptide across the blood brain barrier, comprising the above-described recombinant molecule, and instructions for administering the recombinant molecule to a subject.

[00144] In one aspect, there is provided a kit for increasing enzyme or protein activity in a cell, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule.

[00145] In one aspect, there is provided a kit for alleviating enzyme or protein deficiency in a cell, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule. In one embodiment, the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity. In another embodiment, the cargo polypeptide compensate for the enzyme or protein deficiency.

[00146] In one aspect, there is provided a kit for treating a disease or disorder caused by enzyme or protein deficiency in a subject, comprising the above-described recombinant molecule, and instructions for administering the recombinant molecule to the subject. In one embodiment, the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity. In another embodiment, the cargo polypeptide compensate for the enzyme or protein deficiency. The disease or disorder may be Rett syndrome, and the cargo polypeptide may comprise MecP2 (e.g. SEQ ID No: 16 or 17). The disease or disorder may be Spinal Muscular Atrophy syndrome, and the cargo polypeptide may comprise SMN (e.g. SEQ ID No: 19). The disease or disorder may be Fragile X syndrome, and the cargo polypeptide may comprise FMRP (e.g. SEQ ID No: 18). The disease or disorder may be PNP-deficiency, and the cargo polypeptide may comprise PNP (e.g. SEQ ID No: 24). The disease or disorder may be Lafora Disease, and the cargo polypeptide may comprise alpha-amylase (e.g. SEQ ID No: 15).

[00147] In the kits described herein, the cargo polypeptide may have a molecular weight of less than 10 kDa, greater than 10 kDa, greater than 20 kDa, greater than 30 kDa, greater than 50 kDa, greater than 100 kDa, or greater than 150 kDa.

[00148] In one aspect, there is provided a kit for manipulating the genome of cell, comprising the above-described recombinant molecule, and instructions for contacting the cell with the recombinant molecule, wherein the cargo polypeptide comprises a genome-modifying protein. Genome-modifying proteins for genetic engineering are widely known. The genome-modifying protein may be, for example, a zinc finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), or a CRISPR

clustered regularly interspaced short palindromic repeat) protein. For example, the CRISPR protein may be Cas9 (e.g. SEQ ID No: 22). In some embodiments, these nucleic acids, such as guide RNAs, may be separately delivered to cells. In others, a pre-complex of protein and nucleic acid may be formed for delivery into a cell.

[00149] For applications of the above kits involving subjects or therapy, a recombinant molecule comprising non-toxic, catalytically inactive DTA (dta) may be preferred. For example, a DTA having K51E and E148K mutations may be useful in such applications. A skilled person could generate and test other mutations, e.g. using cellular assays such as those described herein, to determine which have desirable properties in this regard. The DTA may comprise a sequence as set forth in SEQ ID No: 2. The DTA may comprise variants or modification of this sequence, such as those discussed above.

[00150] For some therapeutic applications, it may be desirable to reduce the antigenicity of the fusion protein or the DT domains. They may be accomplished in a number of ways. For example, an amino acid sequence could be PEGylated. The amino acid sequence may also be mutated, e.g. to reduce antigenicity, for example by removing B- and/or T-cell epitopes. Humanization is one example mode of sequence modification.

[00151] **EXAMPLE 1**

[00152] **Generation of Cargo-DT Chimera**

[00153] DT plasmid carrying the E148S mutation was a gift Dr. R. John Collier (Harvard Medical School, Boston, MA). Point mutations were made in the DT E148S plasmid using QuikChange™ lightning multi-mutagenesis kit (Agilent Technologies) to prepare wt-DT (E148), catalytically inactive DT (K51E/E148K), and the pore-formation defective DT (L350K). Cargo proteins were fused to different DT variants using the In-Fusion™ HD Cloning Kits (Clontech).

[00154] As referred to herein, dtA refers to the wildtype DTA sequence, whereas dta refers to the DTA sequence containing the inactivating mutations K51E and E148K.

[00155] Various fluorescent fusion proteins were created as DT fusion proteins. Both enhanced green fluorescent protein (eGFP) and monomeric cherry (mCherry) proteins were used in various constructs. Both eGFP-dtA-dtB and mCherry-dtA-dtB were created. EGFP and dtA were linked via a GSG linker, while mCherry and dtA were linked via a (G4S)2 linker. Further, both eGFP and mCherry were created as dtA-eGFP-dta-dtB and dtA-mCherry-dta-dtB fusion proteins, where dta contains the inactivating mutations K51E and E148K. In both cases, the dtA and cargo are linked via a GSG linker, and cargo and dta are also linked via a GSG linker. Both eGFP and mCherry contain the

mutation V1G to enhance cleavage by the SUMO protease during purification in all constructs except dtA-mCherry-dta-dtB, where first residue is the native valine.

[00156] The alpha-amylase enzyme from *Bacillus megaterium* was linked to dtA via a GSG linker. A mutation was made in the alpha-amylase sequence (V1G) to enhance cleavage by the SUMO protease for purification purposes. Another construct, dtA-Amylase-dta-dtB was also made. In this case, dtA is linked to amylase via a GSG linker, and amylase is linked to dta via a GSG linker.

[00157] **Table 1** lists sequences of domains, linkers, and cargo.

Table 1

Diphtheria Toxin Sequences (Full-length DT = dtA-dtB; dtB = dtT + dtR)		
	SEQ ID	
dtA Domain	1	GADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWKGFYS TDNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSL TEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQAKALSVELEI NFETRGKRGQDAMYEYMAQACAGNRVRRSVGSSL
dta Domain (K51E, E148K)	2	GADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWEGFYS TDNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSL TEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVKYINNWEQAKALSVELEI NFETRGKRGQDAMYEYMAQACAGNRVRRSVGSSL
dtB Domain	3	SCINIDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTA LEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILPG IGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESI INLFQVVHNSYNRPAYSPGHKTQPFLHDGYAVSWNTVEDSIIRTGFQGESGHDIK ITAENTPLPIAGVLLPTIPGKLDVNKSKTHISVNGRKIRMRCRAIDGDVTFCRPK SPVYVGNGVHANLHVAFHRSSSEKIHSNEISSDSIGVLGYQKTVDHTKVNSKLSL FFEIKSRQA
dtT (dtB Translocation Domain)	4	SCINIDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTA LEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILPG IGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESI INLFQVVHNSYNRP
Translocation-deficient dtT (L350K)	5	SCINIDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTA LEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILPG IGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGEKVDIGFAAYNFVESI INLFQVVHNSYNRP
dtR (dtB Receptor-binding Domain)	6	AYSPGHKTQPFLHDGYAVSWNTVEDSIIRTGFQGESGHDIKITAENTPLPIAGVL LPTIPGKLDVNKSKTHISVNGRKIRMRCRAIDGDVTFCRPKSPVYVGNGVHANLH VAFHRSSSEKIHSNEISSDSIGVLGYQKTVDHTKVNSKLSLFFEIKSRQA
Δdta	26	CAGNRVRRSVGSSL
Tag and Linker Sequences		
	SEQ ID	
Polyhistidine-SUMO	7	MGSSHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSE IFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDII EAHREQIGG
MYC	8	EQKLISEEDL
SV40 NLS	9	SPPKKKRKV
(G4S) linker	10	GGGGS
$(G4S)_2$ linker	11	GGGGSGGGGS
$(G4S)_3$ linker	12	GGGGSGGGGSGGGGS
GSG Linker	n/a	GSG
Strep Tag™ II	27	LVPRGSAWSHPQFEK

Cargo Sequences

	SEQ ID	
Enhanced Green Fluorescent Protein (eGFP)	13	GSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPV PWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRA EVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNF KIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVL LEFVTAAGITLGMDELYK
Monomeric Cherry (mCherry)	14	GSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKG GPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVT VTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEI KQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYER AEGRHSTGGMDELYK
α-amylase (*B. megaterium*)	15	GHKGKSPTADKNGVFYEVYVNSFYDANKDGHGDLKGLIQKLDYLNDGNSHTKNDL QVNGIWMMPVNPSPSYHKYDVTDYYNIDPQYGNLQDFRKLVKEADKRDVKVIMDL VVNHTSSEHPWFQAALKDKNSKYRDYYIWADKNTDLNEKGSWGQQVWHKAPNGEY FYGTFWEGMPDLNYDNPEVRKEMINVGKFWLNQGVDGFRLDAALHIFKGQTPEGA KKNILWWNEFRDAMKKENPNVYLTGEVWDQPEVVAPYYQSLDSLFNFDLAGKIVS SVKAGNDQGIATAAAATDELFKSYNPNKIDGIFLTNHDQNRVMSELSGDVNKAKS AASILLTLPGNPYIYYGEEIGMTGEKPDELIREPFRWYEGNGLGQTSWETPIYNK GGNGVSIEAQTKQKDSLLNHYREMIRVRQQHEELVKGLQSISLDQKEVVAYSRT YKGKSISVYHNISNQPIKVSVAAKGKLIFSSEKGVKKVKNQLVIPANITILIK
MeCP2 (e1 isoform)	16	AAAAAAAPSGGGGGGEEERLEEKSEDQDLQGLKDKPLKFKKVKKDKKEEKEGKHE PVQPSAHHSAEPAEAGKAETSEGSGSAPAVPEASASPKQRRSIRDRGPMYDDPT LPEGWTRKLKQRKSGRSAGKYDVYLINPQGKAFRSKVELIAYFEKVGDTSLDPND FDFTVTGRGSPSRREQKPPKKPKSPKAPGTGRGRGRPKGSGTIRPKAATSEGVQV KRVLEKSPGKLLVKMPFQTSPGGKAEGGGATTSTQVMVIKRPGRKRKAEADPQAI PKKRGRKPGSVVAAAAAEAKKKAVKESSIRSVQETVLPIKKRKTRETVSIEVKEV VKPLLVSTLGEKSGKGLKTCKSPGRKSKESSPKGRSSSASSPPKKEHHHHHHHSE SPKAPVPLLPPLPPPPPEPESSEDPTSPPEPQDLSSSVCKEEKMPRGGSLESDGC PKEPAKTQPAVATAATAAEKYKHRGEGERKDIVSSSMPRPNREEPVDSRIPVTER VS
MeCP2 (e2 isoform)	17	VAGMLGLREEKSEDQDLQGLKDKPLKFKKVKKDKKEEKEGKHEPVQPSAHHSAEP AEAGKAETSEGSGSAPAVPEASASPKQRRSIRDRGPMYDDPTLPEGWTRKLKQR KSGRSAGKYDVYLINPQGKAFRSKVELIAYFEKVGDTSLDPNDFDFTVTGRGSPS RREQKPPKKPKSPKAPGTGRGRGRPKGSGTTRPKAATSEGVQVKRVLEKSPGKLL VKMPFQTSPGGKAEGGGATTSTQVMVIKRPGRKRKAEADPQAIPKKRGRKPGSVV AAAAAEAKKKAVKESSIRSVQETVLPIKKRKTRETVSIEVKEVVKPLLVSTLGEK SGKGLKTCKSPGRKSKESSPKGRSSSASSPPKKEHHHHHHHSESPKAPVPLLPPL PPPPPEPESSEDPTSPPEPQDLSSSVCKEEKMPRGGSLESDGCPKEPAKTQPAVA TAATAAEKYKHRGEGERKDIVSSSMPRPNREEPVDSRIPVTERVS
FMRP	18	EELVVEVRGSNGAFYKAFVKDVHEDSITVAFENNWQPDRQIPFHDVRFPPPVGYN KDINESDEVEVYSRANEKEPCCWWLAKVRMIKGEFYVIEYAACDATYNEIVTIER LRSVNPNKPATKDTFHKIKLDVPEDLRQMCAKEAAHKDFKKAVGAFSVTYDPENY QLVILSINEVTSKRAHMLIDMHFRSLRTKLSLIYRNEEASKQLESSRQLASRFHE QFIVREDLMGLAIGTHGANIQQARKVPGVTAIDLDEDTCTFHIYGEDQDAVKKAR SFLEFAEDVIQVPRNLVGKVIGKNGKLIQEIVDKSGVVRVRIEAENEKNVPQEEE IMPPNSLPSNNSRVGPNAPEEKKHLDIKENSTHFSQPNSTKVQRGMVPFVFVGTK DSIANATVLLDYHLNYLKEVDQLRLERLQIDEQLRQIGASSRPPPNRTDKEKSYV TDDGQGMGRGSRPYRNRGHGRRGPGYTSAPTEEERESFLRRGDGRRRGGGGRGQG GRGRGGGFKGNDDHSRTDNRPRNPREAKGRTTDGSLQIRVDCNNERSVHIKILQN TSSEGSRLRTGKDRNQKKEKPDSVDGQQPLVNGVP
SMN	19	MAMSSGGSGGGVPEQEDSVLFRRGTGQSDDSDIWDDTALIKAYDKAVASFKHALK NGDICETSGKPKTTPKRKPAKKNKSQKKNTAASLQQWKVGDKCSAIWSEDGCIYP ATIASIDFKRETCVVVYTGYGNREEQNLSDLLSPICEVANNIEQNAQENENESQV STDESENSRSPGNKSDNIKPKSAPWNSFLPPPPPMPGPRLGPGKPGLKFNGPPPP PPPPPPHLLSCWLPPFPSGPPIIPPPPPICPDSLDDADALGSMLISWYMSGYHTG YYMGFRQNQKEGRCSHSLN
CPD (*C. difficile*)	20	EGSLGEDDNLDFSQNIVVDKEYLLEKISSLARSSERGYIHYIVQLQGDKISYEAA

		CNLFAKTPYDSVLFQKNIEDSEIAYYYNPGDGEIQEIDKYKIPSIISDRPKIKLI FIGHGKDEFNTDIFAGFDVDSLSTEIEAAIDLAKEDISPKSIEINLLGCNMFSYS INVEETYPGKLLKVKDKISELMPSISQDSIIVSANQYEVRINSEGRRELLDHSG EWINKEESIIKDISSKEYISFNPKENKITVKSKNLPELSTL
CPD (*V. cholera*)	21	KEALADGKILHNQNVNSWGPITVTPTTDGGETRFDGQIIVQMENDPVVAKAAANL AGKHAESSVVVQLDSDGNYRVVYGDPSKLDGKLRWQLVGHGRDHSETNNTRLSGY SADELAVKLAKFQQSFNQAENINNKPDHISIVGCSLVSDDKQKGFGHQFIKAMDA NGLRVDVSVRSSELAVDEAGRKHTKDANGDWVQKAENNKVSLSWDAQ
Cas9 (*S. pyogenes*)	22	MDKKYSIGLDIGTNSVGWAVITDDYKVPSKKFKVLGNTDRHSIKKNLIGALLFGS GETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEED KKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLADSTDKADLRLIYLALAHMIKFR GHFLIEGDLNPDNSDVDKLFIQLVQIYNQLFEENPINASRVDAKAILSARLSKSR RLENLIAQLPGEKRNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDL DNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNSEITKAPLSASMIKRYDEHHQ DLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDG TEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKI EKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERM TNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVD LLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGAYHDLLKIIKDKD FLDNEENEDILEDIVLTLTLFEDRGMIEERLKTYAHLFDDKVMKQLKRRRYTGWG RLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSG QGHSLHEQIANLAGSPAIKKGILQTVKIVDELVKVMGHKPENIVIEMARENQTTQ KGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQ ELDINRLSDYDVDHIVPQSFIKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKN YWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILD SRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNA VVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFF KTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQ TGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKK LKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRK RMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYL DEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDSPVR
Cas9 (*S. pyogenes*) with N-terminal His, SV40 and C-terminal SV40 sequences	23	MHHHHHHGSGATMASPPKKKRKVGSMDKKYSIGLDIGTNSVGWAVITDDYKVPSKK FKVLGNTDRHSIKKNLIGALLFGSGETAEATRLKRTARRRYTRRKNRICYLQEIF SNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKK LADSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQIYNQLF EENPINASRVDAKAILSARLSKSRRLENLIAQLPGEKRNGLFGNLIALSLGLTPN FKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDIL RVNSEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAG YIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHL GELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEE TITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTK VKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEIS GVEDRFNASLGAYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDRGMIEERL KTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANR NFMQLIHDDSLTFKEDIQKAQVSGQGHSLHEQIANLAGSPAIKKGILQTVKIVDE LVKVMGHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVE NTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFIKDDSIDNKVL TRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELD KAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFR KDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMI AKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKG RDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYG GFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGY KEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHY EKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKH RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSI

		TGLYETRIDLSQLGGDSPVRSPKKKRKV
PNP	24	MENGYTYEDYKNTAEWLLSHTKHRPQVAIICGSGLGGLTDKLTQAQIFDYSEIPN FPRSTVPGHAGRLVFGFLNGRACVMMQGRFHMYEGYPLWKVTFPVRVFHLLGVDT LVVTNAAGGLNPKFEVGDIMLIRDHINLPGFSGQNPLRGPNDERFGDRFPAMSDA YDRTMRQRALSTWKQMGEQRELQEGTYVMVAGPSFETVAECRVLQKLGADAVGMS TVPEVIVARHCGLRVFGFSLITNKVIMDYESLEKANHEEVLAAGKQAAQKLEQFV SILMASIPLPDKAS
SUMO	25	MSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKR QGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG
GTD	28	MSLVNRKQLEKMANVRFRTQEDEYVAILDALEEYHNMSENIVVEKYLKLKDINSL TDIYIDTYKKSGRNKALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDT AINYINQWKDVNSDYNVNVFYDSNAFLINTLKKIVVESAINDILESFRENLNDPR FDYNKFFRKRMEIIYDKQKNFINYYKAQREENPELIIDDIVKIYLSNEYSKEIDE LNTYIEESLNKITQNSGNDVRNFEEFKNGESFNLYEQELVERWNLAAASDILRIS ALKEIGGMYLDVDMLPGIQPDIFESIEKPSSVTVDFWEMTKLEAIMKYKEYIPEV TSEHFDMLDEEVQSSFESVLASKSDKSEIFSSLGDMEASPLEVKIAFNSKGIINQ GLISVKDSYCSNLIVKQIENRYKILNNSLNPAISEDNDFNITINIFIDSIMAEAN ADNGRFMMELGKYLRVGFFPDVKTTINLSGPEAYAAAYQDLLMFKEGSMNIHLIE ADLRNFEISKTNISQSTEQEMASLWSFDDARAKAQFEEYKRNYFEGSL

[00158] **Table 2** contains a non-exhaustive list of constructs generated and tested in ensuing Examples.

