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(54) Title: METHODS OF TREATING CANCER USING NEUROTROPHIN RETARGETED ENDOPEPTIDASES

(57) Abstract: The present specification discloses TVEMPs, compositions comprising such TVEMPs and methods of treating cancer in a mammal using such TVEMP compositions.



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Methods of Treating Cancer Using Neurotrophin Retargeted Endopeptidases

[01]This patent application claims priority pursuant to 35 U.S.C. § 119(e) to U. S. Provisional Patent Application Serial No. 61/233,969 filed August 14, 2009, which is hereby incorporated by reference in its entirety.

[02]Cancer is a group of more than 100 diseases in which a group of cells display uncontrolled growth (cell division beyond the normal limits). In most cases, cancer cells form a clump of cells called a tumor, although in some cancers, like leukemia, the cells do not form tumors. Tumors may be malignant or benign. Besides, malignant tumors (or cancers) comprise cells with abnormal genetic material and usually undergo rapid uncontrolled cell growth, invade and destroy adjacent tissue, and sometimes spread to other locations in the body via lymph or blood (*i.e.*, metastasis). Cancer is associated with a high incidence of mortality because if the invasion and metastasis of the cancer cells throughout the body are not stopped, cancer cells will invade vital organs and lead to the dysfunction of the organs and eventual death. The malignant properties of cancers differentiate them from benign tumors, which are usually slow-growing and self-limited, do not invade or metastasize, and as such, are generally not life-threatening. Cancers at the local, regional or distant stage are considered invasive. A very early cancer found in only a few layers of cells, called in situ cancer, is considered non-invasive.

[03]Cancer is a diverse class of diseases which differ widely in their causes and biology. Cancers are caused by a variety of factors working alone or in combination. Some cancers are caused by external factors such as tobacco, diet, certain chemicals, radiation, and viruses. Other cancers are caused by internal factors such as hormones, immune conditions, and inherited genetic mutations. Usually ten or more years pass between exposure to a factor that causes cancer and detectable disease.

[04]Cancers are generally classified by the type of cell that resembles the tumor and, therefore, the tissue presumed to be the origin of the tumor. Carcinomas are malignant tumors derived from epithelial cells. This group represents the most common cancers, including the common forms of breast, prostate, lung and colon cancer. Sarcomas are malignant tumors derived from connective tissue, or mesenchymal cells. Blastomas are usually malignant tumors which resembles an immature or embryonic tissue. Many of these tumors are most common in children. Lymphomas and leukemias are malignancies derived from hematopoietic (blood-forming) cells. Lastly, germ cell tumors are tumors derived from totipotent cells. In adults most often found in the testicle and ovary; in fetuses, babies, and young children most often found on the body midline, particularly at the tip of the tailbone.

[05]Cancer is the second leading cause of death in the U.S., with 1,228,600 new cases and 564,800 deaths estimated for 1998. Over the past 50 years, the death rate from cancer has increased steadily, due mainly to a large rise in lung cancer death rates resulting from smoking. Cancer occurs in people of all ages, but its occurrence increases greatly in people over 45 years of age. However, cancer is the leading cause of death in the United States for people between the ages of 35 and 65 and it is also the

leading cause of non-accidental death among U.S. children under age 15. Men have a higher mortality rate due to cancer than women, and blacks have the highest cancer mortality rate of any major racial group. In the U.S., men have about a 1 in 2 lifetime risk of developing cancer and women have about a 1 in 3 lifetime risk. With the anticipated continued decrease in deaths from heart disease and strokes, cancer will become the overall leading cause of death for the entire American population by the year 2010.

[06]Diagnosis of cancer usually requires a histological examination of a tissue biopsy specimen by a pathologist, although the initial indication of malignancy can be symptoms or radiographic imaging abnormalities. Once diagnosed, cancer is commonly treated by surgery, chemotherapy, radiotherapy, or targeted therapies like immunotherapy, hormonal therapy, or angiogenesis inhibitor therapy. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient (performance status). Furthermore, depending on the type and stage of the cancer, two or more of these types of cancer treatments may be combined at the same time or used after one another. Although complete removal of the cancer without damage to the rest of the body is the goal of treatment, current approaches to treating cancer have met with limited success. With respect to surgery, this is due, in part, to the propensity of individual or small numbers of cancer cells to invade adjacent tissue or metastasis to distant sites, thereby limiting the effectiveness of local surgical treatments. The effectiveness of chemotherapy and radiotherapy is often limited by toxicity to or damage of normal tissues in the body. Although targeted therapies are promising, as implied by their name, these treatments are usually specific for one particular type of cancer. Therefore, compounds and methods that can target all cancer cells, regardless of their location would be highly desirable for the treatment of cancer. In addition, compounds and methods that can target a particular type of cancer for which no current targeted therapy exists would also be highly desirable.

[07]The ability of Clostridial toxins, such as, *e.g.*, Botulinum neurotoxins (BoNTs), BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F and BoNT/G, and Tetanus neurotoxin (TeNT), to inhibit neuronal transmission are being exploited in a wide variety of therapeutic and cosmetic applications, see *e.g.*, William J. Lipham, COSMETIC AND CLINICAL APPLICATIONS OF BOTULINUM TOXIN (Slack, Inc., 2004). Clostridial toxins commercially available as pharmaceutical compositions include, BoNT/A preparations, such as, *e.g.*, BOTOX® (Allergan, Inc., Irvine, CA), DYSPORT®/RELOXIN®, (Beaufour Ipsen, Porton Down, England), NEURONOX® (Medy-Tox, Inc., Ochang-myeon, South Korea) BTX-A (Lanzhou Institute Biological Products, China) and XEOMIN® (Merz Pharmaceuticals, GmbH., Frankfurt, Germany); and BoNT/B preparations, such as, *e.g.*, MYOBLOC™/NEUROBLOC™ (Solstice Neurosciences, Inc. San Francisco, CA). As an example, BOTOX® is currently approved in one or more countries for the following indications: achalasia, adult spasticity, anal fissure, back pain, blepharospasm, bruxism, cervical dystonia, essential tremor, glabellar lines or hyperkinetic facial lines, headache, hemifacial spasm, hyperactivity of bladder, hyperhidrosis, juvenile cerebral palsy, multiple sclerosis, myoclonic disorders, nasal labial lines, spasmodic dysphonia, strabismus and VII nerve disorder.

[08]A Clostridial toxin treatment inhibits neurotransmitter release by disrupting the exocytotic process used to secrete the neurotransmitter into the synaptic cleft. This disruption is ultimately accomplished by intracellular delivery of a Clostridial toxin light chain comprising an enzymatic domain where it cleaves a SNARE protein essential for the exocytotic process. There is a great desire by the pharmaceutical industry to expand the use of Clostridial toxin therapies beyond its current myo-relaxant applications to treat other ailments, such as, e.g., various kinds of sensory nerve-based ailments like chronic pain, neurogenic inflammation and urogenital disorders, as well as non-nerve-based disorders, such as, e.g., pancreatitis and cancer. One approach that is currently being exploited to expand Clostridial toxin-based therapies involves modifying a Clostridial toxin so that the modified toxin has an altered cell targeting capability for a non-Clostridial toxin target cell. This re-targeted capability is achieved by replacing a naturally-occurring targeting domain of a Clostridial toxin with a targeting domain showing a selective binding activity for a non-Clostridial toxin receptor present in a non-Clostridial toxin target cell. Such modifications to a targeting domain result in a modified toxin that is able to selectively bind to a non-Clostridial toxin receptor (target receptor) present on a non-Clostridial toxin target cell (re-targeted). A modified Clostridial toxin with a targeting activity for a non-Clostridial toxin target cell can bind to a receptor present on the non-Clostridial toxin target cell, translocate into the cytoplasm, and exert its proteolytic effect on the SNARE complex of the non-Clostridial toxin target cell. In essence, a Clostridial toxin light chain comprising an enzymatic domain is intracellularly delivered to any desired cell by selecting the appropriate targeting domain.

[09]The present specification discloses a class of modified Clostridial toxins retargeted to a non-Clostridial toxin receptor called Targeted Vesicular Exocytosis Modulating Proteins (TVEMPs), compositions comprising TVEMPs, and methods for treating an individual suffering from a cancer. A TVEMP is a recombinantly produced protein that comprises a targeting domain, and a translocation domain and enzymatic domain of a Clostridial toxin. The targeting is selected for its ability to bind to a receptor present on a target cancer cell of interest. The Clostridial toxin translocation domain and enzymatic domain serve to deliver the enzymatic domain into the cytoplasm of the target cell where it cleaves its cognate SNARE substrate. SNARE protein cleavage disrupts exocytosis, the process of cellular secretion or excretion in which substances contained in intracellular vesicles are discharged from the cell by fusion of the vesicular membrane with the outer cell membrane. This disruption prevents many fundamental processes of the cell, including, without limitation, insertion of transmembrane proteins including cell-surface receptors and signal transduction proteins; transportation of extracellular matrix proteins into the extracellular space; secretion of proteins including growth factors, angiogenic factors, neurotransmitters, hormones, and any other molecules involved in cellular communication; and expulsion of material including waste products, metabolites, and other unwanted or detrimental molecules. As such, exocytosis disruption severely affects cellular metabolism and ultimately cell viability. Thus a therapeutic molecule that reduces or inhibits exocytosis of a cell decreases the ability of a cell to survive. Based on this premise, the TVEMPs disclosed herein are designed to target cancer cells, where

subsequent translocation of the enzymatic domain disrupts exocytosis by SNARE protein cleavage, thereby reducing the ability of a cancer cell to survive.

[010] Thus, aspects of the present invention provide a composition comprising a TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain. TVEMPs useful for the development of such compositions are described in, e.g., Steward, L.E. et al., *Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity For Non-Clostridial Toxin Target Cells*, U.S. Patent Application No. 11/776,075 (Jul. 11, 2007); Dolly, J.O. et al., *Activatable Clostridial Toxins*, U.S. Patent Application No. 11/829,475 (Jul. 27, 2007); Foster, K.A. et al., *Fusion Proteins*, International Patent Publication WO 2006/059093 (Jun. 8, 2006); and Foster, K.A. et al., *Non-Cytotoxic Protein Conjugates*, International Patent Publication WO 2006/059105 (Jun. 8, 2006), each of which is incorporated by reference in its entirety. A composition comprising a TVEMP can be a pharmaceutical composition. Such a pharmaceutical composition can comprise, in addition to a TVEMP, a pharmaceutical carrier, a pharmaceutical component, or both.

[011] Other aspects of the present invention provide a method of treating cancer in a mammal, the method comprising the step of administering to the mammal in need thereof a therapeutically effective amount of a composition including a TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain, wherein administration of the composition reduces a symptom associated with cancer. It is envisioned that any TVEMP disclosed herein can be used, including those disclosed in, e.g., Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); and Foster, *supra*, WO 2006/059105 (Jun. 8, 2006). The disclosed methods provide a safe, inexpensive, out patient-based treatment for the treatment of cancer.

[012] Other aspects of the present invention provide a method of treating cancer in a mammal, the method comprising the step of administering to the mammal in need thereof a therapeutically effective amount of a composition including a TVEMP comprising a targeting domain, a Clostridial toxin translocation domain, a Clostridial toxin enzymatic domain, and an exogenous protease cleavage site, wherein administration of the composition reduces a symptom associated with cancer. It is envisioned that any TVEMP disclosed herein can be used, including those disclosed in, e.g., Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); and Foster, *supra*, WO 2006/059105 (Jun. 8, 2006).

[013] Still other aspects of the present invention provide a use of a TVEMP in the manufacturing a medicament for treating cancer in a mammal in need thereof, wherein the TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain and wherein administration of a therapeutically effective amount of the medicament to the mammal reduces a symptom associated with cancer. It is envisioned that any TVEMP disclosed herein can be used,

including those disclosed in, *e.g.*, Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); and Foster, *supra*, WO 2006/059105 (Jun. 8, 2006).

[014] Still other aspects of the present invention provide a use of a TVEMP in the treatment of cancer in a mammal in need thereof, the use comprising the step of administering to the mammal a therapeutically effective amount of the TVEMP, wherein the TVEMP comprising a targeting domain, a Clostridial toxin translocation domain, a Clostridial toxin enzymatic domain and wherein administration of the TVEMP reduces a symptom associated with cancer. It is envisioned that any TVEMP disclosed herein can be used, including those disclosed in, *e.g.*, Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); and Foster, *supra*, WO 2006/059105 (Jun. 8, 2006).

BRIEF DESCRIPTION OF THE DRAWINGS

[015] FIG. 1 shows a schematic of the current paradigm of neurotransmitter release and Clostridial toxin intoxication in a central and peripheral neuron. FIG. 1A shows a schematic for the neurotransmitter release mechanism of a central and peripheral neuron. The release process can be described as comprising two steps: 1) vesicle docking, where the vesicle-bound SNARE protein of a vesicle containing neurotransmitter molecules associates with the membrane-bound SNARE proteins located at the plasma membrane; and 2) neurotransmitter release, where the vesicle fuses with the plasma membrane and the neurotransmitter molecules are exocytosed. FIG. 1B shows a schematic of the intoxication mechanism for tetanus and botulinum toxin activity in a central and peripheral neuron. This intoxication process can be described as comprising four steps: 1) receptor binding, where a Clostridial toxin binds to a Clostridial receptor system and initiates the intoxication process; 2) complex internalization, where after toxin binding, a vesicle containing the toxin/receptor system complex is endocytosed into the cell; 3) light chain translocation, where multiple events are thought to occur, including, *e.g.*, changes in the internal pH of the vesicle, formation of a channel pore comprising the HN domain of the Clostridial toxin heavy chain, separation of the Clostridial toxin light chain from the heavy chain, and release of the active light chain and 4) enzymatic target modification, where the activate light chain of Clostridial toxin proteolytically cleaves its target SNARE substrate, such as, *e.g.*, SNAP-25, VAMP or Syntaxin, thereby preventing vesicle docking and neurotransmitter release.

[016] FIG. 2 shows the domain organization of naturally-occurring Clostridial toxins. The single-chain form depicts the amino to carboxyl linear organization comprising an enzymatic domain, a translocation domain, and a targeting domain. The di-chain loop region located between the translocation and enzymatic domains is depicted by the double SS bracket. This region comprises an endogenous di-chain loop protease cleavage site that upon proteolytic cleavage with a naturally-occurring protease, such as, *e.g.*, an endogenous Clostridial toxin protease or a naturally-occurring protease produced in the environment, converts the single-chain form of the toxin into the di-chain form. Above the single-chain form, the HCC region of the Clostridial toxin binding domain is depicted. This region comprises the β -

trefoil domain which comprises in an amino to carboxyl linear organization an α -fold, a β 4/ β 5 hairpin turn, a β -fold, a β 8/ β 9 hairpin turn and a γ -fold.

[017] FIG. 3 shows TVEMPs with a targeting domain located at the amino terminus. FIG. 3A depicts the single-chain polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising a targeting domain, a translocation domain, a di-chain loop region comprising an exogenous protease cleavage site (P), and an enzymatic domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form. FIG. 3B depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising a targeting domain, an enzymatic domain, a di-chain loop region comprising an exogenous protease cleavage site (P), and a translocation domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form.

[018] FIG. 4 shows TVEMPs with a targeting domain located between the other two domains. FIG. 4A depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising an enzymatic domain, a di-chain loop region comprising an exogenous protease cleavage site (P), a targeting domain, and a translocation domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form. FIG. 4B depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising a translocation domain, a di-chain loop region comprising an exogenous protease cleavage site (P), a targeting domain, and an enzymatic domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form. FIG. 4C depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising an enzymatic domain, a targeting domain, a di-chain loop region comprising an exogenous protease cleavage site (P), and a translocation domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form. FIG. 4D depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising a translocation domain, a targeting domain, a di-chain loop region comprising an exogenous protease cleavage site (P), and an enzymatic domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form.

[019] FIG. 5 shows TVEMPs with a targeting domain located at the carboxyl terminus. FIG. 5A depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising an enzymatic domain, a di-chain loop region comprising an exogenous protease cleavage site (P), a translocation domain, and a targeting domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form. FIG. 5B depicts the single polypeptide form of a TVEMP with an amino to carboxyl linear organization comprising a translocation domain, a di-chain loop region comprising an exogenous protease cleavage site (P), an enzymatic domain, and a targeting domain. Upon proteolytic cleavage with a P protease, the single-chain form of the toxin is converted to the di-chain form.

DETAILED DESCRIPTION

[020] Cancer refers to the uncontrolled growth of cells in a mammalian body, and as such is fundamentally a disease that affects the regulatory mechanism the body uses to control cell growth. In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered. Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide. The vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: 1) self-sufficiency in growth signals; 2) insensitivity to growth-inhibitory (antigrowth) signals; 3) evasion of programmed cell death (apoptosis); 4) limitless replicative potential; 5) sustained angiogenesis; and 6) tissue invasion and metastasis. Hanahan and Weinberg, *The Hallmarks of Cancer*, Cell 100(1): 57–70 (2000).

[021] One way cancer cells exhibit self-sufficiency in growth signals is by the expression of oncogenes. Oncogenes may be normal genes which are expressed at inappropriately high levels, or altered genes which have novel properties. In either case, expression of these genes promote the malignant phenotype of cell growth exhibited by cancer cells through a variety of ways. Many can produce secreted factors between cells, like hormones, which encourage mitosis, the effect of which depends on the signal transduction of the receiving tissue or cells. Thus, when a hormone receptor on a recipient cell is stimulated, the signal is conducted from the surface of the cell to the cell nucleus to effect some change in gene transcription regulation at the nuclear level. Some oncogenes are part of the signal transduction system itself, or the signal receptors in cells and tissues themselves, thus controlling the sensitivity to such hormones. Oncogenes often produce mitogens, or are involved in transcription of DNA in protein synthesis, which creates the proteins and enzymes responsible for producing the products and biochemicals cells use and interact with. Mutations in proto-oncogenes, which are the normally quiescent counterparts of oncogenes, can modify their expression and function, increasing the amount or activity of the product protein. When this happens, the proto-oncogenes become oncogenes, and this transition upsets the normal balance of cell cycle regulation in the cell, making uncontrolled growth possible. The chance of cancer cannot be reduced by removing proto-oncogenes from the genome, even if this were possible, as they are critical for growth, repair and homeostasis of the organism. It is only when they become mutated that the signals for growth become excessive. Therefore, therapeutic strategies to inhibit cell growth signals in cancer cells have the potential to provide powerful tools to treat cancers exhibiting self-sufficiency in growth signals due to oncogene expression. Moreover, many cancer cells express growth factor receptors and the ligands that activate those receptors (autocrine loops). In normal tissue one type of cell expresses the growth factor receptor and another type the ligand (paracrine loops) in an effort to maintain homeostasis. Cancer cells by expressing ligand and receptor acquire self-sufficiency for growth.

[022] One way that cancer cells display an insensitivity to growth-inhibitory (antigrowth) signals is by the inhibition of expression of tumor suppressor genes. Tumor suppressor genes are genes which inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer-promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell. Generally, tumor suppressors are transcription factors that are activated by cellular stress or DNA damage. Often DNA damage will cause the presence of free-floating genetic material as well as other signs, and will trigger enzymes and pathways which lead to the activation of tumor suppressor genes. The functions of such genes is to arrest the progression of the cell cycle in order to carry out DNA repair, preventing mutations from being passed on to daughter cells. Therefore, therapeutic strategies to inhibit cell division signals in cancer cells have the potential to provide powerful tools to treat cancers displaying insensitivity to growth-inhibitory signals due to the suppression of tumor suppressor gene expression.

[023] One way that cancer cells evade programmed cell death (apoptosis) is by continuous exposure to cell survival signals (antiapoptotic signals). Signals to induce cell survival or cell death are provided by sensors in the plasma membrane (i.e. death receptors) and by intracellular sensors. Intracellular sensors monitor the cell's health and in response to detecting abnormalities like DNA damage, oncogene action, survival factor insufficiency, or hypoxia, they activate the death pathway. Therefore, cancer cells should undergo apoptosis as they have DNA damage, activated oncogene, or hypoxia in the center of the tumor. Several types of cancer cells are dependent on survival signals delivered by autocrine loops to counteract apoptotic signals triggered by DNA damage present in these cells. These autocrine loops are established by cancer cells through the expression of growth factor ligands and their cognate receptors. Therefore, therapeutic strategies to inhibit the reception of cell survival signals by cancer cells have the potential to provide powerful tools to treat cancers with overactivation of antiapoptotic signals. In fact, there is evidence in the literature that hormone and/or growth factor withdraw can produce apoptosis in cancer cells as the balance between survival and apoptotic signals is restored.

[024] Another acquired capability of cancer cells is the limitless replicative potential of the tumor cells. Cancer cells overcome the limits of proliferation by maintaining integrity of the telomeres and avoiding the crisis state that results from continue multiplication that erodes the telomeres. Cancer cells overexpress the enzyme telomerase that maintains the size of the telomeres and allow for limitless replicative potential. But another important step is the ability to deliver membrane to the plasma membrane to complete the mitotic process.

[025] As cells proliferate within a tumor they also face other challenges like the limited supply of oxygen and nutrients that would induce apoptosis. So to be able to sustain growth and proliferation the tumor needs to encourage the growth of existing blood vessels as well as the growth of new blood vessels, a process highly regulated in mature tissues. Cancer cells secrete pro-angiogenic factors to activate receptors in endothelial cells. In addition, pro-angiogenic factors sequestered in the extracellular matrix

can be released by digestion of the matrix performed by proteases secreted by tumor cells. Inhibition of angiogenesis is a validated therapeutic target as several approved drugs target this pathway as a treatment for cancer and other pro-angiogenesis diseases.

[026] Finally, tumor cells acquire the capability to invade adjacent tissues and metastasize to distant sites. To accomplish that, tumor cells may first be able to change their adhesion capabilities by altering the expression of adhesion proteins and integrins. More importantly, to be able to migrate cancer cells need to be able to degrade the extracellular matrix that surround them. Cancer cells overexpress matrix degrading proteases either as secreted factors or as membrane anchored proteases and downregulate the expression of protease inhibitors.

[027] As uncontrolled cell growth is the underlying cause of all cancers, compounds and methods that can reduce or prevent this uncontrolled cell growth would be an effective treatment for cancer. The present specification discloses compounds and methods that can reduce or prevent the uncontrolled cell growth displayed by cancer cells. The novel retargeted endopeptidases comprise, in part, a binding domain and an enzymatic domain. The binding domain directs the retargeted endopeptidase to a specific cancer cell type that is expressing the cognate receptor for the binding domain. The endopeptidase activity of the enzymatic domain inhibits exocytosis by cleaving the appropriate target SNARE protein, thereby disrupting exocytosis and delivery of receptors and membrane to the plasma membrane. Preventing exocytosis in cancers cells is therapeutically useful because disruption would, e.g., 1) prevent the release of secreting growth factors by cancer cells which encourage mitosis; or 2) prevent delivery of receptors to the plasma membrane of cancer cells which would interfere with the cancer cell's ability to receive cancer-promoting signals, such as, e.g., receiving a growth stimulating signal or a cell survival signal. The later would be useful in eliminating cancer cells by tilting the balance towards apoptosis of the cancer cells; 3) prevent delivery of membrane to the plasma membrane and thus stopping the process of mitosis that can only occur with a net gain of membrane to produce daughter cells; 4) reduce angiogenesis by inhibiting the release of pro-angiogenic factors by tumor cells or the extracellular matrix; 5) inhibit invasion and metastasis by inhibiting the release of proteases and by interfering with the switch of adhesion proteins and integrins.

[028] Thus, while current cancer therapeutics in the market target only one pathway at a time and are therefore only partially effective and allow cancer cells to acquire resistance to the treatment, A TEVMP-based therapy by means of inhibition of exocytosis, receptor delivery, and membrane delivery, will target several pathways with a single drug delivering a stronger punch to tumor cells and therefore being more effective. Moreover, as normal cells are not proliferating and are not so depending on survival signals they were not be affected by the therapy.

[029] Aspects of the present invention provide, in part, a TVEMP. As used herein, a "TVEMP" means any molecule comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial

toxin enzymatic domain. Exemplary TVEMPs useful to practice aspects of the present invention are disclosed in, e.g., Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); Foster, *supra*, WO 2006/059105 (Jun. 8, 2006).

[030] Clostridial toxins are each translated as a single chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring protease (FIG. 1). This cleavage occurs within the discrete di-chain loop region created between two cysteine residues that form a disulfide bridge. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by the single disulfide bond and non-covalent interactions between the two chains. The naturally-occurring protease used to convert the single chain molecule into the di-chain is currently not known. In some serotypes, such as, e.g., BoNT/A, the naturally-occurring protease is produced endogenously by the bacteria serotype and cleavage occurs within the cell before the toxin is released into the environment. However, in other serotypes, such as, e.g., BoNT/E, the bacterial strain appears not to produce an endogenous protease capable of converting the single chain form of the toxin into the di-chain form. In these situations, the toxin is released from the cell as a single-chain toxin which is subsequently converted into the di-chain form by a naturally-occurring protease found in the environment.

[031] Each mature di-chain molecule comprises three functionally distinct domains: 1) an enzymatic domain located in the LC that includes a metalloprotease region containing a zinc-dependent endopeptidase activity which specifically targets core components of the neurotransmitter release apparatus; 2) a translocation domain contained within the amino-terminal half of the HC (H_N) that facilitates release of the LC from intracellular vesicles into the cytoplasm of the target cell; and 3) a binding domain found within the carboxyl-terminal half of the HC (H_C) that determines the binding activity and binding specificity of the toxin to the receptor complex located at the surface of the target cell. D. B. Lacy and R. C. Stevens, Sequence Homology and Structural Analysis of the Clostridial Neurotoxins, J. Mol. Biol. 291: 1091-1104 (1999). The H_C domain comprises two distinct structural features of roughly equal size, separated by an α -helix, designated the H_{CN} and H_{CC} subdomains. Table 1 gives approximate boundary regions for each domain and subdomain found in exemplary Clostridial toxins.

Table 1. Clostridial Toxin Reference Sequences and Regions

Toxin	SEQ ID NO:	LC	Di-Chain Loop	H_N	H_C		
					H_{CN}	α -Linker	H_{CC}
BoNT/A	1	M1/P2-L429	C430-C454	I455-I873	I874-N1080	E1081-Q1091	S1092-L1296
BoNT/B	6	M1/P2-M436	C437-C446	I447-I860	L861-S1067	Q1068-Q1078	S1079-E1291
BoNT/C1	11	M1/P2-F436	C437-C453	R454-I868	N869-D1081	G1082-L1092	Q1093-E1291
BoNT/D	13	M1/T2-V436	C437-C450	I451-I864	N865-S1069	N1069-Q1079	I1080-E1276
BoNT/E	15	M1/P2-F411	C412-C426	I427-I847	K848-D1055	E1056-E1066	P1067-K1252

Table 1. Clostridial Toxin Reference Sequences and Regions							
Toxin	SEQ ID NO:	LC	Di-Chain Loop	H _N	H _C		
					H _{CN}	α -Linker	H _{CC}
BoNT/F	18	M1/P2-F428	C429-C445	I446-I865	K866-D1075	K1076-E1086	P1087-E1274
BoNT/G	21	M1/P2-M435	C436-C450	I451-I865	S866-N1075	A1076-Q1086	S1087-E1297
TeNT	22	M1/P2-L438	C439-C467	I468-L881	K882-N1097	P1098-Y1108	L1109-D1315
BaNT	23	M1/P2-L420	C421-C435	I436-I857	I858-D1064	K1065-E1075	P1076-E1268
BuNT	24	M1/P2-F411	C412-C426	I427-I847	K848-D1055	E1056-E1066	P1067-K1251

[032] The binding, translocation, and enzymatic activity of these three functional domains are all necessary for toxicity. While all details of this process are not yet precisely known, the overall cellular intoxication mechanism whereby Clostridial toxins enter a neuron and inhibit neurotransmitter release is similar, regardless of serotype or subtype. Although the applicants have no wish to be limited by the following description, the intoxication mechanism can be described as comprising at least four steps: 1) receptor binding, 2) complex internalization, 3) light chain translocation, and 4) enzymatic target modification (FIG. 3). The process is initiated when the H_C domain of a Clostridial toxin binds to a toxin-specific receptor system located on the plasma membrane surface of a target cell. The binding specificity of a receptor complex is thought to be achieved, in part, by specific combinations of gangliosides and protein receptors that appear to distinctly comprise each Clostridial toxin receptor complex. Once bound, the toxin/receptor complexes are internalized by endocytosis and the internalized vesicles are sorted to specific intracellular routes. The translocation step appears to be triggered by the acidification of the vesicle compartment. This process seems to initiate two important pH-dependent structural rearrangements that increase hydrophobicity and promote formation di-chain form of the toxin. Once activated, light chain endopeptidase of the toxin is released from the intracellular vesicle into the cytosol where it appears to specifically target one of three known core components of the neurotransmitter release apparatus. These core proteins, vesicle-associated membrane protein (VAMP)/synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25) and Syntaxin, are necessary for synaptic vesicle docking and fusion at the nerve terminal and constitute members of the soluble *N*-ethylmaleimide-sensitive factor-attachment protein-receptor (SNARE) family. BoNT/A and BoNT/E cleave SNAP-25 in the carboxyl-terminal region, releasing a nine or twenty-six amino acid segment, respectively, and BoNT/C1 also cleaves SNAP-25 near the carboxyl-terminus. The botulinum serotypes BoNT/B, BoNT/D, BoNT/F and BoNT/G, and tetanus toxin, act on the conserved central portion of VAMP, and release the amino-terminal portion of VAMP into the cytosol. BoNT/C1 cleaves syntaxin at a single site near the cytosolic membrane surface. The selective proteolysis of synaptic SNAREs accounts for the block of neurotransmitter release caused by Clostridial toxins *in vivo*. The SNARE protein targets of Clostridial toxins are common to exocytosis in a variety of non-neuronal types; in these cells, as in neurons, light chain peptidase activity inhibits exocytosis, see, e.g., Yann Humeau et al., *How Botulinum and Tetanus Neurotoxins Block Neurotransmitter Release*, 82(5) *Biochimie*. 427-446 (2000); Kathryn Turton et al., *Botulinum and Tetanus Neurotoxins: Structure, Function and Therapeutic Utility*, 27(11) *Trends Biochem.*

Sci. 552-558. (2002); Giovanna Lalli et al., *The Journey of Tetanus and Botulinum Neurotoxins in Neurons*, 11(9) Trends Microbiol. 431-437, (2003).

[033] Aspects of the present specification provide, in part, a TVEMP comprising a Clostridial toxin enzymatic domain. As used herein, the term "Clostridial toxin enzymatic domain" refers to any Clostridial toxin polypeptide that can execute the enzymatic target modification step of the intoxication process. Thus, a Clostridial toxin enzymatic domain specifically targets a Clostridial toxin substrate and encompasses the proteolytic cleavage of a Clostridial toxin substrate, such as, e.g., SNARE proteins like a SNAP-25 substrate, a VAMP substrate, and a Syntaxin substrate. Non-limiting examples of a Clostridial toxin enzymatic domain include, e.g., a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, and a BuNT enzymatic domain.

[034] A Clostridial toxin enzymatic domain includes, without limitation, naturally occurring Clostridial toxin enzymatic domain variants, such as, e.g., Clostridial toxin enzymatic domain isoforms and Clostridial toxin enzymatic domain subtypes; and non-naturally occurring Clostridial toxin enzymatic domain variants, such as, e.g., conservative Clostridial toxin enzymatic domain variants, non-conservative Clostridial toxin enzymatic domain variants, active Clostridial toxin enzymatic domain fragments thereof, or any combination thereof.

[035] As used herein, the term "Clostridial toxin enzymatic domain variant," whether naturally-occurring or non-naturally-occurring, refers to a Clostridial toxin enzymatic domain that has at least one amino acid change from the corresponding region of the disclosed reference sequences (Table 1) and can be described in percent identity to the corresponding region of that reference sequence. Unless expressly indicated, Clostridial toxin enzymatic domain variants useful to practice disclosed embodiments are variants that execute the enzymatic target modification step of the intoxication process. As non-limiting examples, a BoNT/A enzymatic domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 1/2-429 of SEQ ID NO: 1; a BoNT/B enzymatic domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 1/2-436 of SEQ ID NO: 6; a BoNT/C1 enzymatic domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 1/2-436 of SEQ ID NO: 11; a BoNT/D enzymatic domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 1/2-436 of SEQ ID NO: 13; a BoNT/E enzymatic domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 1/2-411 of SEQ ID NO: 15; a BoNT/F enzymatic domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 1/2-428 of SEQ ID NO: 18; a BoNT/G

enzymatic domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 1/2-438 of SEQ ID NO: 21; a TeNT enzymatic domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 1/2-438 of SEQ ID NO: 22; a BaNT enzymatic domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 1/2-420 of SEQ ID NO: 23; and a BuNT enzymatic domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 1/2-411 of SEQ ID NO: 24.

[036] It is recognized by those of skill in the art that within each serotype of Clostridial toxin there can be naturally occurring Clostridial toxin enzymatic domain variants that differ somewhat in their amino acid sequence, and also in the nucleic acids encoding these proteins. For example, there are presently five BoNT/A subtypes, BoNT/A1, BoNT/A2, BoNT/A3, BoNT/A4, and BoNT/A5, with specific enzymatic domain subtypes showing about 80% to 95% amino acid identity when compared to the BoNT/A enzymatic domain of SEQ ID NO: 1. As used herein, the term “naturally occurring Clostridial toxin enzymatic domain variant” refers to any Clostridial toxin enzymatic domain produced by a naturally-occurring process, including, without limitation, Clostridial toxin enzymatic domain isoforms produced from alternatively-spliced transcripts, Clostridial toxin enzymatic domain isoforms produced by spontaneous mutation and Clostridial toxin enzymatic domain subtypes. A naturally occurring Clostridial toxin enzymatic domain variant can function in substantially the same manner as the reference Clostridial toxin enzymatic domain on which the naturally occurring Clostridial toxin enzymatic domain variant is based, and can be substituted for the reference Clostridial toxin enzymatic domain in any aspect of the present specification.