Table 2

Cargo family	Delivered cargo	Cargo MW (kDa)
DT-based	(wildtype) = dtA	21
	(K51E/E148K) = dta	22
	(L350K) = dtb	21
	dtA	21
Sumo-based	Sumo-dtA	35
eGFP-based	eGFP-dtA	49
	eGFP-(G4S)1-dtA	49
	eGFP-(G4S)2-dtA	49
	eGFP-(G4S)3-dtA	49
	dtA-eGFP-dta	71
	dta-eGFP-dtA	71
	Sumo-eGFP-dtA	62
	Sumo-dtA-eGFP-dta	84
	dtA-eGFP-dtA-dtb	71
mCherry-based	mCherry-dtA	48
	dtA-mCherry-dta	70
	Sumo-dtA-mCherry-dta	83
Ubiquitin-based	Sumo-Ub-dtA	43
	Sumo-Ub-eGFP-dtA	70
	Sumo-Ub-dtA-eGFP-dta	92
α-amylase-based	α-amylase-dtA	78
	dtA-α-amylase-dta	100
	Sumo-dtA-α-amylase-dta	113
	α-amylase-dta	78
	α-amylase	57
TAT-based	TAT-dta	21
	dta-TAT	21

[00159] **EXAMPLE 2**

[00160] **Expression and Purification of Recombinant Diphtheria Toxin (DT)**

[00161] Recombinant DT and cargo-DT chimeras were expressed as N-terminal His-tagged proteins in *E. coli* BL21(DE3) cells, induced with 1 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG) for 4 hours at 37°C (DT) or 21°C (cargo-DT), using the Champion™ pet-SUMO expression system (Invitrogen). Cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 20 mM

imidazole, benzonase, lysozyme and Protease inhibitor cocktail) and lysed by an EmulsiFlex C3 microfluidizer (Avestin) at 15,000 psi. The lysates were centrifuged at 18,000 × g for 20 minutes. His-Sumo-tagged proteins were purified by Ni-affinity chromatography using a His-Trap FF column (GE-Healthcare). After purification, the His-Sumo tag was removed by adding 1U of Sumo protease (Life Sensor) to 90 µg of purified protein in 20 mM Tris-HCl pH 8.0 containing 150 mM NaCl and 2 mM DTT. The cleavage reaction mixture was incubated at 30°C for 1 hour followed by purification using His-Pure™ Ni-NTA resin (Thermo Scientific) to remove the His-Sumo protease and His-Sumo tag from the purified DT and cargo-DT samples.

[00162] **EXAMPLE 3**

[00163] **Cellular DTA Intoxication Assay**

[00164] Protein synthesis inhibition was used to measure the ability of DT and cargo-DT chimera to deliver DTA to the cytosol. VERO cells (6,000 cells per well in a 96 well plate) were exposed to 3-fold serial dilutions of DT or cargo-DT. The cells were incubated with the toxin overnight (17 hours) at 37°C. The next day, toxin-containing medium was removed, and the cells were incubated for 2 hours at 37°C in leucine-deficient medium supplemented with 1 µCi of [^{3}H]leucine/ml (PerkinElmer). The cells were washed twice with cold phosphate-buffered saline (PBS) before precipitation of cellular protein with 10% trichloroacetic acid (TCA). Protein synthesis was measured by the amount of [^{3}H]leucine incorporated into total cellular protein, as determined by scintillation counting with a TopCount NXT™ (Perkin Elmer). Percent protein synthesis was plotted versus the log concentration of DT or cargo-DT. Protein synthesis kinetic experiments were performed as described above except that the toxin concentration was fixed at 1 nM and the toxin was exposed to cells for 1, 2, 3, 4, 5 or 17 hrs. Protein synthesis competition experiments were performed as described above but a fixed concentration (1 nM) of a non-toxic variant of DT (a-B or DT_K51E/E148K) was also added to cells to compete with the toxic DT variant (A-EGFP-a-B) which was added to cells using a 3-fold serial dilution pattern (starting concentration 1 nM).

[00165] **EXAMPLE 4**

[00166] **Real-time protein unfolding using Differential Scanning Fluorimetry**

[00167] Purified eGFP (40 ng) and mCherry (7 µg) (20 mM Tris-HCl, 150 mM NaCl, pH8.0) were diluted in citrate-phosphate buffer at pH's that ranged from 3.6 to 7.6 in a 96-well PCR plate. The proteins at various pH's were placed in a Real-Time PCR Detection System (BioRad CFX96™) to measure protein unfolding. Intrinsic fluorescence of eGFP (494 – 519 nm) and mCherry (595 – 613 nm) were captured over a wide range

of temperature (15 to 95°C). The melting temperatures of eGFP and mCherry were calculated by the software provided with the detection system (Bio-Rad CFX Manager™ 3.1).

[00168] **EXAMPLE 5**

[00169] **Amylase delivery of intracellular glycogen**

[00170] 24h prior to treatment HEK293 cells were seeded on 6-well plates (BD amine coated) at 0.5 x 10^6 (for 24 h incubation) or 0.25 x 10^6 (for 48 h incubation) cells per well and cultivated at 37 °C and 5% CO_2 in DMEM (10% FBS, 1% Penicillin/Streptomycin). For treatment the medium was changed, the new medium containing additionally 0.1 or 1.0 uM of DT, DT-amylase, or amylase protein. After incubation as indicated for harvesting cells were put on ice, washed once with ice-cold PBS, and in a cold room (4 °C) transferred to 1.5 mL vials subsequent to scraping and re-suspending them in 1 mL ice-cold PBS. The cells, being henceforth kept on ice or at 4 °C, were pelleted, the supernatant being removed. After washing the cells again with 1 mL PBS the cell sample was split for separate glycogen and protein determination.

[00171] For protein determination cells were lysed on ice in RIPA buffer (150 mM NaCl, 20 mM Tris, 12.1 mM deoxycholate, 1% triton X-100, 0.1% SDS). Following centrifugation (14,000 x g, 4 °C, 15 min) the supernatant was subjected to protein determination using the DC™ protein assay (Bio-Rad) following the manufacturer's instructions.

[00172] For glycogen determination cells were incubated for 45 min in 0.5 M KOH at 98 °C with intermittent mixing to lyse cells and extract glycogen. Following neutralization with 2 M acetic acid glycogen was digested in an aliquot overnight at 55 °C with 0.5 U amyloglucosidase (Sigma) and subsequently determined as free glucose according to Lowry and Passonneau (1972) with an enzymatic assay that detects NADPH by incubating the sample with hexokinase (Roche), glucose 6-phosphate dehydrogenase (Roche), ATP (Sigma), and NADP (Roche). Glucose in undigested extracts was consistently below the limit of detection.

[00173] Glycogen was based on protein levels to account for cell loss or growth variances due to treatment. Protein-based glycogen levels were normalized to controls treated with identical amounts of either DT or amylase protein (as indicated). Significance was tested using a T-test (two-tailed, homoscedastic since variances between sample populations were not significantly different). Significance levels: $0.05>p\geq0.01$ (*), $0.01>p\geq0.001$ (**), $p<0.001$ (***).

[00174] **EXAMPLE 6**

[00175] **Amino-terminal protein fusions dramatically decrease the apparent cytotoxicity of DT**

[00176] To evaluate the ability of the diphtheria toxin translocation apparatus to co-deliver proteins into mammalian cells, a series of model passenger proteins were cloned, in accordance with Example 1, as amino terminal fusions to DT with an intervening Gly-Ser-Gly linker.

[00177] **Figure 1** depicts these constructs. Initially, three distinct passenger proteins were chosen, spanning a range of sizes, structures and physical properties with which to evaluate intracellular delivery: the 13-kDa globular Small Ubiquitin-like Modifier (SUMO; PDB: 3pge) protein; the 27-kDa enhanced green fluorescent protein (eGFP; PDB: 1gfl); and the 57-kDa α-amylase enzyme from *B.megaterium* (in Figure 1, the structure of alpha-amylase of *H. orenni* – PDB: 1wza – Is shown an *example* structure from the alpha-amylase family). The proteins were fused to DT via a GSG linker. These constructs were expressed and purified in accordance with Example 2. To quantify delivery of the chimeric constructs to the cytosol, the intracellular action of the co-delivered A-chain of DT (dtA), which catalyzes the ADP-ribosylation of EF-2 and inhibits protein synthesis (*i.e.*, incorporation of ^{3}H-Leu in the cellular proteome), was measured over a 2h period in VERO cells that had been treated overnight with the chimeric toxins, in general accordance with Example 3.

[00178] **Figure 2** depicts a schematic of first generation chimeric fusions of different passenger proteins to the amino terminus of native diphtheria toxin (DT) via a flexible GSG linker. The enzymatic A domain (dtA) and translocation/ receptor-binding B domain (dtB) have an intervening furin-like recognition site (black triangle) and are further joined by an intra-molecular disulfide bond. DT is internalized into endocytic vesicles by a receptor-mediated process. Within endosomes, a membrane-bound furin-like protease cleaves between dtA and dtB. Upon vesicular acidification, dtB undergoes a major conformational change, resulting in the formation of a membrane-spanning pore. dtA (and any associated passenger proteins) would then translocate into the cytosol starting with dtA, followed by any amino-terminal passenger proteins. Once in the cytosol, the dtA fragment catalyzes ADP-ribosylation of EF-2, resulting in the inhibition of protein synthesis. This straightforward measure of delivery is well established and provides a universal readout of delivery across different studies and different passenger proteins.

[00179] **Figure 3** depicts dose titration curves of chimeric constructs on cells with wt-DT, Sumo-DT, Amylase-DT, and eGFP-DT (EC_{50} values are at the right), and shows

that, in the absence of passenger proteins, (*i.e.*, wildtype DT), protein synthesis was dose-dependently inhibited with an EC_{50} = 1.3 ± 0.7 pM. Figure 3 further shows that, when Sumo, eGFP and α-amylase fusions were tested for intracellular delivery, protein synthesis was dose-dependently inhibited in all cases indicating that passenger proteins were delivered into the cytosol. Comparing the doses at which protein synthesis was inhibited by 50% (EC_{50}) for each chimera however, revealed significant shifts in their relative abilities to inhibit protein synthesis: 65-fold for Sumo; 260-fold for α-amylase; and 1200-fold for eGFP. These shifts, which are consistent with what has been observed previously with smaller cargo[4,6,7,9], suggest that passenger proteins disrupt the natural process of cellular intoxication somehow. Two fundamentally linked questions remain: at what exact step do passenger proteins disrupt intoxication; and, do these observed shifts directly correspond to reduced efficiency of intracellular delivery by DT.

[00180] It was hypothesized that the observed decreases in apparent potency might be due to the cargo differentially affecting the intracellular enzymatic activity of dtA after the chimeras had already entered the cytosol, rather than due to affecting upstream phenomena such as receptor binding or translocation *per se*. Support for this hypothesis came from a set of experiments that had been designed to investigate the effect of increasing the linker size between Cargo and dtA on expression and stability.

[00181] **Figure 4** shows the effect of linker size between eGFP and dtA on cells, with error bars, SD (*n*=2). The consequent effects on the potency of inhibition of protein synthesis for each construct are shown on the right. With eGFP as the passenger protein, increasing the linker size GSG to GGGGS (*i.e.*, G_4S) to $(G_4S)_2$ to $(G_4S)_3$, resulted in increases in potency on cells, consistent with the idea that the passenger protein was affecting a step other than translocation.

[00182] **EXAMPLE 7**

[00183] **Passenger proteins are 'invisible' to the translocation machinery of DT**

[00184] To explore the hypothesis that the passenger cargo was indirectly impacting dtA by proximity effects in a more direct way, a new construct was generated per Example 1 in which the active dtA reporter was placed upstream of eGFP (with a free amino terminus as it is in the WT toxin). The existing dtA attached to dtB was rendered catalytically inactive by the double mutation, $K_{51}E/E_{148}K$[10], signified as dta, to yield the final construct: dtA-eGFP-dta-dtB; or for simplification: A-eGFP-a-B. Cellular assays were carried out per Example 3 to study positional effects of passenger proteins on dtA activity in cells.

[00185] **Figure 5** depicts the construct and shows, remarkably, that A-eGFP-a-B inhibited protein synthesis such that it was indistinguishable from wildtype-like toxin. This shows that the shifts in potency are due to proximity effects on dtA activity. Further, Figure 5 shows that addition of Sumo onto the amino terminus of this construct shifted the apparent activity back to levels observed with amino-terminal cargo constructs. Bars represent average EC_{50} ± SD (n = 3).

[00186] **Figure 6** corroborates certain findings depicted in Figure 5. To rule out the possibility that the amino terminal dtA fragment was affecting translocation, it is shown that a-eGFP-A is shifted similar to eGFP-A. Bars represent average EC_{50} ± SD (n = 3).

[00187] To show that this phenomenon was not specific to eGFP, a similar set of constructs were generated, using α-amylase as the passenger domain.

[00188] **Figure 7** shows that the same positional dependence of dtA on activity was observed when using amylase as the passenger protein. Bars represent average EC_{50} ± SD (n = 3).

[00189] The 'wildtype-like' potencies observed for A-cargo-a-B constructs have several important implications for DT delivery. In addition to strongly supporting the hypothesis that amino-terminal passenger proteins affect dtA activity after they reach the cytosol, rather than impeding receptor binding or translocation, these data indicate that passenger proteins are virtually invisible to the translocation machinery of DT. Also, because translocation initiates with the C-terminal end of the A-domain that is adjacent to the B-moiety and proceeds such that the amino terminus is last to enter the cytosol[11], these findings show unequivocally that passenger proteins fully enter the cytosol. Finally, these constructs eliminate any possibility that the inhibition of protein synthesis observed for chimeric toxins is from breakdown products in which cargo was removed prior to or during intoxication, since amino terminal truncations would result in the loss of dtA and would be nontoxic.

[00190] Building on these findings, the predictable shifts observed with amino terminal fusions to dtA were exploited, and the unique properties of ubiquitin and deubiquitinating enzymes found only in the cytosol, to demonstrate intracellular delivery through an independent measure. Since cytosolic deubiquitinating enzymes cleave at the C-terminus of Ub, Ub was inserted between passenger proteins and dtA in two different contexts so that the amino terminus of dtA will be liberated only if the entire payload was translocated into the cytosol.

[00191] **Figure 8** depicts these constructions, and shows that both Ub-containing constructs were more potent on cells than their des-Ub counterparts, albeit not back to

wildtype levels, which may reflect the kinetics of removal of Ub by DUBs. Ub was placed between Sumo and dtA (left panel of Figure 8) and was found to be more potent on cells than Sumo-A, consistent with deubiquitinating enzymes removing amino terminal cargo and relieving the proximity effect on dtA activity. Using more extensive cargo, the ubiquitin entry assay confirms that large protein cargo enter the cytosol (right panel of Figure 8).

[00192] **Figure 9** depicts a time course of inhibition of protein synthesis of all three constructs using 1nM of each toxin. Symbols ± SD (*n* = 3) are shown.

[00193] In addition to demonstrating that passenger proteins are in the cytosolic compartment, these data show that DT can simultaneously deliver multiple different proteins, akin to beads on a string – that combined, are over 100-kDa in size – into the cytosol *en masse*.

[00194] **Example 8**

[00195] **The DT translocation machinery can deliver a folded protein into cells**

[00196] The unexpected plasticity of the DT translocation machinery observed with large and diverse protein cargo prompted the question of whether DT could transport stably folded proteins into cells. To this end, the fluorescent protein variant derived from *Discosoma* sp. "DsRed" called monomeric Cherry (mCherry) was used. Though similar in size and structure to eGFP, mCherry been shown to possess dramatically increased conformational stability relative to eGFP *in vitro* and *in vivo*. Constructs were generated per Example 1.

[00197] **Figure 10** shows that, using differential scanning fluorimetry, mCherry was indeed dramatically more stable to thermal- and pH-induced unfolding than eGFP. Shaded region shows pH levels within early to late endosomes where translocation takes place. Above pH 4.6, mCherry does not unfold up to 95°C. Symbols represent average T_m ± SD (*n* = 3).

[00198] **Figure 11** shows that mCherry was also more stable than dtA.

[00199] In fact, an unfolding transition for mCherry could only begin to be measured below pH 4.6, strongly suggesting that unfolding of mCherry is not likely to occur within endosomal compartments, where membrane translocation occurs. Zornetta et al.[22] recently investigated anthrax toxin translocation in cells and found that whereas eGFP fusions to lethal factor (LF) were efficiently transported through the narrow and fixed protective antigen (PA) pore, similar fusions with mCherry were unable to translocate into cells, supporting the notion that eGFP, but not mCherry unfolds in early endosomes prior to translocation.

[00200] To test whether the DT translocation apparatus could deliver stably folded mCherry into cells, mCherry-DT chimeras were generated using the same platform designs as above, in Example 1.

[00201] **Figure 12** depicts the results of cell toxicity assays indicating that mCherry is efficiently delivered into cells by DT. Surprisingly, unlike the anthrax toxin translocation system, diphtheria toxin was able to deliver mCherry into cells with wildtype like efficiency. mCherry, like eGFP and amylase are invisible to the DT translocation machinery. Though the possibility cannot be excluded that mCherry is somehow mechanically unfolded immediately prior to, or during translocation, these data indicate, at the very least that DT can accommodate and transport hyper-stable proteins. Furthermore these findings show that DT is distinct from and has a broader substrate profile than the anthrax toxin pore suggesting that not all toxin translocation systems are alike.

[00202] **EXAMPLE 9**

[00203] **Comparison and characterization of the DT delivery platform**

[00204] With the observed differentiation from anthrax toxin, further benchmarking of DT against a non-toxin derived protein delivery platform was sought. To this end, the ability of the cell-penetrating TAT peptide from HIV-1[1] to deliver dtA into the cytosol was evaluated. Given the effects observed here on dtA activity in the presence of amino-terminal extensions, both TAT-dtA and dtA-TAT were generated and compared their ability to inhibit protein synthesis with the translocation machinery of DT (*i.e.*, dtB).

[00205] **Figure 13** shows results comparing the delivery of dtA by dtB and TAT peptides, and shows that both Tat-dtA and dtA-Tat were able to penetrate cells and inhibit protein synthesis. Symbols ± SD (n = 3) are shown. However, as reported previously for TAT-dtA, the concentrations required for both TAT constructs were at least four orders-of-magnitude higher than those required for DT. Beyond this clear efficiency advantage for DT, an important conceptual advantage of the DT system over existing protein delivery platforms such as TAT, is the target-cell specificity conferred by a receptor-binding domain.

[00206] To confirm that cargo translocation by DT was receptor-dependent, a competition experiment between A-eGFP-a-B and catalytically inactive DT was performed.

[00207] **Figure 14** shows that, in the presence of 1 nM nontoxic DT, the potency of A-eGFP-a-B was shifted from 3.8 pM to 215 pM, confirming that cargo delivery was

receptor-dependent, and that the cargo itself did not mediate its own uptake. Symbols ± SD (*n* = 3) are shown.

[00208] **Figure 15** and **Figure 16** show that, using a pore-formation mutant in the translocation domain of DT, which prevents translocation, it is shown that cargo delivery into cells requires a functional translocation domain, and that cargo did not mediate its own entry. Symbols ± SD (*n* = 3) are shown.

[00209] **EXAMPLE 10**

[00210] **Direct evidence of functional entry of α-amylase by DT**

[00211] Having demonstrated that passenger proteins are delivered into cells by DT in a receptor- and translocation-dependent manner with high efficiency, it was next desirable to test whether the delivered cargo was folded and functional within the cytosol. Rather than use the more qualitative measurements of intracellular fluorescence using eGFP or mCherry as cargo, it was desirable to measure the ability of delivered α-amylase to enzymatically digest cytosolic glycogen. An amino-terminal extension of nontoxic DT (*i.e.*, α-amylase-dta-dtB) was prepared in general accordance with Example 1.

[00212] **Figure 17** confirms that the specific activity of α-amylase was equivalent to α-amylase alone using a quenched fluorescence substrate-based assay. The EnzChek™ Ultra Amylase Assay was used to measure the activity of α-amylase-dtA (curve marked with square data points), α-amylase (curve marked with triangular data points), and dtA alone (curve marked with circular data points) over 2 hours.

[00213] **Figure 18** depicts the experimental design for α-amylase-DT treatment of HEK 293 cells. HEK293 cells were treated for 24 or 48 h with α-amylase-dta-dtB at two different concentrations to establish conditions where decreases in protein-based glycogen could be detected.

[00214] **Figure 19** shows protein-based glycogen content in HEK cells after 24 h or 48 h treatment normalized on content in cells treated with either DT alone or amylase alone, respectively (n=1). Using DT alone or α-amylase as controls, dose-dependent decreases in protein-based glycogen content in cells were observed at both time points, with a slightly more pronounced effect apparent at 24 h.

[00215] **Figure 20** shows protein-based glycogen content in HEK cells after 24 h treatment with 1.0 uM DT, amylase-DT, or amylase alone. Error bars, SD (n=4; Significance as determined with STUDENT t-test ($p<0.01$, **; $p<0.001$, ***). A highly significant decrease in glycogen observed was using 1 μM α-amylase-dta-dtB, which demonstrates that the translocated α-amylase-dta is folded and active in the cytosol, and

shows that the amounts delivered are sufficient not only to degrade existing glycogen, but also to compete with on-going cellular glycogen synthesis. The measured breakdown of glycogen is thus likely an underestimation of the intracellular activity. Moreover, because maltose or any longer glucose oligomer - though also products of the amylase-mediated glycogen degradation - would still be determined as 'glycogen' in our biochemical glycogen quantification method, the possibility also exists that even greater amounts of glycogen were degraded by the delivered α-amylase. Nevertheless, these results provide an important proof-of-delivery of a large and functionally active protein into cells by the diphtheria toxin platform.

[00216] **EXAMPLE 11**

[00217] **Discussion**

[00218] In its 'protective' role, the plasma membrane that encases all human cells unwittingly excludes proteins from entering that might otherwise be effective therapeutics. Though several vectors for intracellular protein delivery into cells have been described, few if any combine the attributes of efficiency, target-cell specificity and low toxicity into a single platform. In this study, a protein-delivery vector is described, which based on the versatile diphtheria toxin that is capable of translocating proteins of varying structural motifs, stabilities and sizes including those that are over 100-kDa in size, with high efficiency into specific receptor-bearing cells (see **Table 2**, above, for a list of chimeric constructs with molecular weights). Engineering protein toxins to create 'designer chimeras' is not a new concept, however, efforts thus far have largely focused on delivering the toxic A-fragment enzymes into specific target cells through modifications to the receptor-binding domain of toxins. Denileukin difitox (Ontak®), an FDA-approved antineoplastic agent created through the fusion of human Interleukin-2 (IL2) to a truncated form of DT (*viz.* dtA-dtB$_{389}$-IL2), binds the IL2 receptor that is overexpressed in various malignancies and translocates dtA into the cytosol to ultimately specifically ablate cancerous cells[12,13]. Recent breakthroughs that serve to improve the safety profile and specificity of immunotoxins have intensified interest in these therapeutics with several now in clinical development, largely for cancer-related indications[13-15].

[00219] This study investigated creating a different type of toxin-based 'designer chimera', taking advantage of the cell-penetrating properties of DT, rather than its cytotoxic features. Previous studies investigating passenger protein delivery with toxins have had mixed success. Using anthrax toxin, there have been a number of reports of successful delivery of peptide and protein cargo into cells[16-19]. A limitation of anthrax toxin as a delivery system – aside from it being composed of two separate proteins that must

find each other on the cell surface to become delivery competent – is the rigid β-barrel translocation pore[20,21] of the B-fragment of anthrax toxin[22] that necessitates complete unfolding of passenger proteins. It has been shown here that DT, on the other hand, can deliver large and structurally diverse proteins into cells with relative ease. Previous studies investigating the utility of DT as a protein delivery vector were inconclusive, in large part because the addition of cargo to the amino terminus decreased the apparent efficiency of intracellular delivery as determined using the 'gold-standard' measure of protein synthesis inhibition. An important discovery here is that the translocation step itself was not impeded for all passenger proteins tested, but rather the activity of dtA was diminished by the juxtaposition of amino terminal proteins. Of equal importance is the finding that DT was able to transport hyper stable, potentially folded, proteins into cells.

[00220] An important consideration for future development of DT- and other toxin-based protein delivery vectors is the extent to which immunogenicity can be addressed for *in vivo* applications. Recent breakthroughs in identifying and eliminating both B-cell[23] and T-cell[15,24] epitopes on toxin-based proteins suggest that there are manageable strategies to redesign the surfaces of toxins without disrupting their cell-penetrating properties. With the growing library of chimeric toxins targeting different cell types, next generation toxin designs incorporating different features that target particular proteins to specific cells/tissues will serve to further expand the potential of DT-based systems as intracellular protein delivery platforms, ultimately for therapeutic delivery of proteins into cells *in vivo*.

[00221] **EXAMPLE 12**

[00222] **Delivery of Additional Cargo**

[00223] Additional constructs were made to test the ability of the DT platform to delivery other proteins, including cargo of therapeutic significance (e.g. MecP2, SMN, FMRP, FMRP), cargo for genome editing applications (e.g. Cas9), and an auto-processing release domain (e.g. CPD).

[00224] **Materials and Methods**

[00225] ***Cell lines***

[00226] Vero cells are grown in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. Vero-NlucP cells are Vero cells stably expressing a Nanoluciferase-PEST fusion protein (NlucP; Promega) delivered via lentiviral vector and subsequent puromycin selection and clonal selection. Both WT and RTTΔ3-4 were derived from fibroblasts of a single Rett Syndrome patient (Cheung et al 2011). RTTΔ3-4

neurons lack exons 3 and 4 of the MeCP2 gene, resulting in a MeCP2-null cell line. WT neurons are isogenic controls. Neurons were kindly provided by Dr. James Ellis.

[00227] *Expression and Purification of fusion proteins*

[00228] Recombinant DT fusion proteins were expressed as N-terminal His-tagged proteins using the Champion pET-SUMO expression system (Invitrogen), except Myc-MeCP2-DT, which does not have either an N-terminal His or SUMO tag. Fusion proteins were expressed in E. coli BL21(DE3) cells. Cells were transformed with the individual plasmids and grown to an OD of ~ 0.6. Myc-MeCP2-DT was induced with 0.5 mM IPTG and expressed at 28°C for 6 hours. Myc-SMN-DT and Myc-FMRP-DT were induced with 1 mM IPTG and expressed at 16°C for 18 hours. PNP-DT was expressed with 1 mM IPTG and expressed for 4 hours at 21°C. Cas9-DT was induced with 0.2 mM IPTG and expressed at 18°C for 18 hours. eGFP-CPD_{Vc}-DT was induced with 1mM IPTG and expressed at 21°C for 5 hours. All lysates were purified on HisTrap FF Crude (GE Healthcare) chromatography columns. Cas9-DT was further purified on a GE Heparin FF column, while eGFP-CPD_{Vc}-DT was further purified on a GE Superdex pg75 gel filtration column. All SUMO-tagged proteins were treated with 1U of SUMO protease (Life Sensor) per 90 µg of purified protein in 20 mM Tris-HCl pH 8 containing 150 mM NaCl and 2 mM DTT. The cleavage reaction was incubated at 30°C for 1 hour followed by purification with His-Pure Ni-NTA resin (Thermo Scientific) to remove the His-SUMO tag and SUMO protease from the purified fusion proteins.

[00229] ***DT Toxicity Assays***

[00230] *Cell Viability Assay*

[00231] Vero cells were plated at 4000 cells/well in a 96-well cell culture plate and allowed to attach overnight at 37°C and 5% $C0_2$. The next day, fusion toxins were added at various concentrations in DMEM (10% FBS, 1% penicillin/streptomycin). After 48 hours, 100 µl of Presto-Blue (Life Technologies) cell viability dye was added to all wells and incubated at 37°C for 2 hours. Fluoresence was measured in a SpectraMax M5e microplate reader (Molecular Devices) (Ex/Em 555/585 nm). Results were quantified and fit to a sigmoidal function in GraphPad Prism.

[00232] *^{3}H-Leucine Incorporation Assay*

[00233] Vero cells or neurons were plated as above (neurons were used after 2 weeks at 30,000 cells/well). Cells were treated with various concentrations of fusion toxins in either DMEM (10% FBS, 1% penicillin/streptomycin [cDMEM]) (Vero cells) or neurobasal media supplemented with cAMP (1 µM), BDNF (10 ng/ml) GDNF (10 ng/ml) and ascorbic acid (200 ng/ml) (neurons). After 15 hours, cells were washed with 200 µl

leucine-free cDMEM then incubated in 50 µl leucine-free cDMEM supplemented with 5 µCi/ml of tritiated leucine for 2 hours at 37°C. Cells were washed with 200 µl of ice-cold PBS, and cellular protein was precipitated with 100 µl ice-cold 10% TCA for 10 minutes at room temperature. Cells were then washed with 100 µl of ice-cold 5% TCA and dissolved in 0.1N NaOH before being transferred into a 96-well polystyrene plate and mixed with 200 µl of scintillation fluid. Total incorporated ^{3}H-leucine was measured by scintillation counting using a TopCount NXT.

[00234] *NanoGlo Assay*

[00235] Vero cells were transduced with a lentivirus containing the Nanoluc luciferase gene (Promega) fused to a C-terminal PEST degradation domain. Positive clones were selected by puromycin selection followed by clonal selection to make Vero-NlucP cells. These cells were treated with various concentrations of fusion toxins as above for 15 hours. NanoGlo assay (Promega) was carried out per the manufacturer's instructions. Luminescence was read on a SpectraMax M5e microplate reader and data was fit to a sigmoidal function (GraphPad Prism).

[00236] **Results and Discussion**

[00237] ***Cargo of Therapeutic Significance***

[00238] A primary theme of the development of diphtheria toxin (DT) as a protein delivery platform is the delivery of proteins implicated in recessive monogenic disorders, especially those with a neurological component, as a form of enzyme replacement therapy (ERT). Typically, ERT regimens rely on proteins that are active in the extracellular environment or in the endosomal/lysosomal pathway due to their inability to penetrate the cellular plasma membrane. Others rely on cell-penetrating peptides (CPP) such as the HIV-derived TAT peptide, but these suffer from a lack of specificity, and typically do not cross the blood-brain-barrier (BBB).

[00239] The fundamental platform on which all fusion proteins are built is the dtA-dtB, wildtype diphtheria toxin. The dtB domain is composed of the translocation (dtT) domain, and the receptor-binding (dtR) domain. Two inactivating mutations in dtA (K51E and E148K) render the toxin completely non-toxic (referred to as dta herein). All DT fusion proteins were also created with these inactivating mutations as non-toxic versions. A further mutation in the dtT domain (L350K) abrogates toxicity by preventing pore-formation and translocation. All fusion proteins are expressed with an N-terminal polyhistidine tag and a SUMO tag. Removal of the His-SUMO tag is accomplished during purification with treatment with SUMO protease.

[00240] Four proteins implicated in childhood genetic brain disorders have been cloned, expressed and purified f. Namely, methyl-CpG-Binding Protein 2 (MeCP2; Rett Syndrome), Survival of Motor Neuron (SMN; Spinal Muscular Atrophy), Fragile X Mental Retardation Protein (FMRP; Fragile X Syndrome), and Purine Nucleoside Phosphorylase (PNP; PNP-deficiency). Cloned, expressed and purified are alpha-amylase from *Bacillus megaterium* as a therapeutic treatment for Lafora Disease, the Cas9 nuclease from *Streptococcus pyogenes*, as well as the fluorescent proteins eGFP and mCherry. Cytoplasm-sensing autorelease domains have been engineered into the DT platform in the form of cysteine protease domains from both *Clostridium difficile* toxin B and *Vibrio cholerae* MARTX toxin.

[00241] *MecP2*

[00242] The primary cause of Rett Syndrome, mutations in the MeCP2 gene result in a non-functional protein product. MeCP2 is a DNA-binding protein and acts as a global transcriptional regulator. Myc-MeCP2-dtA-dtB has been expressed and purified. The DNA sequence for MeCP2e1-dtA-dtB was synthesized and codon optimized for *E. coli* expression from GenScript. The Myc tag is linked to MeCP2 with a GSG linker. MeCP2 is linked to dtA with a (G4S)2 linker. MeCP2 can exist in two main isoforms, e1 and e2.

[00243] **Figure 21** demonstrates proof of cystosolic delivery of MeCP2e1 into cells by fusion protein toxicity in Vero cells. Vero cell toxicity based on Presto-Blue cell viability assay. EC_{50} values for WT DT and Myc-MeCP2-DT were 0.93 ± 1.16 and 37.33 ± 1.13 pM, respectively.

[00244] **Figure 22** shows proof of cystosolic delivery of MecP2e1 into iPSC-derived neurons from Rett Syndrome patient fibroblasts. It shows the effect of Myc-MeCP2-DT on protein synthesis in 2 week old RTTΔ3-4 neurons as measured by ^{3}H-leucine incorporation assay. EC_{50} values for WT and Myc-MeCP2-DT were 4.72 ± 1.71 and 29.56 ± 1.39 pM, respectively.

[00245] *SMN1*

[00246] Spinal Muscular Atrophy (SMA) is caused by mutations in the SMN1 gene, resulting in a defective or missing protein product. Disease severity is moderated by a gene duplication event unique to humans that resulted in SMN2, a gene identical to SMN1 except for a C to T transition resulting in alternative splicing and exclusion of exon 7 from most SMN2 transcripts. Myc-SMN-dtA-dtB has been expressed and purified. The N-terminal Myc tag is linked to SMN with a GSG linker.

[00247] **Figure 23** depicts results of fusion protein toxicity assays indicating that SMN is delivered into the cytosol Vero cells. Vero cell toxicity was based on the Presto-

Blue cell viability assay. The EC_{50} value for SUMO-Myc-SMN-DT was 648.2 ± 1.09 pM. Constructs can also contain the inactivating mutations K51E and E148K in dtA, when a non-toxic version is desirable.