[037] A non-limiting examples of a naturally occurring Clostridial toxin enzymatic domain variant is a Clostridial toxin enzymatic domain isoform such as, *e.g.*, a BoNT/A enzymatic domain isoform, a BoNT/B enzymatic domain isoform, a BoNT/C1 enzymatic domain isoform, a BoNT/D enzymatic domain isoform, a BoNT/E enzymatic domain isoform, a BoNT/F enzymatic domain isoform, a BoNT/G enzymatic domain isoform, a TeNT enzymatic domain isoform, a BaNT enzymatic domain isoform, and a BuNT enzymatic domain isoform. Another non-limiting examples of a naturally occurring Clostridial toxin enzymatic domain variant is a Clostridial toxin enzymatic domain subtype such as, *e.g.*, an enzymatic domain from subtype BoNT/A1, BoNT/A2, BoNT/A3, BoNT/A4, or BoNT/A5; an enzymatic domain from subtype BoNT/B1, BoNT/B2, BoNT/Bbv, or BoNT/Bnp; an enzymatic domain from subtype BoNT/C1-1 or BoNT/C1-2; an enzymatic domain from subtype BoNT/E1, BoNT/E2 and BoNT/E3; an enzymatic domain from subtype BoNT/F1, BoNT/F2, or BoNT/F3; and an enzymatic domain from subtype BuNT-1 or BuNT-2.

[038] As used herein, the term “non-naturally occurring Clostridial toxin enzymatic domain variant” refers to any Clostridial toxin enzymatic domain produced with the aid of human manipulation, including,

without limitation, Clostridial toxin enzymatic domains produced by genetic engineering using random mutagenesis or rational design and Clostridial toxin enzymatic domains produced by chemical synthesis. Non-limiting examples of non-naturally occurring Clostridial toxin enzymatic domain variants include, *e.g.*, conservative Clostridial toxin enzymatic domain variants, non-conservative Clostridial toxin enzymatic domain variants, Clostridial toxin enzymatic domain chimeric variants, and active Clostridial toxin enzymatic domain fragments.

[039] As used herein, the term “conservative Clostridial toxin enzymatic domain variant” refers to a Clostridial toxin enzymatic domain that has at least one amino acid substituted by another amino acid or an amino acid analog that has at least one property similar to that of the original amino acid from the reference Clostridial toxin enzymatic domain sequence (Table 1). Examples of properties include, without limitation, similar size, topography, charge, hydrophobicity, hydrophilicity, lipophilicity, covalent-bonding capacity, hydrogen-bonding capacity, a physicochemical property, of the like, or any combination thereof. A conservative Clostridial toxin enzymatic domain variant can function in substantially the same manner as the reference Clostridial toxin enzymatic domain on which the conservative Clostridial toxin enzymatic domain variant is based, and can be substituted for the reference Clostridial toxin enzymatic domain in any aspect of the present specification. Non-limiting examples of a conservative Clostridial toxin enzymatic domain variant include, *e.g.*, conservative BoNT/A enzymatic domain variants, conservative BoNT/B enzymatic domain variants, conservative BoNT/C1 enzymatic domain variants, conservative BoNT/D enzymatic domain variants, conservative BoNT/E enzymatic domain variants, conservative BoNT/F enzymatic domain variants, conservative BoNT/G enzymatic domain variants, conservative TeNT enzymatic domain variants, conservative BaNT enzymatic domain variants, and conservative BuNT enzymatic domain variants.

[040] As used herein, the term “non-conservative Clostridial toxin enzymatic domain variant” refers to a Clostridial toxin enzymatic domain in which 1) at least one amino acid is deleted from the reference Clostridial toxin enzymatic domain on which the non-conservative Clostridial toxin enzymatic domain variant is based; 2) at least one amino acid added to the reference Clostridial toxin enzymatic domain on which the non-conservative Clostridial toxin enzymatic domain is based; or 3) at least one amino acid is substituted by another amino acid or an amino acid analog that does not share any property similar to that of the original amino acid from the reference Clostridial toxin enzymatic domain sequence (Table 1). A non-conservative Clostridial toxin enzymatic domain variant can function in substantially the same manner as the reference Clostridial toxin enzymatic domain on which the non-conservative Clostridial toxin enzymatic domain variant is based, and can be substituted for the reference Clostridial toxin enzymatic domain in any aspect of the present specification. Non-limiting examples of a non-conservative Clostridial toxin enzymatic domain variant include, *e.g.*, non-conservative BoNT/A enzymatic domain variants, non-conservative BoNT/B enzymatic domain variants, non-conservative BoNT/C1 enzymatic domain variants, non-conservative BoNT/D enzymatic domain variants, non-conservative BoNT/E enzymatic domain variants, non-conservative BoNT/F enzymatic domain variants, non-

conservative BoNT/G enzymatic domain variants, and non-conservative TeNT enzymatic domain variants, non-conservative BaNT enzymatic domain variants, and non-conservative BuNT enzymatic domain variants.

[041] As used herein, the term “active Clostridial toxin enzymatic domain fragment” refers to any of a variety of Clostridial toxin fragments comprising the enzymatic domain can be useful in aspects of the present specification with the proviso that these enzymatic domain fragments can specifically target the core components of the neurotransmitter release apparatus and thus participate in executing the overall cellular mechanism whereby a Clostridial toxin proteolytically cleaves a substrate. The enzymatic domains of Clostridial toxins are approximately 420-460 amino acids in length and comprise an enzymatic domain (Table 1). Research has shown that the entire length of a Clostridial toxin enzymatic domain is not necessary for the enzymatic activity of the enzymatic domain. As a non-limiting example, the first eight amino acids of the BoNT/A enzymatic domain are not required for enzymatic activity. As another non-limiting example, the first eight amino acids of the TeNT enzymatic domain are not required for enzymatic activity. Likewise, the carboxyl-terminus of the enzymatic domain is not necessary for activity. As a non-limiting example, the last 32 amino acids of the BoNT/A enzymatic domain are not required for enzymatic activity. As another non-limiting example, the last 31 amino acids of the TeNT enzymatic domain are not required for enzymatic activity. Thus, aspects of this embodiment include Clostridial toxin enzymatic domains comprising an enzymatic domain having a length of, e.g., at least 350, 375, 400, 425, or 450 amino acids. Other aspects of this embodiment include Clostridial toxin enzymatic domains comprising an enzymatic domain having a length of, e.g., at most 350, 375, 400, 425, or 450 amino acids.

[042] Any of a variety of sequence alignment methods can be used to determine percent identity, including, without limitation, global methods, local methods and hybrid methods, such as, e.g., segment approach methods. Protocols to determine percent identity are routine procedures within the scope of one skilled in the art and from the teaching herein.

[043] Global methods align sequences from the beginning to the end of the molecule and determine the best alignment by adding up scores of individual residue pairs and by imposing gap penalties. Non-limiting methods include, e.g., CLUSTAL W, see, e.g., Julie D. Thompson et al., *CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice*, 22(22) Nucleic Acids Research 4673-4680 (1994); and iterative refinement, see, e.g., Osamu Gotoh, *Significant Improvement in Accuracy of Multiple Protein Sequence Alignments by Iterative Refinement as Assessed by Reference to Structural Alignments*, 264(4) J. Mol. Biol. 823-838 (1996).

[044] Local methods align sequences by identifying one or more conserved motifs shared by all of the input sequences. Non-limiting methods include, e.g., Match-box, see, e.g., Eric Depiereux and Ernest Feytmans, *Match-Box: A Fundamentally New Algorithm for the Simultaneous Alignment of Several*

Protein Sequences, 8(5) CABIOS 501-509 (1992); Gibbs sampling, see, e.g., C. E. Lawrence et al., *Detecting Subtle Sequence Signals: A Gibbs Sampling Strategy for Multiple Alignment*, 262(5131) *Science* 208-214 (1993); Align-M, see, e.g., Ivo Van Walle et al., *Align-M – A New Algorithm for Multiple Alignment of Highly Divergent Sequences*, 20(9) *Bioinformatics*,:1428-1435 (2004).

[045] Hybrid methods combine functional aspects of both global and local alignment methods. Non-limiting methods include, e.g., segment-to-segment comparison, see, e.g., Burkhard Morgenstern et al., *Multiple DNA and Protein Sequence Alignment Based On Segment-To-Segment Comparison*, 93(22) *Proc. Natl. Acad. Sci. U.S.A.* 12098-12103 (1996); T-Coffee, see, e.g., Cédric Notredame et al., *T-Coffee: A Novel Algorithm for Multiple Sequence Alignment*, 302(1) *J. Mol. Biol.* 205-217 (2000); MUSCLE, see, e.g., Robert C. Edgar, *MUSCLE: Multiple Sequence Alignment With High Score Accuracy and High Throughput*, 32(5) *Nucleic Acids Res.* 1792-1797 (2004); and DIALIGN-T, see, e.g., Amarendran R Subramanian et al., *DIALIGN-T: An Improved Algorithm for Segment-Based Multiple Sequence Alignment*, 6(1) *BMC Bioinformatics* 66 (2005).

[046] The present specification describes various polypeptide variants where one amino acid is substituted for another, such as, e.g., Clostridial toxin enzymatic domain variants, Clostridial toxin translocation domain variants, targeting domain variants, and protease cleavage site variants. A substitution can be assessed by a variety of factors, such as, e.g., the physic properties of the amino acid being substituted (Table 2) or how the original amino acid would tolerate a substitution (Table 3). The selections of which amino acid can be substituted for another amino acid in a polypeptide are known to a person of ordinary skill in the art.

TABLE 2. Amino Acid Properties	
Property	Amino Acids
Aliphatic	G, A, I, L, M, P, V
Aromatic	F, H, W, Y
C-beta branched	I, V, T
Hydrophobic	C, F, I, L, M, V, W
Small polar	D, N, P
Small non-polar	A, C, G, S, T
Large polar	E, H, K, Q, R, W, Y
Large non-polar	F, I, L, M, V
Charged	D, E, H, K, R
Uncharged	C, S, T
Negative	D, E
Positive	H, K, R
Acidic	D, E
Basic	K, R
Amide	N, Q

TABLE 3. Amino Acid Substitutions			
Amino Acid	Favored Substitution	Neutral Substitutions	Disfavored substitution
A	G, S, T	C, E, I, K, M, L, P, Q, R, V	D, F, H, N, Y, W
C	F, S, Y, W	A, H, I, M, L, T, V	D, E, G, K, N, P, Q, R
D	E, N	G, H, K, P, Q, R, S, T	A, C, I, L,
E	D, K, Q	A, H, N, P, R, S, T	C, F, G, I, L, M, V, W, Y
F	M, L, W, Y	C, I, V	A, D, E, G, H, K, N, P, Q, R, S, T
G	A, S	D, K, N, P, Q, R	C, E, F, H, I, L, M, T, V, W, Y
H	N, Y	C, D, E, K, Q, R, S, T, W	A, F, G, I, L, M, P, V
I	V, L, M	A, C, T, F, Y	D, E, G, H, K, N, P, Q, R, S, W
K	Q, E, R	A, D, G, H, M, N, P, S, T	C, F, I, L, V, W, Y
L	F, I, M, V	A, C, W, Y	D, E, G, H, K, N, P, Q, R, S, T
M	F, I, L, V	A, C, R, Q, K, T, W, Y	D, E, G, H, N, P, S
N	D, H, S	E, G, K, Q, R, T	A, C, F, I, L, M, P, V, W, Y
P	—	A, D, E, G, K, Q, R, S, T	C, F, H, I, L, M, N, V, W, Y
Q	E, K, R	A, D, G, H, M, N, P, S, T	C, F, I, L, V, W, Y
R	K, Q	A, D, E, G, H, M, N, P, S, T	C, F, I, L, V, W, Y
S	A, N, T	C, D, E, G, H, K, P, Q, R, T	F, I, L, M, V, W, Y
T	S	A, C, D, E, H, I, K, M, N, P, Q, R, V	F, G, L, W, Y
V	I, L, M	A, C, F, T, Y	D, E, G, H, K, N, P, Q, R, S, W
W	F, Y	H, L, M	A, C, D, E, G, I, K, N, P, Q, R, S, T, V
Y	F, H, W	C, I, L, M, V	A, D, E, G, K, N, P, Q, R, S, T

Matthew J. Betts and Robert, B. Russell, Amino Acid Properties and Consequences of Substitutions, pp. 289-316, In Bioinformatics for Geneticists, (eds Michael R. Barnes, Ian C. Gray, Wiley, 2003).

[047] Thus, in an embodiment, a TVEMP disclosed herein comprises a Clostridial toxin enzymatic domain. In an aspect of this embodiment, a Clostridial toxin enzymatic domain comprises a naturally occurring Clostridial toxin enzymatic domain variant, such as, *e.g.*, a Clostridial toxin enzymatic domain isoform or a Clostridial toxin enzymatic domain subtype. In another aspect of this embodiment, a Clostridial toxin enzymatic domain comprises a non-naturally occurring Clostridial toxin enzymatic domain variant, such as, *e.g.*, a conservative Clostridial toxin enzymatic domain variant, a non-conservative Clostridial toxin enzymatic domain variant, an active Clostridial toxin enzymatic domain fragment, or any combination thereof.

[048] In another embodiment, a hydrophobic amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another hydrophobic amino acid. Examples of hydrophobic amino acids include, *e.g.*, C, F, I, L, M, V and W. In another aspect of this embodiment, an aliphatic amino acid at one particular position in the polypeptide chain of the Clostridial

toxin enzymatic domain can be substituted with another aliphatic amino acid. Examples of aliphatic amino acids include, *e.g.*, A, I, L, P, and V. In yet another aspect of this embodiment, an aromatic amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another aromatic amino acid. Examples of aromatic amino acids include, *e.g.*, F, H, W and Y. In still another aspect of this embodiment, a stacking amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another stacking amino acid. Examples of stacking amino acids include, *e.g.*, F, H, W and Y. In a further aspect of this embodiment, a polar amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another polar amino acid. Examples of polar amino acids include, *e.g.*, D, E, K, N, Q, and R. In a further aspect of this embodiment, a less polar or indifferent amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another less polar or indifferent amino acid. Examples of less polar or indifferent amino acids include, *e.g.*, A, H, G, P, S, T, and Y. In a yet further aspect of this embodiment, a positive charged amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another positive charged amino acid. Examples of positive charged amino acids include, *e.g.*, K, R, and H. In a still further aspect of this embodiment, a negative charged amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another negative charged amino acid. Examples of negative charged amino acids include, *e.g.*, D and E. In another aspect of this embodiment, a small amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another small amino acid. Examples of small amino acids include, *e.g.*, A, D, G, N, P, S, and T. In yet another aspect of this embodiment, a C-beta branching amino acid at one particular position in the polypeptide chain of the Clostridial toxin enzymatic domain can be substituted with another C-beta branching amino acid. Examples of C-beta branching amino acids include, *e.g.*, I, T and V.

[049] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/A enzymatic domain. In an aspect of this embodiment, a BoNT/A enzymatic domain comprises the enzymatic domains of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In other aspects of this embodiment, a BoNT/A enzymatic domain comprises amino acids 1/2-429 of SEQ ID NO: 1. In another aspect of this embodiment, a BoNT/A enzymatic domain comprises a naturally occurring BoNT/A enzymatic domain variant, such as, *e.g.*, an enzymatic domain from a BoNT/A isoform or an enzymatic domain from a BoNT/A subtype. In another aspect of this embodiment, a BoNT/A enzymatic domain comprises a naturally occurring BoNT/A enzymatic domain variant of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5, such as, *e.g.*, a BoNT/A isoform enzymatic domain or a BoNT/A subtype enzymatic domain. In another aspect of this embodiment, a BoNT/A enzymatic domain comprises amino acids 1/2-429 of a naturally occurring BoNT/A enzymatic domain variant of SEQ ID NO: 1, such as, *e.g.*, a BoNT/A isoform enzymatic domain or a BoNT/A subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/A enzymatic domain comprises a non-naturally occurring BoNT/A enzymatic domain variant, such as, *e.g.*, a conservative BoNT/A enzymatic domain variant, a

non-conservative BoNT/A enzymatic domain variant, an active BoNT/A enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/A enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/A enzymatic domain variant of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5, such as, e.g., a conservative BoNT/A enzymatic domain variant, a non-conservative BoNT/A enzymatic domain variant, an active BoNT/A enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/A enzymatic domain comprises amino acids 1/2-429 of a non-naturally occurring BoNT/A enzymatic domain variant of SEQ ID NO: 1, such as, e.g., a conservative BoNT/A enzymatic domain variant, a non-conservative BoNT/A enzymatic domain variant, an active BoNT/A enzymatic domain fragment, or any combination thereof.

[050] In other aspects of this embodiment, a BoNT/A enzymatic domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In yet other aspects of this embodiment, a BoNT/A enzymatic domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-429 of SEQ ID NO: 1; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-429 of SEQ ID NO: 1.

[051] In other aspects of this embodiment, a BoNT/A enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In yet other aspects of this embodiment, a BoNT/A enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-429 of SEQ ID NO: 1; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-429 of SEQ ID NO: 1. In still other aspects of this embodiment, a BoNT/A enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In further other aspects of this embodiment, a BoNT/A enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions,

and/or substitutions relative to amino acids 1/2-429 of SEQ ID NO: 1; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-429 of SEQ ID NO: 1.

[052] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/B enzymatic domain. In an aspect of this embodiment, a BoNT/B enzymatic domain comprises the enzymatic domains of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In other aspects of this embodiment, a BoNT/B enzymatic domain comprises amino acids 1/2-436 of SEQ ID NO: 6. In another aspect of this embodiment, a BoNT/B enzymatic domain comprises a naturally occurring BoNT/B enzymatic domain variant, such as, *e.g.*, an enzymatic domain from a BoNT/B isoform or an enzymatic domain from a BoNT/B subtype. In another aspect of this embodiment, a BoNT/B enzymatic domain comprises a naturally occurring BoNT/B enzymatic domain variant of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10, such as, *e.g.*, a BoNT/B isoform enzymatic domain or a BoNT/B subtype enzymatic domain. In another aspect of this embodiment, a BoNT/B enzymatic domain comprises amino acids 1/2-436 of a naturally occurring BoNT/B enzymatic domain variant of SEQ ID NO: 6, such as, *e.g.*, a BoNT/B isoform enzymatic domain or a BoNT/B subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/B enzymatic domain comprises a non-naturally occurring BoNT/B enzymatic domain variant, such as, *e.g.*, a conservative BoNT/B enzymatic domain variant, a non-conservative BoNT/B enzymatic domain variant, an active BoNT/B enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/B enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/B enzymatic domain variant of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10, such as, *e.g.*, a conservative BoNT/B enzymatic domain variant, a non-conservative BoNT/B enzymatic domain variant, an active BoNT/B enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/B enzymatic domain comprises amino acids 1/2-436 of a non-naturally occurring BoNT/B enzymatic domain variant of SEQ ID NO: 6, such as, *e.g.*, a conservative BoNT/B enzymatic domain variant, a non-conservative BoNT/B enzymatic domain variant, an active BoNT/B enzymatic domain fragment, or any combination thereof.

[053] In other aspects of this embodiment, a BoNT/B enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In yet other aspects of this embodiment, a BoNT/B enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-436 of SEQ ID NO: 6; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-436 of SEQ ID NO: 6.

[054] In other aspects of this embodiment, a BoNT/B enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In yet other aspects of this embodiment, a BoNT/B enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 6; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 6. In still other aspects of this embodiment, a BoNT/B enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In further other aspects of this embodiment, a BoNT/B enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 6; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 6.

[055] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/C1 enzymatic domain. In an aspect of this embodiment, a BoNT/C1 enzymatic domain comprises the enzymatic domains of SEQ ID NO: 11 or SEQ ID NO: 12. In other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises amino acids 1/2-436 of SEQ ID NO: 11. In another aspect of this embodiment, a BoNT/C1 enzymatic domain comprises a naturally occurring BoNT/C1 enzymatic domain variant, such as, e.g., an enzymatic domain from a BoNT/C1 isoform or an enzymatic domain from a BoNT/C1 subtype. In another aspect of this embodiment, a BoNT/C1 enzymatic domain comprises a naturally occurring BoNT/C1 enzymatic domain variant of SEQ ID NO: 11 or SEQ ID NO: 12, such as, e.g., a BoNT/C1 isoform enzymatic domain or a BoNT/C1 subtype enzymatic domain. In another aspect of this embodiment, a BoNT/C1 enzymatic domain comprises amino acids 1/2-436 of a naturally occurring BoNT/C1 enzymatic domain variant of SEQ ID NO: 11, such as, e.g., a BoNT/C1 isoform enzymatic domain or a BoNT/C1 subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/C1 enzymatic domain comprises a non-naturally occurring BoNT/C1 enzymatic domain variant, such as, e.g., a conservative BoNT/C1 enzymatic domain variant, a non-conservative BoNT/C1 enzymatic domain variant, an active BoNT/C1 enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/C1 enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/C1 enzymatic domain variant of SEQ ID NO: 11 or SEQ ID

NO: 12, such as, *e.g.*, a conservative BoNT/C1 enzymatic domain variant, a non-conservative BoNT/C1 enzymatic domain variant, an active BoNT/C1 enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/C1 enzymatic domain comprises amino acids 1/2-436 of a non-naturally occurring BoNT/C1 enzymatic domain variant of SEQ ID NO: 11, such as, *e.g.*, a conservative BoNT/C1 enzymatic domain variant, a non-conservative BoNT/C1 enzymatic domain variant, an active BoNT/C1 enzymatic domain fragment, or any combination thereof.

[056] In other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 11 or SEQ ID NO: 12; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 11 or SEQ ID NO: 12. In yet other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-436 of SEQ ID NO: 11; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-436 of SEQ ID NO: 11.

[057] In other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 11 or SEQ ID NO: 12; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 11 or SEQ ID NO: 12. In yet other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 11; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 11. In still other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 11 or SEQ ID NO: 12; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 11 or SEQ ID NO: 12. In further other aspects of this embodiment, a BoNT/C1 enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 11; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 11.

[058] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/D enzymatic domain. In an aspect of this embodiment, a BoNT/D enzymatic domain comprises the enzymatic

domains of SEQ ID NO: 13 or SEQ ID NO: 14. In other aspects of this embodiment, a BoNT/D enzymatic domain comprises amino acids 1/2-436 of SEQ ID NO: 13. In another aspect of this embodiment, a BoNT/D enzymatic domain comprises a naturally occurring BoNT/D enzymatic domain variant, such as, *e.g.*, an enzymatic domain from a BoNT/D isoform or an enzymatic domain from a BoNT/D subtype. In another aspect of this embodiment, a BoNT/D enzymatic domain comprises a naturally occurring BoNT/D enzymatic domain variant of SEQ ID NO: 13 or SEQ ID NO: 14, such as, *e.g.*, a BoNT/D isoform enzymatic domain or a BoNT/D subtype enzymatic domain. In another aspect of this embodiment, a BoNT/D enzymatic domain comprises amino acids 1/2-436 of a naturally occurring BoNT/D enzymatic domain variant of SEQ ID NO: 13, such as, *e.g.*, a BoNT/D isoform enzymatic domain or a BoNT/D subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/D enzymatic domain comprises a non-naturally occurring BoNT/D enzymatic domain variant, such as, *e.g.*, a conservative BoNT/D enzymatic domain variant, a non-conservative BoNT/D enzymatic domain variant, an active BoNT/D enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/D enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/D enzymatic domain variant of SEQ ID NO: 13 or SEQ ID NO: 14, such as, *e.g.*, a conservative BoNT/D enzymatic domain variant, a non-conservative BoNT/D enzymatic domain variant, an active BoNT/D enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/D enzymatic domain comprises amino acids 1/2-436 of a non-naturally occurring BoNT/D enzymatic domain variant of SEQ ID NO: 13, such as, *e.g.*, a conservative BoNT/D enzymatic domain variant, a non-conservative BoNT/D enzymatic domain variant, an active BoNT/D enzymatic domain fragment, or any combination thereof.

[059] In other aspects of this embodiment, a BoNT/D enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 13 or SEQ ID NO: 14; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 13 or SEQ ID NO: 14. In yet other aspects of this embodiment, a BoNT/D enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-436 of SEQ ID NO: 13; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-436 of SEQ ID NO: 13.

[060] In other aspects of this embodiment, a BoNT/D enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 13 or SEQ ID NO: 14; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 13 or SEQ ID NO: 14. In yet other aspects of this embodiment, a BoNT/D enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions,

and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 13; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 13. In still other aspects of this embodiment, a BoNT/D enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 13 or SEQ ID NO: 14; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 13 or SEQ ID NO: 14. In further other aspects of this embodiment, a BoNT/D enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 13; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-436 of SEQ ID NO: 13.

[061] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/E enzymatic domain. In an aspect of this embodiment, a BoNT/E enzymatic domain comprises the enzymatic domains of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In other aspects of this embodiment, a BoNT/E enzymatic domain comprises amino acids 1/2-411 of SEQ ID NO: 15. In another aspect of this embodiment, a BoNT/E enzymatic domain comprises a naturally occurring BoNT/E enzymatic domain variant, such as, *e.g.*, an enzymatic domain from a BoNT/E isoform or an enzymatic domain from a BoNT/E subtype. In another aspect of this embodiment, a BoNT/E enzymatic domain comprises a naturally occurring BoNT/E enzymatic domain variant of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17, such as, *e.g.*, a BoNT/E isoform enzymatic domain or a BoNT/E subtype enzymatic domain. In another aspect of this embodiment, a BoNT/E enzymatic domain comprises amino acids 1/2-411 of a naturally occurring BoNT/E enzymatic domain variant of SEQ ID NO: 15, such as, *e.g.*, a BoNT/E isoform enzymatic domain or a BoNT/E subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/E enzymatic domain comprises a non-naturally occurring BoNT/E enzymatic domain variant, such as, *e.g.*, a conservative BoNT/E enzymatic domain variant, a non-conservative BoNT/E enzymatic domain variant, an active BoNT/E enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/E enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/E enzymatic domain variant of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17, such as, *e.g.*, a conservative BoNT/E enzymatic domain variant, a non-conservative BoNT/E enzymatic domain variant, an active BoNT/E enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/E enzymatic domain comprises amino acids 1/2-411 of a non-naturally occurring BoNT/E enzymatic domain variant of SEQ ID NO: 15, such as, *e.g.*, a conservative BoNT/E enzymatic domain variant, a non-conservative BoNT/E enzymatic domain variant, an active BoNT/E enzymatic domain fragment, or any combination thereof.

[062] In other aspects of this embodiment, a BoNT/E enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least

90%, or at least 95% to the enzymatic domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In yet other aspects of this embodiment, a BoNT/E enzymatic domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-411 of SEQ ID NO: 15; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-411 of SEQ ID NO: 15.

[063] In other aspects of this embodiment, a BoNT/E enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In yet other aspects of this embodiment, a BoNT/E enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 15; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 15. In still other aspects of this embodiment, a BoNT/E enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In further other aspects of this embodiment, a BoNT/E enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 15; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 15.

[064] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/F enzymatic domain. In an aspect of this embodiment, a BoNT/F enzymatic domain comprises the enzymatic domains of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In other aspects of this embodiment, a BoNT/F enzymatic domain comprises amino acids 1/2-428 of SEQ ID NO: 18. In another aspect of this embodiment, a BoNT/F enzymatic domain comprises a naturally occurring BoNT/F enzymatic domain variant, such as, e.g., an enzymatic domain from a BoNT/F isoform or an enzymatic domain from a BoNT/F subtype. In another aspect of this embodiment, a BoNT/F enzymatic domain comprises a naturally occurring BoNT/F enzymatic domain variant of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20, such as, e.g., a BoNT/F isoform enzymatic domain or a BoNT/F subtype enzymatic domain. In another aspect of this embodiment, a BoNT/F enzymatic domain comprises amino acids 1/2-428 of a

naturally occurring BoNT/F enzymatic domain variant of SEQ ID NO: 18, such as, *e.g.*, a BoNT/F isoform enzymatic domain or a BoNT/F subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/F enzymatic domain comprises a non-naturally occurring BoNT/F enzymatic domain variant, such as, *e.g.*, a conservative BoNT/F enzymatic domain variant, a non-conservative BoNT/F enzymatic domain variant, an active BoNT/F enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/F enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/F enzymatic domain variant of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20, such as, *e.g.*, a conservative BoNT/F enzymatic domain variant, a non-conservative BoNT/F enzymatic domain variant, an active BoNT/F enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/F enzymatic domain comprises amino acids 1/2-428 of a non-naturally occurring BoNT/F enzymatic domain variant of SEQ ID NO: 18, such as, *e.g.*, a conservative BoNT/F enzymatic domain variant, a non-conservative BoNT/F enzymatic domain variant, an active BoNT/F enzymatic domain fragment, or any combination thereof.

[065] In other aspects of this embodiment, a BoNT/F enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In yet other aspects of this embodiment, a BoNT/F enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-428 of SEQ ID NO: 18; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-428 of SEQ ID NO: 18.

[066] In other aspects of this embodiment, a BoNT/F enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In yet other aspects of this embodiment, a BoNT/F enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-428 of SEQ ID NO: 18; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-428 of SEQ ID NO: 18. In still other aspects of this embodiment, a BoNT/F enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO:

20. In further other aspects of this embodiment, a BoNT/F enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-428 of SEQ ID NO: 18; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-428 of SEQ ID NO: 18.

[067] In another embodiment, a Clostridial toxin enzymatic domain comprises a BoNT/G enzymatic domain. In an aspect of this embodiment, a BoNT/G enzymatic domain comprises the enzymatic domains of SEQ ID NO: 21. In other aspects of this embodiment, a BoNT/G enzymatic domain comprises amino acids 1/2-4435 of SEQ ID NO: 21. In another aspect of this embodiment, a BoNT/G enzymatic domain comprises a naturally occurring BoNT/G enzymatic domain variant, such as, *e.g.*, an enzymatic domain from a BoNT/G isoform or an enzymatic domain from a BoNT/G subtype. In another aspect of this embodiment, a BoNT/G enzymatic domain comprises a naturally occurring BoNT/G enzymatic domain variant of SEQ ID NO: 21, such as, *e.g.*, a BoNT/G isoform enzymatic domain or a BoNT/G subtype enzymatic domain. In another aspect of this embodiment, a BoNT/G enzymatic domain comprises amino acids 1/2-4435 of a naturally occurring BoNT/G enzymatic domain variant of SEQ ID NO: 21, such as, *e.g.*, a BoNT/G isoform enzymatic domain or a BoNT/G subtype enzymatic domain. In still another aspect of this embodiment, a BoNT/G enzymatic domain comprises a non-naturally occurring BoNT/G enzymatic domain variant, such as, *e.g.*, a conservative BoNT/G enzymatic domain variant, a non-conservative BoNT/G enzymatic domain variant, an active BoNT/G enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/G enzymatic domain comprises the enzymatic domain of a non-naturally occurring BoNT/G enzymatic domain variant of SEQ ID NO: 21, such as, *e.g.*, a conservative BoNT/G enzymatic domain variant, a non-conservative BoNT/G enzymatic domain variant, an active BoNT/G enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/G enzymatic domain comprises amino acids 1/2-4435 of a non-naturally occurring BoNT/G enzymatic domain variant of SEQ ID NO: 21, such as, *e.g.*, a conservative BoNT/G enzymatic domain variant, a non-conservative BoNT/G enzymatic domain variant, an active BoNT/G enzymatic domain fragment, or any combination thereof.

[068] In other aspects of this embodiment, a BoNT/G enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 21; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 21. In yet other aspects of this embodiment, a BoNT/G enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-4435 of SEQ ID NO: 21; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-4435 of SEQ ID NO: 21.

[069] In other aspects of this embodiment, a BoNT/G enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 21. In yet other aspects of this embodiment, a BoNT/G enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-4435 of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-4435 of SEQ ID NO: 21. In still other aspects of this embodiment, a BoNT/G enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 21. In further other aspects of this embodiment, a BoNT/G enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-4435 of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-4435 of SEQ ID NO: 21.

[070] In another embodiment, a Clostridial toxin enzymatic domain comprises a TeNT enzymatic domain. In an aspect of this embodiment, a TeNT enzymatic domain comprises the enzymatic domains of SEQ ID NO: 22. In other aspects of this embodiment, a TeNT enzymatic domain comprises amino acids 1/2-438 of SEQ ID NO: 22. In another aspect of this embodiment, a TeNT enzymatic domain comprises a naturally occurring TeNT enzymatic domain variant, such as, e.g., an enzymatic domain from a TeNT isoform or an enzymatic domain from a TeNT subtype. In another aspect of this embodiment, a TeNT enzymatic domain comprises a naturally occurring TeNT enzymatic domain variant of SEQ ID NO: 22, such as, e.g., a TeNT isoform enzymatic domain or a TeNT subtype enzymatic domain. In another aspect of this embodiment, a TeNT enzymatic domain comprises amino acids 1/2-438 of a naturally occurring TeNT enzymatic domain variant of SEQ ID NO: 22, such as, e.g., a TeNT isoform enzymatic domain or a TeNT subtype enzymatic domain. In still another aspect of this embodiment, a TeNT enzymatic domain comprises a non-naturally occurring TeNT enzymatic domain variant, such as, e.g., a conservative TeNT enzymatic domain variant, a non-conservative TeNT enzymatic domain variant, an active TeNT enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a TeNT enzymatic domain comprises the enzymatic domain of a non-naturally occurring TeNT enzymatic domain variant of SEQ ID NO: 22, such as, e.g., a conservative TeNT enzymatic domain variant, a non-conservative TeNT enzymatic domain variant, an active TeNT enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a TeNT enzymatic domain comprises amino acids 1/2-438 of a non-naturally occurring TeNT enzymatic domain variant of SEQ ID

NO: 22, such as, e.g., a conservative TeNT enzymatic domain variant, a non-conservative TeNT enzymatic domain variant, an active TeNT enzymatic domain fragment, or any combination thereof.