[00248] *FMRP*

[00249] Fragile X Syndrome is characterized by the lack of the FMRP protein product due to the expansion of a CGG trinucleotide repeat region in the 5'UTR of the FMR1 gene. This results in hypermethylation and silencing of FMR1. FMRP is a translational regulator with many downstream gene targets. Myc-FMRP-dtA-dtB was expressed and purified. The N-terminal Myc tag is linked to FMRP with a GSG linker.

[00250] **Figure 24** depicts results of fusion protein toxicity assays indicating that SMN is delivered into the cytosol Vero cells. Vero cell toxicity was based on the Presto-Blue cell viability assay. The EC_{50} value for SUMO-Myc-FMRP-DT was 4.95 ± 1.15 pM.

[00251] *PNP*

[00252] PNP-deficiency is a metabolic disorder that results in immunodeficiency, as well as neurological symptoms such as developmental decline and mental retardation. The PNP enzyme catalyzes the conversion of inosine and guanosine into hypoxanthine. PNP-dtA-dtB has been exprsessed and purified, wherein PNP is linked to dtA with a $(G4S)_2$ linker.

[00253] **Figure 25** depicts results indicating that PNP fusion proteins have been delivered into the cytosol of Vero cells. Vero cell toxicity was based on the ^{3}H-leucine incorporation assay. EC50 values for WT DT and PNP-DT were 1.31 ± 1.11 and 145.8 ± 1.15 pM, respectively.

[00254] **Figure 26** depicts results indicating that PNP fusion proteins have been delivered into the e cytosol of two-week old wild type (WT) neurons. Toxicity was again based on the ^{3}H-leucine incorporation assay. The EC_{50} value for PNP-DT was 61.34 ± 1.59 pM.

[00255] The construct dtA-PNP-dta-dtB has also been expressed and purified, wherein dtA is linked to PNP via a GSG linker and PNP is linked to dta using a GSG linker.

[00256] ***Cargo for Genome Editing***

[00257] *Cas9*

[00258] The recent application of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system to targeted genome editing has radically changed the field of molecular biology. The system involves the DNA endonuclease CRISPR-associated protein 9 (Cas9), which is guided to its DNA target sequence by a ~97nt RNA

molecule (gRNA). *In vitro*, both Cas9 and gRNA are delivered on a plasmid by transfection, while *in vivo*, the use of viral vectors is the preferred delivery method. There have also been reports of other delivery methods for Cas9, such as CPPs, or lipid-based methods. The construct Cas9-dtA-dtB has been expressed and purified. The Cas9 sequence is from *S. pyogenes*. Cas9 is linked to dtA with a $(G4S)_2$ linker. Cas9 is flanked on both sides by SV40 nuclear localization sequences (NLS). Cas9 is preceded by an N-terminal His tag (6xHis).

[00259] **Figure 27** demonstrates delivery of Cas9 into the cytosol of Vero cells by fusion protein toxicity, as above. Vero cell toxicity was based on the 3H-leucine incorporation assay. The EC_{50} values for WT DT and Cas9-DT was 1.63 ± 1.10 and 21.74 ± 1.09 pM, respectively.

[00260] A further version of this construct contains the inactivating mutations K51E and E148K. Cas9 is the largest cargo protein yet delivered (160 kDa).

[00261] ***Cargo Release***

[00262] *CPD*

[00263] In order to release the native cargo protein upon delivery into the cytosol, a cysteine protease domain (CPD) from each of *Clostridium difficile* toxin B (CPD_{Cd}) and *Vibrio cholera* MARTX toxin (CPD_{Vc}) has been employed. The construct eGFP-CPD_{xx}-dtA-dtB as been expressed and purified, wherein CPDxx is either CPD_{Vc} or CPD_{Cd}. CPD is linked to dtA with a GSG linker. Both domains undergo self-cleavage at their own N-termini upon binding of the small molecule inositol hexakisphosphate (IP6), which is exclusively located in the cytoplasm of mammalian cells. They are autoprocessing, and "self-clearing".

[00264] **Figure 28** depicts delivery of eGFP-CPD_{Vc}-DT into the cytosol of Vero-NlucP cells by fusion protein toxicity. Vero-NlucP cell toxicity was based on the NanoGlo assay. The EC_{50} values for eGFP-CPD_{Vc}-DT was 54.44 ± 1.06 pM.

[00265] Similar release constructs can be made for other cargo proteins, for example with either CPD_{Vc} or CPD_{Cd} and the cargo proteins described above. To date, the following CPD constructs have been made:

[00266] CPD_{cd}-$(G4S)_2$-dtA-dtB;

[00267] eGFP-CPD_{vc}-(GSG)-dtA-dtB; and

[00268] eGFP-CPD_{cd}-(GSG)-dtA-dtB;

[00269] Data generated to date indicates that the presence of CPD results in release of (eGFP) in the presence of InsP6 *in vitro* (data not shown). Western blotting data indicates that that cargo is similarly released in vivo (data not shown).

[00270] Other constructs that have been made to date include:

[00271] MeCP2-CPD_{vc}-$(G4S)_2$-dtA-dtB; and

[00272] PNP-$(G4S)_2$-CPD_{cd}-$(G4S)_3$-dtA-dtB.

[00273] These constructs are expected to be capable of releasing their cargo upon delivery to the cytosol. Non-toxic variants can be readily made, using dta in place of dtA.

[00274] **EXAMPLE 13**

[00275] For some therapeutic applications, it may be desirable to reduce the immunogenicity of DT domains. To this end, DT domains could be mutated, e.g., to reduce their antigenicity, for example by removing T-cell epitopes.

[00276] **EXAMPLE 14**

[00277] For some applications, it may be advantageous to reduce the size of the construct, e.g. to provide a smaller construct and/or to reduce potential for antigenicity. Experiments were conducted to assess the function of the DTA domain.

[00278] **Materials and Methods**

[00279] ***Constructs***

[00280] The glucosyl transferase domain (GTD; SEQ ID NO: 29) from *Clostridium difficile* toxin B was linked to dta or Δdta-dtB via a GSG linker to generate the constructs GTD-dta-dtB and GTD-Δdta-dtB. The latter retains a small DTA fragment (Δdta); most of the functional domain proper has been deleted, leaving SEQ ID NO: 26. Δdta thus extends from a cysteine corresponding to position 186 of SEQ ID NO: 1 through its C-terminus. This cysteine residue was retained as it is involved in disulphide bond formation. The CPD domain from *Vibrio cholerae* was subsequently cloned between GTD and dtB upstream of the linker yielding the construct GTD-CPD-Δdta-dtB with no linker sequence between the GTD and CPD domains. All three constructs were cloned with an N-terminal polyhistidine tag and a C-terminal Strep-tag™ II sequence (SEQ ID NO: 27) for affinity purification using the GE-Healthcare StrepTactin™ purification system.

[00281] ***Expression and Purification***

[00282] GTD DT chimeras were expressed as N-terminal His-tagged proteins in *E. coli* BL21(DE3) cells, induced with 1 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG) for 4 hours at 21°C. Cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 20 mM imidazole, benzonase, lysozyme and Protease inhibitor cocktail) and lysed by an EmulsiFlex C3 microfluidizer (Avestin) at 15,000 psi. The lysates were centrifuged at 18,000 × g for 20 minutes. His -tagged proteins were purified by Ni-affinity chromatography using a His-Trap FF column (GE-Healthcare). Protein was eluted in 20 mM Tris-HCl pH 8.0, 0.5 M NaCl and 125 mM imidazole and

loaded directly onto a 5 mL StrepTrap HP column (GE-Healthcare). Pure protein was then eluted from the StrepTrap HP column in 20 mM Tris-HCl pH 8.0, 150 mM NaCl and 2.0 mM desthiobiotin.

[00283] ***Cell Viability Assay***

[00284] Vero cells were plated at 4000 cells/well in a 96-well cell culture plate and allowed to attach overnight at 37°C and 5% $C0_2$. The next day, fusion toxins were added at various concentrations in DMEM (10% FBS, 1% penicillin/streptomycin). After 48 hours, 100 µl of Presto-Blue (Life Technologies) cell viability dye was added to all wells and incubated at 37°C for 2 hours. Fluorescence was measured in a SpectraMax M5e microplate reader (Molecular Devices) (Ex/Em 555/585 nm). Results were quantified and fit to a sigmoidal function in GraphPad Prism.

[00285] **Results and Discussion**

[00286] ***Delivery of cargo in the absence of the catalytic A domain of DT***

[00287] Inhibition of protein synthesis by DTA has been a useful tool to demonstrate delivery of cargo-DTA chimeras to the cytosol, but tethering to DTA may interfere with certain cargo protein's activity through steric interference and/or cellular localization in some applications. While inclusion of a cysteine protease domain between cargo and DTA would allow for release of native cargo protein, a question remained of whether cargo could be fused more directly to the DTB domain, i.e. with less of DTA, thereby decreasing the size and complexity of cargo being delivered into the cell. Reducing or eliminating the A domain would have the additional benefit of reducing the potential for immunogenicity in future *in vivo* applications of this technology. Fusion proteins containing the glucosyltransferase domain (GTD) from *Clostridium difficile* fused to dta-dtB, CPD-Δdta -dtB or simply Δdta-dtB were cloned, expressed and purified. Upon reaching the cytosol, the GTD inactivates small Rho family GTPases (Rac1, RhoA, Cdc42), thereby disrupting actin cytoskeleton organization resulting in an acute rounding phenotype and eventual apoptosis (Just 1995). In the absence most of the DTA domain, the cytotoxicity of the GTD was used to compare its cytosolic entry in these three different delivery paradigms.

[00288] **Figure 29** shows the effect of removing most of the A domain, which appears to have no effect on the ability of the GTD cargo to reach the cytosol. Remarkably, there is a small increase in toxicity of the two constructs lacking most of the DTA domain, however, this difference is small and could be due to an effect on the enzymatic activity of GTD as both GTD-Δdta-CPD-dtB and GTD-Δdta-dtB result in delivery of free GTD while GTD-dta remain fused upon delivery.

[00289] The dispensable nature of the DTA domain in cargo translocation has important implications for the DT delivery platform and speaks to the versatility and modularity of this system. This finding also deviates significantly from the widely accepted model of DT translocation, in which DTA is absolutely required and is thought to make up part of the translocation machinery.

[00290] In the preceding description, for purposes of explanation, numerous details are set forth in order to provide a thorough understanding of the embodiments. However, it will be apparent to one skilled in the art that these specific details are not required. In other instances, well-known electrical structures and circuits are shown in block diagram form in order not to obscure the understanding. For example, specific details are not provided as to whether the embodiments described herein are implemented as a software routine, hardware circuit, firmware, or a combination thereof.

[00291] The above-described embodiments are intended to be examples only. Alterations, modifications and variations can be effected to the particular embodiments by those of skill in the art. The scope of the claims should not be limited by the particular embodiments set forth herein, but should be construed in a manner consistent with the specification as a whole.

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[00318] All references are incorporated by reference herein to the same extent as if set forth verbatim herein.

CLAIMS:

1. A recombinant molecule comprising a cargo polypeptide, a diphtheria toxin enzymatic fragment (DTA), and a diphtheria toxin translocation fragment (DTB).

2. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of less than 10 kDa.

3. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of greater than 10 kDa.

4. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of greater than 20 kDa.

5. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of greater than 30 kDa.

6. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of greater than 50 kDa.

7. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of greater than 100 kDa.

8. The recombinant molecule of claim 1, wherein the cargo polypeptide has a molecular weight of greater than 150 kDa.

9. The recombinant molecule of any one of claims 1 to 8, wherein the cargo polypeptide is positioned at or upstream of the amino terminus of the diphtheria toxin enzymatic fragment.

10. The recombinant molecule of claim 1, having a general structure:

x-C-y-DTA-DTB

wherein:

x is a polypeptide or absent,

C is the cargo polypeptide, and
y is a polypeptide, a linker, or absent.

11. The recombinant molecule of claim 10, wherein the diphtheria toxin enzymatic fragment is linked to the diphtheria toxin translocation fragment by way of a disulphide linkage.

12. The recombinant molecule of claim 10 or 11, wherein y is an autoprocessing domain.

13. The recombinant molecule of claim 12, wherein the autoprocessing domain comprises a cysteine protease domain.

14. The recombinant molecule of claim 13, wherein the cysteine protease domain is derived from a bacterium.

15. The recombinant molecule of claim 14, wherein the bacterium is Vibrio cholerae or Clostridium difficile.

16. The recombinant molecule of claim 15, wherein the cysteine protease domain comprises an amino acid sequence as set forth in SEQ ID No: 20 or 21.

17. The recombinant molecule of claim 16, wherein y is a linker.

18. The recombinant molecule of claim 17, wherein the linker is an amino acid linker.

19. The recombinant molecule of claim 18, wherein the amino acid linker comprises at least five amino acid residues.

20. The recombinant molecule of claim 18, wherein the amino acid linker comprises (G4S)n, wherein n is 1 to 3.

21. The recombinant molecule of claim 20, wherein n is 3.

22. The recombinant molecule of any one of claims 10 to 21, wherein x is absent.

23. The recombinant molecule of any one of claims 10 to 22, wherein the diphtheria toxin translocation fragment comprises an amino acid sequence as set forth in SEQ ID No: 3.

24. The recombinant molecule of any one of claims 10 to 23, wherein the diphtheria toxin enzymatic fragment is catalytically active.

25. The recombinant molecule of claim 24, wherein the diphtheria toxin enzymatic fragment comprises an amino acid sequence as set forth in SEQ ID No: 1.

26. The recombinant molecule of any one of claims 10 to 23, wherein the diphtheria toxin enzymatic fragment is catalytically inactive.

27. The recombinant molecule of claim 26, wherein the diphtheria toxin enzymatic fragment comprises an amino acid sequence bearing the mutations K51E and E148K, as numbered with respect to wild type sequence.

28. The recombinant molecule of claim 27, wherein the diphtheria toxin enzymatic fragment comprises an amino acid sequence as set forth in SEQ ID No: 2.

29. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide comprises an enzyme, or an active fragment thereof having substantially the same activity.

30. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide comprises a stably folded protein.

31. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises a therapeutic protein.

32. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises MecP2.

33. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises SMN.

34. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises FMRP.

35. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises PNP.

36. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises alpha-amylase.

37. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises a genome-modifying protein.

38. The recombinant molecule of claim 37, wherein the genome-modifying protein comprises a zinc finger nuclease (ZFN).

39. The recombinant molecule of claim 37, wherein the genome-modifying protein comprises a transcription activator-like effector nuclease (TALEN).

40. The recombinant molecule of claim 37, wherein the genome-modifying protein comprises a CRISPR (clustered regularly interspaced short palindromic repeat) protein.

41. The recombinant molecule of claim 40, wherein the CRISPR protein is Cas9.

42. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of less than 10 kDa.

43. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of greater than 10 kDa.

44. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of greater than 20 kDa.

45. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of greater than 30 kDa.

46. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of greater than 50 kDa.

47. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of greater than 100 kDa.

48. The recombinant molecule of any one of claims 10 to 28, wherein the cargo polypeptide has a molecular weight of greater than 150 kDa.

49. The recombinant molecule of any one of claims 26 to 28, wherein the cargo comprises an ubiquitin or a variant thereof.

50. The recombinant molecule of any one of claims 26 to 28, wherein the cargo polypeptide comprises a therapeutic polypeptide.

51. The recombinant molecule of any one of claim 1 to 50, wherein y comprises a ligation site.

52. The recombinant molecule of claim 51, wherein the ligation site is a sortase ligation site.

53. The recombinant molecule of any one of claims 1 to 23, 26, and 29 to 52, wherein the DTA is a C-terminal fragment comprising a cysteine corresponding to the cysteine at position 186 of SEQ ID NO: 1.

54. The recombinant molecule of claim 53, wherein the C-terminal fragment comprises a polypeptide having a sequence CAGNRVRRSVGSSL (SEQ ID NO: 26).

55. The recombinant molecule of claim 53, wherein the C-terminal fragment consists of a polypeptide having a sequence CAGNRVRRSVGSSL (SEQ ID NO: 26).

56. A nucleic acid encoding the recombinant molecule of any one of claims 1 to 55.

57. The nucleic acid of claim 56, wherein diphtheria toxin enzymatic fragment and diphtheria toxin translocation fragment are encoded separately.

58. A recombinant cell comprising at least one nucleic acid of claim 56 or 57.

59. A vector comprising at least one nucleic acid of claim 56 or 57.

60. A cell transformed with the vector according to claim 59.

61. A pharmaceutical composition comprising the recombinant molecule of any one of claims 26 to 28, 31 to 41, 49, 50, and 53 to 55; and a pharmaceutically acceptable carrier.

62. A method of delivering a cargo polypeptide to a cell, comprising contacting the cell with the recombinant molecule of any one of claims 1 to 55.

63. A method of delivery a cargo polypeptide to a cell of a subject, comprising contacting the cell with the recombinant molecule of any one of claims 26 to 28 and 31 to 41.

64. A method of delivering a cargo polypeptide across the blood brain barrier, comprising administering to a subject the recombinant molecule of any one of claims 26 to 28, 31 to 41, 49, 50, and 53 to 55.

65. A method of increasing enzyme or protein activity in a cell, comprising contacting the cell with the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55.

66. A method of alleviating enzyme or protein deficiency in a cell, comprising contacting the cell with the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55.

67. The method of claim 66, wherein the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity.

68. The method of claim 66, wherein the cargo polypeptide compensates for the enzyme or protein deficiency.

69. A method of treating a disease or disorder caused by enzyme or protein deficiency in a subject, comprising administering to the subject the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55.

70. The method of claim 69, wherein the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity.

71. The method of claim 69, wherein the cargo polypeptide compensates for the enzyme or protein deficiency.

72. The method of claim 69, wherein the disease or disorder is Rett syndrome, and the cargo polypeptide comprises MecP2.

73. The method of claim 69, wherein the disease or disorder is Spinal Muscular Atrophy syndrome, and the cargo polypeptide comprises SMN.

74. The method of claim 69, wherein the disease or disorder is Fragile X syndrome, and the cargo polypeptide comprises FMRP.

75. The method of claim 69, wherein the disease or disorder is PNP-deficiency, and the cargo polypeptide comprises PNP.

76. The method of claim 69, wherein the disease or disorder is Lafora Disease, and the cargo polypeptide comprises alpha-amylase.

77. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of less than 10 kDa.

78. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of greater than 10 kDa.

79. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of greater than 20 kDa.

80. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of greater than 30 kDa.

81. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of greater than 50 kDa.

82. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of greater than 100 kDa.

83. The method of any one of claims 66 to 71, wherein the cargo polypeptide has a molecular weight of greater than 150 kDa.

84. A method of manipulating the genome of a cell, comprising contacting the cell with the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55, wherein the cargo polypeptide comprises a genome-modifying protein.

85. The method of claim 84, wherein the genome-modifying protein comprises a zinc finger nuclease (ZFN).

86. The method of claim 84, wherein the genome-modifying protein comprises a transcription activator-like effector nuclease (TALEN).

87. The method of claim 84, wherein the genome-modifying protein comprises a CRISPR clustered regularly interspaced short palindromic repeat) protein.

88. The method of claim 87, wherein the CRISPR protein is Cas9.

89. A use of the recombinant molecule of any one of claims 1 to 55 for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide to a cell.

90. A use of the recombinant molecule of any one of claims 26 to 28 and 31 to 41, 49, 50, and 53 to 55 for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide to a cell of a subject.

91. A use of the recombinant molecule of any one of claims 26 to 28 and 31 to 41, 49, 50, and 53 to 55 for delivery, or for preparation of a medicament for delivery, of the cargo polypeptide across the blood brain barrier.

92. A use of the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55 for increasing, or for preparation of a medicament for increasing, enzyme or protein activity in a cell.

93. A use of the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55 for alleviating, or for preparation of a medicament for alleviating, enzyme or protein deficiency in a cell.

94. The use of claim 93, wherein the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity.

95. The use of claim 93, wherein the cargo polypeptide compensates for the enzyme or protein deficiency.

96. A use of the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55 for treating, or for preparation of a medicament for treating, a disease or disorder caused by enzyme or protein deficiency in a subject.

97. The use of claim 96, wherein the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity.

98. The use of claim 96, wherein the cargo polypeptide compensates for the enzyme or protein deficiency.

99. The use of claim 96, wherein the disease or disorder is Rett syndrome, and the cargo polypeptide comprises MecP2.

100. The use of claim 96, wherein the disease or disorder is Spinal Muscular Atrophy, and the cargo polypeptide comprises SMN.

101. The use of claim 96, wherein the disease or disorder is Fragile X syndrome, and the cargo polypeptide comprises FMRP.

102. The use of claim 96, wherein the disease or disorder is PNP-deficiency, and the cargo polypeptide comprises PNP.

103. The use of claim 96, wherein the disease or disorder is Lafora Disease, and the cargo polypeptide comprises alpha-amylase.

104. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of less than 10 kDa.

105. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of greater than 10 kDa.

106. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of greater than 20 kDa.

107. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of greater than 30 kDa.

108. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of greater than 50 kDa.

109. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of greater than 100 kDa.

110. The use of any one of claims 90 to 98, wherein the cargo polypeptide has a molecular weight of greater than 150 kDa.

111. A use of the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55 for manipulating the genome of a cell, wherein the cargo polypeptide comprises a genome-modifying protein.

112. The use of claim 111, wherein the genome-modifying protein comprises a zinc finger nuclease (ZFN).

113. The use of claim 111, wherein the genome-modifying protein comprises a transcription activator-like effector nuclease (TALEN).

114. The use of claim 111, wherein the genome-modifying protein comprises a CRISPR (clustered regularly interspaced short palindromic repeat) protein.

115. The use of claim 114, wherein the CRISPR protein is Cas9.

116. A kit for delivering a cargo polypeptide to a cell comprising the recombinant molecule of any one of claims 1 to 55, and instructions for contacting the cell with the recombinant molecule.

117. A kit for delivering a cargo polypeptide to a cell of a subject, comprising the recombinant molecule of any one of claims 26 to 28 and 31 to 41, 49, 50, and 53 to 55; and instructions for contacting the cell with the recombinant molecule.

118. A kit for delivering a cargo polypeptide across the blood brain barrier, comprising the recombinant molecule of any one of claims 26 to 28 and 31 to 41, 49, 50, and 53 to 55; and instructions for administering the recombinant molecule to a subject.

119. A kit for increasing enzyme or protein activity in a cell, comprising the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55; and instructions for contacting the cell with the recombinant molecule.

120. A kit for alleviating enzyme or protein deficiency in a cell, comprising the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55; and instructions for contacting the cell with the recombinant molecule.

121. The kit of claim 120, wherein the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity.

122. The kit of claim 120, wherein the cargo polypeptide compensates for the enzyme or protein deficiency.

123. A kit for treating a disease or disorder caused by enzyme or protein deficiency in a subject, comprising the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55and instructions for administering the recombinant molecule to the subject.

124. The kit of claim 123, wherein the cargo polypeptide comprises the enzyme or protein, or an active fragment thereof having substantially the same activity.

125. The kit of claim 123, wherein the cargo polypeptide compensates for the enzyme or protein deficiency.

126. The kit of claim 123, wherein the disease or disorder is Rett syndrome, and the cargo polypeptide comprises MecP2.

127. The kit of claim 123, wherein the disease or disorder is Spinal Muscular Atrophy syndrome, and the cargo polypeptide comprises SMN.

128. The kit of claim 123, wherein the disease or disorder is Fragile X syndrome, and the cargo polypeptide comprises FMRP.

129. The kit of claim 123, wherein the disease or disorder is PNP-deficiency, and the cargo polypeptide comprises PNP.

130. The kit of claim 123, wherein the disease or disorder is Lafora Disease, and the cargo polypeptide comprises alpha-amylase.

131. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of less than 10 kDa.

132. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of greater than 10 kDa.

133. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of greater than 20 kDa.

134. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of greater than 30 kDa.

135. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of greater than 50 kDa.

136. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of greater than 100 kDa.

137. The kit of any one of claims 117 to 125, wherein the cargo polypeptide has a molecular weight of greater than 150 kDa.

138. A kit for manipulating the genome of cell, comprising the recombinant molecule of any one of claims 26 to 28, 49, 50, and 53 to 55 and instructions for contacting the cell with the recombinant molecule, wherein the cargo polypeptide comprises a genome-modifying protein.

139. The kit of claim 138, wherein the genome-modifying protein comprises a zinc finger nuclease (ZFN).

140. The kit of claim 138, wherein the genome-modifying protein comprises a transcription activator-like effector nuclease (TALEN).

141. The kit of claim 138, wherein the genome-modifying protein comprises a CRISPR (clustered regularly interspaced short palindromic repeat) protein.

142. The kit of claim 138, wherein the CRISPR protein is Cas9.

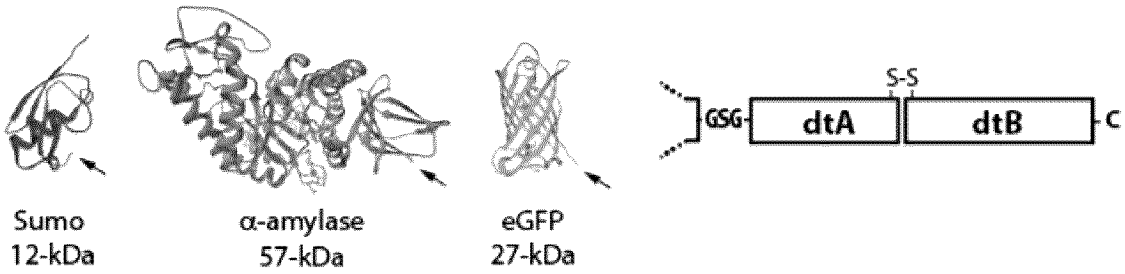


Figure 1

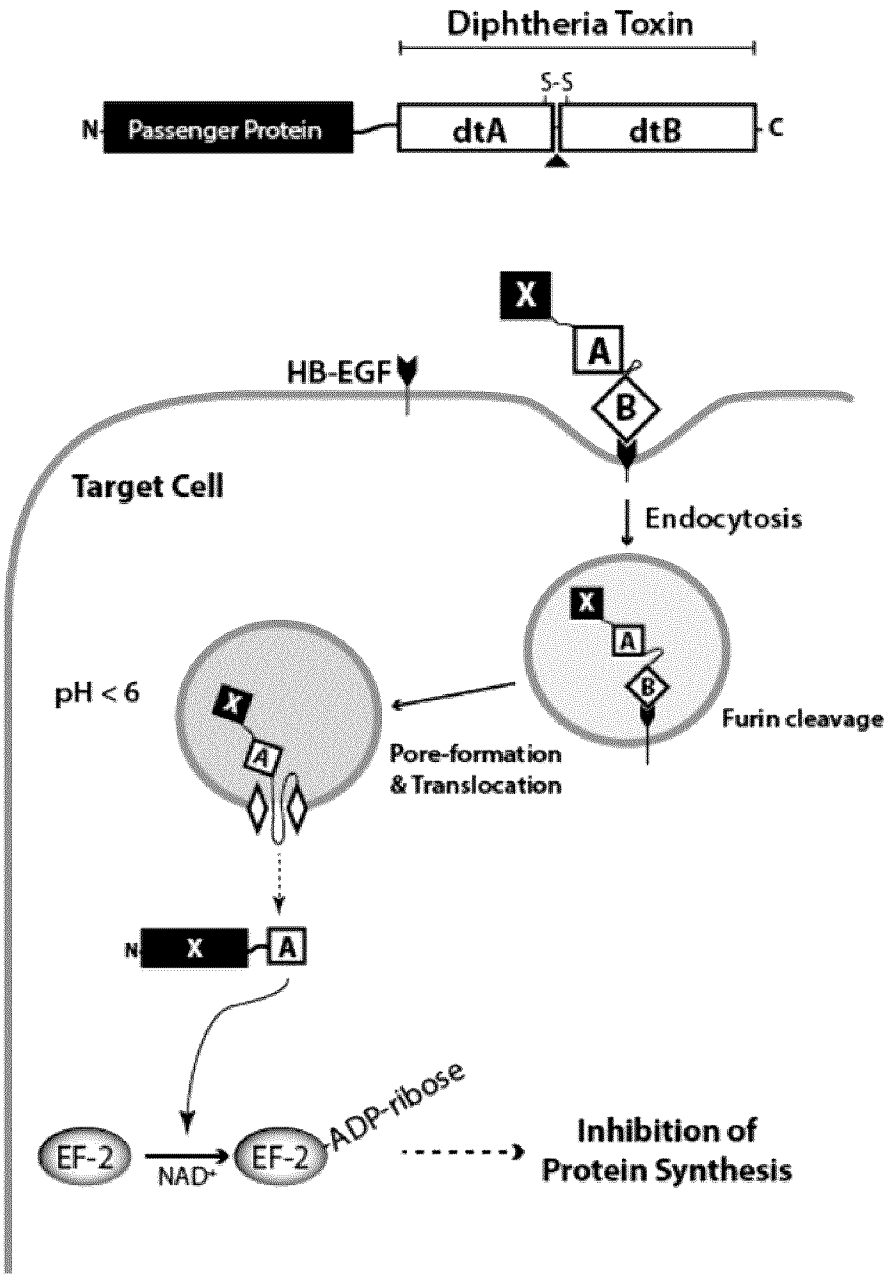
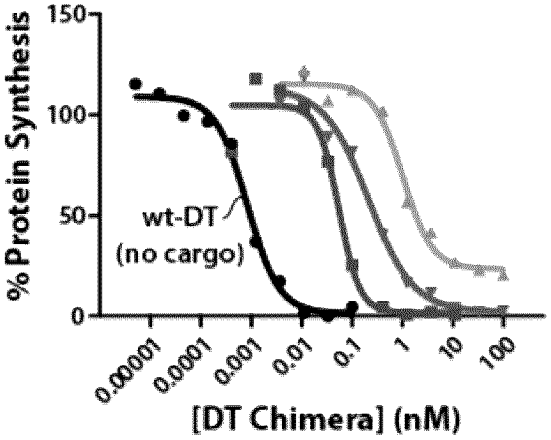

Diphtheria Toxin
S-S
N
Passenger Protein
dtA
dtB
C
X
A
B
HB-EGF
Target Cell
Endocytosis
pH < 6
Furin cleavage
Pore-formation
& Translocation
N
X
A
EF-2
NAD+
EF-2
ADP-ribose
Inhibition of
Protein Synthesis

Figure 2



Symbol	X =	EC_{50} (pM)
	-	0.8
	Sumo	55
	Amylase	216
	eGFP	1022

Figure 3

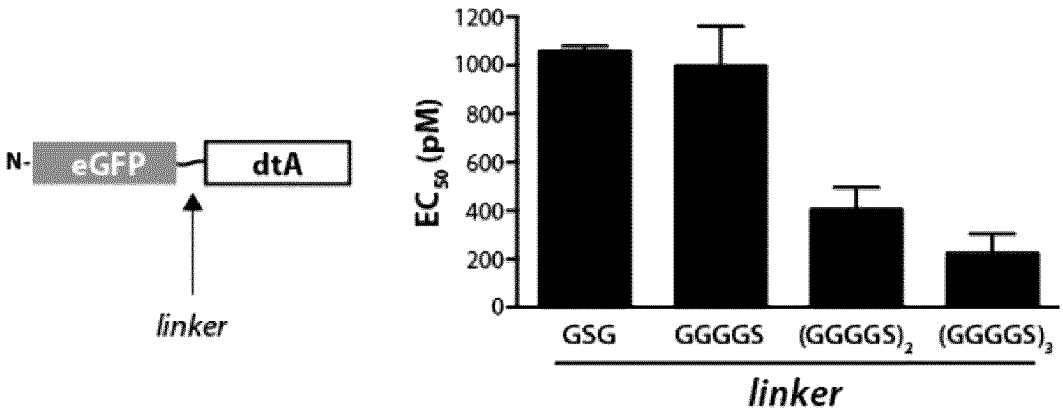


Figure 4

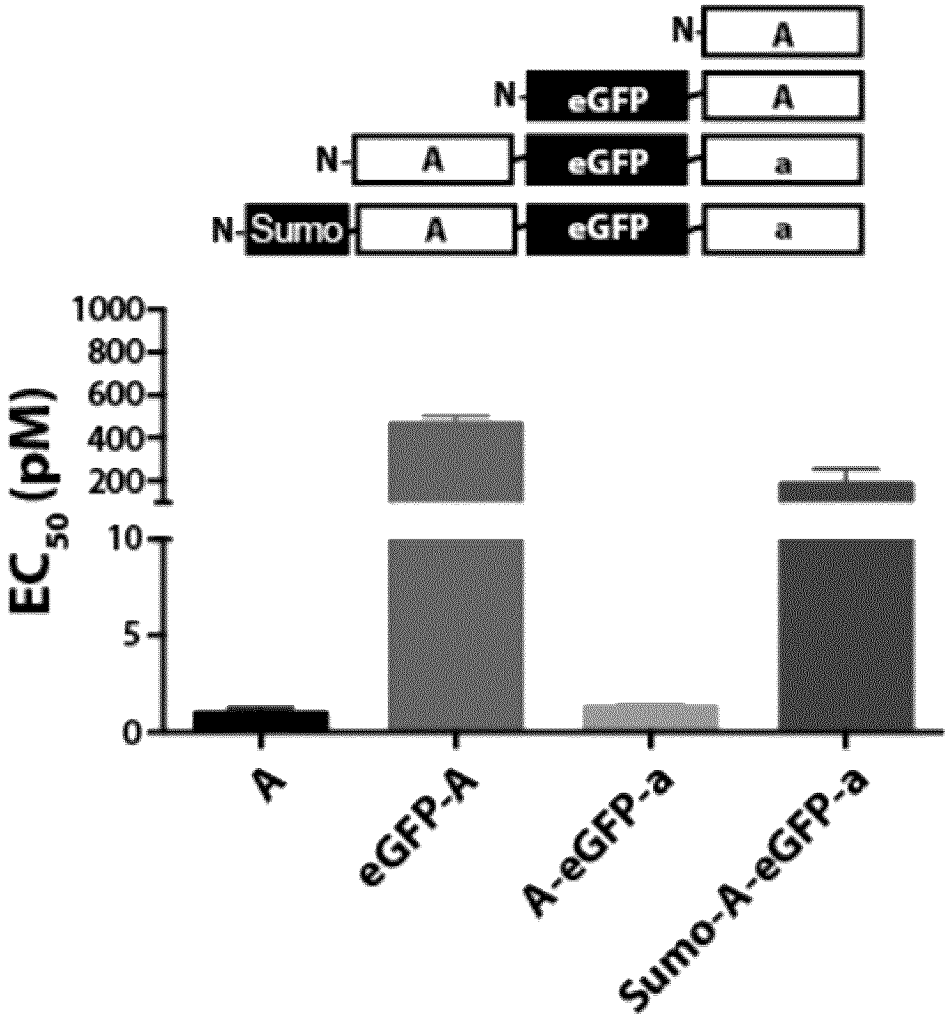

N A
N eGFP A
N A eGFP a
N Sumo A eGFP a
EC50 (pM)
1000
800
600
400
200
10
5
0
A
eGFP-A
A-eGFP-a
Sumo-A-eGFP-a