[071] In other aspects of this embodiment, a TeNT enzymatic domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 22; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 22. In yet other aspects of this embodiment, a TeNT enzymatic domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-438 of SEQ ID NO: 22; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-438 of SEQ ID NO: 22.

[072] In other aspects of this embodiment, a TeNT enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 22. In yet other aspects of this embodiment, a TeNT enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-438 of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-438 of SEQ ID NO: 22. In still other aspects of this embodiment, a TeNT enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 22. In further other aspects of this embodiment, a TeNT enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-438 of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-438 of SEQ ID NO: 22.

[073] In another embodiment, a Clostridial toxin enzymatic domain comprises a BaNT enzymatic domain. In an aspect of this embodiment, a BaNT enzymatic domain comprises the enzymatic domains of SEQ ID NO: 23. In other aspects of this embodiment, a BaNT enzymatic domain comprises amino acids 1/2-420 of SEQ ID NO: 23. In another aspect of this embodiment, a BaNT enzymatic domain comprises a naturally occurring BaNT enzymatic domain variant, such as, e.g., an enzymatic domain from a BaNT isoform or an enzymatic domain from a BaNT subtype. In another aspect of this embodiment, a BaNT enzymatic domain comprises a naturally occurring BaNT enzymatic domain variant

of SEQ ID NO: 23, such as, *e.g.*, a BaNT isoform enzymatic domain or a BaNT subtype enzymatic domain. In another aspect of this embodiment, a BaNT enzymatic domain comprises amino acids 1/2-420 of a naturally occurring BaNT enzymatic domain variant of SEQ ID NO: 23, such as, *e.g.*, a BaNT isoform enzymatic domain or a BaNT subtype enzymatic domain. In still another aspect of this embodiment, a BaNT enzymatic domain comprises a non-naturally occurring BaNT enzymatic domain variant, such as, *e.g.*, a conservative BaNT enzymatic domain variant, a non-conservative BaNT enzymatic domain variant, an active BaNT enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BaNT enzymatic domain comprises the enzymatic domain of a non-naturally occurring BaNT enzymatic domain variant of SEQ ID NO: 23, such as, *e.g.*, a conservative BaNT enzymatic domain variant, a non-conservative BaNT enzymatic domain variant, an active BaNT enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BaNT enzymatic domain comprises amino acids 1/2-420 of a non-naturally occurring BaNT enzymatic domain variant of SEQ ID NO: 23, such as, *e.g.*, a conservative BaNT enzymatic domain variant, a non-conservative BaNT enzymatic domain variant, an active BaNT enzymatic domain fragment, or any combination thereof.

[074] In other aspects of this embodiment, a BaNT enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 23; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 23. In yet other aspects of this embodiment, a BaNT enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-420 of SEQ ID NO: 23; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-420 of SEQ ID NO: 23.

[075] In other aspects of this embodiment, a BaNT enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 23. In yet other aspects of this embodiment, a BaNT enzymatic domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-420 of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-420 of SEQ ID NO: 23. In still other aspects of this embodiment, a BaNT enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 23. In further other aspects of this

embodiment, a BaNT enzymatic domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-420 of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-420 of SEQ ID NO: 23.

[076] In another embodiment, a Clostridial toxin enzymatic domain comprises a BuNT enzymatic domain. In an aspect of this embodiment, a BuNT enzymatic domain comprises the enzymatic domains of SEQ ID NO: 24 or SEQ ID NO: 25. In other aspects of this embodiment, a BuNT enzymatic domain comprises amino acids 1/2-411 of SEQ ID NO: 24. In another aspect of this embodiment, a BuNT enzymatic domain comprises a naturally occurring BuNT enzymatic domain variant, such as, *e.g.*, an enzymatic domain from a BuNT isoform or an enzymatic domain from a BuNT subtype. In another aspect of this embodiment, a BuNT enzymatic domain comprises a naturally occurring BuNT enzymatic domain variant of SEQ ID NO: 24 or SEQ ID NO: 25, such as, *e.g.*, a BuNT isoform enzymatic domain or a BuNT subtype enzymatic domain. In another aspect of this embodiment, a BuNT enzymatic domain comprises amino acids 1/2-411 of a naturally occurring BuNT enzymatic domain variant of SEQ ID NO: 24, such as, *e.g.*, a BuNT isoform enzymatic domain or a BuNT subtype enzymatic domain. In still another aspect of this embodiment, a BuNT enzymatic domain comprises a non-naturally occurring BuNT enzymatic domain variant, such as, *e.g.*, a conservative BuNT enzymatic domain variant, a non-conservative BuNT enzymatic domain variant, an active BuNT enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BuNT enzymatic domain comprises the enzymatic domain of a non-naturally occurring BuNT enzymatic domain variant of SEQ ID NO: 24 or SEQ ID NO: 25, such as, *e.g.*, a conservative BuNT enzymatic domain variant, a non-conservative BuNT enzymatic domain variant, an active BuNT enzymatic domain fragment, or any combination thereof. In still another aspect of this embodiment, a BuNT enzymatic domain comprises amino acids 1/2-411 of a non-naturally occurring BuNT enzymatic domain variant of SEQ ID NO: 24, such as, *e.g.*, a conservative BuNT enzymatic domain variant, a non-conservative BuNT enzymatic domain variant, an active BuNT enzymatic domain fragment, or any combination thereof.

[077] In other aspects of this embodiment, a BuNT enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the enzymatic domain of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the enzymatic domain of SEQ ID NO: 24 or SEQ ID NO: 25. In yet other aspects of this embodiment, a BuNT enzymatic domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 1/2-411 of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 1/2-411 of SEQ ID NO: 24 or SEQ ID NO: 25.

[078] In other aspects of this embodiment, a BuNT enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 24 OR SEQ ID NO: 25. In yet other aspects of this embodiment, a BuNT enzymatic domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 24 or SEQ ID NO: 25. In still other aspects of this embodiment, a BuNT enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the enzymatic domain of SEQ ID NO: 24 or SEQ ID NO: 25. In further other aspects of this embodiment, a BuNT enzymatic domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 1/2-411 of SEQ ID NO: 24 or SEQ ID NO: 25.

[079] The “translocation domain” comprises a portion of a Clostridial neurotoxin heavy chain having a translocation activity. By “translocation” is meant the ability to facilitate the transport of a polypeptide through a vesicular membrane, thereby exposing some or all of the polypeptide to the cytoplasm. In the various botulinum neurotoxins translocation is thought to involve an allosteric conformational change of the heavy chain caused by a decrease in pH within the endosome. This conformational change appears to involve and be mediated by the N terminal half of the heavy chain and to result in the formation of pores in the vesicular membrane; this change permits the movement of the proteolytic light chain from within the endosomal vesicle into the cytoplasm. See e.g., Lacy, et al., *Nature Struct. Biol.* 5:898-902 (October 1998).

[080] The amino acid sequence of the translocation-mediating portion of the botulinum neurotoxin heavy chain is known to those of skill in the art; additionally, those amino acid residues within this portion that are known to be essential for conferring the translocation activity are also known. It would therefore be well within the ability of one of ordinary skill in the art, for example, to employ the naturally occurring N-terminal peptide half of the heavy chain of any of the various *Clostridium tetanus* or *Clostridium botulinum* neurotoxin subtypes as a translocation domain, or to design an analogous translocation domain by aligning the primary sequences of the N-terminal halves of the various heavy chains and selecting a

consensus primary translocation sequence based on conserved amino acid, polarity, steric and hydrophobicity characteristics between the sequences.

[081] Aspects of the present specification provide, in part, a TVEMP comprising a Clostridial toxin translocation domain. As used herein, the term “Clostridial toxin translocation domain” refers to any Clostridial toxin polypeptide that can execute the translocation step of the intoxication process that mediates Clostridial toxin light chain translocation. Thus, a Clostridial toxin translocation domain facilitates the movement of a Clostridial toxin light chain across a membrane and encompasses the movement of a Clostridial toxin light chain through the membrane an intracellular vesicle into the cytoplasm of a cell. Non-limiting examples of a Clostridial toxin translocation domain include, *e.g.*, a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, and a BuNT translocation domain.

[082] A Clostridial toxin translocation domain includes, without limitation, naturally occurring Clostridial toxin translocation domain variants, such as, *e.g.*, Clostridial toxin translocation domain isoforms and Clostridial toxin translocation domain subtypes; non-naturally occurring Clostridial toxin translocation domain variants, such as, *e.g.*, conservative Clostridial toxin translocation domain variants, non-conservative Clostridial toxin translocation domain variants, active Clostridial toxin translocation domain fragments thereof, or any combination thereof.

[083] As used herein, the term “Clostridial toxin translocation domain variant,” whether naturally-occurring or non-naturally-occurring, refers to a Clostridial toxin translocation domain that has at least one amino acid change from the corresponding region of the disclosed reference sequences (Table 1) and can be described in percent identity to the corresponding region of that reference sequence. Unless expressly indicated, Clostridial toxin translocation domain variants useful to practice disclosed embodiments are variants that execute the translocation step of the intoxication process that mediates Clostridial toxin light chain translocation. As non-limiting examples, a BoNT/A translocation domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 455-873 of SEQ ID NO: 1; a BoNT/B translocation domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 447-860 of SEQ ID NO: 6; a BoNT/C1 translocation domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 454-868 of SEQ ID NO: 11; a BoNT/D translocation domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 451-864 of SEQ ID NO: 13; a BoNT/E translocation domain variant will have at least one amino acid difference, such as, *e.g.*, an amino acid substitution, deletion or addition, as compared to amino acids 427-847 of SEQ ID NO: 15; a BoNT/F translocation domain variant will have at

least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 446-865 of SEQ ID NO: 18; a BoNT/G translocation domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 451-865 of SEQ ID NO: 21; a TeNT translocation domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 468-881 of SEQ ID NO: 22; a BaNT translocation domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 436-857 of SEQ ID NO: 23; and a BuNT translocation domain variant will have at least one amino acid difference, such as, e.g., an amino acid substitution, deletion or addition, as compared to amino acids 427-847 of SEQ ID NO: 24.

[084] It is recognized by those of skill in the art that within each serotype of Clostridial toxin there can be naturally occurring Clostridial toxin translocation domain variants that differ somewhat in their amino acid sequence, and also in the nucleic acids encoding these proteins. For example, there are presently five BoNT/A subtypes, BoNT/A1, BoNT/A2, BoNT/A3, BoNT/A4, and BoNT/A5, with specific translocation domain subtypes showing about 85-87% amino acid identity when compared to the BoNT/A translocation domain subtype of SEQ ID NO: 1. As used herein, the term “naturally occurring Clostridial toxin translocation domain variant” refers to any Clostridial toxin translocation domain produced by a naturally-occurring process, including, without limitation, Clostridial toxin translocation domain isoforms produced from alternatively-spliced transcripts, Clostridial toxin translocation domain isoforms produced by spontaneous mutation and Clostridial toxin translocation domain subtypes. A naturally occurring Clostridial toxin translocation domain variant can function in substantially the same manner as the reference Clostridial toxin translocation domain on which the naturally occurring Clostridial toxin translocation domain variant is based, and can be substituted for the reference Clostridial toxin translocation domain in any aspect of the present specification.

[085] A non-limiting examples of a naturally occurring Clostridial toxin translocation domain variant is a Clostridial toxin translocation domain isoform such as, e.g., a BoNT/A translocation domain isoform, a BoNT/B translocation domain isoform, a BoNT/C1 translocation domain isoform, a BoNT/D translocation domain isoform, a BoNT/E translocation domain isoform, a BoNT/F translocation domain isoform, a BoNT/G translocation domain isoform, a TeNT translocation domain isoform, a BaNT translocation domain isoform, and a BuNT translocation domain isoform. Another non-limiting examples of a naturally occurring Clostridial toxin translocation domain variant is a Clostridial toxin translocation domain subtype such as, e.g., a translocation domain from subtype BoNT/A1, BoNT/A2, BoNT/A3, BoNT/A4, and BoNT/A5; a translocation domain from subtype BoNT/B1, BoNT/B2, BoNT/B bivalent and BoNT/B nonproteolytic; a translocation domain from subtype BoNT/C1-1 and BoNT/C1-2; a translocation domain from subtype BoNT/E1, BoNT/E2 and BoNT/E3; a translocation domain from subtype BoNT/F1, BoNT/F2, BoNT/F3; and a translocation domain from subtype BuNT-1 and BuNT-2.

[086] As used herein, the term “non-naturally occurring Clostridial toxin translocation domain variant” refers to any Clostridial toxin translocation domain produced with the aid of human manipulation, including, without limitation, Clostridial toxin translocation domains produced by genetic engineering using random mutagenesis or rational design and Clostridial toxin translocation domains produced by chemical synthesis. Non-limiting examples of non-naturally occurring Clostridial toxin translocation domain variants include, *e.g.*, conservative Clostridial toxin translocation domain variants, non-conservative Clostridial toxin translocation domain variants, and active Clostridial toxin translocation domain fragments.

[087] As used herein, the term “conservative Clostridial toxin translocation domain variant” refers to a Clostridial toxin translocation domain that has at least one amino acid substituted by another amino acid or an amino acid analog that has at least one property similar to that of the original amino acid from the reference Clostridial toxin translocation domain sequence (Table 1). Examples of properties include, without limitation, similar size, topography, charge, hydrophobicity, hydrophilicity, lipophilicity, covalent-bonding capacity, hydrogen-bonding capacity, a physicochemical property, of the like, or any combination thereof. A conservative Clostridial toxin translocation domain variant can function in substantially the same manner as the reference Clostridial toxin translocation domain on which the conservative Clostridial toxin translocation domain variant is based, and can be substituted for the reference Clostridial toxin translocation domain in any aspect of the present specification. Non-limiting examples of a conservative Clostridial toxin translocation domain variant include, *e.g.*, conservative BoNT/A translocation domain variants, conservative BoNT/B translocation domain variants, conservative BoNT/C1 translocation domain variants, conservative BoNT/D translocation domain variants, conservative BoNT/E translocation domain variants, conservative BoNT/F translocation domain variants, conservative BoNT/G translocation domain variants, conservative TeNT translocation domain variants, conservative BaNT translocation domain variants, and conservative BuNT translocation domain variants.

[088] As used herein, the term “non-conservative Clostridial toxin translocation domain variant” refers to a Clostridial toxin translocation domain in which 1) at least one amino acid is deleted from the reference Clostridial toxin translocation domain on which the non-conservative Clostridial toxin translocation domain variant is based; 2) at least one amino acid added to the reference Clostridial toxin translocation domain on which the non-conservative Clostridial toxin translocation domain is based; or 3) at least one amino acid is substituted by another amino acid or an amino acid analog that does not share any property similar to that of the original amino acid from the reference Clostridial toxin translocation domain sequence (Table 1). A non-conservative Clostridial toxin translocation domain variant can function in substantially the same manner as the reference Clostridial toxin translocation domain on which the non-conservative Clostridial toxin translocation domain variant is based, and can be substituted for the reference Clostridial toxin translocation domain in any aspect of the present specification. Non-limiting examples of a non-conservative Clostridial toxin translocation domain variant include, *e.g.*, non-conservative BoNT/A translocation domain variants, non-conservative BoNT/B translocation domain variants, non-conservative BoNT/C1 translocation domain variants, non-conservative BoNT/D

translocation domain variants, non-conservative BoNT/E translocation domain variants, non-conservative BoNT/F translocation domain variants, non-conservative BoNT/G translocation domain variants, and non-conservative TeNT translocation domain variants, non-conservative BaNT translocation domain variants, and non-conservative BuNT translocation domain variants.

[089] As used herein, the term “active Clostridial toxin translocation domain fragment” refers to any of a variety of Clostridial toxin fragments comprising the translocation domain can be useful in aspects of the present specification with the proviso that these active fragments can facilitate the release of the LC from intracellular vesicles into the cytoplasm of the target cell and thus participate in executing the overall cellular mechanism whereby a Clostridial toxin proteolytically cleaves a substrate. The translocation domains from the heavy chains of Clostridial toxins are approximately 410-430 amino acids in length and comprise a translocation domain (Table 1). Research has shown that the entire length of a translocation domain from a Clostridial toxin heavy chain is not necessary for the translocating activity of the translocation domain. Thus, aspects of this embodiment include a Clostridial toxin translocation domain having a length of, *e.g.*, at least 350, 375, 400, or 425 amino acids. Other aspects of this embodiment include a Clostridial toxin translocation domain having a length of, *e.g.*, at most 350, 375, 400, or 425 amino acids.

[090] Any of a variety of sequence alignment methods can be used to determine percent identity of naturally-occurring Clostridial toxin translocation domain variants and non-naturally-occurring Clostridial toxin translocation domain variants, including, without limitation, global methods, local methods and hybrid methods, such as, *e.g.*, segment approach methods. Protocols to determine percent identity are routine procedures within the scope of one skilled in the art and from the teaching herein.

[091] Thus, in an embodiment, a TVEMP disclosed herein comprises a Clostridial toxin translocation domain. In an aspect of this embodiment, a Clostridial toxin translocation domain comprises a naturally occurring Clostridial toxin translocation domain variant, such as, *e.g.*, a Clostridial toxin translocation domain isoform or a Clostridial toxin translocation domain subtype. In another aspect of this embodiment, a Clostridial toxin translocation domain comprises a non-naturally occurring Clostridial toxin translocation domain variant, such as, *e.g.*, a conservative Clostridial toxin translocation domain variant, a non-conservative Clostridial toxin translocation domain variant, an active Clostridial toxin translocation domain fragment, or any combination thereof.

[092] In another embodiment, a hydrophobic amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another hydrophobic amino acid. Examples of hydrophobic amino acids include, *e.g.*, C, F, I, L, M, V and W. In another aspect of this embodiment, an aliphatic amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another aliphatic amino acid. Examples of aliphatic amino acids include, *e.g.*, A, I, L, P, and V. In yet another aspect of this embodiment, an aromatic amino

acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another aromatic amino acid. Examples of aromatic amino acids include, *e.g.*, F, H, W and Y. In still another aspect of this embodiment, a stacking amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another stacking amino acid. Examples of stacking amino acids include, *e.g.*, F, H, W and Y. In a further aspect of this embodiment, a polar amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another polar amino acid. Examples of polar amino acids include, *e.g.*, D, E, K, N, Q, and R. In a further aspect of this embodiment, a less polar or indifferent amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another less polar or indifferent amino acid. Examples of less polar or indifferent amino acids include, *e.g.*, A, H, G, P, S, T, and Y. In a yet further aspect of this embodiment, a positive charged amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another positive charged amino acid. Examples of positive charged amino acids include, *e.g.*, K, R, and H. In a still further aspect of this embodiment, a negative charged amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another negative charged amino acid. Examples of negative charged amino acids include, *e.g.*, D and E. In another aspect of this embodiment, a small amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another small amino acid. Examples of small amino acids include, *e.g.*, A, D, G, N, P, S, and T. In yet another aspect of this embodiment, a C-beta branching amino acid at one particular position in the polypeptide chain of the Clostridial toxin translocation domain can be substituted with another C-beta branching amino acid. Examples of C-beta branching amino acids include, *e.g.*, I, T and V.

[093] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/A translocation domain. In an aspect of this embodiment, a BoNT/A translocation domain comprises the translocation domains of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In other aspects of this embodiment, a BoNT/A translocation domain comprises amino acids 455-873 of SEQ ID NO: 1. In another aspect of this embodiment, a BoNT/A translocation domain comprises a naturally occurring BoNT/A translocation domain variant, such as, *e.g.*, an translocation domain from a BoNT/A isoform or an translocation domain from a BoNT/A subtype. In another aspect of this embodiment, a BoNT/A translocation domain comprises a naturally occurring BoNT/A translocation domain variant of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5, such as, *e.g.*, a BoNT/A isoform translocation domain or a BoNT/A subtype translocation domain. In another aspect of this embodiment, a BoNT/A translocation domain comprises amino acids 455-873 of a naturally occurring BoNT/A translocation domain variant of SEQ ID NO: 1, such as, *e.g.*, a BoNT/A isoform translocation domain or a BoNT/A subtype translocation domain. In still another aspect of this embodiment, a BoNT/A translocation domain comprises a non-naturally occurring BoNT/A translocation domain variant, such as, *e.g.*, a conservative BoNT/A translocation domain variant, a non-conservative BoNT/A translocation domain variant, an active BoNT/A translocation domain fragment, or any

combination thereof. In still another aspect of this embodiment, a BoNT/A translocation domain comprises the translocation domain of a non-naturally occurring BoNT/A translocation domain variant of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5, such as, *e.g.*, a conservative BoNT/A translocation domain variant, a non-conservative BoNT/A translocation domain variant, an active BoNT/A translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/A translocation domain comprises amino acids 455-873 of a non-naturally occurring BoNT/A translocation domain variant of SEQ ID NO: 1, such as, *e.g.*, a conservative BoNT/A translocation domain variant, a non-conservative BoNT/A translocation domain variant, an active BoNT/A translocation domain fragment, or any combination thereof.

[094] In other aspects of this embodiment, a BoNT/A translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In yet other aspects of this embodiment, a BoNT/A translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 455-873 of SEQ ID NO: 1; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 455-873 of SEQ ID NO: 1.

[095] In other aspects of this embodiment, a BoNT/A translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In yet other aspects of this embodiment, a BoNT/A translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 455-873 of SEQ ID NO: 1; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 455-873 of SEQ ID NO: 1. In still other aspects of this embodiment, a BoNT/A translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In further other aspects of this embodiment, a BoNT/A translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 455-873 of SEQ ID NO: 1; or

at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 455-873 of SEQ ID NO: 1.

[096] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/B translocation domain. In an aspect of this embodiment, a BoNT/B translocation domain comprises the translocation domains of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In other aspects of this embodiment, a BoNT/B translocation domain comprises amino acids 447-860 of SEQ ID NO: 6. In another aspect of this embodiment, a BoNT/B translocation domain comprises a naturally occurring BoNT/B translocation domain variant, such as, *e.g.*, an translocation domain from a BoNT/B isoform or an translocation domain from a BoNT/B subtype. In another aspect of this embodiment, a BoNT/B translocation domain comprises a naturally occurring BoNT/B translocation domain variant of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10, such as, *e.g.*, a BoNT/B isoform translocation domain or a BoNT/B subtype translocation domain. In another aspect of this embodiment, a BoNT/B translocation domain comprises amino acids 447-860 of a naturally occurring BoNT/B translocation domain variant of SEQ ID NO: 6, such as, *e.g.*, a BoNT/B isoform translocation domain or a BoNT/B subtype translocation domain. In still another aspect of this embodiment, a BoNT/B translocation domain comprises a non-naturally occurring BoNT/B translocation domain variant, such as, *e.g.*, a conservative BoNT/B translocation domain variant, a non-conservative BoNT/B translocation domain variant, an active BoNT/B translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/B translocation domain comprises the translocation domain of a non-naturally occurring BoNT/B translocation domain variant of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10, such as, *e.g.*, a conservative BoNT/B translocation domain variant, a non-conservative BoNT/B translocation domain variant, an active BoNT/B translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/B translocation domain comprises amino acids 447-860 of a non-naturally occurring BoNT/B translocation domain variant of SEQ ID NO: 6, such as, *e.g.*, a conservative BoNT/B translocation domain variant, a non-conservative BoNT/B translocation domain variant, an active BoNT/B translocation domain fragment, or any combination thereof.

[097] In other aspects of this embodiment, a BoNT/B translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In yet other aspects of this embodiment, a BoNT/B translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 447-860 of SEQ ID NO: 6; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 447-860 of SEQ ID NO: 6.

[098] In other aspects of this embodiment, a BoNT/B translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In yet other aspects of this embodiment, a BoNT/B translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 447-860 of SEQ ID NO: 6; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 447-860 of SEQ ID NO: 6. In still other aspects of this embodiment, a BoNT/B translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or SEQ ID NO: 10. In further other aspects of this embodiment, a BoNT/B translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 447-860 of SEQ ID NO: 6; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 447-860 of SEQ ID NO: 6.

[099] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/C1 translocation domain. In an aspect of this embodiment, a BoNT/C1 translocation domain comprises the translocation domains of SEQ ID NO: 11 or SEQ ID NO: 12. In other aspects of this embodiment, a BoNT/C1 translocation domain comprises amino acids 454-868 of SEQ ID NO: 11. In another aspect of this embodiment, a BoNT/C1 translocation domain comprises a naturally occurring BoNT/C1 translocation domain variant, such as, *e.g.*, an translocation domain from a BoNT/C1 isoform or an translocation domain from a BoNT/C1 subtype. In another aspect of this embodiment, a BoNT/C1 translocation domain comprises a naturally occurring BoNT/C1 translocation domain variant of SEQ ID NO: 11 or SEQ ID NO: 12, such as, *e.g.*, a BoNT/C1 isoform translocation domain or a BoNT/C1 subtype translocation domain. In another aspect of this embodiment, a BoNT/C1 translocation domain comprises amino acids 454-868 of a naturally occurring BoNT/C1 translocation domain variant of SEQ ID NO: 11, such as, *e.g.*, a BoNT/C1 isoform translocation domain or a BoNT/C1 subtype translocation domain. In still another aspect of this embodiment, a BoNT/C1 translocation domain comprises a non-naturally occurring BoNT/C1 translocation domain variant, such as, *e.g.*, a conservative BoNT/C1 translocation domain variant, a non-conservative BoNT/C1 translocation domain variant, an active BoNT/C1 translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/C1 translocation domain comprises the translocation domain of a non-naturally occurring BoNT/C1

translocation domain variant of SEQ ID NO: 11 or SEQ ID NO: 12, such as, *e.g.*, a conservative BoNT/C1 translocation domain variant, a non-conservative BoNT/C1 translocation domain variant, an active BoNT/C1 translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/C1 translocation domain comprises amino acids 454-868 of a non-naturally occurring BoNT/C1 translocation domain variant of SEQ ID NO: 11, such as, *e.g.*, a conservative BoNT/C1 translocation domain variant, a non-conservative BoNT/C1 translocation domain variant, an active BoNT/C1 translocation domain fragment, or any combination thereof.

[0100] In other aspects of this embodiment, a BoNT/C1 translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 11 or SEQ ID NO: 12; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 11 or SEQ ID NO: 12. In yet other aspects of this embodiment, a BoNT/C1 translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 454-868 of SEQ ID NO: 11; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 454-868 of SEQ ID NO: 11.

[0101] In other aspects of this embodiment, a BoNT/C1 translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 11 or SEQ ID NO: 12; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 11 or SEQ ID NO: 12. In yet other aspects of this embodiment, a BoNT/C1 translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 454-868 of SEQ ID NO: 11; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 454-868 of SEQ ID NO: 11. In still other aspects of this embodiment, a BoNT/C1 translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 11 or SEQ ID NO: 12; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 11 or SEQ ID NO: 12. In further other aspects of this embodiment, a BoNT/C1 translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 454-868 of SEQ ID NO: 11; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 454-868 of SEQ ID NO: 11.

[0102] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/D translocation domain. In an aspect of this embodiment, a BoNT/D translocation domain comprises the translocation domains of SEQ ID NO: 13 or SEQ ID NO: 14. In other aspects of this embodiment, a BoNT/D translocation domain comprises amino acids 451-864 of SEQ ID NO: 13. In another aspect of this embodiment, a BoNT/D translocation domain comprises a naturally occurring BoNT/D translocation domain variant, such as, *e.g.*, an translocation domain from a BoNT/D isoform or an translocation domain from a BoNT/D subtype. In another aspect of this embodiment, a BoNT/D translocation domain comprises a naturally occurring BoNT/D translocation domain variant of SEQ ID NO: 13 or SEQ ID NO: 14, such as, *e.g.*, a BoNT/D isoform translocation domain or a BoNT/D subtype translocation domain. In another aspect of this embodiment, a BoNT/D translocation domain comprises amino acids 451-864 of a naturally occurring BoNT/D translocation domain variant of SEQ ID NO: 13, such as, *e.g.*, a BoNT/D isoform translocation domain or a BoNT/D subtype translocation domain. In still another aspect of this embodiment, a BoNT/D translocation domain comprises a non-naturally occurring BoNT/D translocation domain variant, such as, *e.g.*, a conservative BoNT/D translocation domain variant, a non-conservative BoNT/D translocation domain variant, an active BoNT/D translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/D translocation domain comprises the translocation domain of a non-naturally occurring BoNT/D translocation domain variant of SEQ ID NO: 13 or SEQ ID NO: 14, such as, *e.g.*, a conservative BoNT/D translocation domain variant, a non-conservative BoNT/D translocation domain variant, an active BoNT/D translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/D translocation domain comprises amino acids 451-864 of a non-naturally occurring BoNT/D translocation domain variant of SEQ ID NO: 13, such as, *e.g.*, a conservative BoNT/D translocation domain variant, a non-conservative BoNT/D translocation domain variant, an active BoNT/D translocation domain fragment, or any combination thereof.

[0103] In other aspects of this embodiment, a BoNT/D translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 13 or SEQ ID NO: 14; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 13 or SEQ ID NO: 14. In yet other aspects of this embodiment, a BoNT/D translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 451-864 of SEQ ID NO: 13; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 451-864 of SEQ ID NO: 13.

[0104] In other aspects of this embodiment, a BoNT/D translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 13 or SEQ ID NO: 14; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid

deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 13 or SEQ ID NO: 14. In yet other aspects of this embodiment, a BoNT/D translocation domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-864 of SEQ ID NO: 13; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-864 of SEQ ID NO: 13. In still other aspects of this embodiment, a BoNT/D translocation domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 13 or SEQ ID NO: 14; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 13 or SEQ ID NO: 14. In further other aspects of this embodiment, a BoNT/D translocation domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-864 of SEQ ID NO: 13; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-864 of SEQ ID NO: 13.

[0105] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/E translocation domain. In an aspect of this embodiment, a BoNT/E translocation domain comprises the translocation domains of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In other aspects of this embodiment, a BoNT/E translocation domain comprises amino acids 427-847 of SEQ ID NO: 15. In another aspect of this embodiment, a BoNT/E translocation domain comprises a naturally occurring BoNT/E translocation domain variant, such as, e.g., an translocation domain from a BoNT/E isoform or an translocation domain from a BoNT/E subtype. In another aspect of this embodiment, a BoNT/E translocation domain comprises a naturally occurring BoNT/E translocation domain variant of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17, such as, e.g., a BoNT/E isoform translocation domain or a BoNT/E subtype translocation domain. In another aspect of this embodiment, a BoNT/E translocation domain comprises amino acids 427-847 of a naturally occurring BoNT/E translocation domain variant of SEQ ID NO: 15, such as, e.g., a BoNT/E isoform translocation domain or a BoNT/E subtype translocation domain. In still another aspect of this embodiment, a BoNT/E translocation domain comprises a non-naturally occurring BoNT/E translocation domain variant, such as, e.g., a conservative BoNT/E translocation domain variant, a non-conservative BoNT/E translocation domain variant, an active BoNT/E translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/E translocation domain comprises the translocation domain of a non-naturally occurring BoNT/E translocation domain variant of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17, such as, e.g., a conservative BoNT/E translocation domain variant, a non-conservative BoNT/E translocation domain variant, an active BoNT/E translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/E translocation domain comprises amino acids 427-847 of a non-naturally occurring BoNT/E translocation domain variant of SEQ ID NO: 15, such as, e.g., a conservative

BoNT/E translocation domain variant, a non-conservative BoNT/E translocation domain variant, an active BoNT/E translocation domain fragment, or any combination thereof.

[0106] In other aspects of this embodiment, a BoNT/E translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In yet other aspects of this embodiment, a BoNT/E translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 427-847 of SEQ ID NO: 15; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 427-847 of SEQ ID NO: 15.

[0107] In other aspects of this embodiment, a BoNT/E translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In yet other aspects of this embodiment, a BoNT/E translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 15; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 15. In still other aspects of this embodiment, a BoNT/E translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 15, SEQ ID NO: 16, or SEQ ID NO: 17. In further other aspects of this embodiment, a BoNT/E translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 15; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 15.

[0108] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/F translocation domain. In an aspect of this embodiment, a BoNT/F translocation domain comprises the translocation domains of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In other aspects of this embodiment, a BoNT/F translocation domain comprises amino acids 446-865 of SEQ ID NO: 18. In another aspect of this embodiment, a BoNT/F translocation domain comprises a naturally occurring

BoNT/F translocation domain variant, such as, *e.g.*, an translocation domain from a BoNT/F isoform or an translocation domain from a BoNT/F subtype. In another aspect of this embodiment, a BoNT/F translocation domain comprises a naturally occurring BoNT/F translocation domain variant of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20, such as, *e.g.*, a BoNT/F isoform translocation domain or a BoNT/F subtype translocation domain. In another aspect of this embodiment, a BoNT/F translocation domain comprises amino acids 446-865 of a naturally occurring BoNT/F translocation domain variant of SEQ ID NO: 18, such as, *e.g.*, a BoNT/F isoform translocation domain or a BoNT/F subtype translocation domain. In still another aspect of this embodiment, a BoNT/F translocation domain comprises a non-naturally occurring BoNT/F translocation domain variant, such as, *e.g.*, a conservative BoNT/F translocation domain variant, a non-conservative BoNT/F translocation domain variant, an active BoNT/F translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/F translocation domain comprises the translocation domain of a non-naturally occurring BoNT/F translocation domain variant of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20, such as, *e.g.*, a conservative BoNT/F translocation domain variant, a non-conservative BoNT/F translocation domain variant, an active BoNT/F translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/F translocation domain comprises amino acids 446-865 of a non-naturally occurring BoNT/F translocation domain variant of SEQ ID NO: 18, such as, *e.g.*, a conservative BoNT/F translocation domain variant, a non-conservative BoNT/F translocation domain variant, an active BoNT/F translocation domain fragment, or any combination thereof.