Figure 5

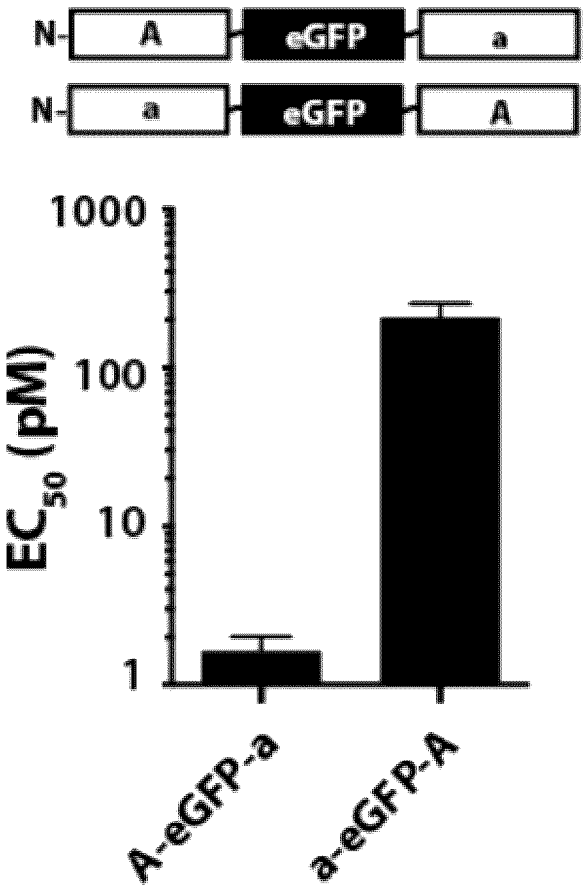


Figure 6

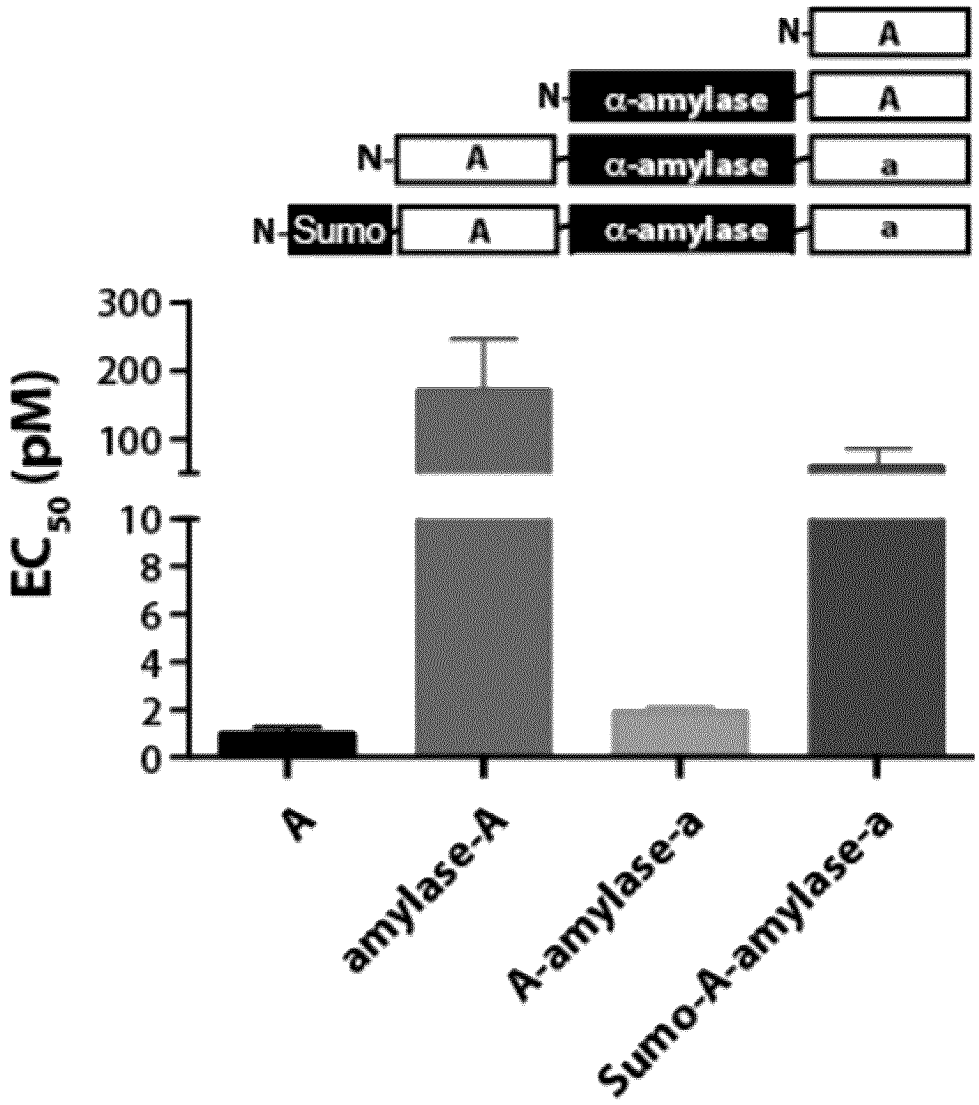

N- A
N- α-amylase A
N- A α-amylase a
N- Sumo A α-amylase a
EC50 (pM)
300
200
100
10
8
6
4
2
0
A
amylase-A
A-amylase-a
Sumo-A-amylase-a

Figure 7

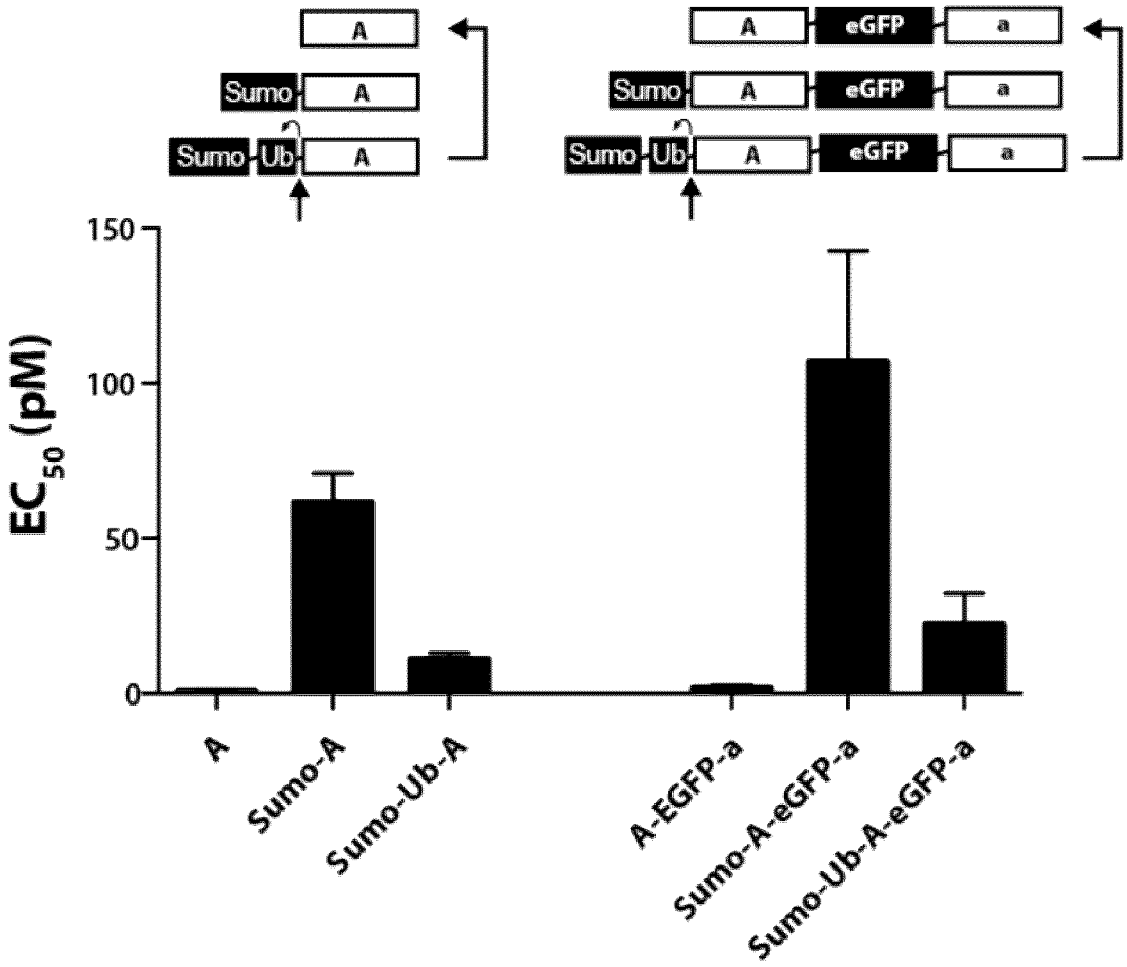

A
Sumo
A
Sumo
Ub
A
A
eGFP
a
Sumo
A
eGFP
a
Sumo
Ub
A
eGFP
a
150
100
50
0
EC50 (pM)
A
Sumo-A
Sumo-Ub-A
A-EGFP-a
Sumo-A-eGFP-a
Sumo-Ub-A-eGFP-a

Figure 8

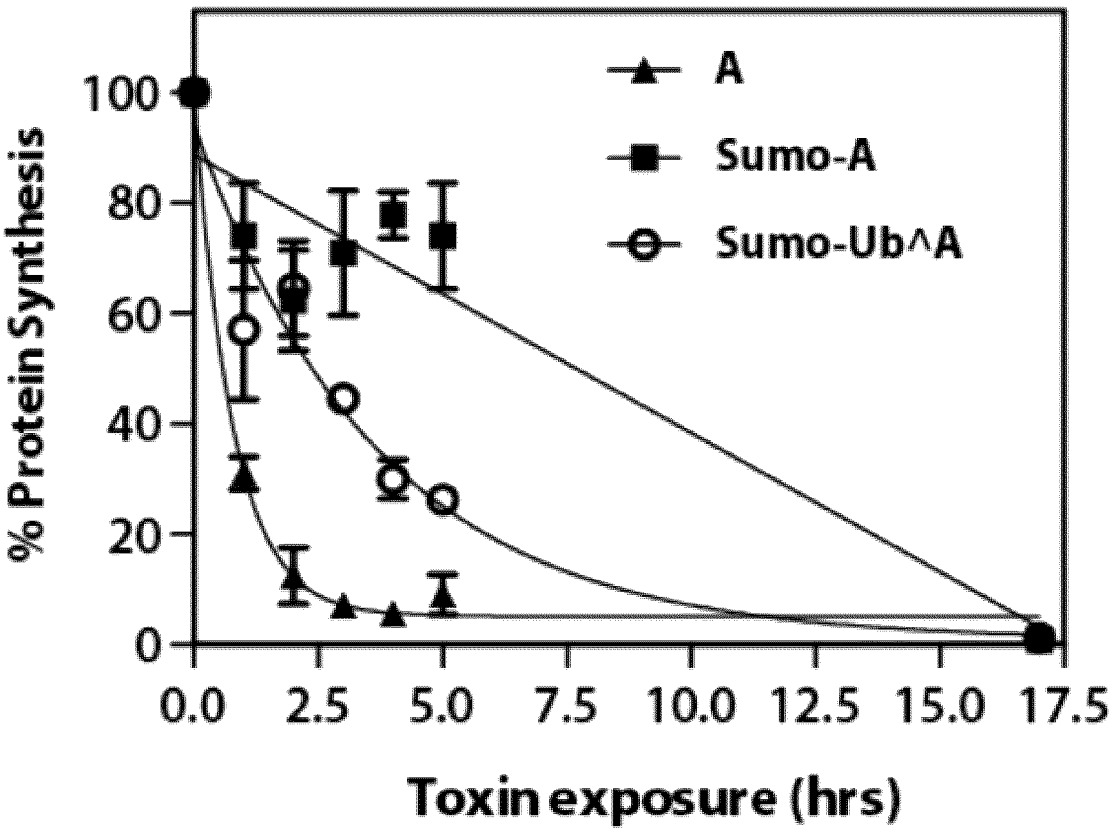

A
Sumo-A
Sumo-Ub^A
% Protein Synthesis
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Toxin exposure (hrs)

Figure 9

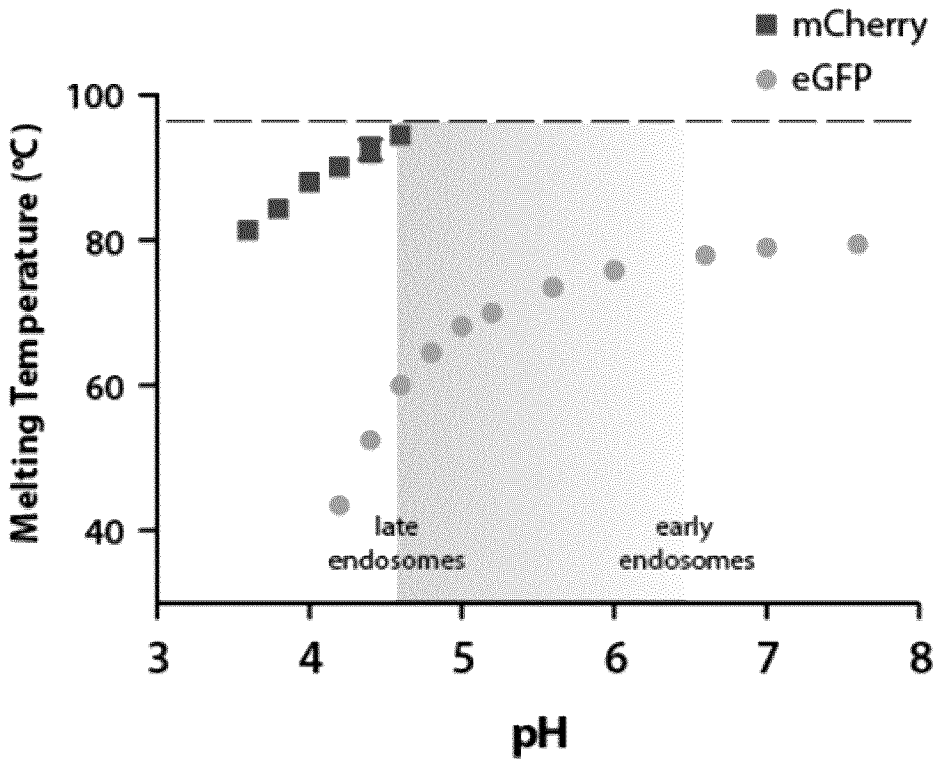


Figure 10

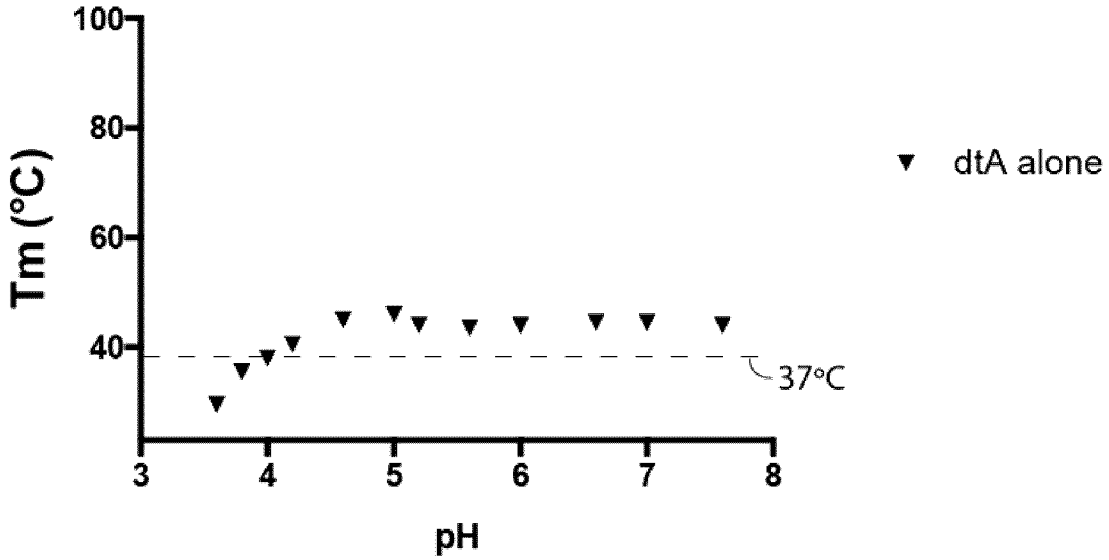


Figure 11

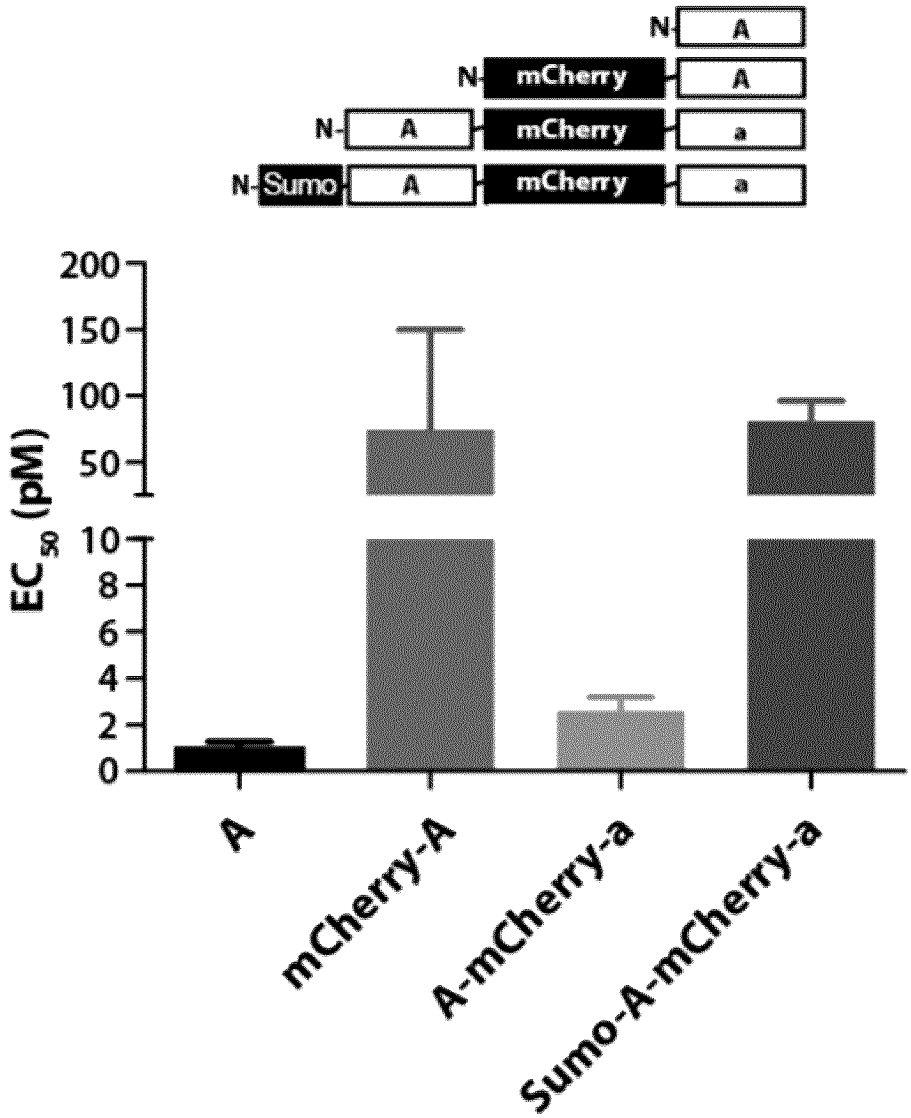

N A
N mCherry A
N A mCherry a
N Sumo A mCherry a
200
150
100
50
10
8
6
4
2
0
EC50 (pM)
A
mCherry-A
A-mCherry-a
Sumo-A-mCherry-a