[0109] In other aspects of this embodiment, a BoNT/F translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In yet other aspects of this embodiment, a BoNT/F translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 446-865 of SEQ ID NO: 18; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 446-865 of SEQ ID NO: 18.

[0110] In other aspects of this embodiment, a BoNT/F translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In yet other aspects of this embodiment, a BoNT/F translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 446-865 of SEQ ID NO: 18; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid

deletions, additions, and/or substitutions relative to amino acids 446-865 of SEQ ID NO: 18. In still other aspects of this embodiment, a BoNT/F translocation domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20. In further other aspects of this embodiment, a BoNT/F translocation domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 446-865 of SEQ ID NO: 18; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 446-865 of SEQ ID NO: 18.

[0111] In another embodiment, a Clostridial toxin translocation domain comprises a BoNT/G translocation domain. In an aspect of this embodiment, a BoNT/G translocation domain comprises the translocation domains of SEQ ID NO: 21. In other aspects of this embodiment, a BoNT/G translocation domain comprises amino acids 451-865 of SEQ ID NO: 21. In another aspect of this embodiment, a BoNT/G translocation domain comprises a naturally occurring BoNT/G translocation domain variant, such as, e.g., an translocation domain from a BoNT/G isoform or an translocation domain from a BoNT/G subtype. In another aspect of this embodiment, a BoNT/G translocation domain comprises a naturally occurring BoNT/G translocation domain variant of SEQ ID NO: 21, such as, e.g., a BoNT/G isoform translocation domain or a BoNT/G subtype translocation domain. In another aspect of this embodiment, a BoNT/G translocation domain comprises amino acids 451-865 of a naturally occurring BoNT/G translocation domain variant of SEQ ID NO: 21, such as, e.g., a BoNT/G isoform translocation domain or a BoNT/G subtype translocation domain. In still another aspect of this embodiment, a BoNT/G translocation domain comprises a non-naturally occurring BoNT/G translocation domain variant, such as, e.g., a conservative BoNT/G translocation domain variant, a non-conservative BoNT/G translocation domain variant, an active BoNT/G translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/G translocation domain comprises the translocation domain of a non-naturally occurring BoNT/G translocation domain variant of SEQ ID NO: 21, such as, e.g., a conservative BoNT/G translocation domain variant, a non-conservative BoNT/G translocation domain variant, an active BoNT/G translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BoNT/G translocation domain comprises amino acids 451-865 of a non-naturally occurring BoNT/G translocation domain variant of SEQ ID NO: 21, such as, e.g., a conservative BoNT/G translocation domain variant, a non-conservative BoNT/G translocation domain variant, an active BoNT/G translocation domain fragment, or any combination thereof.

[0112] In other aspects of this embodiment, a BoNT/G translocation domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 21; or at most 70%, at most 75%, at

most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 21. In yet other aspects of this embodiment, a BoNT/G translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 451-865 of SEQ ID NO: 21; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 451-865 of SEQ ID NO: 21.

[0113] In other aspects of this embodiment, a BoNT/G translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 21. In yet other aspects of this embodiment, a BoNT/G translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-865 of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-865 of SEQ ID NO: 21. In still other aspects of this embodiment, a BoNT/G translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 21. In further other aspects of this embodiment, a BoNT/G translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-865 of SEQ ID NO: 21; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 451-865 of SEQ ID NO: 21.

[0114] In another embodiment, a Clostridial toxin translocation domain comprises a TeNT translocation domain. In an aspect of this embodiment, a TeNT translocation domain comprises the translocation domains of SEQ ID NO: 22. In other aspects of this embodiment, a TeNT translocation domain comprises amino acids 468-881 of SEQ ID NO: 22. In another aspect of this embodiment, a TeNT translocation domain comprises a naturally occurring TeNT translocation domain variant, such as, *e.g.*, an translocation domain from a TeNT isoform or an translocation domain from a TeNT subtype. In another aspect of this embodiment, a TeNT translocation domain comprises a naturally occurring TeNT translocation domain variant of SEQ ID NO: 22, such as, *e.g.*, a TeNT isoform translocation domain or a TeNT subtype translocation domain. In another aspect of this embodiment, a TeNT translocation domain comprises amino acids 468-881 of a naturally occurring TeNT translocation domain variant of SEQ ID NO: 22, such as, *e.g.*, a TeNT isoform translocation domain or a TeNT subtype translocation domain. In still another aspect of this embodiment, a TeNT translocation domain comprises a non-naturally occurring TeNT translocation domain variant, such as, *e.g.*, a conservative TeNT translocation domain variant, a

non-conservative TeNT translocation domain variant, an active TeNT translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a TeNT translocation domain comprises the translocation domain of a non-naturally occurring TeNT translocation domain variant of SEQ ID NO: 22, such as, *e.g.*, a conservative TeNT translocation domain variant, a non-conservative TeNT translocation domain variant, an active TeNT translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a TeNT translocation domain comprises amino acids 468-881 of a non-naturally occurring TeNT translocation domain variant of SEQ ID NO: 22, such as, *e.g.*, a conservative TeNT translocation domain variant, a non-conservative TeNT translocation domain variant, an active TeNT translocation domain fragment, or any combination thereof.

[0115] In other aspects of this embodiment, a TeNT translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 22; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 22. In yet other aspects of this embodiment, a TeNT translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 468-881 of SEQ ID NO: 22; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 468-881 of SEQ ID NO: 22.

[0116] In other aspects of this embodiment, a TeNT translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 22. In yet other aspects of this embodiment, a TeNT translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 468-881 of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 468-881 of SEQ ID NO: 22. In still other aspects of this embodiment, a TeNT translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 22. In further other aspects of this embodiment, a TeNT translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 468-881 of SEQ ID NO: 22; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 468-881 of SEQ ID NO: 22.

[0117] In another embodiment, a Clostridial toxin translocation domain comprises a BaNT translocation domain. In an aspect of this embodiment, a BaNT translocation domain comprises the translocation domains of SEQ ID NO: 23. In other aspects of this embodiment, a BaNT translocation domain comprises amino acids 436-857 of SEQ ID NO: 23. In another aspect of this embodiment, a BaNT translocation domain comprises a naturally occurring BaNT translocation domain variant, such as, *e.g.*, an translocation domain from a BaNT isoform or an translocation domain from a BaNT subtype. In another aspect of this embodiment, a BaNT translocation domain comprises a naturally occurring BaNT translocation domain variant of SEQ ID NO: 23, such as, *e.g.*, a BaNT isoform translocation domain or a BaNT subtype translocation domain. In another aspect of this embodiment, a BaNT translocation domain comprises amino acids 436-857 of a naturally occurring BaNT translocation domain variant of SEQ ID NO: 23, such as, *e.g.*, a BaNT isoform translocation domain or a BaNT subtype translocation domain. In still another aspect of this embodiment, a BaNT translocation domain comprises a non-naturally occurring BaNT translocation domain variant, such as, *e.g.*, a conservative BaNT translocation domain variant, a non-conservative BaNT translocation domain variant, an active BaNT translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BaNT translocation domain comprises the translocation domain of a non-naturally occurring BaNT translocation domain variant of SEQ ID NO: 23, such as, *e.g.*, a conservative BaNT translocation domain variant, a non-conservative BaNT translocation domain variant, an active BaNT translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BaNT translocation domain comprises amino acids 436-857 of a non-naturally occurring BaNT translocation domain variant of SEQ ID NO: 23, such as, *e.g.*, a conservative BaNT translocation domain variant, a non-conservative BaNT translocation domain variant, an active BaNT translocation domain fragment, or any combination thereof.

[0118] In other aspects of this embodiment, a BaNT translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to the translocation domain of SEQ ID NO: 23; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 23. In yet other aspects of this embodiment, a BaNT translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 436-857 of SEQ ID NO: 23; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 436-857 of SEQ ID NO: 23.

[0119] In other aspects of this embodiment, a BaNT translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 23. In yet other aspects of this embodiment, a BaNT translocation domain comprises a polypeptide having, *e.g.*, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions

relative to amino acids 436-857 of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 436-857 of SEQ ID NO: 23. In still other aspects of this embodiment, a BaNT translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 23. In further other aspects of this embodiment, a BaNT translocation domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 436-857 of SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 436-857 of SEQ ID NO: 23.

[0120] In another embodiment, a Clostridial toxin translocation domain comprises a BuNT translocation domain. In an aspect of this embodiment, a BuNT translocation domain comprises the translocation domains of SEQ ID NO: 24 or SEQ ID NO: 25. In other aspects of this embodiment, a BuNT translocation domain comprises amino acids 427-847 of SEQ ID NO: 24. In another aspect of this embodiment, a BuNT translocation domain comprises a naturally occurring BuNT translocation domain variant, such as, *e.g.*, a translocation domain from a BuNT isoform or a translocation domain from a BuNT subtype. In another aspect of this embodiment, a BuNT translocation domain comprises a naturally occurring BuNT translocation domain variant of SEQ ID NO: 24 or SEQ ID NO: 25, such as, *e.g.*, a BuNT isoform translocation domain or a BuNT subtype translocation domain. In another aspect of this embodiment, a BuNT translocation domain comprises amino acids 427-847 of a naturally occurring BuNT translocation domain variant of SEQ ID NO: 24, such as, *e.g.*, a BuNT isoform translocation domain or a BuNT subtype translocation domain. In still another aspect of this embodiment, a BuNT translocation domain comprises a non-naturally occurring BuNT translocation domain variant, such as, *e.g.*, a conservative BuNT translocation domain variant, a non-conservative BuNT translocation domain variant, an active BuNT translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BuNT translocation domain comprises the translocation domain of a non-naturally occurring BuNT translocation domain variant of SEQ ID NO: 24 or SEQ ID NO: 25, such as, *e.g.*, a conservative BuNT translocation domain variant, a non-conservative BuNT translocation domain variant, an active BuNT translocation domain fragment, or any combination thereof. In still another aspect of this embodiment, a BuNT translocation domain comprises amino acids 427-847 of a non-naturally occurring BuNT translocation domain variant of SEQ ID NO: 24, such as, *e.g.*, a conservative BuNT translocation domain variant, a non-conservative BuNT translocation domain variant, an active BuNT translocation domain fragment, or any combination thereof.

[0121] In other aspects of this embodiment, a BuNT translocation domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least

90%, or at least 95% to the translocation domain of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to the translocation domain of SEQ ID NO: 24 or SEQ ID NO: 25. In yet other aspects of this embodiment, a BuNT translocation domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% to amino acids 427-847 of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90%, or at most 95% to amino acids 427-847 of SEQ ID NO: 24 or SEQ ID NO: 25.

[0122] In other aspects of this embodiment, a BuNT translocation domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 24 OR SEQ ID NO: 25. In yet other aspects of this embodiment, a BuNT translocation domain comprises a polypeptide having, e.g., at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 24 or SEQ ID NO: 25. In still other aspects of this embodiment, a BuNT translocation domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to the translocation domain of SEQ ID NO: 24 or SEQ ID NO: 25. In further other aspects of this embodiment, a BuNT translocation domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 24 or SEQ ID NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 427-847 of SEQ ID NO: 24 or SEQ ID NO: 25.

[0123] Aspects of the present specification provide, in part, a TVEMP comprising a targeting domain. As used herein, the term “targeting domain” is synonymous with “binding domain”, “ligand”, or “targeting moiety” and refers to an amino acid sequence region able to preferentially bind to a cell surface marker, like a receptor, characteristic of the target cell under physiological conditions. The cell surface marker may comprise a polypeptide, a polysaccharide, a lipid, a glycoprotein, a lipoprotein, or may have structural characteristics of more than one of these. As used herein, the term “preferentially interacts” refers to a molecule capable of binding to its target cell surface marker under physiological conditions, or in vitro conditions substantially approximating physiological conditions, to a statistically significantly greater degree relative to other, non-target cell surface marker. With reference to a targeting domain disclosed herein, there is a discriminatory binding of the targeting domain to its cognate receptor relative

to other receptors. Examples of binding domains are described in, *e.g.*, Steward, L.E. et al., Modified Clostridial Toxins with Enhanced Translocation Capability and Enhanced Targeting Activity, U.S. Patent Application No. 11/776,043 (Jul. 11, 2007); Steward, L.E. et al., Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity For Clostridial Toxin Target Cells, U.S. Patent Application No. 11/776,052 (Jul. 11, 2007); and Steward, L.E. et al., Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity For Non-Clostridial Toxin Target Cells, U.S. Patent Application No. 11/776,075 (Jul. 11, 2007), each of which is incorporated by reference in its entirety.

[0124] In an embodiment, a targeting domain that selectively binds a target receptor has a dissociation equilibrium constant (K_D) that is greater for the target receptor relative to a non-target receptor by, *e.g.*, at least one-fold, at least two-fold, at least three-fold, at least four fold, at least five-fold, at least 10 fold, at least 50 fold, at least 100 fold, at least 1000, at least 10,000, or at least 100,000 fold.

[0125] An example of a targeting domain disclosed herein is a neurotrophin peptide targeting domain. Non-limiting examples of a neurotrophin peptide targeting domain include a nerve growth factor (NGF), a brain derived neurotrophic factor (BDNF), a neurotrophin-3 (NT-3), a neurotrophin-4/5 (NT-4/5), Tumor Necrosis Factor (TNF), and amyloid beta (A4) precursor protein neurotrophin (APP). Neurotrophin peptides bind to a family of protein receptors. For example, NGF, BDNF, NTF3, NTF4, TNF and APP bind to nerve growth factor receptor (NGFR); NGF, BDNF, NTF3, and NTF4 bind to neurotrophic tyrosine kinase, receptor, type 1 (NTRK1); NGF, BDNF, NTF3, NTF4 bind to neurotrophic tyrosine kinase, receptor, type 2 (NTRK2); and NGF, BDGF, NTF3, and NTF4 bind to neurotrophic tyrosine kinase, receptor, type 3 (NTRK3).

[0126] Neurotrophin peptide receptors have been detected on the surface of several different types of cancer cells. For example, NGFR is expressed in breast cancer, neuroblastomas, melanomas, schwannomas, insulinomas, hepatomas, prolactinomas, colon cancer, chronic myelogenous leukemias, mast cell leukemias, endometrial cancer, and pheochromocytomas. See, *e.g.*, A. Chiarenza, et al., Tamoxifen inhibits nerve growth factor-induced proliferation of the human breast cancerous cell line MCF-7, *Cancer Res.* 61(7): 3002-3008 (2001); S. Descamps, et al., Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells, *J. Biol. Chem.* 273(27): 16659-16662 (1998); G. Perini, et al., Role of p75 neurotrophin receptor in the neurotoxicity by beta-amyloid peptides and synergistic effect of inflammatory cytokines, *J. Exp. Med.* 195(7): 907-918 (2002); M. Kajiya, et al., Brain-derived neurotrophic factor stimulates bone/cementum-related protein gene expression in cementoblasts, *J. Biol. Chem.* 283(23): 16259-16267 (2008); A. Nakagawara, et al., Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma, *N. Engl. J. Med.* 328(12): 847-854 (1993); C. Azar, et al., Multiple defects of the nerve growth factor receptor in human neuroblastomas, *Prog. Clin. Biol. Res.* 366: 219-26 (1991); S.Y. Tam, et al., Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells, *Blood* 90(5): 1807-

1820 (1997); O. Shonukan, et al., Neurotrophin-induced melanoma cell migration is mediated through the actin-bundling protein fascin, *Oncogene* 22(23): 3616-3623 (2003); J.A. Reed, et al., Divergent cellular differentiation pathways during the invasive stage of cutaneous malignant melanoma progression, *Am. J. Pathol.* 155(2): 549-555 (1999); K.J. Busam, et al., Distinction of desmoplastic melanoma from non-desmoplastic melanoma by gene expression profiling, *J. Invest. Dermatol.* 124(2): 412-418 (2005); D.G. Menter, et al., Involvement of neurotrophins and growth factors in brain metastasis formation, *Invasion Metastasis* 14(1-6): 372-384 (1994); J.J. Gentry, et al., Nerve growth factor activation of nuclear factor kappaB through its p75 receptor is an anti-apoptotic signal in RN22 schwannoma cells, *J. Biol. Chem.* 275(11): 7558-7565 (2000); M. Polak, et al., Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line, *Proc. Natl. Acad. Sci. USA* 90(12): 5781-5785 (1993); S. Preiss, et al., Characterization of the innate immune signalling pathways in hepatocyte cell lines, *J. Viral Hepat.* 15(12): 888-900 (2008); C. Fiorentini, et al., Nerve growth factor regulates dopamine D(2) receptor expression in prolactinoma cell lines via p75(NGFR)-mediated activation of nuclear factor-kappaB, *Mol. Endocrinol.* 16(2): 353-366 (2002); L. Monlauzeur, et al., Putative O-glycosylation sites and a membrane anchor are necessary for apical delivery of the human neurotrophin receptor in Caco-2 cells, *J. Biol. Chem.* 273(46): 30263-30270 (1998); E.K. Innis, et al., Growth of the endometrial adenocarcinoma cell line AN3 CA is modulated by tumor necrosis factor and its receptor is up-regulated by estrogen in vitro, *Endocrinology* 130(4): 1852-1856 (1992); J.L. Urdiales, et al., Cell cycle phase-specific surface expression of nerve growth factor receptors TrkA and p75(NTR), *J. Neurosci.* 18(17): 6767-6775 (1998).

[0127] As another example, NTRK1 is expressed in breast cancer, neuroblastomas, schwannomas, prolactinomas, leukemias, prostate cancer, thyroid cancer, squamous lung cell carcinomas, and pheochromocytoma. See, e.g., E. Tagliabue, et al., Nerve growth factor cooperates with p185(HER2) in activating growth of human breast carcinoma cells, *J. Biol. Chem.* 275(8): 5388-5394 (2000); S. Descamps, et al., Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells, *J. Biol. Chem.* 273(27):16659-16662 (1998); S. Descamps, et al., Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways, *J. Biol. Chem.* 276(21): 17864-17870 (2001); D. Melck, et al., Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation, *Endocrinology* 141(1): 118-126 (2000); S.J. Pollack and S.J. Harper, Small molecule Trk receptor agonists and other neurotrophic factor mimetics, *Curr. Drug Targets CNS Neurol. Disord.* 1(1): 59-80 (2002); D.S. Middlemas, et al., Brain-derived neurotrophic factor promotes survival and chemoprotection of human neuroblastoma cells, *J. Biol. Chem.* 274(23): 16451-16460 (1999); K. Matsumoto, et al., Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cells, *Cancer Res.* 55(8): 1798-1806 (1999); K. Kramer, et al., Prognostic value of TrkA protein detection by monoclonal antibody 5C3 in neuroblastoma, *Clin. Cancer Res.* 2(8): 1361 (1996); M. Polak, et al., Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line, *Proc. Natl. Acad. Sci. USA* 90(12): 5781-5785 (1993); C. Fiorentini, et al., Nerve growth factor regulates dopamine D(2) receptor expression in

prolactinoma cell lines via p75(NGFR)-mediated activation of nuclear factor-kappaB, *Mol. Endocrinol.* 16(2): 353-366 (2002); M. Eguchi, et al., Fusion of ETV6 to neurotrophin-3 receptor TRKC in acute myeloid leukemia with t(12;15)(p13;q25), *Blood* 93(4): 1355-1363 (1999); M.A. Sortino, et al., Mitogenic effect of nerve growth factor (NGF) in LNCaP prostate adenocarcinoma cells: role of the high- and low-affinity NGF receptors, *Mol. Endocrinol.* 14(1): 124-136 (2000); B.R. Pflug, et al., Expression of a Trk high affinity nerve growth factor receptor in the human prostate, *Endocrinology* 136(1): 262-268 (1995); R.A. Segal, Selectivity in neurotrophin signaling: theme and variations, *Annu. Rev. Neurosci.* 26: 299-330 (2003); L.M. McGregor, et al., Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression, *Proc. Natl. Acad. Sci. USA* 96(8): 4540-4545 (1999); S. Amatschek, et al., Tissue-wide expression profiling using cDNA subtraction and microarrays to identify tumor-specific genes, *Cancer Res.* 64(3): 844-856 (2004); X. Lou, et al., GIPC and GAIP form a complex with TrkA: a putative link between G protein and receptor tyrosine kinase pathways, *Mol. Biol. Cell* 12(3): 615-627 (2001); and J.L. Urdiales, et al., Cell cycle phase-specific surface expression of nerve growth factor receptors TrkA and p75(NTR), *J. Neurosci.* 18(17): 6767-6775 (1998).

[0128] As another example, NTRK2 is expressed in Neuroblastomas, colon cancer, leukemias, prostate cancer, thyroid cancer, lung cancer, astrocytomas, and glioblastomas. See, e.g., S.J. Pollack and S.J. Harper, Small molecule Trk receptor agonists and other neurotrophic factor mimetics, *Curr. Drug Targets CNS Neurol. Disord.* 1(1): 59-80 (2002); D.S. Middlemas, et al., Brain-derived neurotrophic factor promotes survival and chemoprotection of human neuroblastoma cells, *J. Biol. Chem.* 274(23): 16451-16460 (1999); S. Scala, et al. Brain-derived neurotrophic factor protects neuroblastoma cells from vinblastine toxicity, *Cancer Res.* 56(16): 3737-3742 (1996); J. Zhang, et al., The studies on the correlation for gene expression of tyrosine-kinase receptors and vascular endothelial growth factor in human neuroblastomas, *J. Pediatr. Hematol. Oncol.* 32(3): 180 (2010); J.B. Easton, et al., Brain-derived neurotrophic factor induces phosphorylation of fibroblast growth factor receptor substrate 2, *J. Biol. Chem.* 274(16): 11321-11327 (1999); W. Jin, et al., TrkC Binds to the Bone Morphogenetic Protein Type II Receptor to Suppress Bone Morphogenetic Protein Signaling, *Cancer Res.* 67(20): 9869-9877 (2007); T. Powles, et al., Cannabis-induced cytotoxicity in leukemic cell lines: the role of the cannabinoid receptors and the MAPK pathway, *Blood* 105(3): 1214-12121 (2005); B.R. Pflug, et al., Expression of a Trk high affinity nerve growth factor receptor in the human prostate, *Endocrinology* 136(1): 262-268 (1995); L. M. McGregor, et al., Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression, *Proc. Natl. Acad. Sci. USA* 96(8): 4540-4545 (1999); J. Shen, et al., Identification and validation of differences in protein levels in normal, premalignant, and malignant lung cells and tissues using high-throughput Western array and immunohistochemistry, *Cancer Res.* 66(23): 11194-11206 (2006); and J.Y. Ljubimova, et al., Overexpression of alpha4 chain-containing laminins in human glial tumors identified by gene microarray analysis, *Cancer Res.* 61(14):5601-5610 (2001).

[0129] As another example, NTRK3 is expressed in breast cancer, neuroblastomas, insulinomas, medulloblastomas, colon cancer, leukemias, prostate cancer, thyroid cancer, astrocytomas,

glioblastomas, and fibrosarcoma. See, e.g., Jin W, et al. c-Src Is Required for Tropomyosin Receptor Kinase C (TrkC)-induced Activation of the Phosphatidylinositol 3-Kinase (PI3K)-AKT Pathway. *J Biol Chem* 2008 Jan 18;283(3):1391-400; Pollack SJ, Harper SJ. Small molecule Trk receptor agonists and other neurotrophic factor mimetics. *Curr Drug Targets CNS Neurol Disord* 2002 Feb 01;1(1):59-80; Laneve P, et al. The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. *Proc Natl Acad Sci U S A* 2007 May 08;104(19):7957-62; Fu AK, et al. Muscle-derived neurotrophin-3 increases the aggregation of acetylcholine receptors in neuron-muscle co-cultures. *Neuroreport* 1997 Dec 22;8(18):3895-900; Tazi A, et al. Neurotrophin-3 increases intracellular calcium in a rat insulin-secreting cell line through its action on a functional TrkC receptor. *J Biol Chem* 1996 Apr 26;271(17):10154-60; Segal RA, et al. Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. *Proc Natl Acad Sci U S A* 1994 Dec 20;91(26):12867-71; Jin W, et al. TrkC Binds to the Bone Morphogenetic Protein Type II Receptor to Suppress Bone Morphogenetic Protein Signaling. *Cancer Res* 2007 Oct 15;67(20):9869-77; Eguchi M, et al. Fusion of ETV6 to neurotrophin-3 receptor TRKC in acute myeloid leukemia with t(12;15)(p13;q25). *Blood* 1999 Feb 15;93(4):1355-63; Pflug BR, et al. Expression of a Trk high affinity nerve growth factor receptor in the human prostate. *Endocrinology* 1995 Jan;136(1):262-8; McGregor LM, et al. Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression. *Proc Natl Acad Sci U S A* 1999 Apr 13;96(8):4540-5; Bruce AW, et al. Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc Natl Acad Sci U S A* 2004 Jul 13;101(28):10458-63; and Segal RA. Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* 2003 Jan 01;26:299-330.

[0130] As such, a TVEMP comprising a neurotrophin peptide targeting domain would be effective in treating cancer, including a breast cancer, a neuroblastoma, a melanoma, a schwannoma, an insulinoma, a hepatoma, a medulloblastoma, a prolactinoma, a colon cancer, a leukemia, a chronic myelogenous leukemia, mast cell leukemia, an endometrial cancer, a prostate cancer, a thyroid cancer, a squamous-cell lung carcinoma, a lung cancer, an astrocytoma, a glioblastoma, a fibrosarcoma, or a pheochromocytoma.

[0131] Thus, in an embodiment, a retargeted targeting domain comprises a neurotrophin peptide targeting domain. In aspects of this embodiment, a neurotrophin peptide targeting domain comprises a NGF, a BDNF, a NT-3, or a NT-4/5. In other aspects of this embodiment, a neurotrophin peptide targeting domain comprises SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85. In other aspects of this embodiment, a neurotrophin peptide targeting domain comprises amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85.

[0132] In other aspects of this embodiment, a neurotrophin targeting domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85. In yet other aspects of this embodiment, a neurotrophin targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85. In still other aspects of this embodiment, a neurotrophin targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, or SEQ ID NO: 85.

[0133] In other aspects of this embodiment, a neurotrophin targeting domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85. In yet other aspects of this embodiment, a neurotrophin targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85. In still other aspects of this embodiment, a neurotrophin targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85.

[0134] Another example of a targeting domain disclosed herein is a head activator (HA) peptide. Thus, in an embodiment, a retargeted targeting domain comprises a HA peptide targeting domain. In aspects of this embodiment, a neurotrophin peptide targeting domain comprises SEQ ID NO: 86. HA peptides bind to a family of protein receptors. For example, HA peptides bind to the orphan G-protein-coupled receptor GPR37 (also known as Endothelin receptor type B-like) and the Sortilin-related receptor (SRR).

[0135] Head activator peptide receptors have been detected on the surface of several different types of cancer cells. For example, GPR37 is expressed in breast cancer. See, e.g., G. Deblois, et al., Genome-wide identification of direct target genes implicates estrogen-related receptor alpha as a determinant of breast cancer heterogeneity, *Cancer Res.* 69(15): 6149-6157 (2009). As another example, SRR is expressed in breast cancer. See, e.g., S. Ashida, et al., Molecular Features of the Transition from Prostatic Intraepithelial Neoplasia (PIN) to Prostate Cancer: Genome-wide Gene-expression Profiles of Prostate Cancers and PINs, *Cancer Res.* 64(17): 5963-5972 (2004).

[0136] As such, a TVEMP comprising a head activator peptide targeting domain would be effective in treating cancer, including a breast cancer.

[0137] In other aspects of this embodiment, a HA targeting domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 86; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 86. In yet other aspects of this embodiment, a HA targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, or 5 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 86; or at most 1, 2, 3, 4, or 5 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 86. In still other aspects of this embodiment, a HA targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, or 5 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 86; or at most 1, 2, 3, 4, or 5 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 86.

[0138] Another example of a targeting domain disclosed herein is a glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) peptide targeting domain. Non-limiting examples of a GFL peptide targeting domain include a GDNF, a Neurturin (NRTN), a Persephrin (PSPN), or an Artemin (ARTN). GDNF-GFL peptides bind to a family of G-coupled protein receptors. For example, GDNF and NRTN bind to GDNF family receptor alpha 1 and 2 (GFRA1 and GFRA2); GDNF and ARTN bind to GDNF family receptor alpha 3 (GFRA3); GDNF and PSPN bind to GDNF family receptor alpha 4 (GFRA4); and GDNF, NRTN, and GFRA1 bind Ret proto-oncogene (RET).

[0139] GDNF-GFL peptide receptors have been detected on the surface of several different types of cancer cells. For example, GFRA1 is expressed in breast cancer, pancreatic cancer, and thyroid cancer.

See, e.g., Naderi A, et al. BEX2 is overexpressed in a subset of primary breast cancers and mediates nerve growth factor/nuclear factor-kappaB inhibition of apoptosis in breast cancer cell lines. *Cancer Res* 2007 Jul 15;67(14):6725-36; Funahashi, H., et al. The role of glial cell line-derived neurotrophic factor (GDNF) and integrins for invasion and metastasis in human pancreatic cancer cells. *J Surg Oncol*. 2005 Jul 1;91(1):77-83; Borrego S, et al. Evaluation of germline sequence variants of GFRA1, GFRA2, and GFRA3 genes in a cohort of Spanish patients with sporadic medullary thyroid cancer. *Thyroid*. 2002 Nov;12(11):1017-22 ; Gimm O, et al. Over-representation of a germline variant in the gene encoding RET co-receptor GFRalpha-1 but not GFRalpha-2 or GFRalpha-3 in cases with sporadic medullary thyroid carcinoma. *Oncogene*. 2001 Apr 19;20(17):2161-70; and Wells, et al. Targeting the RET pathway in thyroid cancer. *Clin Cancer Res*. 2009 Dec 1;15(23):7119-23.

[0140] As another example, GFRA2 is expressed in thyroid cancer. See, e.g., S. Borrego, et al., Evaluation of germline sequence variants of GFRA1, GFRA2, and GFRA3 genes in a cohort of Spanish patients with sporadic medullary thyroid cancer, *Thyroid* 12(11): 1017-1022 (2002).

[0141] As yet another example, GFRA3 is expressed in thyroid cancer. See, e.g., S. Borrego, et al., Evaluation of germline sequence variants of GFRA1, GFRA2, and GFRA3 genes in a cohort of Spanish patients with sporadic medullary thyroid cancer, *Thyroid* 12(11): 1017-1022 (2002).

[0142] As another example, GFRA4 is expressed in thyroid cancer. See, e.g., Lindahl, et al., Human glial cell line-derived neurotrophic factor receptor alpha 4 is the receptor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells, *J. Biol. Chem.* 276(12): 9344-9351 (2001).

[0143] As another example, RET is expressed in thyroid cancer, renal cancer, adenocarcinoma, melanoma, and bladder cancer. See, e.g., La Perle KM, et al. Loss of p53 promotes anaplasia and local invasion in ret/PTC1-induced thyroid carcinomas. *Am J Pathol* 2000 Aug 1;157(2):671-7; Blume-Jensen P, et al. Oncogenic kinase signalling. *Nature* 2001 May 17;411(6835):355-65; Schmid M, et al. *Cell Cycle Regulation and Hematological Disorders Williams Hematology 6th Edition*, Chapter 12:131-140; Kitamura Y, et al. Novel germline RET proto-oncogene mutations associated with medullary thyroid carcinoma (MTC): mutation analysis in Japanese patients with MTC. *Oncogene* 1997 Jun 26;14(25):3103-6; Gimm O, et al. Over-representation of a germline RET sequence variant in patients with sporadic medullary thyroid carcinoma and somatic RET codon 918 mutation. *Oncogene* 1999 Feb 11;18(6):1369-73; Michiels FM, et al. Development of medullary thyroid carcinoma in transgenic mice expressing the RET protooncogene altered by a multiple endocrine neoplasia type 2A mutation. *Proc Natl Acad Sci U S A* 1997 Apr 1;94(7):3330-5; Jhiang SM, et al. Targeted expression of the ret/PTC1 oncogene induces papillary thyroid carcinomas. *Endocrinology* 1996 Jan;137(1):375-8; van der Geer P, et al. Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol* 1994;10:251-337; Qiao S, et al. Differential effects of leukocyte common antigen-related protein on biochemical and

biological activities of RET-MEN2A and RET-MEN2B mutant proteins. J Biol Chem 2001 Mar 23;276(12):9460-7; Sweetser DA, et al. Ganglioneuromas and renal anomalies are induced by activated RET(MEN2B) in transgenic mice. Oncogene 1999 Jan 28;18(4):877-86; Kawai K, et al. Tissue-specific carcinogenesis in transgenic mice expressing the RET proto-oncogene with a multiple endocrine neoplasia type 2A mutation. Cancer Res 2000 Sep 15;60(18):5254-60; Kato M, et al. Transgenic mouse model for skin malignant melanoma. Oncogene 1998 Oct 8;17(14):1885-8; Kumasaka MY, et al. A novel mouse model for de novo Melanoma. Cancer Res 2010 Jan 01;70(1):24-9; and Staack A, et al. Combined determination of plasma MMP2, MMP9, and TIMP1 improves the non-invasive detection of transitional cell carcinoma of the bladder. BMC Urol 2006 Jan 01;6:19.

[0144] As such, a TVEMP comprising a CDNF-GFL peptide targeting domain would be effective in treating cancer, including a breast cancer, a pancreatic cancer, a thyroid cancer, a renal cancer, an adenocarcinoma, a melanoma, or a bladder cancer.

[0145] Thus, in an embodiment, a targeting domain comprises a GFL peptide targeting domain. In aspects of this embodiment, a GFL peptide targeting domain comprises a GDNF, a NRTN, a PSPN, or an ARTN. In other aspects of this embodiment, a GFL peptide targeting domain comprises SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90. In other aspects of this embodiment, a GFL peptide targeting domain comprises amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90.

[0146] In other aspects of this embodiment, a GFL targeting domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90. In yet other aspects of this embodiment, a GFL targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90. In still other aspects of this embodiment, a GFL targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90.

[0147] In other aspects of this embodiment, a GFL targeting domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least

95% to amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90. In yet other aspects of this embodiment, a GFL targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90. In still other aspects of this embodiment, a GFL targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90.

[0148] Another example of a targeting domain disclosed herein is a RF-amide related peptide (RFRP) peptide targeting domain. Non-limiting examples of a RFRP peptide targeting domain include a RFRP-1, a RFRP-2, a RFRP-3, neuropeptide AF (NPAF), and neuropeptide FF (NPFF). RFRP peptides bind to a family of G-coupled protein receptors. For example, NPVF, 1DME-NPFF, NPFF bind to neuropeptide FF receptor 1 (NPFFR1); and NPFF, Pancreatic polypeptide, NPY, NPVF, PRLH (prolactin releasing hormone), 1DME-NPFF, EYFSLAAPQRF-NH₂ bind to neuropeptide FF receptor 2 (NPFFR2).

[0149] RFRP peptide receptors have been detected on the surface of several different types of cancer cells. For example, NPFFR2 is expressed in breast cancer. See, *e.g.*, K.B. Hendricks, et al., Role for BRG1 in cell cycle control and tumor suppression, *Mol. Cell Biol.* 24(1):362-376 (2004).

[0150] As such, a TVEMP comprising a RFRP peptide targeting domain would be effective in treating cancer, including a breast cancer.

[0151] Thus, in an embodiment, a targeting domain comprises a RFRP peptide targeting domain. In aspects of this embodiment, a RFRP peptide targeting domain comprises a RFRP-1, a RFRP-2, a RFRP-3, a NPAF, or a NPFF. In other aspects of this embodiment, a RFRP peptide targeting domain comprises SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94. In other aspects of this embodiment, a RFRP peptide targeting domain comprises amino acids 81-92, amino acids 101-112, or

amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94.

[0152] In other aspects of this embodiment, a RFRP targeting domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94. In yet other aspects of this embodiment, a RFRP targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94. In still other aspects of this embodiment, a RFRP targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, or SEQ ID NO: 94.

[0153] In other aspects of this embodiment, a RFRP targeting domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94. In yet other aspects of this embodiment, a RFRP targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, or 5 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94; or at most 1, 2, 3, 4, or 5 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94. In still other aspects of this embodiment, a RFRP targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, or 5 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of

SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94; or at most 1, 2, 3, 4, or 5 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94.

[0154] Another example of a targeting domain disclosed herein is a neurohormone peptide targeting domain. Non-limiting examples of a neurohormone peptide targeting domain include a corticotropin-releasing hormone (CRH), a parathyroid hormone (PTH), a parathyroid hormone-like hormone (PTHLH), a PHYH, a thyrotropin-releasing hormone (TRH), an urocortin-1 (UCN1), an urocortin-2 (UCN2), an urocortin-3 (UCN3), or an urotensin 2 (UTS2). Neurohormone peptides bind to a family of G-coupled protein receptors. For example, CRH, PTH, and UCN bind to corticotropin releasing hormone receptor 1 (CRHR1); CRH, UTS2, UCN, UCN2, UCN3 bind to corticotropin releasing hormone receptor 2 (CRHR2); PTH and PTHLH bind to parathyroid hormone 1 receptor (PTH1R); PTH, PTHLH, and PHYH bind to parathyroid hormone 2 receptor (PTH2R); and TRH binds to thyrotropin-releasing hormone receptor (TRHR) and thyrotropin releasing hormone receptor 2 (TRHR2).

[0155] Neurohormone peptide receptors have been detected on the surface of several different types of cancer cells. For example, CRHR1 is expressed in breast cancer, uterine cancer, melanoma, basal-cell skin carcinomas, pituitary cancer, neuroblastomas, small cell lung carcinomas, endometrial cancer, pheochromocytomas, and squamous cell carcinoma. See, e.g., Yuan R, et al. Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage. *Cancer Res* 2001 Nov 1;61(21):8022-31; Graziani G, et al. CRH inhibits cell growth of human endometrial adenocarcinoma cells via CRH-receptor 1-mediated activation of cAMP-PKA pathway. *Endocrinology* 2002 Mar;143(3):807-13; Pisarchik A, Slominski AT. Alternative splicing of CRH-R1 receptors in human and mouse skin: identification of new variants and their differential expression. *FASEB J* 2001 Dec 1;15(14):2754-6; Pisarchik A, Slominski AT. Alternative splicing of CRH-R1 receptors in human and mouse skin: identification of new variants and their differential expression. *FASEB J* 2001 Dec 1;15(14):2754-6; Peeters PJ, et al. Transcriptional Response to Corticotropin-Releasing Factor in AtT-20 Cells. *Mol Pharmacol* 2004 Nov 01;66(5):1083-92; Brar BK, et al. Specificity and regulation of ERK1/2 phosphorylation through CRF receptors 1 and 2{beta} by the CRF/Ucn family of peptides. *Endocrinology* 2004 04 1;145(4):1718-29; Dieterich KD, et al. Corticotropin-releasing factor receptors in human small cell lung carcinoma cells: radioligand binding, second messenger, and northern blot analysis data. *Endocrinology* 1994 Oct;135(4):1551-8; Graziani G, et al. CRH inhibits cell growth of human endometrial adenocarcinoma cells via CRH-receptor 1-mediated activation of cAMP-PKA pathway. *Endocrinology* 2002 Mar;143(3):807-13; Dermitzaki E, et al. Corticotropin-Releasing Factor (CRF) and the Urocortins Differentially Regulate Catecholamine Secretion in Human and Rat Adrenals, in a CRF Receptor Type-Specific Manner. *Endocrinology* 2007 Apr 01;148(4):1524-38; and Pisarchik A,

Slominski AT. Alternative splicing of CRH-R1 receptors in human and mouse skin: identification of new variants and their differential expression. *FASEB J* 2001 Dec 1;15(14):2754-6.

[0156] As another example, CRHR2 is expressed in neuroblastoma, pheochromocytoma, and colon cancer. See, e.g., Brar BK, et al. Specificity and regulation of ERK1/2 phosphorylation through CRF receptors 1 and 2{beta} by the CRF/Ucn family of peptides. *Endocrinology* 2004 04 1;145(4):1718-29; Dermitzaki E, et al. Corticotropin-Releasing Factor (CRF) and the Urocortins Differentially Regulate Catecholamine Secretion in Human and Rat Adrenals, in a CRF Receptor Type-Specific Manner. *Endocrinology* 2007 Apr 01;148(4):1524-38; and Kokkotou E, et al. Corticotropin-releasing hormone receptor 2-deficient mice have reduced intestinal inflammatory responses. *J Immunol* 2006 Sep 01;177(5):3355-61.

[0157] As yet another example, PTH1R is expressed in alveolar basal epithelial carcinomas, testicular cancer, and osteosarcomas. See, e.g., Hastings RH, et al. Parathyroid hormone-related protein, an autocrine regulatory factor in alveolar epithelial cells. *Am J Physiol* 1996 Mar 1;270(3 Pt 1):L353-61; Tfelt-Hansen J, et al. Calcium-Sensing Receptor Induces Proliferation through p38 Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase But Not Extracellularly Regulated Kinase in a Model of Humoral Hypercalcemia of Malignancy. *Endocrinology* 2004 Mar 01;145(3): 1211-7; and Williams LJ, Abou-Samra AB. The transcription factors SP1 and MAZ regulate expression of the parathyroid hormone/parathyroid hormone-related peptide receptor gene. *J Mol Endocrinol* 2000 12 1;25(3):309-19.

[0158] As still another example, TRHR is expressed in pituitary cancer. See, e.g., Yang J, Tashjian AH Jr. Transcriptional regulation by dexamethasone of endogenous thyrotropin-releasing hormone receptor messenger ribonucleic acid in rat pituitary GH4C1 cells. *Endocrinology* 1993 Aug;133(2):487-90; Yamada M, et al. Pituitary adenomas of patients with acromegaly express thyrotropin-releasing hormone receptor messenger RNA: cloning and functional expression of the human thyrotropin-releasing hormone receptor gene. *Biochem Biophys Res Commun* 1993 Sep 15;195(2): 737-45; and Sortino M, et al. Protein kinase C inhibits TRH-stimulated phosphoinositide hydrolysis in GH3 cells. *Eur J Pharmacol* 1987 Mar 3;135(1):77-83.

[0159] As such, a TVEMP comprising a neurohormone peptide targeting domain would be effective in treating cancer, including a breast cancer, an uterine cancer, a melanoma, a basal-cell skin carcinoma, a pituitary cancer, a neuroblastoma, a small cell lung carcinoma, an endometrial cancer, a pheochromocytoma, a squamous cell carcinoma, a colon cancer, an alveolar basal epithelial carcinoma, a testicular cancer, or an osteosarcoma.

[0160] Thus, in an embodiment, a targeting domain comprises a neurohormone peptide targeting domain. In aspects of this embodiment, a neurohormone peptide targeting domain comprises a CCRH, a PTH, or a TRH. In other aspects of this embodiment, a neurohormone peptide targeting domain

comprises SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97. In other aspects of this embodiment, a neurohormone peptide targeting domain comprises amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97.

[0161] In other aspects of this embodiment, a neurohormone targeting domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97. In yet other aspects of this embodiment, a neurohormone targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97. In still other aspects of this embodiment, a neurohormone targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 95, SEQ ID NO: 96, or SEQ ID NO: 97.

[0162] In other aspects of this embodiment, a neurohormone targeting domain comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97. In yet other aspects of this embodiment, a neurohormone targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, or 5 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97; or at most 1, 2, 3, 4, or 5 non-contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97. In still other aspects of this embodiment, a neurohormone targeting domain comprises a polypeptide having, *e.g.*, at least 1, 2, 3, 4, or 5 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97; or at most 1, 2, 3, 4, or 5 contiguous amino acid deletions, additions, and/or substitutions relative to amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97.

[0163] Another example of a targeting domain disclosed herein is a neuroregulatory cytokine peptide targeting domain. Non-limiting examples of a neuroregulatory cytokine peptide targeting domain include a ciliary neurotrophic factor (CNTF), a glycoprotein-A (GPA), a leukemia inhibitory factor (LIF), also known as cholinergic differentiation factor (CDF), a cardiotrophin-1 (CT-1), a cardiotrophin-like cytokine (CLC), a neuroleukin, also known as glucose phosphate isomerase (GPI), autocrine motility factor (AMF), maturation and differentiation factor (MF), and an onostatin M (OSM). Neuroregulatory cytokine peptides bind to a family of G-coupled protein receptors. For example, CNTF binds to CNTF receptor (CNTFR), Interleukin 6 signal transducer (IL6ST) also known as gp130, oncostatin M receptor, Leukemia inhibitory factor receptor alpha (LIFR), and Interleukin 6 receptor (IL6R); LIF binds to LIFR, IL6ST, and Insulin-like growth factor 2 receptor (IGF2R); CTF1 binds to LIFR and IL6ST; CLCF1 binds to CNTFR, IL6ST, and LIFR; AMF and VCP bind to autocrine motility factor receptor (AMFR); and OSM binds to a heterodimer of LIFR and IL6ST.

[0164] Neuroregulatory cytokine peptide receptors have been detected on the surface of several different types of cancer cells. For example, CNTFR is expressed in liver cancer. See, e.g., X. Hu, et al., Ciliary neurotrophic factor receptor alpha subunit-modulated multiple downstream signaling pathways in hepatic cancer cell lines and their biological implications, *Hepatology* 47(4):1298-1308 (2008).

[0165] As another example, IL6ST is expressed in stomach cancer. See, e.g., Tebbutt NC, et al. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT- mediated trefoil gene activation in gp130 mutant mice. *Nat Med* 2002 10 1;8(10):1089-97.

[0166] As yet another example, LIFR is expressed in lymphomas. See, e.g., Piccaluga PP, et al. Gene expression analysis of peripheral T cell lymphoma, unspecified, reveals distinct profiles and new potential therapeutic targets. *J Clin Invest* 2007 Mar 01;117(3):823-34; and Piccaluga PP, et al. Gene expression analysis of angioimmunoblastic lymphoma indicates derivation from T follicular helper cells and vascular endothelial growth factor deregulation. *Cancer Res* 2007 Nov 15;67(22):10703-10.

[0167] As still another example, IL-6R is expressed in colon cancer. See, e.g., A.M. Sääf, et al, Parallels between global transcriptional programs of polarizing Caco-2 intestinal epithelial cells in vitro and gene expression programs in normal colon and colon cancer, *Mol. Biol. Cell.* 18(11): 4245-4260 (2007).

[0168] As a further example, IGF2R is expressed in gastric cancer and liver cancer. See, e.g., L. Ottini, et al., Mutations at coding mononucleotide repeats in gastric cancer with the microsatellite mutator phenotype, *Oncogene* 16(21): 2767-2772 (1998); and Y.J. Chung, et al., Evidence of genetic progression in human gastric carcinomas with microsatellite instability, *Oncogene* 15(14): 1719-1726 (1997); and J.J. Mills, et al., Imprinted M6p/Igf2 receptor is mutated in rat liver tumors, *Oncogene* 16(21): 2797-2802 (1998).

[0169] As such, a TVEMP comprising a neuroregulatory cytokine peptide targeting domain would be effective in treating cancer, including a liver cancer, a stomach cancer, a lymphoma, a colon cancer, a gastric cancer.

[0170] Thus, in an embodiment, a targeting domain comprises a neuroregulatory cytokine peptide targeting domain. In aspects of this embodiment, a neuroregulatory cytokine peptide targeting domain comprises a CNTF, a GPA, a LIF, a CT-1, a CLC, a neuroleukin, or an OSM. In other aspects of this embodiment, a neurohormone peptide targeting domain comprises SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103.

[0171] In other aspects of this embodiment, a neuroregulatory cytokine targeting domain comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103. In yet other aspects of this embodiment, a neuroregulatory cytokine targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103. In still other aspects of this embodiment, a neuroregulatory cytokine targeting domain comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103.

[0172] Clostridial toxins are each translated as a single-chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring protease (FIG. 18). This cleavage occurs within the discrete di-chain loop region created between two cysteine residues that form a disulfide bridge. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by the single disulfide bond and non-covalent interactions between the two chains (FIG. 2). To facilitate recombinant production of a TVEMP, an exogenous protease cleavage site can be used to convert the single-chain polypeptide form of a TVEMP disclosed herein into the di-chain form. See, e.g., Steward, L.E. et al., *Modified Clostridial Toxins with Enhanced Targeting Capabilities For Endogenous Clostridial Toxin Receptor Systems*, U.S. Patent Publication No. US 2008/0096248 (Apr. 24, 2008); Steward, L.E. et al., *Activatable Clostridial Toxins*, U.S. Patent Publication No. US 2008/0032930 (Feb. 7,

2008); Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); and Foster, *supra*, WO 2006/059105 (2006), each of which is hereby incorporated by reference in its entirety.

[0173] It is envisioned that any and all protease cleavage sites can be used to convert the single-chain polypeptide form of a Clostridial toxin into the di-chain form, including, without limitation, endogenous di-chain loop protease cleavage sites and exogenous protease cleavage sites. Thus, in an aspect of the invention, a TVEMP comprises, in part, an endogenous protease cleavage site within a di-chain loop region. In another aspect of the invention, a TVEMP comprises, in part, an exogenous protease cleavage site within a di-chain loop region. As used herein, the term “di-chain loop region” means the amino acid sequence of a Clostridial toxin containing a protease cleavage site used to convert the single-chain form of a Clostridial toxin into the di-chain form. Non-limiting examples of a Clostridial toxin di-chain loop region, include, a di-chain loop region of BoNT/A comprising amino acids 430-454 of SEQ ID NO: 1; a di-chain loop region of BoNT/B comprising amino acids 437-446 of SEQ ID NO: 2; a di-chain loop region of BoNT/C1 comprising amino acids 437-453 of SEQ ID NO: 3; a di-chain loop region of BoNT/D comprising amino acids 437-450 of SEQ ID NO: 4; a di-chain loop region of BoNT/E comprising amino acids 412-426 of SEQ ID NO: 5; a di-chain loop region of BoNT/F comprising amino acids 429-445 of SEQ ID NO: 6; a di-chain loop region of BoNT/G comprising amino acids 436-450 of SEQ ID NO: 7; and a di-chain loop region of TeNT comprising amino acids 439-467 of SEQ ID NO: 8 (Table 4).

Table 4. Di-chain Loop Region of Clostridial Toxins		
Toxin	SEQ ID NO:	Di-chain Loop Region Containing the Naturally-occurring Protease Cleavage Site
BoNT/A	26	CVRGIITSKTKSLDKGYNK*---ALNDLC
BoNT/B	27	CKSVK*-----APGIC
BoNT/C1	28	CHKAIDGRSLYNK*-----TLDC
BoNT/D	29	CLRLTKNSR*-----DDSTC
BoNT/E	30	CKNIVSVKGIR*-----KSIC
BoNT/F	31	CKSVIPRKGTK*-----APPRLC
BoNT/G	32	CKPVMYKNTGK*-----SEQC
TeNT	33	CKKIIPPTNIRENLNRTA*SLTDLGGELC
BaNT	34	CKS-IVSKKGTK*-----NSLC
BuNT	35	CKN-IVSVKGIR*-----KSIC
The amino acid sequence displayed are as follows: BoNT/A, residues 430-454 of SEQ ID NO: 1; BoNT/B, residues 437-446 of SEQ ID NO: 2; BoNT/C1, residues 437-453 of SEQ ID NO: 3; BoNT/D, residues 437-450 of SEQ ID NO: 4; BoNT/E, residues 412-426 of SEQ ID NO: 5; BoNT/F, residues 429-445 of SEQ ID NO: 6; BoNT/G, residues 436-450 of SEQ ID NO: 7; TeNT, residues 439-467 of SEQ ID NO: 8; BaNT, residues 421-435 of SEQ ID NO: 9; and BuNT, residues 412-426 of SEQ ID NO: 10. An asterisks (*) indicates the peptide bond that is cleaved by a Clostridial toxin protease.		

[0174] As used herein, the term “endogenous di-chain loop protease cleavage site” is synonymous with a “naturally occurring di-chain loop protease cleavage site” and means a naturally occurring protease cleavage site found within the di-chain loop region of a naturally occurring Clostridial toxin and includes,

without limitation, naturally occurring Clostridial toxin di-chain loop protease cleavage site variants, such as, *e.g.*, Clostridial toxin di-chain loop protease cleavage site isoforms and Clostridial toxin di-chain loop protease cleavage site subtypes. Non-limiting examples of an endogenous protease cleavage site, include, *e.g.*, a BoNT/A di-chain loop protease cleavage site, a BoNT/B di-chain loop protease cleavage site, a BoNT/C1 di-chain loop protease cleavage site, a BoNT/D di-chain loop protease cleavage site, a BoNT/E di-chain loop protease cleavage site, a BoNT/F di-chain loop protease cleavage site, a BoNT/G di-chain loop protease cleavage site and a TeNT di-chain loop protease cleavage site.

[0175] As mentioned above, Clostridial toxins are translated as a single-chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring protease. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by a single disulphide bond and noncovalent interactions. While the identity of the protease is currently unknown, the di-chain loop protease cleavage site for many Clostridial toxins has been determined. In BoNTs, cleavage at K448-A449 converts the single polypeptide form of BoNT/A into the di-chain form; cleavage at K441-A442 converts the single polypeptide form of BoNT/B into the di-chain form; cleavage at K449-T450 converts the single polypeptide form of BoNT/C1 into the di-chain form; cleavage at R445-D446 converts the single polypeptide form of BoNT/D into the di-chain form; cleavage at R422-K423 converts the single polypeptide form of BoNT/E into the di-chain form; cleavage at K439-A440 converts the single polypeptide form of BoNT/F into the di-chain form; and cleavage at K446-S447 converts the single polypeptide form of BoNT/G into the di-chain form. Proteolytic cleavage of the single polypeptide form of TeNT at A457-S458 results in the di-chain form. Proteolytic cleavage of the single polypeptide form of BaNT at K431-N432 results in the di-chain form. Proteolytic cleavage of the single polypeptide form of BuNT at R422-K423 results in the di-chain form. Such a di-chain loop protease cleavage site is operably-linked in-frame to a TVEMP as a fusion protein. However, it should also be noted that additional cleavage sites within the di-chain loop also appear to be cleaved resulting in the generation of a small peptide fragment being lost. As a non-limiting example, BoNT/A single-chain polypeptide cleave ultimately results in the loss of a ten amino acid fragment within the di-chain loop.

[0176] Thus, in an embodiment, a protease cleavage site comprising an endogenous Clostridial toxin di-chain loop protease cleavage site is used to convert the single-chain toxin into the di-chain form. In aspects of this embodiment, conversion into the di-chain form by proteolytic cleavage occurs from a site comprising, *e.g.*, a BoNT/A di-chain loop protease cleavage site, a BoNT/B di-chain loop protease cleavage site, a BoNT/C1 di-chain loop protease cleavage site, a BoNT/D di-chain loop protease cleavage site, a BoNT/E di-chain loop protease cleavage site, a BoNT/F di-chain loop protease cleavage site, a BoNT/G di-chain loop protease cleavage site, a TeNT di-chain loop protease cleavage site, a BaNT di-chain loop protease cleavage site, or a BuNT di-chain loop protease cleavage site.

[0177] In other aspects of this embodiment, conversion into the di-chain form by proteolytic cleavage occurs from a site comprising, e.g., a di-chain loop region of BoNT/A comprising amino acids 430-454 of SEQ ID NO: 1; a di-chain loop region of BoNT/B comprising amino acids 437-446 of SEQ ID NO: 2; a di-chain loop region of BoNT/C1 comprising amino acids 437-453 of SEQ ID NO: 3; a di-chain loop region of BoNT/D comprising amino acids 437-450 of SEQ ID NO: 4; a di-chain loop region of BoNT/E comprising amino acids 412-426 of SEQ ID NO: 5; a di-chain loop region of BoNT/F comprising amino acids 429-445 of SEQ ID NO: 6; a di-chain loop region of BoNT/G comprising amino acids 436-450 of SEQ ID NO: 7; or a di-chain loop region of TeNT comprising amino acids 439-467 of SEQ ID NO: 8. a di-chain loop region of BaNT comprising amino acids 421-435 of SEQ ID NO: 9; or a di-chain loop region of BuNT comprising amino acids 412-426 of SEQ ID NO: 10.

[0178] It is also envisioned that an exogenous protease cleavage site can be used to convert the single-chain polypeptide form of a TVEMP disclosed herein into the di-chain form. As used herein, the term “exogenous protease cleavage site” is synonymous with a “non-naturally occurring protease cleavage site” or “non-native protease cleavage site” and means a protease cleavage site that is not normally present in a di-chain loop region from a naturally occurring Clostridial toxin, with the proviso that the exogenous protease cleavage site is not a human protease cleavage site or a protease cleavage site that is susceptible to a protease being expressed in the host cell that is expressing a construct encoding an activatable polypeptide disclosed herein. It is envisioned that any and all exogenous protease cleavage sites can be used to convert the single-chain polypeptide form of a Clostridial toxin into the di-chain form are useful to practice aspects of the present invention. Non-limiting examples of exogenous protease cleavage sites include, e.g., a plant papain cleavage site, an insect papain cleavage site, a crustacean papain cleavage site, an enterokinase cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a tobacco etch virus (TEV) protease cleavage site, a Tobacco Vein Mottling Virus (TVMV) cleavage site, a subtilisin cleavage site, a hydroxylamine cleavage site, or a Caspase 3 cleavage site.

[0179] It is envisioned that an exogenous protease cleavage site of any and all lengths can be useful in aspects of the present invention with the proviso that the exogenous protease cleavage site is capable of being cleaved by its respective protease. Thus, in aspects of this embodiment, an exogenous protease cleavage site can have a length of, e.g., at least 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, or at least 60 amino acids; or at most 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, or at least 60 amino acids.

[0180] In an embodiment, an exogenous protease cleavage site is located within the di-chain loop of a TVEMP. In aspects of this embodiment, a TVEMP comprises an exogenous protease cleavage site comprises, e.g., a plant papain cleavage site, an insect papain cleavage site, a crustacean papain cleavage site, a non-human enterokinase protease cleavage site, a Tobacco Etch Virus protease cleavage site, a Tobacco Vein Mottling Virus protease cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a subtilisin cleavage site, a hydroxylamine

cleavage site, a SUMO/ULP-1 protease cleavage site, and a non-human Caspase 3 cleavage site. In other aspects of this embodiment, an exogenous protease cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0181] In an aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a non-human enterokinase cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a bovine enterokinase protease cleavage site located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a bovine enterokinase protease cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 36. In still other aspects of this embodiment, a bovine enterokinase protease cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0182] In another aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a Tobacco Etch Virus protease cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a Tobacco Etch Virus protease cleavage site located within the di-chain loop of a TVEMP comprises the consensus sequence E-P5-P4-Y-P2-Q*-G (SEQ ID NO: 377) or E-P5-P4-Y-P2-Q*-S (SEQ ID NO: 38), where P2, P4 and P5 can be any amino acid. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a Tobacco Etch Virus protease cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 48. In still other aspects of this embodiment, a Tobacco Etch Virus protease cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0183] In another aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a Tobacco Vein Mottling Virus protease cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a Tobacco Vein Mottling Virus protease cleavage site located within the di-chain loop of a TVEMP comprises the consensus sequence P6-P5-V-R-F-Q*-G (SEQ ID NO: 49) or P6-P5-V-R-F-Q*-S (SEQ ID NO: 50), where P5 and P6 can be any amino acid. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a Tobacco Vein Mottling Virus protease cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, or SEQ ID NO: 54. In still other aspects of this embodiment, a Tobacco Vein Mottling Virus protease cleavage site is located within

the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0184] In still another aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a human rhinovirus 3C protease cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a human rhinovirus 3C protease cleavage site located within the di-chain loop of a TVEMP comprises the consensus sequence P5-P4-L-F-Q*-G-P (SEQ ID NO: 55), where P4 is G, A, V, L, I, M, S or T and P5 can any amino acid, with D or E preferred. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a human rhinovirus 3C protease cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60 or SEQ ID NO: 61. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a human rhinovirus 3C protease located within the di-chain loop of a TVEMP that can be cleaved by PRESSION®. In still other aspects of this embodiment, a human rhinovirus 3C protease cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0185] In yet another aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a subtilisin cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a subtilisin cleavage site located within the di-chain loop of a TVEMP comprises the consensus sequence P6-P5-P4-P3-H*-Y (SEQ ID NO: 62) or P6-P5-P4-P3-Y-H* (SEQ ID NO: 63), where P3, P4 and P5 and P6 can be any amino acid. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a subtilisin cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 64, SEQ ID NO: 65, or SEQ ID NO: 66. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a subtilisin cleavage site located within the di-chain loop of a TVEMP that can be cleaved by GENENASE®. In still other aspects of this embodiment, a subtilisin cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0186] In yet another aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a hydroxylamine cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a hydroxylamine cleavage site comprising multiples of the dipeptide N*G. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a hydroxylamine cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 67, or SEQ ID NO: 68. In still other aspects of this embodiment, a

hydroxylamine cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0187] In yet another aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a SUMO/ULP-1 protease cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a SUMO/ULP-1 protease cleavage site located within the di-chain loop of a TVEMP comprising the consensus sequence G-G*-P1'-P2'-P3' (SEQ ID NO: 69), where P1', P2', and P3' can be any amino acid. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a SUMO/ULP-1 protease cleavage site located within the di-chain loop of a TVEMP comprises SEQ ID NO: 70. In still other aspects of this embodiment, a SUMO/ULP-1 protease cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0188] In an aspect of this embodiment, an exogenous protease cleavage site can comprise, e.g., a non-human Caspase 3 cleavage site is located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a mouse Caspase 3 protease cleavage site located within the di-chain loop of a TVEMP. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a non-human Caspase 3 protease cleavage site located within the di-chain loop of a TVEMP comprises the consensus sequence D-P3-P2-D*P1' (SEQ ID NO: 71), where P3 can be any amino acid, with E preferred, P2 can be any amino acid and P1' can any amino acid, with G or S preferred. In other aspects of the embodiment, an exogenous protease cleavage site can comprise, e.g., a non-human Caspase 3 protease cleavage site located within the di-chain loop of a TVEMP comprising SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, or SEQ ID NO: 77. In still other aspects of this embodiment, a bovine enterokinase protease cleavage site is located within the di-chain loop of, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0189] A di-chain loop region is modified to replace a naturally-occurring di-chain loop protease cleavage site for an exogenous protease cleavage site. In this modification, the naturally-occurring di-chain loop protease cleavage site is made inoperable and thus can not be cleaved by its protease. Only the exogenous protease cleavage site can be cleaved by its corresponding exogenous protease. In this type of modification, the exogenous protease site is operably-linked in-frame to a TVEMP as a fusion protein and the site can be cleaved by its respective exogenous protease. Replacement of an endogenous di-chain loop protease cleavage site with an exogenous protease cleavage site can be a substitution of the sites where the exogenous site is engineered at the position approximating the cleavage site location of

the endogenous site. Replacement of an endogenous di-chain loop protease cleavage site with an exogenous protease cleavage site can be an addition of an exogenous site where the exogenous site is engineered at the position different from the cleavage site location of the endogenous site, the endogenous site being engineered to be inoperable. The location and kind of protease cleavage site may be critical because certain targeting domains require a free amino-terminal or carboxyl-terminal amino acid. For example, when a peptide targeting domain is placed between two other domains, e.g., see FIG. 4, a criterion for selection of a protease cleavage site could be whether the protease that cleaves its site leaves a flush cut, exposing the free amino-terminal or carboxyl-terminal of the targeting domain necessary for selective binding of the targeting domain to its receptor.

[0190] A naturally-occurring protease cleavage site can be made inoperable by altering at least one of the two amino acids flanking the peptide bond cleaved by the naturally-occurring di-chain loop protease. More extensive alterations can be made, with the proviso that the two cysteine residues of the di-chain loop region remain intact and the region can still form the disulfide bridge. Non-limiting examples of an amino acid alteration include deletion of an amino acid or replacement of the original amino acid with a different amino acid. Thus, in one embodiment, a naturally-occurring protease cleavage site is made inoperable by altering at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 amino acids including at least one of the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease. In another embodiment, a naturally-occurring protease cleavage site is made inoperable by altering at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 amino acids including at least one of the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease.

[0191] It is understood that a TVEMP disclosed herein can optionally further comprise a flexible region comprising a flexible spacer. A flexible region comprising flexible spacers can be used to adjust the length of a polypeptide region in order to optimize a characteristic, attribute or property of a polypeptide. As a non-limiting example, a polypeptide region comprising one or more flexible spacers in tandem can be used to better expose a protease cleavage site thereby facilitating cleavage of that site by a protease. As another non-limiting example, a polypeptide region comprising one or more flexible spacers in tandem can be used to better present a peptide targeting domain, thereby facilitating the binding of that targeting domain to its receptor.

[0192] A flexible space comprising a peptide is at least one amino acid in length and comprises non-charged amino acids with small side-chain R groups, such as, e.g., glycine, alanine, valine, leucine or serine. Thus, in an embodiment a flexible spacer can have a length of, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In still another embodiment, a flexible spacer can be, e.g., between 1-3 amino acids, between 2-4 amino acids, between 3-5 amino acids, between 4-6 amino acids, or between 5-7 amino acids. Non-limiting examples of a flexible spacer include, e.g., a G-spacers such as GGG, GGGG (SEQ ID NO: 78), and GGGGS (SEQ ID NO: 79) or an

A-spacers such as AAA, AAAA (SEQ ID NO: 80) and AAAAV (SEQ ID NO: 81). Such a flexible region is operably-linked in-frame to the TVEMP as a fusion protein.

[0193] Thus, in an embodiment, a TVEMP disclosed herein can further comprise a flexible region comprising a flexible spacer. In another embodiment, a TVEMP disclosed herein can further comprise flexible region comprising a plurality of flexible spacers in tandem. In aspects of this embodiment, a flexible region can comprise in tandem, e.g., at least 1, 2, 3, 4, or 5 G-spacers; or at most 1, 2, 3, 4, or 5 G-spacers. In still other aspects of this embodiment, a flexible region can comprise in tandem, e.g., at least 1, 2, 3, 4, or 5 A-spacers; or at most 1, 2, 3, 4, or 5 A-spacers. In another aspect of this embodiment, a TVEMP can comprise a flexible region comprising one or more copies of the same flexible spacers, one or more copies of different flexible-spacer regions, or any combination thereof.

[0194] In other aspects of this embodiment, a TVEMP comprising a flexible spacer can be, e.g., a modified BoNT/A, a modified BoNT/B, a modified BoNT/C1, a modified BoNT/D, a modified BoNT/E, a modified BoNT/F, a modified BoNT/G, a modified TeNT, a modified BaNT, or a modified BuNT.

[0195] It is envisioned that a TVEMP disclosed herein can comprise a flexible spacer in any and all locations with the proviso that TVEMP is capable of performing the intoxication process. In aspects of this embodiment, a flexible spacer is positioned between, e.g., an enzymatic domain and a translocation domain, an enzymatic domain and a peptide targeting domain, an enzymatic domain and an exogenous protease cleavage site. In other aspects of this embodiment, a G-spacer is positioned between, e.g., an enzymatic domain and a translocation domain, an enzymatic domain and a peptide targeting domain, an enzymatic domain and an exogenous protease cleavage site. In other aspects of this embodiment, an A-spacer is positioned between, e.g., an enzymatic domain and a translocation domain, an enzymatic domain and a peptide targeting domain, an enzymatic domain and an exogenous protease cleavage site.