Figure 12

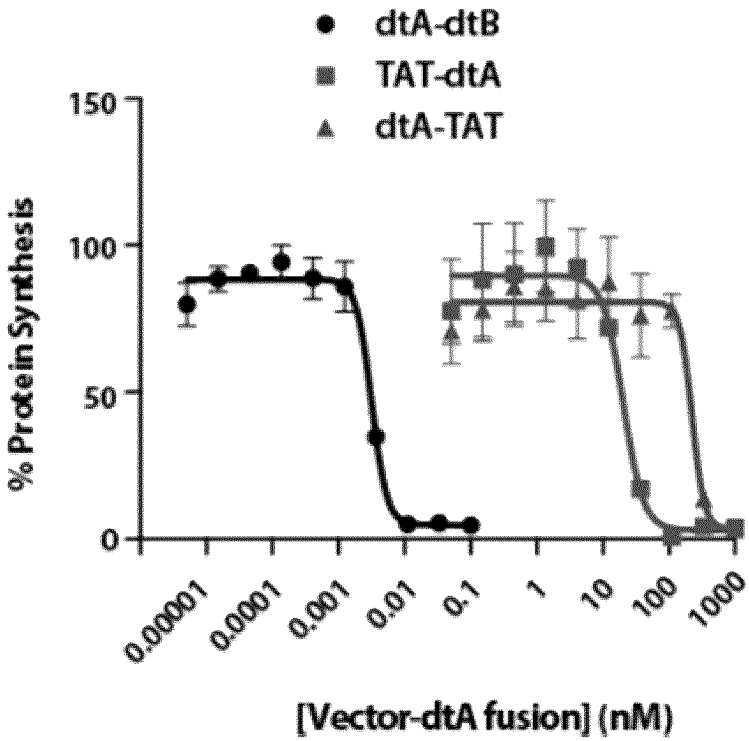

dtA-dtB
TAT-dtA
dtA-TAT
150
100
50
0
% Protein Synthesis
0.00001
0.0001
0.001
0.01
0.1
1
10
100
1000
[Vector-dtA fusion] (nM)

Figure 13

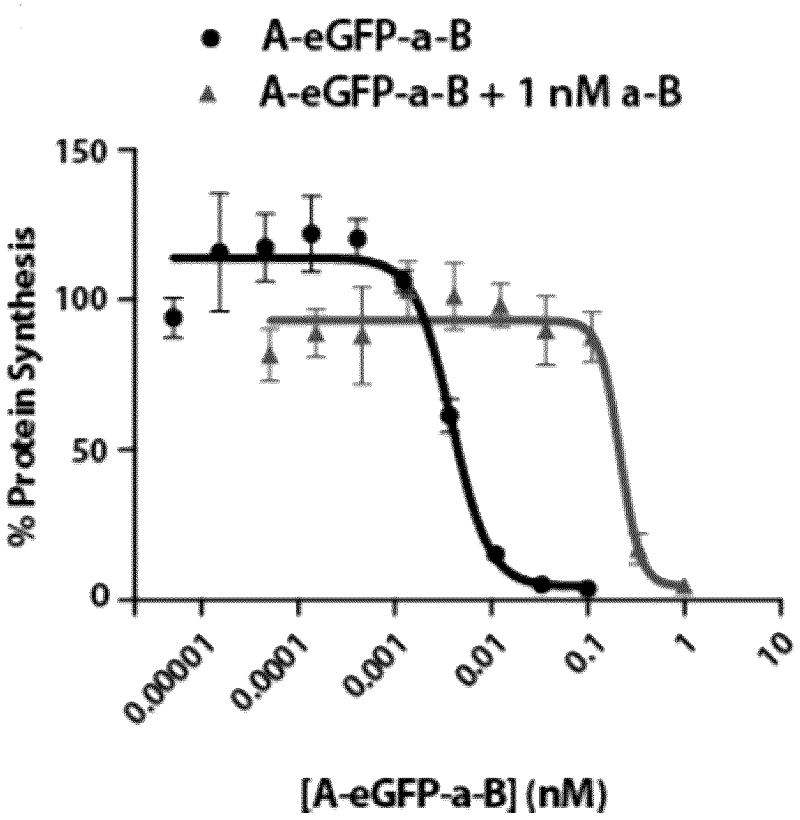

A-eGFP-a-B
A-eGFP-a-B + 1 nM a-B
% Protein Synthesis
150
100
50
0
0.00001
0.0001
0.001
0.01
0.1
1
10
[A-eGFP-a-B] (nM)

Figure 14

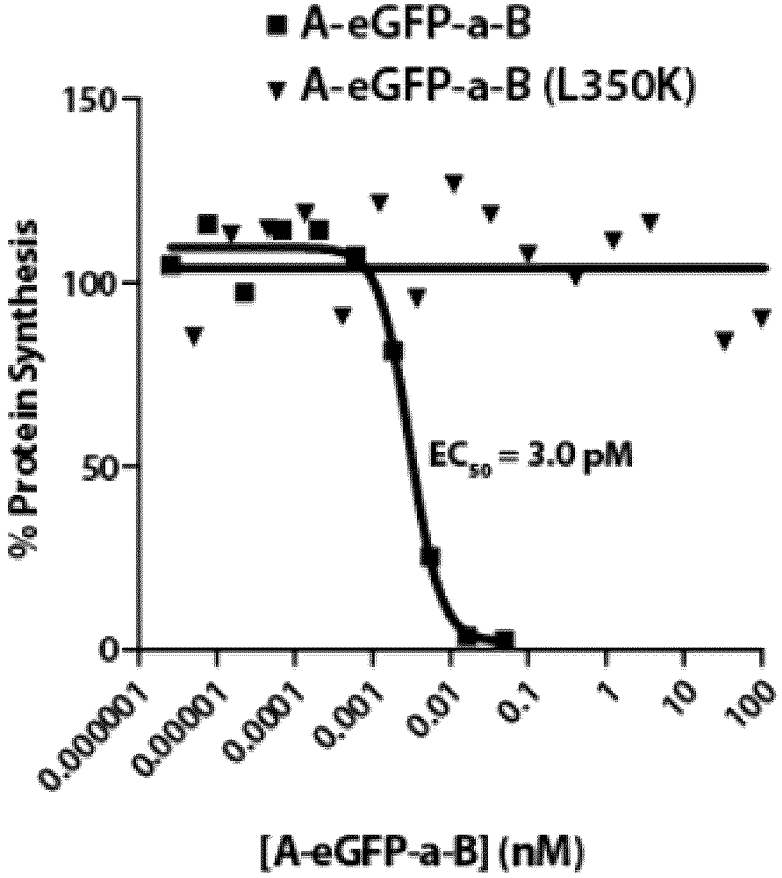

A-eGFP-a-B
A-eGFP-a-B (L350K)
150
100
50
0
% Protein Synthesis
EC50 = 3.0 pM
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
[A-eGFP-a-B] (nM)

Figure 15

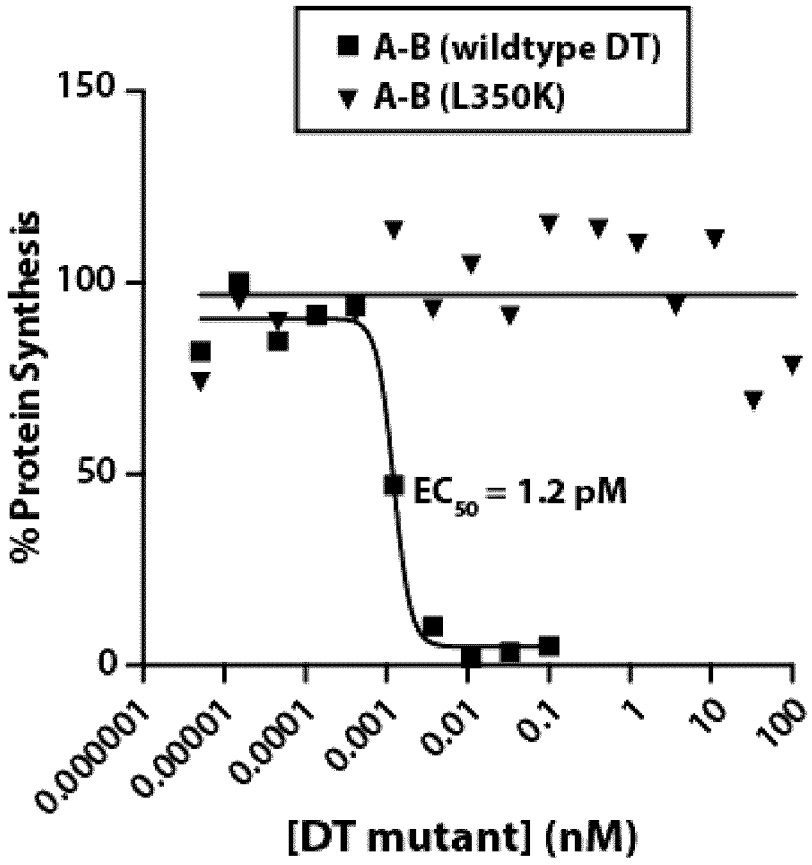

A-B (wildtype DT)
A-B (L350K)
150
100
50
0
% Protein Synthesis
EC50 = 1.2 pM
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
[DT mutant] (nM)