[0196] In other aspects of this embodiment, a flexible spacer is positioned between, e.g., a peptide targeting domain and a translocation domain, a peptide targeting domain and an enzymatic domain, a peptide targeting domain and an exogenous protease cleavage site. In other aspects of this embodiment, a G-spacer is positioned between, e.g., a peptide targeting domain and a translocation domain, a peptide targeting domain and an enzymatic domain, a peptide targeting domain and an exogenous protease cleavage site. In other aspects of this embodiment, an A-spacer is positioned between, e.g., a peptide targeting domain and a translocation domain, a peptide targeting domain and an enzymatic domain, a peptide targeting domain and an exogenous protease cleavage site.

[0197] In yet other aspects of this embodiment, a flexible spacer is positioned between, e.g., a translocation domain and an enzymatic domain, a translocation domain and a peptide targeting domain, a translocation domain and an exogenous protease cleavage site. In other aspects of this embodiment, a G-spacer is positioned between, e.g., a translocation domain and an enzymatic domain, a translocation

domain and a peptide targeting domain, a translocation domain and an exogenous protease cleavage site. In other aspects of this embodiment, an A-spacer is positioned between, e.g., a translocation domain and an enzymatic domain, a translocation domain and a peptide targeting domain, a translocation domain and an exogenous protease cleavage site.

[0198] It is envisioned that a TVEMP disclosed herein can comprise a peptide targeting domain in any and all locations with the proviso that TVEMP is capable of performing the intoxication process. Non-limiting examples include, locating a peptide targeting domain at the amino terminus of a TVEMP; locating a peptide targeting domain between a Clostridial toxin enzymatic domain and a translocation domain of a TVEMP; and locating a peptide targeting domain at the carboxyl terminus of a TVEMP. Other non-limiting examples include, locating a peptide targeting domain between a Clostridial toxin enzymatic domain and a Clostridial toxin translocation domain of a TVEMP. The enzymatic domain of naturally-occurring Clostridial toxins contains the native start methionine. Thus, in domain organizations where the enzymatic domain is not in the amino-terminal location an amino acid sequence comprising the start methionine should be placed in front of the amino-terminal domain. Likewise, where a peptide targeting domain is in the amino-terminal position, an amino acid sequence comprising a start methionine and a protease cleavage site may be operably-linked in situations in which a peptide targeting domain requires a free amino terminus, see, e.g., Shengwen Li et al., *Degradable Clostridial Toxins*, U.S. Patent Application 11/572,512 (Jan. 23, 2007), which is hereby incorporated by reference in its entirety. In addition, it is known in the art that when adding a polypeptide that is operably-linked to the amino terminus of another polypeptide comprising the start methionine that the original methionine residue can be deleted.

[0199] Thus, in an embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a peptide targeting domain, a translocation domain, an exogenous protease cleavage site and an enzymatic domain (FIG. 3A). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a peptide targeting domain, a Clostridial toxin translocation domain, an exogenous protease cleavage site and a Clostridial toxin enzymatic domain.

[0200] In another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a peptide targeting domain, an enzymatic domain, an exogenous protease cleavage site, and a translocation domain (FIG. 3B). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a peptide targeting domain, a Clostridial toxin enzymatic domain, an exogenous protease cleavage site, a Clostridial toxin translocation domain.

[0201] In yet another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising an enzymatic domain, an exogenous protease cleavage site, a peptide targeting domain, and a translocation domain (FIG. 4A). In an aspect of this embodiment, a TVEMP can comprise

an amino to carboxyl single polypeptide linear order comprising a Clostridial toxin enzymatic domain, an exogenous protease cleavage site, a peptide targeting domain, and a Clostridial toxin translocation domain.

[0202] In yet another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a translocation domain, an exogenous protease cleavage site, a peptide targeting domain, and an enzymatic domain (FIG. 4B). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a Clostridial toxin translocation domain, a peptide targeting domain, an exogenous protease cleavage site and a Clostridial toxin enzymatic domain.

[0203] In another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising an enzymatic domain, a peptide targeting domain, an exogenous protease cleavage site, and a translocation domain (FIG. 4C). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a Clostridial toxin enzymatic domain, a peptide targeting domain, an exogenous protease cleavage site, a Clostridial toxin translocation domain.

[0204] In yet another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a translocation domain, a peptide targeting domain, an exogenous protease cleavage site and an enzymatic domain (FIG. 4D). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a Clostridial toxin translocation domain, a peptide targeting domain, an exogenous protease cleavage site and a Clostridial toxin enzymatic domain.

[0205] In still another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising an enzymatic domain, an exogenous protease cleavage site, a translocation domain, and a peptide targeting domain (FIG. 5A). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a Clostridial toxin enzymatic domain, an exogenous protease cleavage site, a Clostridial toxin translocation domain, and a peptide targeting domain.

[0206] In still another embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a translocation domain, an exogenous protease cleavage site, an enzymatic domain and a peptide targeting domain, (FIG. 5B). In an aspect of this embodiment, a TVEMP can comprise an amino to carboxyl single polypeptide linear order comprising a Clostridial toxin translocation domain, a peptide targeting domain, an exogenous protease cleavage site and a Clostridial toxin enzymatic domain.

[0207] A composition useful in the invention generally is administered as a pharmaceutical acceptable composition comprising a TVEMP. As used herein, the term "pharmaceutically acceptable" means any

molecular entity or composition that does not produce an adverse, allergic or other untoward or unwanted reaction when administered to an individual. As used herein, the term “pharmaceutically acceptable composition” is synonymous with “pharmaceutical composition” and means a therapeutically effective concentration of an active ingredient, such as, *e.g.*, any of the TVEMPs disclosed herein. A pharmaceutical composition comprising a TVEMP is useful for medical and veterinary applications. A pharmaceutical composition may be administered to a patient alone, or in combination with other supplementary active ingredients, agents, drugs or hormones. The pharmaceutical compositions may be manufactured using any of a variety of processes, including, without limitation, conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, and lyophilizing. The pharmaceutical composition can take any of a variety of forms including, without limitation, a sterile solution, suspension, emulsion, lyophilizate, tablet, pill, pellet, capsule, powder, syrup, elixir or any other dosage form suitable for administration.

[0208] Aspects of the present invention provide, in part, a composition comprising a TVEMP. It is envisioned that any of the composition disclosed herein can be useful in a method of treating neurogenic inflammation in a mammal in need thereof, with the proviso that the composition prevents or reduces a symptom associated with neurogenic inflammation. Non-limiting examples of compositions comprising a TVEMP include a TVEMP comprising a peptide targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain. It is envisioned that any TVEMP disclosed herein can be used, including those disclosed in, *e.g.*, Steward, *supra*, (2007); Dolly, *supra*, (2007); Foster, *supra*, WO 2006/059093 (2006); Foster, *supra*, WO 2006/059105 (Jun. 8, 2006). It is also understood that the two or more different TVEMPs can be provided as separate compositions or as part of a single composition.

[0209] It is also envisioned that a pharmaceutical composition comprising a TVEMP can optionally include a pharmaceutically acceptable carriers that facilitate processing of an active ingredient into pharmaceutically acceptable compositions. As used herein, the term “pharmacologically acceptable carrier” is synonymous with “pharmacological carrier” and means any carrier that has substantially no long term or permanent detrimental effect when administered and encompasses terms such as “pharmacologically acceptable vehicle, stabilizer, diluent, additive, auxiliary or excipient.” Such a carrier generally is mixed with an active compound, or permitted to dilute or enclose the active compound and can be a solid, semi-solid, or liquid agent. It is understood that the active ingredients can be soluble or can be delivered as a suspension in the desired carrier or diluent. Any of a variety of pharmaceutically acceptable carriers can be used including, without limitation, aqueous media such as, *e.g.*, water, saline, glycine, hyaluronic acid and the like; solid carriers such as, *e.g.*, mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like; solvents; dispersion media; coatings; antibacterial and antifungal agents; isotonic and absorption delaying agents; or any other inactive ingredient. Selection of a pharmacologically acceptable carrier can depend on the mode of administration. Except insofar as any pharmacologically acceptable carrier is incompatible with the active ingredient, its use in pharmaceutically acceptable compositions is

contemplated. Non-limiting examples of specific uses of such pharmaceutical carriers can be found in PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS (Howard C. Ansel et al., eds., Lippincott Williams & Wilkins Publishers, 7th ed. 1999); REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY (Alfonso R. Gennaro ed., Lippincott, Williams & Wilkins, 20th ed. 2000); GOODMAN & GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS (Joel G. Hardman et al., eds., McGraw-Hill Professional, 10th ed. 2001); and HANDBOOK OF PHARMACEUTICAL EXCIPIENTS (Raymond C. Rowe et al., APhA Publications, 4th edition 2003). These protocols are routine procedures and any modifications are well within the scope of one skilled in the art and from the teaching herein.

[0210] It is further envisioned that a pharmaceutical composition disclosed herein can optionally include, without limitation, other pharmaceutically acceptable components (or pharmaceutical components), including, without limitation, buffers, preservatives, tonicity adjusters, salts, antioxidants, osmolality adjusting agents, physiological substances, pharmacological substances, bulking agents, emulsifying agents, wetting agents, sweetening or flavoring agents, and the like. Various buffers and means for adjusting pH can be used to prepare a pharmaceutical composition disclosed herein, provided that the resulting preparation is pharmaceutically acceptable. Such buffers include, without limitation, acetate buffers, citrate buffers, phosphate buffers, neutral buffered saline, phosphate buffered saline and borate buffers. It is understood that acids or bases can be used to adjust the pH of a composition as needed. Pharmaceutically acceptable antioxidants include, without limitation, sodium metabisulfite, sodium thiosulfate, acetylcysteine, butylated hydroxyanisole and butylated hydroxytoluene. Useful preservatives include, without limitation, benzalkonium chloride, chlorobutanol, thimerosal, phenylmercuric acetate, phenylmercuric nitrate, a stabilized oxy chloro composition and chelants, such as, *e.g.*, DTPA or DTPA-bisamide, calcium DTPA, and CaNaDTPA-bisamide. Tonicity adjusters useful in a pharmaceutical composition include, without limitation, salts such as, *e.g.*, sodium chloride, potassium chloride, mannitol or glycerin and other pharmaceutically acceptable tonicity adjuster. The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. It is understood that these and other substances known in the art of pharmacology can be included in a pharmaceutical composition.

[0211] In an embodiment, a composition comprising a TVEMP is a pharmaceutical composition comprising a TVEMP. In aspects of this embodiment, a pharmaceutical composition comprising a TVEMP further comprises a pharmacological carrier, a pharmaceutical component, or both a pharmacological carrier and a pharmaceutical component. In other aspects of this embodiment, a pharmaceutical composition comprising a TVEMP further comprises at least one pharmacological carrier, at least one pharmaceutical component, or at least one pharmacological carrier and at least one pharmaceutical component.

[0212] Aspects of the present invention provide, in part, a cancer. As used herein, the term “cancer” means cells exhibiting uncontrolled growth that have a pathophysiology effect. It is envisioned that a TVEMP, compositions and methods disclosed herein can be useful to treat any cancer comprising cells that express the cognate receptor for the targeting domain present in the TVEMP. For example, a TVEMP comprising a neurotrophin peptide targeting domain would be useful in treating cancer cells that express a neurotrophin receptor; a TVEMP comprising a head activator peptide targeting domain would be useful in treating cancer cells that express a head activator receptor; a TVEMP comprising a glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) peptide targeting domain would be useful in treating cancer cells that express a GDNF-GFL receptor; a TVEMP comprising a RF-amide related peptide (RFRP) targeting domain would be useful in treating cancer cells that express a RFRP receptor; a TVEMP comprising a neurohormone peptide targeting domain would be useful in treating cancer cells that express a neurohormone receptor; and a TVEMP comprising a neuroregulatory cytokine peptide targeting domain would be useful in treating cancer cells that express a neuroregulatory cytokine receptor.

[0213] Aspects of the present invention provide, in part, reducing a symptom associated with cancer. In an aspect, the symptom reduced is an increase in the growth rate of cancer cells. In another aspect, the symptom reduced is an increase in the cell division rate of cancer cells. In yet another aspect, the symptom reduced is an increase in the extent of invasion of cancer cells into adjacent tissue or organs. In still another aspect, the symptom reduced is an increase in the extent of metastasis. In a further aspect, the symptom reduced is an increase in angiogenesis. In a yet further aspect, the symptom reduced is a decrease in apoptosis. In a still further aspect, the symptom reduced is a decrease in cell death or cell necrosis. Thus, a TVEMP treatment will decrease the growth rate of cancer cells, decrease the cell division rate of cancer cells, decrease the extent of invasion of cancer cells into adjacent tissue or organs, decrease the extent of metastasis, decrease angiogenesis, increase apoptosis, and/or increase cell death and/or cell necrosis.

[0214] Aspects of the present invention provide, in part, a mammal. A mammal includes a human, and a human can be a patient. Other aspects of the present invention provide, in part, an individual. An individual includes a human, and a human can be a patient.

[0215] Aspects of the present invention provide, in part, administering a composition comprising a TVEMP. As used herein, the term “administering” means any delivery mechanism that provides a composition comprising a TVEMP to a patient that potentially results in a clinically, therapeutically, or experimentally beneficial result. A TVEMP can be delivered to a patient using a cellular uptake approach where a TVEMP is delivered intracellular or a gene therapy approach where a TVEMP is express derived from precursor RNAs expressed from an expression vectors.

[0216] A composition comprising a TVEMP as disclosed herein can be administered to a mammal using a cellular uptake approach. Administration of a composition comprising a TVEMP using a cellular uptake approach comprise a variety of enteral or parenteral approaches including, without limitation, oral administration in any acceptable form, such as, e.g., tablet, liquid, capsule, powder, or the like; topical administration in any acceptable form, such as, e.g., drops, spray, creams, gels or ointments; intravascular administration in any acceptable form, such as, e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature; peri- and intra-tissue administration in any acceptable form, such as, e.g., intraperitoneal injection, intramuscular injection, subcutaneous injection, subcutaneous infusion, intraocular injection, retinal injection, or sub-retinal injection or epidural injection; intravesicular administration in any acceptable form, such as, e.g., catheter instillation; and by placement device, such as, e.g., an implant, a patch, a pellet, a catheter, an osmotic pump, a suppository, a bioerodible delivery system, a non-bioerodible delivery system or another implanted extended or slow release system. An exemplary list of biodegradable polymers and methods of use are described in, e.g., *Handbook of Biodegradable Polymers* (Abraham J. Domb et al., eds., Overseas Publishers Association, 1997).

[0217] A composition comprising a TVEMP can be administered to a mammal by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by ionophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors. Delivery mechanisms for administering a composition comprising a TVEMP to a patient are described in, e.g., Leonid Beigelman et al., *Compositions for the Delivery of Negatively Charged Molecules*, U.S. Patent 6,395,713; and Achim Aigner, *Delivery Systems for the Direct Application of siRNAs to Induce RNA Interference (RNAi) in vivo*, 2006(716559) J. Biomed. Biotech. 1-15 (2006); *Controlled Drug Delivery: Designing Technologies for the Future* (Kinam Park & Randy J. Mersny eds., American Chemical Association, 2000); Vernon G. Wong & Mae W. L. Hu, *Methods for Treating Inflammation-mediated Conditions of the Eye*, U.S. Patent No. 6,726,918; David A. Weber et al., *Methods and Apparatus for Delivery of Ocular Implants*, U.S. Patent Publication No. US2004/0054374; Thierry Nivaggioli et al., *Biodegradable Ocular Implant*, U.S. Patent Publication No. US2004/0137059; Patrick M. Hughes et al., *Anti-Angiogenic Sustained Release Intraocular Implants and Related Methods*, U.S. Patent Application 11/364,687; and Patrick M. Hughes et al., *Sustained Release Intraocular Drug Delivery Systems*, U.S. Patent Publication 2006/0182783, each of which is hereby incorporated by reference in its entirety.

[0218] A composition comprising a TVEMP as disclosed herein can also be administered to a patient using a gene therapy approach by expressing a TVEMP within in a cell manifesting a nerve-based etiology that contributes to a cancer. A TVEMP can be expressed from nucleic acid molecules operably-linked to an expression vector, see, e.g., P. D. Good et al., *Expression of Small, Therapeutic RNAs in Human Cell Nuclei*, 4(1) Gene Ther. 45-54 (1997); James D. Thompson, Polymerase III-based expression of therapeutic RNAs, U.S. Patent 6,852,535 (Feb. 8, 2005); Maciej Wiznerowicz et al., *Tuning*

Silence: Conditional Systems for RNA Interference, 3(9) Nat. Methods 682-688m (2006); Ola Snøve and John J. Rossi, *Expressing Short Hairpin RNAi in vivo*, 3(9) Nat. Methods 689-698 (2006); and Charles X. Li et al., *Delivery of RNA Interference*, 5(18) Cell Cycle 2103-2109 (2006). A person of ordinary skill in the art would realize that any TVEMP can be expressed in eukaryotic cells using an appropriate expression vector.

[0219] Expression vectors capable of expressing a TVEMP can provide persistent or stable expression of the TVEMP in a cell manifesting a nerve-based etiology that contributes to a cancer. Alternatively, expression vectors capable of expressing a TVEMP can provide for transient expression of the TVEMP in a cell manifesting a nerve-based etiology that contributes to a cancer. Such transiently expressing vectors can be repeatedly administered as necessary. A TVEMP-expressing vectors can be administered by a delivery mechanism and route of administration discussed above, by administration to target cells ex-planted from a patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell, see, e.g., Larry A. Couture and Dan T. Stinchcomb, *Anti-gene Therapy: The Use of Ribozymes to Inhibit Gene Function*, 12(12) Trends Genet. 510-515 (1996).

[0220] The actual delivery mechanism used to administer a composition comprising a TVEMP to a mammal can be determined by a person of ordinary skill in the art by taking into account factors, including, without limitation, the type of cancer, the location of the cancer, the cause of the cancer, the severity of the cancer, the degree of relief desired, the duration of relief desired, the particular TVEMP used, the rate of excretion of the TVEMP used, the pharmacodynamics of the TVEMP used, the nature of the other compounds to be included in the composition, the particular route of administration, the particular characteristics, history and risk factors of the patient, such as, e.g., age, weight, general health and the like, or any combination thereof.

[0221] In an embodiment, a composition comprising a TVEMP is administered to the site to be treated by injection. In aspects of this embodiment, injection of a composition comprising a TVEMP is by, e.g., intramuscular injection, intraorgan injection, subdermal injection, dermal injection, or injection into any other body area for the effective administration of a composition comprising a TVEMP. In aspects of this embodiment, injection of a composition comprising a TVEMP is a tumor or into the area surrounding the tumor.

[0222] A composition comprising a TVEMP can be administered to a mammal using a variety of routes. Routes of administration suitable for a method of treating a cancer as disclosed herein include both local and systemic administration. Local administration results in significantly more delivery of a composition to a specific location as compared to the entire body of the mammal, whereas, systemic administration results in delivery of a composition to essentially the entire body of the patient. Routes of administration suitable for a method of treating a cancer as disclosed herein also include both central and peripheral

administration. Central administration results in delivery of a composition to essentially the central nervous system of the patient and includes, e.g., intrathecal administration, epidural administration as well as a cranial injection or implant. Peripheral administration results in delivery of a composition to essentially any area of a patient outside of the central nervous system and encompasses any route of administration other than direct administration to the spine or brain. The actual route of administration of a composition comprising a TVEMP used in a mammal can be determined by a person of ordinary skill in the art by taking into account factors, including, without limitation, the type of cancer, the location of the cancer, the cause of the cancer, the severity of the cancer, the degree of relief desired, the duration of relief desired, the particular TVEMP used, the rate of excretion of the TVEMP used, the pharmacodynamics of the TVEMP used, the nature of the other compounds to be included in the composition, the particular route of administration, the particular characteristics, history and risk factors of the mammal, such as, e.g., age, weight, general health and the like, or any combination thereof.

[0223] In an embodiment, a composition comprising a TVEMP is administered systemically to a mammal. In another embodiment, a composition comprising a TVEMP is administered locally to a mammal. In an aspect of this embodiment, a composition comprising a TVEMP is administered to a tumor of a mammal. In another aspect of this embodiment, a composition comprising a TVEMP is administered to the area surrounding a tumor of a mammal.

[0224] Aspects of the present invention provide, in part, administering a therapeutically effective amount of a composition comprising a TVEMP. As used herein, the term “therapeutically effective amount” is synonymous with “therapeutically effective dose” and when used in reference to treating a cancer means the minimum dose of a TVEMP necessary to achieve the desired therapeutic effect and includes a dose sufficient to reduce a symptom associated with a cancer. In aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP reduces a symptom associated with a cancer by, e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or at least 100%. In other aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP reduces a symptom associated with a cancer by, e.g., at most 10%, at most 20%, at most 30%, at most 40%, at most 50%, at most 60%, at most 70%, at most 80%, at most 90% or at most 100%. In yet other aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP reduces a symptom associated with a cancer by, e.g., about 10% to about 100%, about 10% to about 90%, about 10% to about 80%, about 10% to about 70%, about 10% to about 60%, about 10% to about 50%, about 10% to about 40%, about 20% to about 100%, about 20% to about 90%, about 20% to about 80%, about 20% to about 70%, about 20% to about 60%, about 20% to about 50%, about 20% to about 40%, about 30% to about 100%, about 30% to about 90%, about 30% to about 80%, about 30% to about 70%, about 30% to about 60%, or about 30% to about 50%. As used herein, the term “about” when qualifying a value of a stated item, number, percentage, or term refers to a range of plus or minus ten percent of the value of the stated item, percentage, parameter, or term. In still other aspects of this embodiment, a therapeutically effective amount of the TVEMP is the

dosage sufficient to inhibit neuronal activity for, e.g., at least one week, at least one month, at least two months, at least three months, at least four months, at least five months, at least six months, at least seven months, at least eight months, at least nine months, at least ten months, at least eleven months, or at least twelve months.

[0225] The actual therapeutically effective amount of a composition comprising a TVEMP to be administered to a mammal can be determined by a person of ordinary skill in the art by taking into account factors, including, without limitation, the type of cancer, the location of the cancer, the cause of the cancer, the severity of the cancer, the degree of relief desired, the duration of relief desired, the particular TVEMP used, the rate of excretion of the TVEMP used, the pharmacodynamics of the TVEMP used, the nature of the other compounds to be included in the composition, the particular route of administration, the particular characteristics, history and risk factors of the patient, such as, e.g., age, weight, general health and the like, or any combination thereof. Additionally, where repeated administration of a composition comprising a TVEMP is used, the actual effect amount of a composition comprising a TVEMP will further depend upon factors, including, without limitation, the frequency of administration, the half-life of the composition comprising a TVEMP, or any combination thereof. It is known by a person of ordinary skill in the art that an effective amount of a composition comprising a TVEMP can be extrapolated from *in vitro* assays and *in vivo* administration studies using animal models prior to administration to humans. Wide variations in the necessary effective amount are to be expected in view of the differing efficiencies of the various routes of administration. For instance, oral administration generally would be expected to require higher dosage levels than administration by intravenous or intravitreal injection. Variations in these dosage levels can be adjusted using standard empirical routines of optimization, which are well-known to a person of ordinary skill in the art. The precise therapeutically effective dosage levels and patterns are preferably determined by the attending physician in consideration of the above-identified factors.

[0226] As a non-limiting example, when administering a composition comprising a TVEMP to a mammal, a therapeutically effective amount generally is in the range of about 1 fg to about 3.0 mg. In aspects of this embodiment, an effective amount of a composition comprising a TVEMP can be, e.g., about 100 fg to about 3.0 mg, about 100 pg to about 3.0 mg, about 100 ng to about 3.0 mg, or about 100 µg to about 3.0 mg. In other aspects of this embodiment, an effective amount of a composition comprising a TVEMP can be, e.g., about 100 fg to about 750 µg, about 100 pg to about 750 µg, about 100 ng to about 750 µg, or about 1 µg to about 750 µg. In yet other aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP can be, e.g., at least 1 fg, at least 250 fg, at least 500 fg, at least 750 fg, at least 1 pg, at least 250 pg, at least 500 pg, at least 750 pg, at least 1 ng, at least 250 ng, at least 500 ng, at least 750 ng, at least 1 µg, at least 250 µg, at least 500 µg, at least 750 µg, or at least 1 mg. In still other aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP can be, e.g., at most 1 fg, at most 250 fg, at most 500 fg, at most 750 fg, at most 1

pg, at most 250 pg, at most 500 pg, at most 750 pg, at most 1 ng, at most 250 ng, at most 500 ng, at most 750 ng, at most 1 µg, at least 250 µg, at most 500 µg, at most 750 µg, or at most 1 mg.

[0227] As another non-limiting example, when administering a composition comprising a TVEMP to a mammal, a therapeutically effective amount generally is in the range of about 0.00001 mg/kg to about 3.0 mg/kg. In aspects of this embodiment, an effective amount of a composition comprising a TVEMP can be, e.g., about 0.0001 mg/kg to about 0.001 mg/kg, about 0.03 mg/kg to about 3.0 mg/kg, about 0.1 mg/kg to about 3.0 mg/kg, or about 0.3 mg/kg to about 3.0 mg/kg. In yet other aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP can be, e.g., at least 0.00001 mg/kg, at least 0.0001 mg/kg, at least 0.001 mg/kg, at least 0.01 mg/kg, at least 0.1 mg/kg, or at least 1 mg/kg. In yet other aspects of this embodiment, a therapeutically effective amount of a composition comprising a TVEMP can be, e.g., at most 0.00001 mg/kg, at most 0.0001 mg/kg, at most 0.001 mg/kg, at most 0.01 mg/kg, at most 0.1 mg/kg, or at most 1 mg/kg.

[0228] Dosing can be single dosage or cumulative (serial dosing), and can be readily determined by one skilled in the art. For instance, treatment of a cancer may comprise a one-time administration of an effective dose of a composition comprising a TVEMP. As a non-limiting example, an effective dose of a composition comprising a TVEMP can be administered once to a patient, e.g., as a single injection or deposition at or near the site exhibiting a symptom of a cancer. Alternatively, treatment of a cancer may comprise multiple administrations of an effective dose of a composition comprising a TVEMP carried out over a range of time periods, such as, e.g., daily, once every few days, weekly, monthly or yearly. As a non-limiting example, a composition comprising a TVEMP can be administered once or twice yearly to a mammal. The timing of administration can vary from mammal to mammal, depending upon such factors as the severity of a mammal's symptoms. For example, an effective dose of a composition comprising a TVEMP can be administered to a mammal once a month for an indefinite period of time, or until the patient no longer requires therapy. A person of ordinary skill in the art will recognize that the condition of the mammal can be monitored throughout the course of treatment and that the effective amount of a composition comprising a TVEMP that is administered can be adjusted accordingly.

[0229] A composition comprising a TVEMP as disclosed herein can also be administered to a mammal in combination with other therapeutic compounds to increase the overall therapeutic effect of the treatment. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

[0230] Aspects of the present invention can also be described as follows:

1. A method of treating cancer in a mammal, the method comprising the step of administering to the mammal in need thereof a therapeutically effective amount of a composition including a TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin

enzymatic domain, wherein administration of the composition reduces a symptom associated with cancer.

2. A use of a TVEMP in the manufacturing a medicament for treating cancer in a mammal in need thereof, wherein the TVEMP comprises a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain and wherein administration of a therapeutically effective amount of the medicament to the mammal reduces a symptom associated with cancer.
3. A use of a TVEMP for the treatment of cancer in a mammal in need thereof, the use comprising the step of administering to the mammal a therapeutically effective amount of the TVEMP, wherein the TVEMP comprises a targeting domain, a Clostridial toxin translocation domain, a Clostridial toxin enzymatic domain and wherein administration of the TVEMP reduces a symptom associated with cancer.
4. A method of treating cancer in a mammal, the method comprising the step of administering to the mammal in need thereof a therapeutically effective amount of a composition including a TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain, and an exogenous protease cleavage site, wherein administration of the composition reduces a symptom associated with cancer.
5. A use of a TVEMP in the manufacturing a medicament for treating cancer in a mammal in need thereof, wherein the TVEMP comprises a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain, and an exogenous protease cleavage site and wherein administration of a therapeutically effective amount of the medicament to the mammal reduces a symptom associated with cancer.
6. A use of a TVEMP for the treatment of cancer in a mammal in need thereof, the use comprising the step of administering to the mammal a therapeutically effective amount of the TVEMP, wherein the TVEMP comprises a targeting domain, a Clostridial toxin translocation domain, a Clostridial toxin enzymatic domain, and an exogenous protease cleavage site and wherein administration of the TVEMP reduces a symptom associated with cancer.
7. The method of 1-3, wherein the TVEMP comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, the targeting domain, 2) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the targeting domain, the Clostridial toxin translocation domain, 3) the targeting domain, the Clostridial toxin translocation domain, the exogenous protease cleavage site and the Clostridial toxin enzymatic domain, 4) the targeting domain, the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain,

- 5) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the targeting domain, or 6) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the targeting domain and the Clostridial toxin enzymatic domain.
8. The method of 4-6, wherein the TVEMP comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, the targeting domain, 2) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the targeting domain, the Clostridial toxin translocation domain, 3) the targeting domain, the Clostridial toxin translocation domain, the exogenous protease cleavage site and the Clostridial toxin enzymatic domain, 4) the targeting domain, the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, 5) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the targeting domain, or 6) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the targeting domain and the Clostridial toxin enzymatic domain.
 9. The method of 1-8, wherein the targeting domain is a neurotrophin peptide, a head activator peptide, a glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) peptide, a RF-amide related peptide, a neurohormone peptide, or a neuroregulatory cytokine peptide.
 10. The method of 9, wherein the neurotrophin peptide targeting domain is a nerve growth factor (NGF) peptide, a brain derived neurotrophic factor (BDNF) peptide, a neurotrophin-3 (NT-3) peptide, or a neurotrophin-4/5 (NT-4/5) peptide.
 11. The method of 10, wherein the neurotrophin peptide targeting domain comprises amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85.
 12. The method of 10-11, wherein the cancer is a breast cancer, a neuroblastoma, a melanoma, a schwannoma, an insulinoma, a hepatoma, a medulloblastoma, a prolactinoma, a colon cancer, a leukemia, a chronic myelogenous leukemia, mast cell leukemia, an endometrial cancer, a prostate cancer, a thyroid cancer, a squamous-cell lung carcinoma, a lung cancer, an astrocytoma, a glioblastoma, a fibrosarcoma, or a pheochromocytoma.
 13. The method of 9, wherein the head activator peptide targeting domain is a head activator peptide.
 14. The method of 13, wherein the head activator peptide targeting domain comprises SEQ ID NO: 86.
 15. The method of 13-14, wherein the cancer is a breast cancer.

16. The method of 9, wherein the glial cell line-derived neurotrophic factor family of ligands peptide targeting domain is a glial cell line-derived neurotrophic factor peptide, a Neurturin peptide, a Persephrin peptide, or an Artemin peptide.
17. The method of 16, wherein the glial cell line-derived neurotrophic factor family of ligands peptide targeting domain comprises amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90.
18. The method of 16-17, wherein the cancer is a breast cancer, a pancreatic cancer, a thyroid cancer, a renal cancer, an adenocarcinoma, a melanoma, or a bladder cancer.
19. The method of 9, wherein the RF-amide related peptide targeting domain a RF-amide related peptide-1, a RF-amide related peptide-2, a RF-amide related peptide-3, a neuropeptide AF, or a neuropeptide FF
20. The method of 19, wherein the RF-amide related peptide targeting domain comprises amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94.
21. The method of 19-20, wherein the cancer is a breast cancer.
22. The method of 9, wherein the neurohormone peptide targeting domain is a corticotropin-releasing hormone (CCRH), a parathyroid hormone (PTH), a parathyroid hormone-like hormone (PTH LH), a PHYH, a thyrotropin-releasing hormone (TRH), an urocortin-1 (UCN1), an urocortin-2 (UCN2), an urocortin-3 (UCN3), or an urotensin 2 (UTS2).
23. The method of 22, wherein the neurohormone peptide targeting domain comprises amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97.
24. The method of 22-23, wherein the cancer is a breast cancer, an uterine cancer, a melanoma, a basal-cell skin carcinoma, a pituitary cancer, a neuroblastoma, a small cell lung carcinoma, an endometrial cancer, a pheochromocytoma, a squamous cell carcinoma, a colon cancer, an alveolar basal epithelial carcinoma, a testicular cancer, or an osteosarcoma.

25. The method of 9, wherein the neuroregulatory cytokine peptide targeting domain is a ciliary neurotrophic factor peptide, a glycophorin-A peptide, a leukemia inhibitory factor peptide, a cardiotrophin-1 peptide, a cardiotrophin-like cytokine peptide, a neuroleukin peptide, and an onostatin M peptide.
26. The method of 25, wherein the neuroregulatory cytokine peptide targeting domain comprises SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103.
27. The method 25-26, wherein the cancer is a liver cancer, a stomach cancer, a lymphoma, a colon cancer, a gastric cancer.
28. The method of 1-27, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.
29. The method of 1-27, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.
30. The method of 4-6 and 8, wherein the exogenous protease cleavage site is a plant papain cleavage site, an insect papain cleavage site, a crustacean papain cleavage site, an enterokinase cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a tobacco etch virus protease cleavage site, a Tobacco Vein Mottling Virus cleavage site, a subtilisin cleavage site, a hydroxylamine cleavage site, or a Caspase 3 cleavage site.
31. A TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain, wherein administration of the composition reduces a symptom associated with cancer.
32. A TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain, and an exogenous protease cleavage site, wherein administration of the composition reduces a symptom associated with cancer.
33. The TVEMP of 31, wherein the TVEMP comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, the targeting domain, 2) the Clostridial toxin enzymatic domain, the

- exogenous protease cleavage site, the targeting domain, the Clostridial toxin translocation domain, 3) the targeting domain, the Clostridial toxin translocation domain, the exogenous protease cleavage site and the Clostridial toxin enzymatic domain, 4) the targeting domain, the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, 5) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the targeting domain, or 6) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the targeting domain and the Clostridial toxin enzymatic domain.
34. The TVEMP of 32, wherein the TVEMP comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, the targeting domain, 2) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the targeting domain, the Clostridial toxin translocation domain, 3) the targeting domain, the Clostridial toxin translocation domain, the exogenous protease cleavage site and the Clostridial toxin enzymatic domain, 4) the targeting domain, the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, 5) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the targeting domain, or 6) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the targeting domain and the Clostridial toxin enzymatic domain.
35. The TVEMP of 31-34, wherein the targeting domain is a neurotrophin peptide, a head activator peptide, a glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) peptide, a RF-amide related peptide, a neurohormone peptide, or a neuroregulatory cytokine peptide.
36. The TVEMP of 35, wherein the neurotrophin peptide targeting domain is a nerve growth factor (NGF) peptide, a brain derived neurotrophic factor (BDNF) peptide, a neurotrophin-3 (NT-3) peptide, or a neurotrophin-4/5 (NT-4/5) peptide.
37. The TVEMP of 36, wherein the neurotrophin peptide targeting domain comprises amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85.
38. The TVEMP of 35, wherein the head activator peptide targeting domain is a head activator peptide.
39. The TVEMP of 38, wherein the head activator peptide targeting domain comprises SEQ ID NO: 86.
40. The TVEMP of 35, wherein the glial cell line-derived neurotrophic factor family of ligands peptide targeting domain is a glial cell line-derived neurotrophic factor peptide, a Neurturin peptide, a Persephrin peptide, or an Artemin peptide.

41. The TVEMP of 40, wherein the glial cell line-derived neurotrophic factor family of ligands peptide targeting domain comprises amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90.
42. The TVEMP of 35, wherein the RF-amide related peptide targeting domain a RF-amide related peptide-1, a RF-amide related peptide-2, a RF-amide related peptide-3, a neuropeptide AF, or a neuropeptide FF
43. The TVEMP of 42, wherein the RF-amide related peptide targeting domain comprises amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94.
44. The TVEMP of 35, wherein the neurohormone peptide targeting domain is a corticotropin-releasing hormone (CCRH), a parathyroid hormone (PTH), a parathyroid hormone-like hormone (PTH LH), a PHYH, a thyrotropin-releasing hormone (TRH), an urocortin-1 (UCN1), an urocortin-2 (UCN2), an urocortin-3 (UCN3), or an urotensin 2 (UTS2).
45. The TVEMP of 44, wherein the neurohormone peptide targeting domain comprises amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97.
46. The TVEMP of 35, wherein the neuroregulatory cytokine peptide targeting domain is a ciliary neurotrophic factor peptide, a glycoporphin-A peptide, a leukemia inhibitory factor peptide, a cardiotrophin-1 peptide, a cardiotrophin-like cytokine peptide, a neuroleukin peptide, and an onostatin M peptide.
47. The TVEMP of 46, wherein the neuroregulatory cytokine peptide targeting domain comprises SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103.
48. The TVEMP of 31-47, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.

49. The TVEMP of 31-47, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.
50. The TVEMP of 32 and 34, wherein the exogenous protease cleavage site is a plant papain cleavage site, an insect papain cleavage site, a crustacean papain cleavage site, an enterokinase cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a tobacco etch virus protease cleavage site, a Tobacco Vein Mottling Virus cleavage site, a subtilisin cleavage site, a hydroxylamine cleavage site, or a Caspase 3 cleavage site.
51. A composition comprising a TVEMP of 31-50.
52. The composition of 51, wherein the composition is a pharmaceutical composition.
53. The composition of 52, wherein the pharmaceutical composition comprises a pharmaceutical carrier, pharmaceutical excipient, or any combination thereof.

EXAMPLES

[0231] The following examples illustrate representative embodiments now contemplated, but should not be construed to limit the disclosed TVEMPs, compositions including TVEMPs, and methods of treating cancer using such compositions.

Example 1 Light Chain Assays

[0232] This example illustrates how to screen cancer cells in order to determine which Clostridial toxin light chain had an effect sufficient to provide a therapeutic benefit in a cancer treatment.

[0233] To identify which Clostridial toxin light chain or active fragment thereof was useful in making a TVEMP for treating a cancer using a method disclosed herein, a Clostridial toxin light chain cleavage assay was conducted. These assays address two fundamental issues. First, the light chains of the various botulinum neurotoxin serotypes cleave different SNARE substrates. In addition, some cells may only express SNAP-23 which is not cleavable by naturally-occurring botulinum neurotoxins. These cells would not be sensitive to LC/A, but may be sensitive to LC/B and LC/C1 if they express synaptobrevin-2 (VAMP-2) and/or Syntaxin, respectively. Second, this transfection assay allows the examination of the cellular effects of the light chains on cancer cells in a way that is independent of receptor binding and

translocation into the cell. Taken together, this assay allows the examination of the effects of cleaving SNARE proteins on a variety of cancer cell lines encompassing several types of human cancers.

[0234] Mammalian expression constructs encoding a fusion protein comprising a green fluorescent protein (GFP) linked to a light chain of different botulinum neurotoxin serotypes were made using standard procedures. These expression constructs were designated 1) pQBI25/GFP, a construct expressing GFP of SEQ ID NO: 104 encoded by the polynucleotide of SEQ ID NO: 105; 2) pQBI25/GFP-LC/A, a construct expressing GFP-LC/A fusion protein of SEQ ID NO: 106 encoded by the polynucleotide of SEQ ID NO: 107; 3) pQBI/GFP-LC/B, a construct expressing GFP-LC/B fusion protein of SEQ ID NO: 108 encoded by the polynucleotide of SEQ ID NO: 109; 4) pQBI/GFP-LC/C1, a construct expressing GFP-LC/C1 fusion protein of SEQ ID NO: 110 encoded by the polynucleotide of SEQ ID NO: 111; and 5) pQBI/GFP-LC/E, a construct expressing GFP-LC/E fusion protein of SEQ ID NO: 112 encoded by the polynucleotide of SEQ ID NO: 113. The light chains for these particular botulinum toxin serotypes were selected because overall, the light chains cleave one of the three predominant SNARE proteins SNAP-25, VAMP, or Syntaxin.

[0235] To culture cells, an appropriate density of cells were plated into the wells of 6-well tissue culture plates containing 3 mL of an appropriate medium (Table 5). The cells were grown in a 37 °C incubator under 5% carbon dioxide until cells reached the appropriate density (about 1×10^6 cells). A 500 μ L transfection solution was prepared by adding 250 μ L of OPTI-MEM Reduced Serum Medium containing 10 μ L of LipofectAmine 2000 (Invitrogen Inc., Carlsbad, CA), incubated at room temperature for 5 minutes, to 250 μ L of OPTI-MEM Reduced Serum Medium containing 5 μ g of the desired mammalian expression construct. This transfection mixture was incubated at room temperature for approximately 25 minutes. The growth media was replaced with fresh unsupplemented serum-free media and the 500 μ L transfection solution was added to the cells. The cells were then incubated in a 37 °C incubator under 5% carbon dioxide for approximately 8 hours. The transfection media was replaced with fresh unsupplemented serum-free media and the cells then incubated in a 37 °C incubator under 5% carbon dioxide for approximately 48 hours. After this incubation, the cells were washed by aspirating the media and rinsing each well with 3 mL of 1 x PBS.

Table 5. Cell Lines and Media			
Cell Line	Origin	Source	Serum Growth Media Composition
RT4	Human urinary bladder transitional cell carcinoma	ATCC HTB-2	McCoy's 5a media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 μ g/mL Streptomycin
P19	Mouse embryonic carcinoma	ATCC CRL-1825	Alpha Minimal Essential Medium media with 7.5 % bovine calf serum, 2.5% fetal bovine calf serum, 100 U/mL Penicillin, and 100 μ g/mL Streptomycin
NCI H69	Human small lung carcinoma	ATCC HTB-119	RPMLI-1640 media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 μ g/mL Streptomycin

Table 5. Cell Lines and Media			
Cell Line	Origin	Source	Serum Growth Media Composition
NCI H82	Human small lung carcinoma	ATCC HTB-175	RPMI-1640 media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
DU-145	Human prostate carcinoma derived from brain	ATCC HTB-81	Eagle's Minimum Essential Medium with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
T24	Human urinary bladder transitional cell carcinoma	ATCC HTB-4	McCoy's 5a media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
J82	Human urinary bladder transitional cell carcinoma	ATCC HTB-1	Eagle's Minimum Essential Medium with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
HIT-T15	Syrian Golden Hamster, pancreatic islet of Langerhans beta cells	ATCC CRL-1777	Eagle's Minimum Essential Medium (low glucose) with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin

[0236] The cells were first analyzed using fluorescent microscopy for the expression of GFP, which also indicated the simultaneous expression of the attached light chain. To detect the expression and subcellular localization of the GFP-LC fusion proteins, the cells were examined by confocal microscopy. Cells from the cell lines RT4, P19, NCI H69, NCI H82, DU145, T24, and J82, transfected and washed as described above, were fixed with 4% paraformaldehyde. The fixed cells were imaged with a confocal microscope using a 488 nm excitation laser and an emission path of 510-530 nm. The data shows that each cell type was successfully transfected and, that except the small cell lung cancer cell lines NCI H69 and NCI H82, cells from each cell line expressed both GFP and the GFP-light chain fusion proteins (Table 6).

Table 6. Expression of Mammalian Constructs in Cells						
Cell Line	Origin	Expression				
		GFP	GFP-LC/A	GFP-LC/B	GFP-LC/C1	GFP-LC/E
RT4	Bladder carcinoma	+	+	+	+	+
P19	Embryonic carcinoma	+	+	+	+	+
NCI H69	Small Cell Lung carcinoma	–	–	–	–	–
NCI H82	Small Cell Lung carcinoma	–	–	–	–	–
DU145	Prostate carcinoma	+	+	+	+	+
T24	Bladder carcinoma	+	+	+	+	+
J82	Bladder carcinoma	+	+	+	+	+

[0237] In order for cancer cells to be sensitive to the endoproteolytic cleavage, the target SNARE protein must be endogenously expressed and accessible to the light chain cleavage. To detect the presence of cleaved SNARE products a Western blot analysis was performed. Cells from the cell lines RT4, P19, NCI H69, NCI H82, DU145, T24, and J82, transfected and washed as described above, were lysed, by adding 200 μ L of 2 x SDS-PAGE Loading Buffer to each well, and the lysates were transferred to tubes and heated to 95 °C for 5 minutes. A 12 μ L of each sample was separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen Inc., Carlsbad, CA) under denaturing, reducing conditions. Separated peptides were transferred from the gel onto nitrocellulose membranes by Western blotting using an electrophoretic tank transfer apparatus. The membranes were blocked by incubation, at room temperature, for 1 hour with gentle agitation, in a Blocking Solution containing Tris-Buffered Saline (TBS) (25 mM 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloric acid (Tris-HCl)(pH 7.4), 137 mM sodium chloride, 2.7 mM potassium chloride), 0.1% polyoxyethylene (20) sorbitan monolaureate, 2% Bovine Serum Albumin (BSA), and 5% nonfat dry milk. Blocked membranes were incubated at 4 °C over night in TBS, 0.1% polyoxyethylene (20) sorbitan monolaureate, 2% BSA, and either 1) a 1:5,000 dilution of S9684 α -SNAP-25 rabbit polyclonal antiserum as the primary antibody (Sigma, St. Louis, MO); 2) a 1:5,000 dilution of sc17836 α -Syntaxin-1 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); or 3) a 1:5,000 dilution of sc69706 α -VAMP-2 mouse polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibody probed blots were washed three times for 5 minutes each time in TBS, polyoxyethylene (20) sorbitan monolaureate. Washed membranes were incubated at room temperature for 1 hour in TBS, 0.1% polyoxyethylene (20) sorbitan monolaureate, 2% BSA containing either 1) a 1:5,000 dilution of 81-6720 goat polyclonal α -mouse immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Invitrogen, Inc., Carlsbad, CA) as a secondary antibody; or 2) a 1:5,000 dilution of 81-6120 goat polyclonal α -rabbit immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Invitrogen, Inc., Carlsbad, CA) as a secondary antibody. Secondary antibody-probed blots were washed three times for 5 minutes each time in TBS, 0.1% polyoxyethylene (20) sorbitan monolaureate. Signal detection of the labeled SNARE products were visualized using the ECL Plus™ Western Blot Detection System, a chemiluminescence-based detection system, (GE Healthcare-Amersham, Piscataway, NJ). The membranes were imaged and the percent of cleaved SNARE product were quantified with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (GE Healthcare-Amersham, Piscataway, NJ). The data shows that SNAP-25 and VAMP-2 were expressed in some cell types, while Syntaxin was expressed in each cell type tested (Table 7).

Table 7. Presence of SNARE in Cells				
Cell Line	Origin	SNARE Presence in Cells		
		SNAP-25	VAMP-2	Syntaxin-1
RT4	Bladder carcinoma	–	+	+
P19	Embryonic	+	–	+

Table 7. Presence of SNARE in Cells				
Cell Line	Origin	SNARE Presence in Cells		
		SNAP-25	VAMP-2	Syntaxin-1
	carcinoma			
NCI H69	Small cell Lung carcinoma	ND	ND	ND
NCI H82	Small cell Lung carcinoma	ND	ND	ND
DU145	Prostate carcinoma	+	+	+
T24	Bladder carcinoma	–	+	+
J82	Bladder carcinoma	+	–	+

[0238] In addition, the data shows that 1) BoNT/A light chain was able to cleave SNAP-25 present in cells from a P19 embryonic carcinoma cell line, a DU145 prostate carcinoma cell line, and a J82 urinary bladder carcinoma cell line (Table 8); 2) BoNT/E light chain was able to cleave SNAP-25 present in cells from a P19 embryonic carcinoma cell line and a J82 urinary bladder carcinoma cell line (Table 8); 3) BoNT/B light chain was unable to cleave VAMP-2 in all cell lines tested (Table 8); and 4) BoNT/C1 light chain was able to cleave Syntaxin-1 present in cells from a T24 urinary bladder carcinoma cell line (Table 8). These results indicate that treatment of cancer cells with the appropriate Clostridial toxin light chain will cleave one of three SNARE proteins to inhibit exocytosis. This inhibition will prevent the release of growth factors, angiogenic factors, and anti-apoptotic survival factors necessary for cancer cell growth and survival.

Table 8. Cleavage of SNARE by Light Chain					
Cell Line	Origin	SNARE Cleavage by Light Chain			
		SNAP-25		VAMP-2	Syntaxin-1
		LC/A	LC/E	LC/B	LC/C1
RT4	Bladder carcinoma	–	–	–	–
P19	Embryonic carcinoma	+	+	–	–
NCI H69	Small Cell Lung carcinoma	ND	ND	ND	ND
NCI H82	Small Cell Lung carcinoma	ND	ND	ND	ND
DU145	Prostate carcinoma	+	–	–	–
T24	Bladder carcinoma	–	–	–	+
J82	Bladder carcinoma	+	+	–	–

[0239] To further test whether SNARE cleavage disrupts exocytosis, an insulin release assay was performed. HIT-T15 cells release insulin when placed in high concentration of glucose. It has also been shown these cells express SNAP-25, and that SNAP-25 is an integral component of the SNARE complex needed for insulin release. HIT-T15 cells, transfected and washed as described above, were placed in DMEM media containing either 1) 5.6 mM glucose for basal insulin release (low glucose); or 2) 25.2 mM glucose for evoked insulin release (high glucose). Cells were incubated in a 37 °C incubator under 5% carbon dioxide for approximately 1 hour to allow for insulin release. The incubated media was collected and the amount of insulin released was determined using an insulin ELISA kit. The assay was performed according to the manufacturer's instructions (APLCO Diagnostics, Salem, NH). Exocytosis was expressed as the amount of insulin released per 1×10^6 cells per hour.

[0240] The data shows that HIT-T15 cells transfected with GFP-LC/A, GFP-LC/B, and GFP-LC/E released less insulin than untransfected cells or cells transfected with GFP (Table 9). In addition, the basal insulin released in media containing a low glucose concentration (5.6 mM) remained unchanged between the transfected cells. The data indicate that BoNT/A, BoNT/B and BoNT/E light chains inhibited the release of insulin by cleaving SNAP-25 or VAMP-2 in HIT-T15 cells.

Table 9. Insulin Release from HIT-H15 Cells		
Construct	5.6 mM Glucose (Low)	25.2 mM Glucose (High)
Untransfected Control	6.5 +/- 0.1	9.9 +/- 2.9
GFP	4.3 +/- 0.7	10.8 +/- 2.1
GFP-LCA	3.2 +/- 0.4	4.5 +/- 0.6
GFP-LCB	3.4 +/- 0.2	5.5 +/- 0.9
GFP-LCE	4.2 +/- 0.7	4.4 +/- 1.0

[0241] The botulinum toxin light chain activity may also inhibit the trafficking of proteins to and from the plasma membrane. To test whether SNARE cleavage disrupts delivery and localization of receptors to the plasma membrane, the presence or absence of cell membrane proteins was determined in cells transfected with botulinum toxin light chains. Cells from the cell lines DU145 and J82, transfected and washed as described above, were treated with 2 mM NHS-LC-Biotin (Thermo Scientific, Rockford, IL) at 4 °C for 2 hours. The cells were then treated with 250 mM Tris-HCl (pH 7.5) for 30 minutes at 4 °C, and then washed three times in TBS. Membranes proteins were isolated using the Membrane Protein extraction kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. The biotinylated proteins were precipitated with immobilized-avidin (Thermo Scientific, Rockford, IL). After three washes with TBS, the samples were suspended in 50 μ L 2x SDS-PAGE loading buffer and separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen Inc., Carlsbad, CA) under denaturing, reducing conditions. The gel was washed and fixed in 10% methanol and 7% acetic acid for 30 minutes. The wash solution was removed and the gel incubated in SYPRO® Ruby protein gel stain solution (Bio-Rad Laboratories, Hercules, CA) for 3 hours to overnight at room temperature. The stained gel was destained in 10% methanol and 7% acetic acid for 30 minutes.

Chemiluminescence from the destained gel was visualized with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (GE Healthcare-Amersham, Piscataway, NJ). The data show that treatment with a BoNT/A light chain inhibits the trafficking of proteins to and from the plasma membrane, which would necessarily affect the population of receptors located on the surface of the cell. This disrupted trafficking may cause the cancer cells to become more sensitive to apoptotic factors and less sensitive to growth signals and angiogenic factors.

[0242] By establishing the SNARE cleavage effects by the light chains, and which light chains cleaved which SNARE proteins in each cell line, TVEMPs were subsequently designed in a manner that targeted the TVEMP to receptors that were overexpressed or uniquely expressed in cancers cells in order to deliver the catalytic light chain.

Example 2

Presence of Receptor and Target in Cancer Cells

[0243] This example illustrates how to determine the presence of a cognate receptor that can bind with the targeting moiety of a TVEMP disclosed herein as well as the presence of the target SNARE protein of the enzymatic domain of a TVEMP disclosed herein.

[0244] In order for a TVEMP to be an effective agent for the methods of treating cancer disclosed herein, the cancer cells must express the appropriate receptor that can bind with the targeting moiety of a TVEMP as well as the appropriate SNARE protein that can be cleaved by the enzymatic domain of the TVEMP.

[0245] To culture cells, an appropriate density of cells were plated into the wells of 96-well tissue culture plates containing 100 μ L of an appropriate medium (Table 10), but without serum, and with or without 25 μ g/mL of GT1b (Alexis Biochemicals, San Diego, CA). Cells were plated and incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest (approximately 3 days). The media was aspirated from each well and replaced with 100 μ L of fresh media containing various concentrations of the botulinum toxin or TVEMP being tested in order to generate a full dose-response. The assay was done in triplicate. After 24 hrs treatment, the cells were washed, incubated for an additional two days without toxin or TVEMP to allow for the cleavage of the SNARE substrate. After this incubation, the cells were washed by aspirating the media and rinsing each well with 3 mL of 1 x PBS. The cells were harvested by lysing in freshly prepared Lysis Buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% , 4-octylphenol polyethoxylate) at 4°C for 30 minutes with constant agitation. Lysed cells were centrifuged at 4000 rpm for 20 min at 4°C to eliminate debris using a bench-top centrifuge. The total protein concentrations of the cell lysates were measured by Bradford assay.

Table 10. Cell Lines and Media			
Cell Line	Origin	Source	Serum Growth Media Composition
RT4	Human urinary bladder transitional cell carcinoma	ATCC HTB-2	McCoy's 5a media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
P19	Mouse embryonic carcinoma	ATCC CRL-1825	Alpha Minimal Essential Medium media with 7.5 % bovine calf serum, 2.5% fetal bovine calf serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
NCI H69	Human small lung carcinoma	ATCC HTB-119	RPMI-1640 media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
NCI H82	Human small lung carcinoma	ATCC HTB-175	RPMI-1640 media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
DU-145	Human prostate carcinoma derived from brain	ATCC HTB-81	Eagle's Minimum Essential Medium with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
PC-3	Human prostate carcinoma derived from brain	ATCC CRL-1435	F-12K media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
LNCaP clone FGC	Human prostate carcinoma derived from brain	ATCC CRL-1740	RPMI-1640 Eagle's with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
RWPE-1	Human prostate	ATCC CRL-11609	Dulbecco's Minimum Essential Medium with 10% Fetal Bovine Serum, 2 mM GlutaMAX™ I with 0.1 mM Non-Essential Amino-Acids, 10 mM HEPES, 1 mM Sodium Pyruvate, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
T24	Human urinary bladder transitional cell carcinoma	ATCC HTB-4	McCoy's 5a media with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
J82	Human urinary bladder transitional cell carcinoma	ATCC HTB-1	Eagle's Minimum Essential Medium with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
MCF-7	Human breast carcinoma	ATCC HTB-22	Dulbecco's Minimum Essential Medium with 10% Fetal Bovine Serum, 2 mM GlutaMAX™ I with 0.1 mM Non-Essential Amino-Acids, 10 mM HEPES, 1 mM Sodium Pyruvate, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
SiMa	Human neuroblastoma	DSMZ ACC 164	RPMI 1640 with 10% Fetal Bovine Serum, 0.1 mM Non-Essential Amino-Acids, 10 mM HEPES, 1 mM Sodium Pyruvate, 100 U/mL Penicillin, and 100 µg/mL Streptomycin,
266.6	Mouse pancreatic	ATCC CRL -2151	Dulbecco's Minimum Essential Medium with 10% Fetal Bovine Serum, 2 mM GlutaMAX™ I with 0.1 mM Non-Essential Amino-Acids, 10 mM HEPES, 1 mM Sodium Pyruvate, 100 U/mL Penicillin, and 100 µg/mL Streptomycin

Table 10. Cell Lines and Media			
Cell Line	Origin	Source	Serum Growth Media Composition
HIT-T15	Hamster pancreatic islet of Langerhans beta cells	ATCC CRL-1777	Eagle's Minimum Essential Medium (low glucose) with 10 % fetal bovine serum, 100 U/mL Penicillin, and 100 µg/mL Streptomycin
HUVEC	Human Umbilical Vein Endothelial Cells	Cell Applications, Inc., San Diego, CA, Cat. No. 200-05n	Endothelial Cell Growth Medium (Cell Applications, Inc., San Diego, CA, Cat. No. 211-500)

[0246] To determine whether a cancer cell expresses the appropriate receptor and target SNARE protein, a Western blot analysis can be performed.

[0247] In one experiment, cells from the cell lines RT4, P19, NCI H69, NCI H82, DU-145, T24, J82, LNCaP, and PC-3, transfected and washed as described above, were harvested by adding 40 µL of 2 x SDS-PAGE Loading Buffer (Invitrogen, Inc., Carlsbad, CA) and heating the plate to 95 °C for 5 min. A 12 µL of the harvested sample was separated by MOPS polyacrylamide gel electrophoresis under denaturing, reducing conditions using 1) CRITERION® 12% Bis-Tris precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), when separating the SNAP-25₁₉₇ cleavage product; 2) NuPAGE® 12% Bis-Tris precast polyacrylamide gels (Invitrogen Inc., Carlsbad, CA), when separating both the uncleaved SNAP-25₂₀₆ substrate and the SNAP-25₁₉₇ cleavage product; or 3) NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen Inc., Carlsbad, CA), when separating all other proteins. Separated peptides were transferred from the gel onto nitrocellulose membranes by Western blotting using a electrophoretic tank transfer apparatus. The membranes were blocked by incubation at room temperature for 1 hour with gentle agitation in a Blocking Solution containing Tris-Buffered Saline (TBS) (25 mM 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloric acid (Tris-HCl)(pH 7.4), 137 mM sodium chloride, 2.7 mM potassium chloride), 0.1% polyoxyethylene (20) sorbitan monolaureate, 2% Bovine Serum Albumin (BSA), and 5% nonfat dry milk. Blocked membranes were incubated at 4 °C overnight in TBS, 0.1% polyoxyethylene (20) sorbitan monolaureate, 2% BSA, and either 1) a 1:5,000 dilution of S9684 α-SNAP-25 rabbit polyclonal antiserum as the primary antibody (Sigma, St. Louis, MO); 2) a 1:5,000 dilution of sc123 α-Syntaxin-1 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 3) a 1:5,000 dilution of sc13992 α-VAMP-1/2/3 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 4) a 1:5,000 dilution of sc50371 α-SNAP-23 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 5) a 1:5,000 dilution of sc28955 α-SVC2 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 6) a 1:5,000 dilution of sc123 α-FGFR3 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 7) a 1:5,000 dilution of sc9112 α-KOR1 rabbit polyclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 8) a 1:5,000 dilution of H00004987-D01P α-OPRL1 rabbit polyclonal antiserum as the primary antibody (Novus Biologicals, Littleton, CO); and 9) a 1:5,000 dilution of sc47778 α-β-actin mouse monoclonal antiserum as the primary antibody (Santa Cruz Biotechnology, Santa Cruz,

CA). Primary antibody probed blots were washed three times for 5 minutes each time in TBS, polyoxyethylene (20) sorbitan monolaureate. Washed membranes were incubated at room temperature for 1 hour in TBS, 0.1% polyoxyethylene (20) sorbitan monolaureate, 2% BSA containing either 1) a 1:5,000 dilution of 81-6720 goat polyclonal α -mouse immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Invitrogen, Inc., Carlsbad, CA) as a secondary antibody; or 2) a 1:5,000 dilution of 81-6120 goat polyclonal α -rabbit immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Invitrogen, Inc., Carlsbad, CA) as a secondary antibody. Secondary antibody-probed blots were washed three times for 5 minutes each time in TBS, 0.1% polyoxyethylene (20) sorbitan monolaureate. Signal detection of the labeled SNARE products were visualized using the ECL Plus™ Western Blot Detection System, a chemiluminescence-based detection system (GE Healthcare-Amersham, Piscataway, NJ). The membranes were imaged and the percent of cleaved SNARE product was quantified with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (GE Healthcare-Amersham, Piscataway, NJ). The data shows that this approach can identify the receptors and SNARE proteins present in the cells comprising each cell line (Table 11).

Table 11. Expression of Receptors and SNARE Proteins in Cells

Cell Line	Expression							
	SNAP-25	SNAP-23	VAMP-2	Syntaxin-1	FGFR3	SV2C	OPRL-1	KOR-1
RT4	+	—	+	+	+	+	ND	+
P19	+	—	—	+	+	—	ND	+
NCI H69	+	—	+	+	+	—	ND	+
NCI H82	+	—	+	+	+	—	ND	+
DU-145	++	+	++	++	+++	ND	ND	+
PC-3	—	++	+/-	++	+++	ND	ND	+
LNCaP clone FGC	+	+	+	+	+++	+++	++	+
T24	—	++	+	+	++	++	++	+
J82	++	+/-	++	+	+++	++	++	+
ND, not determined								

[0248] Once cell lines comprising cells including the appropriate receptor and SNARE proteins were identified, the ability of a botulinum toxin or TVEMP to intoxicate these cells can be determined by detecting the presence of cleaved SNARE products using Western blot analysis. An appropriate density of cells from each cell line to be tested are plated into the wells of 96-well tissue culture plates containing 100 μ L of an appropriate medium (Table 7) with or without 25 μ g/mL of GT1b (Alexis Biochemicals, San Diego, CA). Cells are plated and incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest (approximately 3 days). The media is aspirated from each well and is replaced with 100 μ L of fresh media containing various concentrations of the botulinum toxin or TVEMP being tested sufficient to generate a full dose-response. The assay is done in triplicate. After 24 hrs treatment, the cells are washed, incubated for an additional two days without toxin or TVEMP to allow for the cleavage of the

SNARE substrate. After this incubation, the cells are washed by aspirating the media and rinsing each well with 3 mL of 1 x PBS. The cells are harvested by lysing in freshly prepared Lysis Buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% , 4-octylphenol polyethoxylate) at 4°C for 30 minutes with constant agitation. Lysed cells are centrifuged at 4000 rpm for 20 min at 4°C to eliminate debris using a bench-top centrifuge. The protein concentrations of cell lysates are measured by Bradford assay. Samples of the cell lysates are analyzed by Western blot analysis as described above.

[0249] In one experiment, differentiated cells from the cell lines LNCaP, J82, and MCF-7, transfected as described above. The media was aspirated from each well and the differentiated cells were treated by replacing with fresh media containing either 1) 0 (untreated sample), 0.12 nM, 0.36 nM, 1.1 nM, 3.3 nM, 10 nM, 30 nM, and 90 nM of a BoNT/A; 2) 0 (untreated sample), and 50 nM of a BoNT/A; 3) 0 (untreated sample), 0.12 nM, 0.36 nM, 1.1 nM, 3.3 nM, 10 nM, 30 nM, and 90 nM of a TVEMP designated Noci-LH_N/A; or 4) 0 (untreated sample), and 166 nM of a TVEMP designated Noci-LHN/A. After 1) 3-15 hours; 2) 6 hours or 3) 24 hours treatment, the cells were washed, incubated for an additional 16 hours without toxin or TVEMP to allow for the cleavage of the SNAP-25 substrate. After this incubation, the cells were washed and harvested as described above. The presence of cleaved SNAP-25 product was detected using Western blot analysis as described above using a 1:5,000 dilution of S9684 α-SNAP-25 rabbit polyclonal antiserum as the primary antibody (Sigma, St. Louis, MO) as the primary antibody and a 1:5,000 dilution of 81-6120 goat polyclonal α-rabbit immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Invitrogen, Inc., Carlsbad, CA) as a secondary antibody. These results are shown in Table 12.

Table 12. Cleavage of SNARE Substrate		
Cell Line	Lowest Concentration and Earliest Time for Cleavage Detection	
	BoNT/A	Noci-LH_N/A
LNCaP	50 nM at 9 hours	166 nM at 9 hours
J82	50 nM at 3 hours 1.1 nM at 24 hours	166 nM at 3 hours
MCF-7	1.1 nM at 6 hours	ND
ND, not determined		

[0250] Taken together, the data shows that 1) BoNT/A was able to cleave SNAP-25 present in cells from a LNCaP prostate carcinoma cell line, a J82 urinary bladder carcinoma cell line, and a MCF-7 breast carcinoma cell line (Table 9); 2) Noci-LH_N/A was able to cleave SNAP-25 present in cells from a LNCaP prostate carcinoma cell line and a J82 urinary bladder carcinoma cell line (Table 9). These results indicate that treatment of cancer cells with the appropriate Clostridial toxin light chain will cleave one of three SNARE proteins to inhibit exocytosis. This inhibition will prevent the release of growth factors, angiogenic factors, and anti-apoptotic survival factors necessary for cancer cell growth and survival. Lastly, these experiments illustrate the validity of the general concept that intracellular delivery of a botulinum light chain into cancer cells results in cleavage of the appropriate SNARE protein not only by

transfecting light chain constructs, but also by using the endogenous signal transduction pathway for the targeting domain.

Example 3

Effects of Light Chain Delivery on Angiogenesis

[0251] This example illustrates that treatment with a botulinum toxin or TVEMP will affect angiogenesis to a degree sufficient to provide a therapeutic benefit in a cancer treatment.

[0252] The blockade of exocytosis resulting from a treatment with botulinum toxin or TVEMP based on LHN/A-G will likely prevent the release of angiogenic factors, including, e.g., Vascular endothelial growth factor (VEGF), Fibroblast Growth Factor-1 (FGF1) and FGF2. Preventing the release of these angiogenic factors will reduce, or altogether inhibit, angiogenesis in the area where the toxin or TVEMP is administered. To test whether such a treatment reduces or inhibits angiogenesis, four different assays were performed: a VEGF release assay, a cell migration assay, an in vitro blood vessel formation assay, and a human angiogenesis protein array assay.

[0253] VEGF is known to be a potent mitogen for vascular endothelial cells and an inducer of physiological and pathological angiogenesis. To validate the potential for a botulinum toxin or TVEMP in inhibiting angiogenesis, the ability of a toxin or TVEMP to inhibit release of VEGF from a cell was assessed. To conduct a VEGF release assay, about 600,000 cells from a SiMa cell line were plated into the wells of 6-well collagen IV tissue culture plates containing 3 mL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 µg/mL GT1b. These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media from the differentiated cells was aspirated from each well and replaced with fresh media containing either 0.77 mg/mL of a BoNT/A or 1 mg/mL of a Noci-LH_N/A TVEMP. As a control, cells were treated with media alone in parallel. After treatment the media was removed and replaced with fresh differentiation media. A 60 µL aliquot of media was removed from each well and replaced with 100 µL differentiation media 1 day, 2 days, 3 days, and 4 days after the addition of fresh differentiation media. The removed media was stored at -20 °C until needed. After the last sample was removed, the cells were trypsinized and the number of cells in each well was counted.