Figure 16

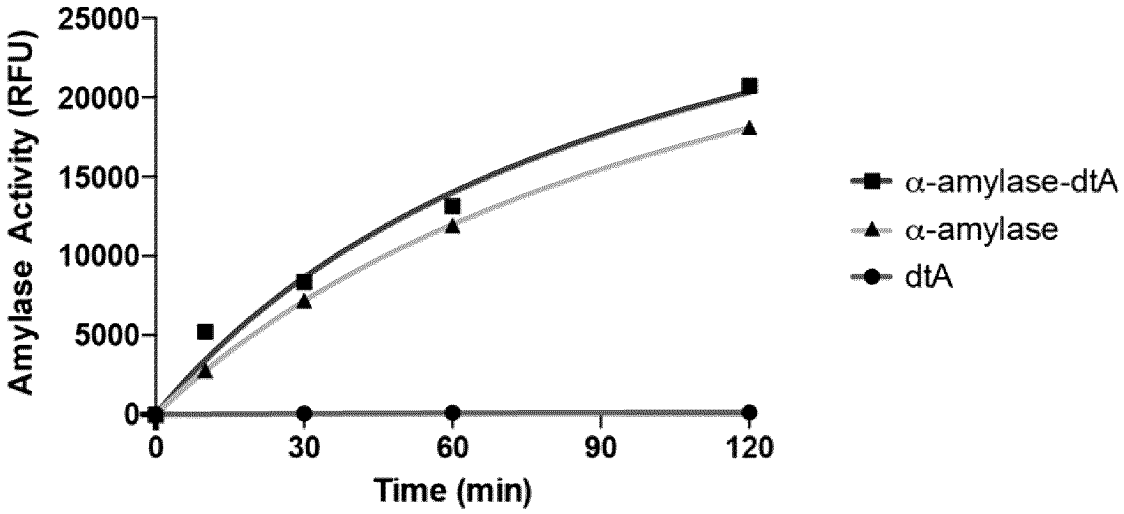


Figure 17

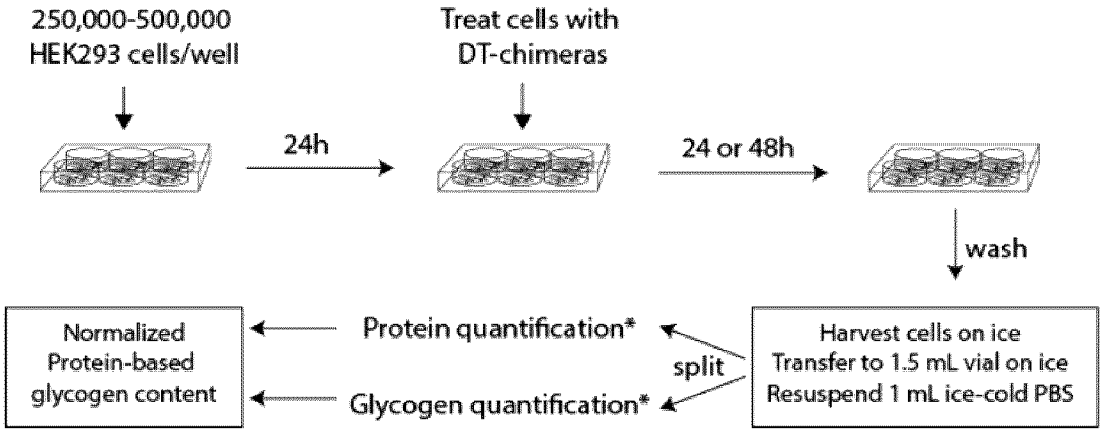


Figure 18

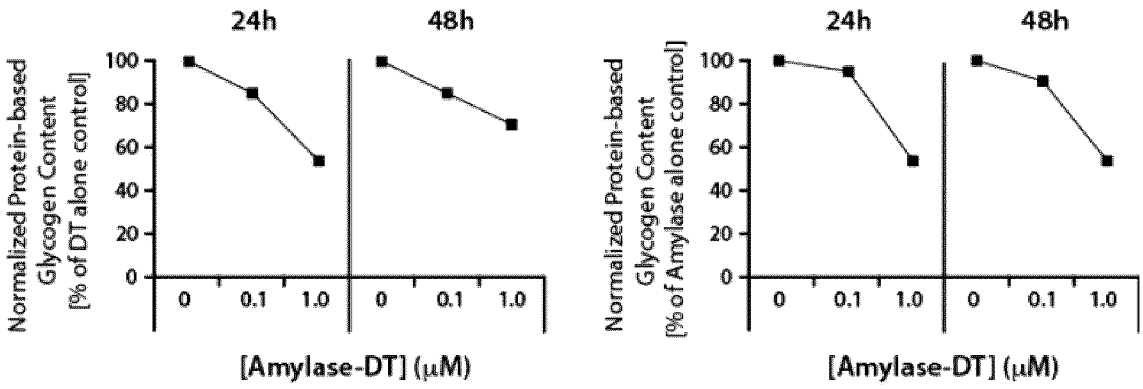


Figure 19

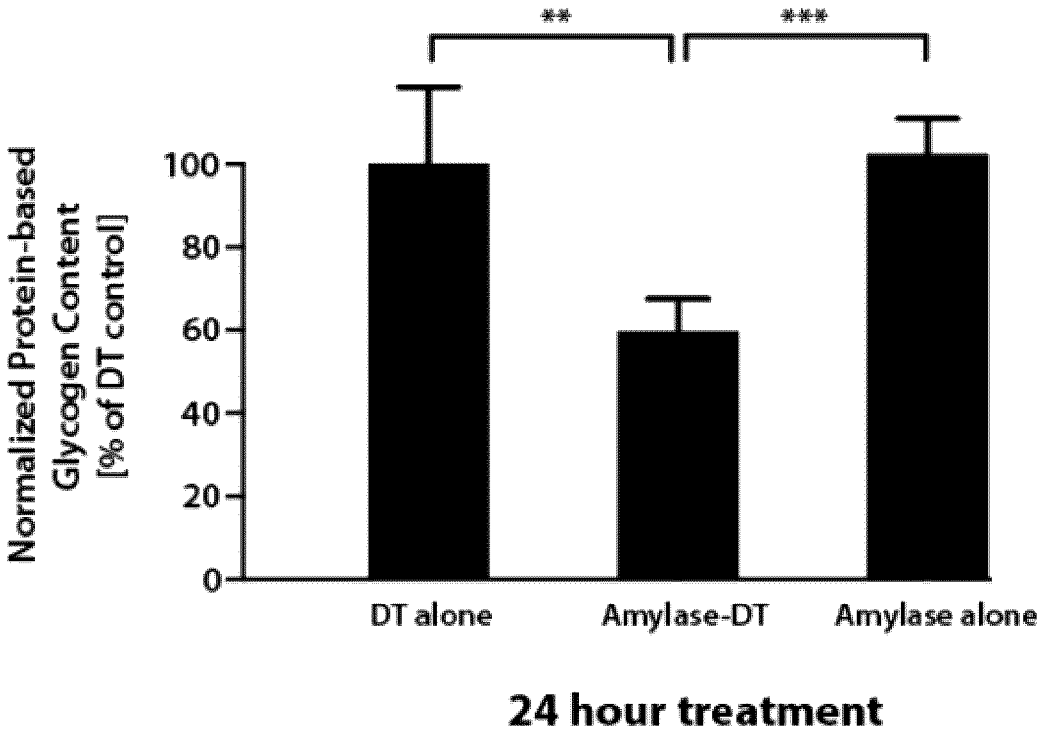


Figure 20

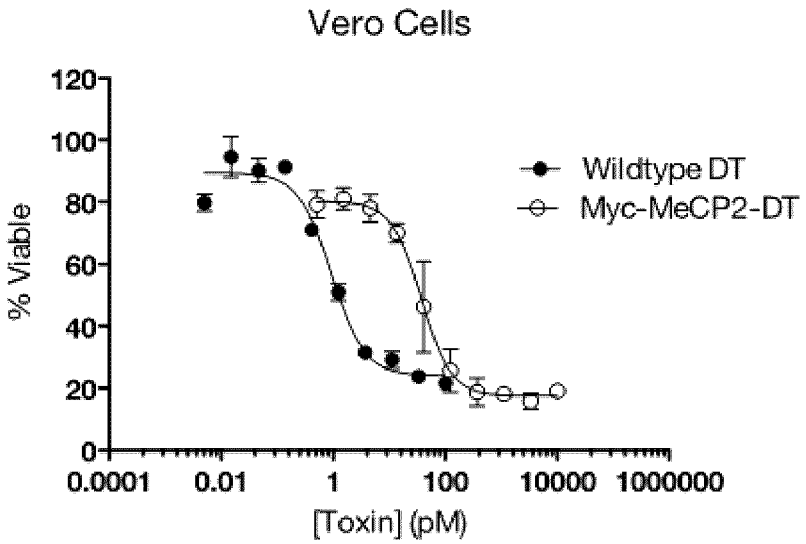


Figure 21

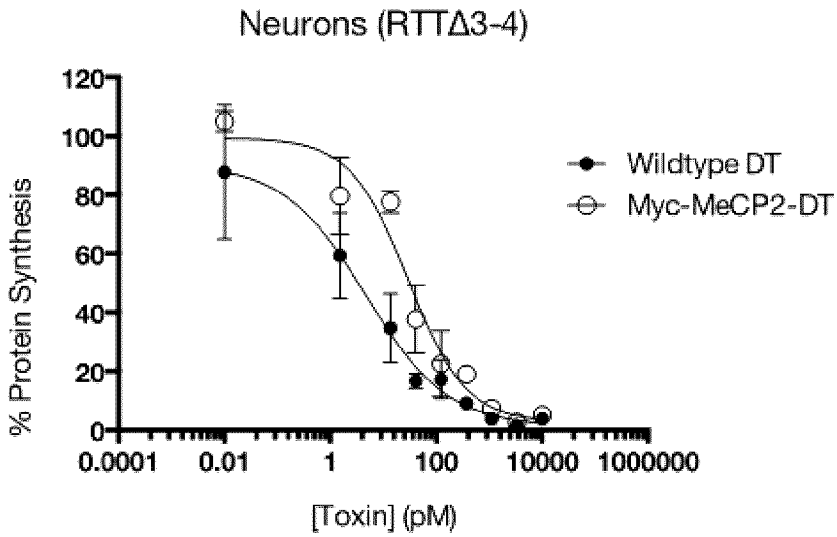


Figure 22

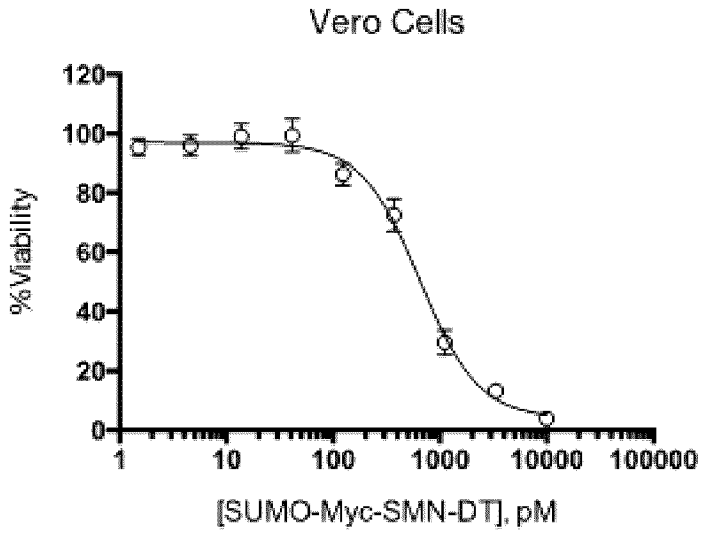


Figure 23

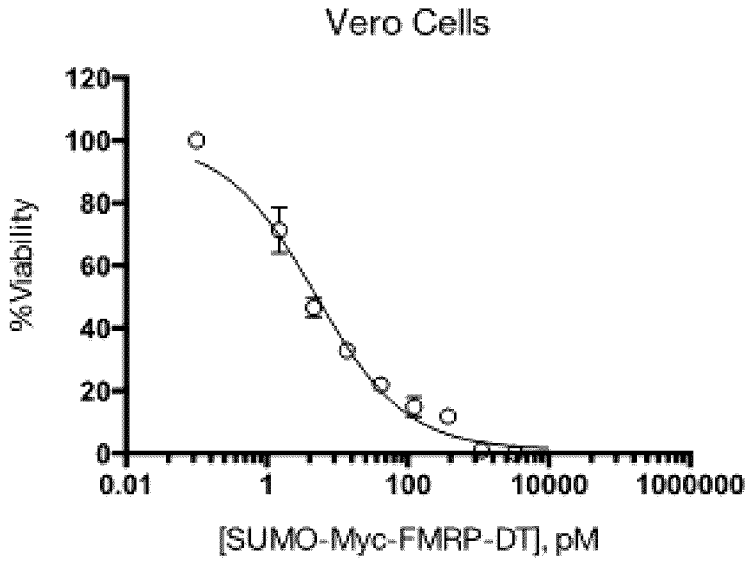


Figure 24

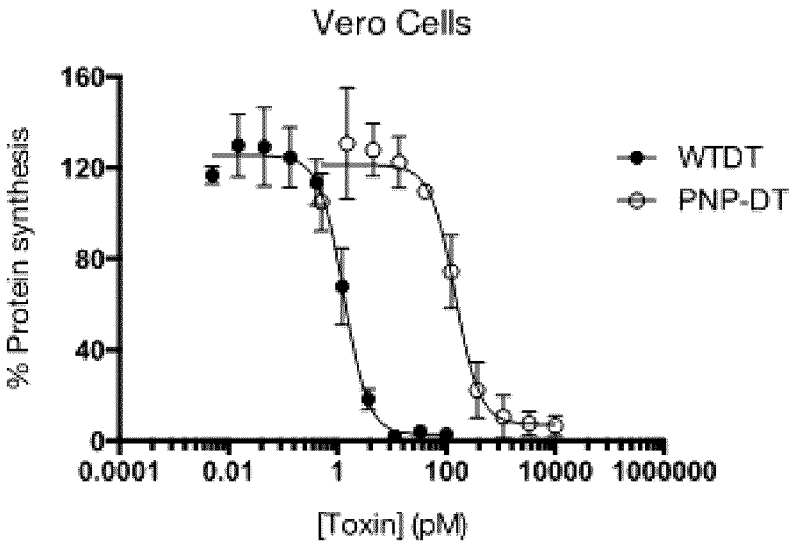


Figure 25

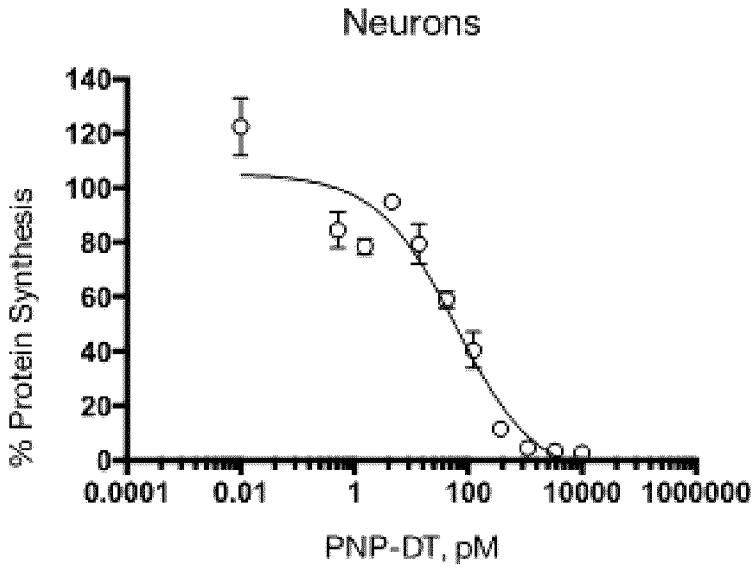


Figure 26

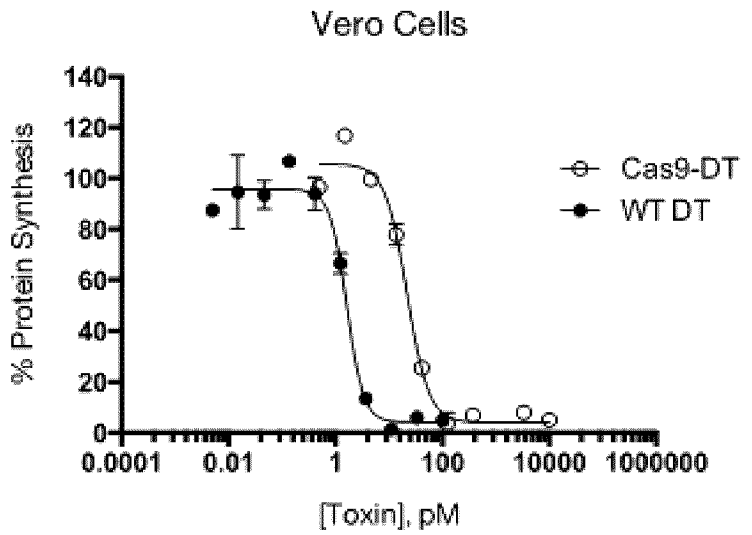


Figure 27

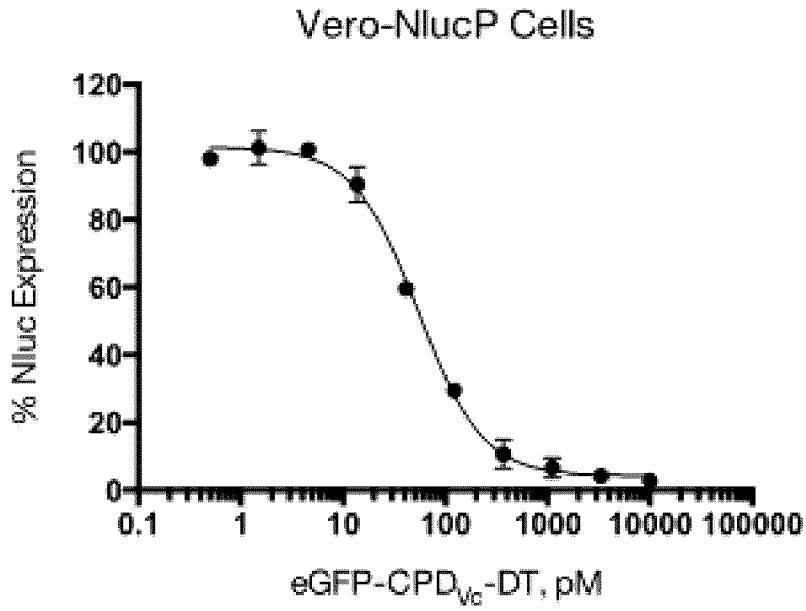


Figure 28

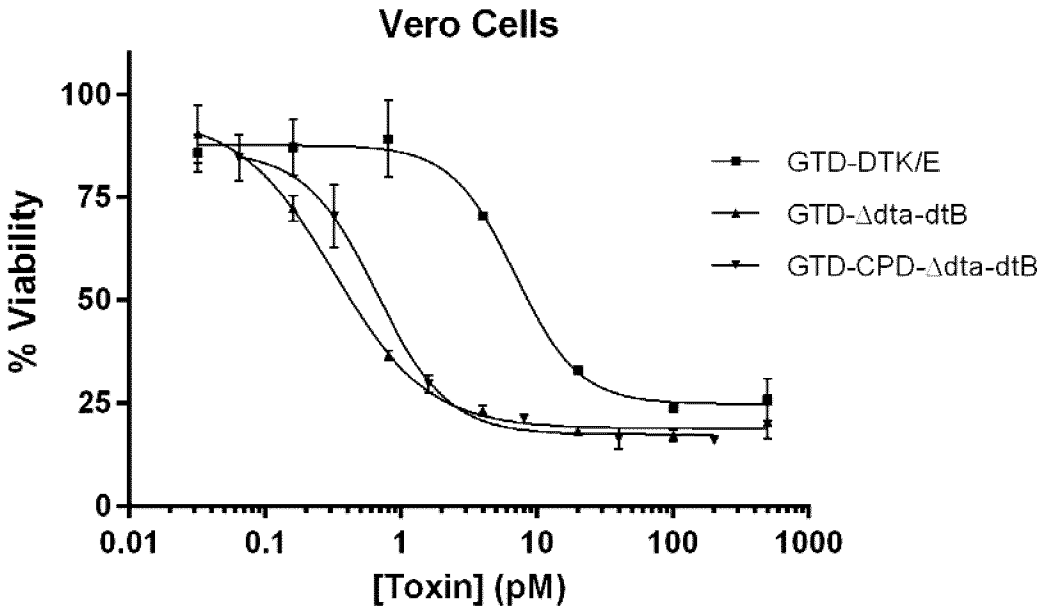

Vero Cells
% Viability
100
75
50
25
0
0.01
0.1
1
10
100
1000
[Toxin] (pM)
GTD-DTK/E
GTD-Δdta-dtB
GTD-CPD-Δdta-dtB

Figure 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2016/050612

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 19/00*** (2006.01) , ***A61K 35/74*** (2015.01) , ***A61K 38/16*** (2006.01) , ***A61K 38/45*** (2006.01) , ***A61K 38/48*** (2006.01) , ***A61K 47/48*** (2006.01) (more IPCs on the last page)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Keywords searched across all IPCs.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Questel-Orbit, Scopus, Pubmed
Keywords: diphtheria, toxin, translocation, blood brain barrier, CRISPR, Cas9, TALEN, ZFN, amylase, FMRP, PNP, SMN, MecP2, Melnyk, Auger, Beilhartz, Minassian, Sugiman-Marangos

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GAILLARD, P.J. et al., "*Diphtheria toxin receptor-targeted brain drug delivery*". International Congress Series, April 2005 (04-2005), Vol. 1277, pp. 185-198, ISSN 05315131 *entire document	1-142
X	WO2011133658 A1 (MURPHY, J. R.) 27 October 2011 (27-10-2011) *Pages 3-6, 12-17, 28, 29, 48, 50-51, Fig. 11	1-142
X	MADSHUS, I.H. et al., "*Membrane translocation of diphtheria toxin carrying passenger protein domains*". Infection and Immunity, August 1992 (08-1992), Vol. 60(8), pp. 3296-3302, ISSN 00199567 *entire document	1-4, 9-31, 42-44, 50-63, 65-71, 77-79, 89, 90, 92-98, 104-106, 116, 117, 119-125, 131-133
P, X	AUGER, A. et al., "*Efficient Delivery of Structurally Diverse Protein Cargo into Mammalian Cells by a Bacterial Toxin*". Molecular Pharmaceutics, 23 June 2015 (23-06-2015), Vol. 12(8), pp. 2962-2971, ISSN 15438384 *entire document	1-142

☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 05 July 2016 (05-07-2016)	Date of mailing of the international search report 08 September 2016 (08-09-2016)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 819-953-2476	Authorized officer Rebecca Minaker (819) 639-6819

CONTINUATION OF CLASSIFICATION OF SUBJECT MATTER

A61P 25/00 (2006.01) , ***C07K 14/34*** (2006.01) , ***C12N 15/09*** (2006.01) , ***C12N 15/62*** (2006.01) , ***C12N 9/10*** (2006.01) , ***C12N 9/48*** (2006.01) , ***C12N 9/52*** (2006.01)

Box No. II **Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claim Nos.: 62-88
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 62-88 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under PCT Rule 39.1(iv). However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 62-88.

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2011133658A1	27 October 2011 (27-10-2011)	None	