[0254] The presence of VEGF in the collected samples was detected using a K151BMB-1 VEGF tissue culture assay (Meso Scale Discovery, Gaithersburg, MD). A MULTI-ARRAY® 96-well Small Spot Plate VEGF plate was blocked with 150 µL Blocking Buffer (PBS with 0.05% polyoxyethylene (20) sorbitan monolaureate, 2% ECL Blocking reagent (GE Healthcare-Amersham, Piscataway, NJ), and 1% goat serum (Rockland Immunochemicals, Gilbertsville, PA) and shaken at 600 rpm for one hour. The blocking

buffer was discharged and 25 μ L of each sample was added to each well of the VEGF plate and the plate was incubated at 4 °C for 2 hours. The plate was washed three times with 200 μ L PBS-T (PBS plus 0.05% Tween-20) and then 25 μ L of SULFO-TAG α -hVEGF mouse monoclonal antibody 5 μ g/mL in 2% antibody buffer (PBS plus 0.05 % polyoxyethylene (20) sorbitan monolaureate, and 2% ECL Blocking reagent (GE Healthcare-Amersham, Piscataway, NJ) added and incubated on a shaker at 600 rpm at RT for 1 hour. Plates were washed three times with PBS-T and then 150 μ L Read Buffer (MSD, Cat# R92TC-1) were added per well. Plates were read in a SECTOR™ Imager 6000 Image Reader (Meso Scale Discovery, Gaithersburg, MD). The data was then exported into Microsoft Office Excel 2007. The amount of VEGF detected was normalized to the number of cells present in the well and the percent VEGF release value was calculated using the control as the 100% value.

[0255] The data shows that treatment with BoNT/A inhibits VEGF release by about 50 % in SiMa cells (Table 13). Although the addition of Noci-LH_N/A TVEMP did not appear to inhibit VEGF release, this result could be due to the lower potency of Noci-LH_N/A TVEMP compared to BoNT/A in SiMa cells. The EC₅₀ of BoNT/A in differentiated SiMa cells is less than about 0.5 nM, while the EC₅₀ of Noci-LH_N/A TVEMP is more than 30 nM. As such, the lack of effect of Noci-LH_N/A TVEMP in SiMa cells is simply due to the low amount of OPRL-1 receptor present in these cells. This lack of effect corroborates the concept that cells expressing low levels of the targeted receptor will not be affected by botulinum toxin or TVEMP treatment (i.e. normal cells surrounding tumors over-expressing a receptor of interest). In addition, the finding that the addition of IL-6, a known transcriptional regulator of VEGF, had no effect on VEGF release is consistent with reports that the addition of exogenous IL-6 does not affect VEGF secretion.

Table 13. VEGF Release Assay

Time Point	VEGF Release		
	Control	BoNT/A	Noci-LH _N /A TVEMP
Day 1	100%	69%	119%
Day 2	100%	57%	123%
Day 3	100%	53%	125%
Day 4	100%	57%	104%

[0256] Since VEGF is an inducer of migration, a compound that affects the release of VEGF should effect migration as well. Moreover, inhibition of exocytosis by a compound will also inhibit the release of additional factors involved in cell migration. To determine whether a botulinum toxin or TVEMP treatment could reduce or inhibit cell migration, a cell migration assay (Essen Bioscience, Ann Arbor, MI) was performed according to the manufacturer's instructions. On day 1, DU-145 cells were plated at 25,000 cells per well in a 96-well Essen ImageLock plate in growth media. On day 2 the cells were treated with either 10 nM BoNT/A, 40 nM Noci-LH_N/A TVEMP, or 90 nM Gal-LH_N/A TVEMP in growth media. As a positive control for inhibition of migration, cells were treated with 0.11 μ M, 0.33 μ M, or 1 μ M Cytochalasin-D. As a negative control, cells were treated with media alone. On day 3, after the cells had reached 100 % confluence, the cells were washed with media and then a 96-pin WoundMaker (Essen Bioscience, Ann

Arbor, MI) was used to simultaneously create wounds in all the wells. After cell wounding, the media was removed and the cells were washed two times with 150 μ L Dulbecco's Phosphate Buffered Saline with Ca^{2+} and Mg^{2+} and then 100 μ L of media was added. The plate was then placed in an INCUCYTE™ scanner (Essen Bioscience, Ann Arbor, MI) and images were taken every 1 hour for 45 consecutive hours. The data was analyzed as relative wound density versus time using the INCUCYTE™ Cell Migration software. Relative wound density is designed to be zero at time zero, and 100% when the cell density inside the wound is the same as the cell density outside the initial wound.

[0257] The results are presented in Table 14. The results showed that cells pre-treated with either Noci-LH_N/A TVEMP or Gal-LH_N/A TVEMP migrated slightly slower than cells treated with media alone. The result showed that treatment with Noci-LH_N/A TVEMP or Gal-LH_N/A TVEMP resulted in a significant reduction in cell migration after 24 hours, about 10 % reduction when compared to cells treated with media alone. Cells treated with BoNT/A did not exhibit an affect on cell migration. The cells treated with Cytochalasin-D did not migrate. When the same experiment was performed with PC-3 cells, that do not contain SNAP-25, rather than a reduction, an increase in migration was observed (data not shown), suggesting that initially, likely via activation of their ligand receptors, BoNT/A, , Noci-LH_N/A TVEMP, and Gal-LH_N/A TVEMP function to increase migration. But after cleavage of SNAP-25 migration is reduced. As such, a longer exposure to a botulinum toxin and/or TVEMP will most likely result in more dramatic reduction in migration of such treated cells.

Table 14. Cell Migration Assay		
Treatment	Relative Wound Density at 24 Hours	
	Mean	Percent Relative to Media
Media Control	78.2 $\pm$ 2.4	100%
BoNT/A	78.6 $\pm$ 1.1	101%
Noci-LH _N /A TVEMP	71.5 $\pm$ 3.3	91%
Gal-LH _N /A TVEMP	69.5 $\pm$ 4.4	89%
Cytochalasin-D	3.3 $\pm$ 0.2	4%

[0258] Angiogenesis involves multiple steps; to achieve new blood vessel formation, endothelial cells must first escape their stable location by breaking through the basement membrane. Once this is achieved, endothelial cells migrate towards an angiogenic stimulus that might be released from cancer cells, or wound-associated macrophages. In addition, endothelial cells proliferate to provide the necessary number of cells for making a new vessel. Subsequent to this proliferation, the new outgrowth of endothelial cells needs to reorganize into a three-dimensionally tubular structure. To determine whether a botulinum toxin or TVEMP treatment could reduce or inhibit blood vessel formation, an in vitro Endothelial Tube Formation assay (Cell Biolabs, Inc., San Diego, CA) was performed according to the manufacturer's instructions. Human Umbilical Vein Endothelial Cells (HUVECs) were grown to 80% confluence in T-75 culture flasks until confluent. Cells were harvested and then plated at 500,000 cells per well for HUVECs in a 6-well plate for 24 hours. After incubation, cells were either kept untreated or

treated with 2 nM or 5 nM of BoNT/A or 6 nM or 25 nM of Noci-LH_N/A TVEMP for 24 hours. As a positive control for inhibition, cells were treated with a collagenase inhibitor. As a negative control for inhibition, cells were treated with media alone. The cells were then harvested again and plated at 35,000 cells per well onto the ECM gel prepared from murine Engelbreth-Holm-Swan (EHS) tumor cells, which contain multiple angiogenic stimulating factors, such as, e.g., laminin, type IV collagen, heparan sulfate proteoglycans, entactin and growth factors such as FGF2 and TGF- β s. The cells were incubated for 3-4 hours on the ECM gels and then inspected under a microscope and photographed, either before or after staining with Calcein AM.

[0259] A Endothelial Tube Formation assay was also modified to use cells from a tumor cell line. In this modified assay, cells from a LNCaP, PC-3, DU-145, T24, and J82 cell lines were grown to 80% confluence in T-75 culture flasks. Cells were then harvested and plated at 400,000 cell per well in a 6-well plate containing 3 mL of an appropriate medium (Table 10), but with 1% serum. Cells were incubated in a 37 °C incubator under 5% carbon dioxide for 3 days. After incubation, cells were either kept untreated or treated with 20 nM of BoNT/A or 40 nM of Noci-LH_N/A TVEMP for 24 hours. The cells were then harvested, plated on ECM gel plates and inspected as described above.

[0260] The results show that in HUVEC, DU145 and J82 cells, and to a lesser degree in T24 and LNCaP cells, tubes formed on ECM plates treated with media alone, whereas treatment with a collagenase inhibitor prevented the formation of tubes (Table 15). No tubes formed in PC-3 cells. BoNT/A and Noci-LH_N/A TVEMP treatment of cells from a LNCaP prostate carcinoma cell line and a J82 bladder carcinoma cell line inhibited the formation of tubes. BoNT/A and Noci-LH_N/A TVEMP treatment had no effect on tube formation from HUVEC cultures. This inhibition of tube formation maybe due to inhibition of migration, delivery of receptors and other proteins to the membrane (motility factors and their receptors), adhesion molecules that interact with the matrix or other cells, and/or secretion of proteases.

Table 15. Endothelial Tube Formation Assay

Cell Line	Inhibition of Endothelial Tube Formation			
	Media	Collagenase Inhibitor	BoNT/A	Noci-LH _N /A
LNCaP	No	Yes	Yes	Yes
PC-3	—	—	—	—
DU-145	No	ND	ND	ND
T24	No	ND	ND	ND
J82	No	Yes	Yes	Yes
HUVEC	No	ND	No	No
ND, not determined				

[0261] To conduct a human angiogenesis protein array screen, cells from a DU-145 prostate cancer cell line were plated in a 100 mm² plate containing Eagle's Minimum Essential Medium with 1% charcoal stripped FBS, 100 U/mL Penicillin, and 100 μ g/mL Streptomycin. Cells were grown to a density of 5×10^6 cells by incubating in a 37 °C incubator under 5% carbon dioxide overnight. After this incubation, the

cells were washed by aspirating the media and rinsing the plate with 10 mL of 1 x PBS. The washed cells were treated by replacing with fresh media containing 50 nM BoNT/A. For comparison, cells treated with media alone were run in parallel. After 24 hour treatment, the cells were washed, and harvested by lysing in freshly prepared Lysis Buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% , 4-octylphenol polyethoxylate) on ice for 30 minutes with constant gentle agitation. Lysed cells were centrifuged at 14,000 g for 5 minutes at 4°C to eliminate debris. The protein concentrations of cell lysates were measured by Bradford assay. To perform an assay, an array was incubated with 250 µL of each cell lysate containing 500 µg of protein. Array images were captured by scanning the blots with a Typhoon 9410 Imager and quantitation of array was performed with Image Quant TL V2005. Fold increased was determined by dividing signal from untreated over treated sample.

[0262] The results show that the majority of the 35 angiogenesis-related proteins detected were up-regulated in the cells treated with BoNT/A, compared to the untreated control (Table 16). Proteins that increased in expression were involved in promoting angiogenesis except for two proteins that are anti-angiogenic (endostatin and angiostatin). There was increased presence of GDNF, PDGF-AA, and FGF1 that promote cell proliferation, differentiation, cell growth and development. Proteins that promote or initiate angiogenesis were; Coagulation Factor III, EG-VEGF, Angiopoetin-1, Angiopoetin-2, and PD-ECGF. Expressions in proteins involved in glucose metabolism were; DPPIV, IGFBP-1, IGFBP-2, and IGFBP-3. Proteins that enhance cell-cell adhesion were also up-regulated; MIP-1, MMP-9, Endothelin-1, Platelet Factor 4 and TGF-β1. The most significant increase was observed for Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), which was almost 100-fold increased. The increase of these proteins in cell lysates may reflect their accumulation in the cytoplasm since exocytosis has been inhibited and the cells cannot release them to the media.

Table 16. Human Angiogenesis Array in DU145 Cell line

Analyte	Mean Pixels Density		Fold Increased	Function
	Untreated	Treated		
External Control	65451	68877	1.1	—
Internal Control	50052	59543	1.2	—
Coagulation Factor III/TF	12736	26726	2.1	Promotes angiogenesis
GDNF	156	428	2.7	Promotes survival and differentiation
MIP-1 alpha	153	535	3.5	Chemotaxis
CXCL 16	3465	2352	0.7	Cytokine
GM-CSF	5001	1457	0.3	Cytokine
Serpin E1	677	2214	3.3	Inhibit proteases
Activin A	552	1672	3.0	Regulate morphogenesis in prostate
DPPIV	3790	8923	2.4	Glucose metabolism
HB-EGF	8990	6717	0.7	Cell proliferation
MMP-9	2454	5050	2.1	Breakdown extracellular matrix
Serpin F1	743	882	1.2	Inhibit proteases
TIMP-1	95918	86280	0.9	Anti-angiogenic
Angiogenin	6022	5468	0.9	Promotes angiogenesis

Table 16. Human Angiogenesis Array in DU145 Cell line				
Analyte	Mean Pixels Density		Fold Increased	Function
	Untreated	Treated		
EG-VEGF	15	1368	88.3	Promotes angiogenesis
IGFBP-1	122	1147	9.4	Insulin growth factor protein
Pentraxin 3	119	732	6.2	Involved in complement-mediated clearance of apoptotic cells
TIMP-4	152	845	5.6	Matrix metalloproteinases inhibitor
Angiopoietin-1	137	807	5.9	Promotes angiogenesis
IGFBP-2	2379	8330	3.5	Insulin growth factor protein
PD-ECGF	942	12924	13.7	Promotes angiogenesis
Thrombospondin-1	2138	12359	5.8	Anti-angiogenic
Angiopoietin-2	129	1985	15.3	Antagonist of angiopoietin 1
Endostatin/Collagen XVIII	2388	6800	2.8	Anti-angiogenic
IGFBP-3	1145	11329	9.9	Insulin like promotes cell survivor
PDGF-AA	202	908	4.5	Regulates cell proliferation, cellular differentiation, cell growth, development
Angiostatin/Plasminogen	142	893	6.3	Anti-angiogenic
Endothelin-1	581	5828	10.0	Vascular homeostasis
uPA	30656	57108	1.9	Serine protease
Amphiregulin	33908	20736	0.6	Interacts with the EGF/TGF- α receptor to promote the growth
FGF1	1189	1875	1.6	Promotes proliferation & differentiation
IL-8	45837	19261	0.4	Angiogenic factor
FGF2	28018	23513	0.8	Promotes proliferation & differentiation
LAP/TGF- β 1	360	1914	5.3	Increases extracellular matrix production
Platelet Factor 4	456	819	1.8	Cytokine
VEGF	33513	31434	0.9	Affects permeability

[0263] Taken together, the experiments described in this Example show an overall decrease in angiogenic potential after treatment with botulinum toxin of TVEMP together with an observed increase in intracellular angiogenic proteins. This could be due to either activation of receptors for botulinum toxin or TVEMP that promotes angiogenesis and/or accumulation of vesicular proteins due to blockage of exocytosis after cleavage of SNARE proteins.

Example 4

Effects of Light Chain Delivery on Apoptosis

[0264] This example illustrates that treatment with a botulinum toxin or TVEMP will affect apoptosis to a degree sufficient to provide a therapeutic benefit in a cancer treatment.

[0265] The blockade of exocytosis resulting from a treatment with botulinum toxin or TVEMP based on LHN/A-G will likely result in decreased metabolic activity and decreased cell viability. As such, cancer

cells with inhibited exocytosis capability due to a toxin or TVEMP effect will have a reduced ability to survive. To test whether such a treatment causes decreased cancer cell viability, three different assays were performed: a cell viability and metabolism assay, a Caspase-3/8 activity assay, and a human apoptotic protein array assay.

[0266] To determine whether a botulinum toxin or TVEMP treatment could decrease cancer cell viability, a CELLTITER 96[®] AQueous One Solution Cell Proliferation Assay cell metabolic activity assay (Promega Corp., Madison, WI) was performed according to the manufacturer's instructions. This assay is a colorimetric assay containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] that is reduced by NADPH or NADH in metabolically active cells. The reduced MTS is a colored formazan product that can be measured at an absorbance of 490nm. An appropriate density of cells from the cell lines MCF-7, SiMa, PC-12, 266.6, RWPE-1, and N2a, were plated into the wells of 96-well tissue culture plates containing 100 μ L of an appropriate medium (Table 7), but without serum, and with or without 25 μ g/mL of GT1b (Alexis Biochemicals, San Diego, CA). Cells were plated and incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest (approximately 3 days). The media was aspirated from each well and the differentiated cells were treated by replacing with fresh media containing 0 (untreated sample), 0.3125 nM, 1.25 nM, and 20 nM of a BoNT/A. After 24 hrs treatment, the cells were washed by aspirating the media and rinsing each well with 100 μ L of 1 x PBS. After washing, 100 μ L of MTS solution was added to each well, incubated for 2 hours, and then the absorbance at 490nm recorded with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. A similar design can be employed to examine the effects of a TVEMP on cell viability.

[0267] The results show that a BoNT/A treatment decreased the metabolic activity in the cancerous cell lines tested (Table 17).

Table 17. Cell Metabolic Activity Assay				
Cell Line	BoNT/A Concentration			
	0 nM	0.3125 nM	1.25 nM	20 nM
MCF-7	1.60	1.45	1.41	1.30
SiMa	1.68	1.40	1.07	0.33
PC-12	1.68	1.66	1.45	1.15
266.6	1.10	1.05	1.02	0.82
RWPE-1	0.99	1.01	0.89	0.67
N2a	1.63	1.50	1.43	1.28

[0268] To further demonstrate that a botulinum toxin or TVEMP treatment could decrease cancer cell viability, a CELLTITER GLO[®] Luminescent Cell Viability Assay (Promega Corp., Madison, WI) was

performed according to the manufacturer's instructions. In this assay, cell viability is quantified on the bases of the presence of ATP, which signals the presence of metabolically active cells. A decreased in ATP content corresponds to less metabolically active cells. Cells from the cell lines LNCaP, J82, T24, and DU-145 were differentiated as described above. The media was aspirated from each well and the differentiated cells were treated by replacing with fresh media containing either 1) 0 (untreated sample), 25 nM, and 50 nM of a BoNT/A; or 2) 0 (untreated sample), 250 nM, and 500 nM of a Noci-LH_N/A TVEMP. After 24 hrs treatment, the cells were washed by aspirating the media and rinsing each well with 100 μ L of 1 x PBS. After washing, 100 μ L of CELLTITER GLO[®] reagent was added to each well. After ten minutes incubation at room temperature, the sample luminescence was measured using a SpectraMAX L luminescence reader (Molecular Devices, Sunnyvale, CA). Assays were performed in triplicate and cell viability was noted every day for four or five days.

[0269] The data shows that decreased viability was observed in cells from both a DU-145 prostate carcinoma cell line and a J82 bladder carcinoma cell line after BoNT/A treatments (Table 18) or Noci-LH_N/A TVEMP treatments (Table 19).

Table 18. Cell Viability Assay for BoNT/A

Time	BoNT/A Concentration							
	DU-145				J82			
	0 nM	25 nM	0 nM	50 nM	0 nM	25 nM	0 nM	50 nM
Day 1	3356	3291 (0.385)	404219	301228 (0.325)	3077	2853 (0.223)	543436	318900 (0.398)
Day 2	2360	2433 (0.433)	649139	394645 (0.174)	5211	4646 (0.016)	741025	493817 (0.129)
Day 4	ND	ND	1277552	809182 (0.058)	ND	ND	1242627	649797 (0.010)
Day 5	4823	2325 (0.0001)	ND	ND	7384	4262 (0.0001)	ND	ND
P value indicating significant difference relative to non-treated control is listed in parenthesis. ND, not determined								

Table 19. Cell Viability Assay for Noci-LH_N/A TVEMP

Time	Noci-LH _N /A TVEMP Concentration							
	DU-145				J82			
	0 nM	250 nM	0 nM	500 nM	0 nM	250 nM	0 nM	500 nM
Day 1	3356	3630 (0.087)	404219	408023 (0.959)	3077	3189 (0.223)	543436	406420 (0.103)
Day 2	2360	2379 (0.876)	649139	622596 (0.802)	5211	4639 (0.015)	741025	677236 (0.581)
Day 4			1277552	1030346 (0.171)			1242627	854124 (0.020)
Day 5	4823	3595 (0.0003)			7384	6349 (0.009)		
P value indicating significant difference relative to non-treated control is listed in parenthesis. ND, not determined								

[0270] To determine whether a botulinum toxin or TVEMP treatment decreased cancer cell viability by an apoptotic process, the activity of Caspase-3/8 was measured in cell treated with BoNT/A. Cells from the cell lines LNCaP, J82, and T24 were differentiated as described above. The media was aspirated from each well and the differentiated cells were treated by replacing with fresh media containing either 1) 0 (untreated sample), 0.5 nM, 5 nM, and 50 nM of a BoNT/A; or 2) 0 (untreated sample), 1.6 nM, 16 nM, and 166 nM of a Noci-LH_N/A TVEMP. After 24 hrs treatment, the cells were washed by aspirating the media and rinsing each well with 100 μ L of 1 x PBS. To measure cellular caspase 9 activity, 50 μ L of CASPASE-GLO[®] 9 (Promega, Corp., Madison, WI) reagent was added to the culture media of each well. After 30 minute incubation at 37 °C, the luminescence of each sample was measured using a Spectramax L luminometer (Molecular Devices, Sunnyvale, CA). T24 does not express SNAP-25 and should not be sensitive to treatment with BoNT/A or Noci-LH_N/A TVEMP.

[0271] The data shows that an effect on Caspase 3/8 activity was most prevalent in LNCaP cell after exposure to BoNT/A, indicating that LNCaP cell line viability decreases with BoNT/A treatment (Table 20). These data are supported by the cell viability assays measuring the number of live and dead cells in populations treated with BoNT/A (Table 18). Although cells from a J82 cell line did not show significant differences in Caspase 3/8 activity, this cell line did contain a higher amount of dead cells after BoNT/A or Noci-LH_N/A TVEMP treatments (Table 19). The reason for the observation of no caspase activity in J82 cells could be due to at least two possibilities: 1) the timing of BoNT/A treatment to detect Caspase 3/8 activity is different for J82 and LNCaP (e.g., Caspase 3/8 activation may have occurred earlier in J82 cells); or 2) the cell death pathway for J82 is independent of Caspase 3/8.

Table 20. Caspase 3/8 Activity Assay

Cell Line	BoNT/A Concentration				Noci-LH _N /A TVEMP			
	0 nM	0.5 nM	5 nM	50 nM	0 nM	1.6 nM	16 nM	166 nM
LNCaP	270	283	239	572	218	232	233	263
T24	656	612	634	646	637	602	623	617
J82	235	146	256	194	132	133	103	98

[0272] To test whether cell death of cells treated with a botulinum toxin or TVEMP was directed by a process independent of Caspase 3/8 pathway, cells were assayed for the presence of cleaved nuclear poly (ADP-ribose) polymerase (PARP). PARP is a 116 kDa nuclear poly (ADP-ribose) polymerase and appears to be involved in DNA repair in response to environmental stress. This protein can be cleaved by many ICE-like caspases in vitro and is one of the main cleavage targets of Caspase-3 in vivo. In human PARP, the cleavage occurs between Asp214 and Gly215, which separates the PARP amino-terminal DNA binding domain (24 kDa) from the carboxy-terminal catalytic domain (89 kDa). PARP helps cells to maintain their viability; cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. To determine whether changes in cell viability are due to cells undergoing apoptosis, cells from the cell lines DU-145 and J82 were differentiated as described above. The media

was aspirated from each well and the differentiated cells were treated by replacing with fresh media containing either 1) 0 (untreated sample) and 50 nM of a BoNT/A; or 2) 0 (untreated sample) and 500 nM of a Noci-LH_N/A TVEMP. After 48 hrs treatment, the cells were washed, harvested and Western blot analysis performed as described in Example 1, except an α -PARP antibodies were used as the primary antibody. Cells from both cell lines showed an increased of cleaved PARP after 2 days of Noci-LH_N/A TVEMP treatment. However, the presence of cleaved PARP was minimal in cells from both cell lines treated with a BoNT/A.

[0273] To conduct a human apoptosis protein array screen, cells from a DU-145 prostate cancer cell line were treated with a BoNT/A, harvested, and assayed as described above in Example 3. The results show that after treatment of cells from the DU-145 cell line with 50 nM BoNT/A for 24 hours, most of apoptosis-related proteins remained unchanged when compared to control. There were only 10 apoptotic-related proteins where expression decreased from 1.5-fold to 2.4-fold (Table 21). A decreased in expression was noted in three anti-apoptotic proteins (Livin, survivin, and BCL-x), two cell cycle related proteins (Claspin and P27), antioxidant related protein (PON2), chaperone protein (clusterin) and two pro-apoptotic related proteins (Bax and Cytochrome C).

Table 21. Human Apoptosis Array in DU-145 Cell line				
	Mean Pixel density			
Analyte	Untreated	Treated	Fold Decrease	Function
Livin	644.1	469.7	1.7	Anti-apoptotic
Cytochrome c	3423	1889	1.9	Pro-apoptotic
XIAP	10099	10045	1.0	Anti-apoptotic
HTRA2/Omi	7542	9368	0.8	IAP antagonist
Clusterin	1139	816	1.6	Chaperones misfolded proteins
TNF rRI/TNFRSF1A	2036	1467	1.5	Activates NFkB
HSP70	7058	9669	0.7	Stress response chaperone
Claspin	6630	3390	2.0	Cell cycle check point
Survivin	8717	3739	2.4	Anti-apoptotic
HSP60	945	855	1.2	Stress response chaperone
cIAP-2	2862	3156	0.9	Inhibitor of Apoptosis (IAP)
SMAC/Diablo	8379	7132	1.2	Promotes caspase activation by interaction with IAP proteins
HSP27	5716	5683	1.0	Stress response chaperone
cIAP-1	16916	15297	1.1	Inhibitor of Apoptosis (IAP)
Phospho-Rad17	1646	999	1.8	cell cycle check point
HO-2/HMOX2	8930	8934	1.0	Microsomal enzyme
Catalase	18742	18710	1.0	Prevent cell damage from oxidative stress
p53	19134	22007	0.9	Induces apoptosis
HO-1/HMOX1/HSP32	9878	11333	0.9	Microsomal enzyme
Cleaved Caspase-3	715	614	1.3	Downstream mediator of apoptotis
p53	8623	11225	0.8	Induces apoptosis

Table 21. Human Apoptosis Array in DU-145 Cell line				
	Mean Pixel density			
Analyte	Untreated	Treated	Fold Decrease	Function
HIF-1 alpha	6832	6703	1.0	Binds to hypoxia response elements
Pro-Caspase-3	36318	42668	0.9	Downstream mediator of apoptosis
p53	20019	24725	0.8	Induces apoptosis
Fas/TNFSF6	34978	35878	1.0	Induces apoptosis
Bcl-x	571	445	1.6	Anti-apoptotic
p27	1293	852	1.7	Cell cycle check point
FADD	9996	8647	1.2	Induces apoptosis
Bcl-2	967	1427	0.7	Anti-apoptotic
p21	1062	1029	1.1	Blocks cell cycle
TRAIL R2/DR5	25985	21477	1.2	Induces apoptosis
Bax	2097	1436	1.6	Apoptotic activator
PON2	2611	1784	1.5	Antioxidant enzyme
TRAIL R1	28443	20518	1.4	Induces apoptosis
Bad	5097	5932	0.9	Pro-apoptotic

[0274] Taken together, the experiments described in this Example show that treatment with a BoNT/A or TVEMP results in decreased metabolic activity and decreased cells viability. Events related to apoptosis were identified following light chain delivery into cancer cells, Caspase 3/8 activity was observed after treatment with BoNT/A in LNCaP cells as well as increased cleavage of PARP, the main substrate for Caspase 3 was observed after treatment with Noci-LH_N/A TVEMP in the DU-145 and J82 cells, showing that cells are pushed towards apoptosis after treatment with a BoNT/A or a TVEMP. Overall, the amounts of proteins involved with apoptosis in the cell lysates did not change after treatment with BoNT/A. Most of the pro-apoptotic and anti-apoptotic proteins exert their function by translocating from the cytoplasm to the mitochondria without changes in total protein amount. The small changes detected may be a short term response of the tumor cells to the inhibition of exocytosis and the interference with the input from the autocrine or paracrine loops that the cancer cell needs to survive. Eventually these cells will be pushed into apoptosis due to the lack of survival signals.

Example 5 Treatment of Cancer

[0275] The following examples are provided by way of describing specific embodiments without intending to limit the scope of the invention in any way.

[0276] A physician examines a 62 year old woman who complains of a lump in her left breast and diagnoses her with breast cancer. The woman is treated by local administration a composition comprising a TVEMP as disclosed herein in the vicinity of the affected area. The patient's condition is

monitored and after about 1-7 days after treatment, the physician notes that the growth of the malignant tumor has slowed down. At one and three month check-ups, the physician determines that the size of the tumor has become smaller. This reduction in tumor size indicates successful treatment with the composition comprising a TVEMP. In addition, a systemic administration of a composition comprising a TVEMP as disclosed herein could also be used to administer a disclosed TVEMP to treat the breast cancer.

[0277] A physician examines a 58 year old man who complains of difficulty in urinating and diagnoses him with prostate cancer. The man is treated systemically by intravenous administration a composition comprising a TVEMP as disclosed herein. The patient's condition is monitored and after about 1-7 days after treatment, the physician determines that the size of the prostate has become smaller. At one and three month check-ups, the physician determines that the size of the prostate has returned to its normal size and that serum PSA levels are within the normal range. This reduction in tumor size and/or reduces serum PSA levels indicates successful treatment with the composition comprising a TVEMP. In addition, a local administration of a composition comprising a TVEMP as disclosed herein could also be used to administer a disclosed TVEMP to treat the prostate cancer.

[0278] A physician examines a 67 year old man who complains of wheezing when he breathes and diagnoses him with lung cancer. The man is treated systemically by intravenous administration a composition comprising a TVEMP as disclosed herein. The patient's condition is monitored and after about 1-7 days after treatment, the physician notes that the growth of the malignant tumor has slowed down. At one and three month check-ups, the man indicates that his breathing has returned to normal and the physician determines that the size of the tumor has become smaller. The normal breathing and/or the reduction in tumor size indicate successful treatment with the composition comprising a TVEMP. In addition, systemic administration could also be used to administer a disclosed TVEMP to treat cancer. In addition, administration by inhalation could also be used to administer a disclosed TVEMP to treat the lung cancer.

[0279] A physician examines a 33 year old woman who complains of pelvic pain and diagnoses her with bladder cancer. The woman is treated by local administration a composition comprising a TVEMP as disclosed herein in the vicinity of the affected area. The patient's condition is monitored and after about 1-7 days after treatment, the physician notes that the growth of the malignant tumor has slowed down. At one and three month check-ups, the woman indicates that the pelvic pain has subsided and the physician determines that the size of the tumor has become smaller. The reduced pain and/or the reduction in tumor size indicate successful treatment with the composition comprising a TVEMP. In addition, a systemic administration of a composition comprising a TVEMP as disclosed herein could also be used to administer a disclosed TVEMP to treat the bladder cancer.

[0280] A physician examines a 73 year old woman who complains of abdominal pain and diagnoses her with colon cancer. The woman is treated by systemically by intravenous administration of a composition comprising a TVEMP as disclosed herein. The patient's condition is monitored and after about 1-7 days after treatment, and the physician notes that the growth of the malignant tumor has slowed down. At one and three month check-ups, the woman indicates that the abdominal pain has subsided and the physician determines that the size of the tumor has become smaller. The reduced pain and/or the reduction in tumor size indicate successful treatment with the composition comprising a TVEMP. In addition, a local administration of a composition comprising a TVEMP as disclosed herein could also be used to administer a disclosed TVEMP to treat the colon cancer.

[0281] A physician examines a 37 year old man who complains of headaches and dizziness and diagnoses him with a neuroblastoma. The man is treated by intracranial administration a composition comprising a TVEMP as disclosed herein in the vicinity of the affected area. The patient's condition is monitored and after about 1-7 days after treatment, the physician determines that the size of the malignant tumor has become smaller. At one and three month check-ups, the man indicates that he no longer suffers form headaches and dizziness and the physician determines that the neuroblastoma is gone. The disappearance of headache, dizziness and/or the neuroblastoma indicates successful treatment with the composition comprising a TVEMP.

[0282] A physician examines a 46 year old man who complains of painful skin moles and discoloration and diagnoses him with a melanoma. The man is treated by topical administration of a composition comprising a TVEMP as disclosed herein. The patient's condition is monitored and after about 1-7 days after treatment, the physician determines that the size of the skin moles has reduced slightly and the skin is not as discolored as before. At one and three month check-ups, the man indicates that he no longer suffers any pain and the physician determines that the skin moles and discoloration has disappeared. The reduced pain and/or the disappearance of the skin moles indicate successful treatment with the composition comprising a TVEMP. In addition, a systemic administration of a composition comprising a TVEMP as disclosed herein could also be used to administer a disclosed TVEMP to treat the bladder cancer.

[0283] In closing, it is to be understood that although aspects of the present specification have been described with reference to the various embodiments, one skilled in the art will readily appreciate that the specific examples disclosed are only illustrative of the principles of the subject matter disclosed herein. Therefore, it should be understood that the disclosed subject matter is in no way limited to a particular methodology, protocol, and/or reagent, etc., described herein. As such, various modifications or changes to or alternative configurations of the disclosed subject matter can be made in accordance with the teachings herein without departing from the spirit of the present specification. Lastly, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the

scope of the present invention, which is defined solely by the claims. Accordingly, the present invention is not limited to that precisely as shown and described.

[0284] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0285] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0286] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” As used herein, the term “about” means that the item, parameter or term so qualified encompasses a range of plus or minus ten percent above and below the value of the stated item, parameter or term. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0287] The terms “a,” “an,” “the” and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values

herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0288] Specific embodiments disclosed herein may be further limited in the claims using consisting of or consisting essentially of language. When used in the claims, whether as filed or added per amendment, the transition term “consisting of” excludes any element, step, or ingredient not specified in the claims. The transition term “consisting essentially of” limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the invention so claimed are inherently or expressly described and enabled herein.

[0289] All patents, patent publications, and other publications referenced and identified in the present specification are individually and expressly incorporated herein by reference in their entirety for the purpose of describing and disclosing, for example, the compositions and methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

CLAIMS

1. A method of treating cancer in a mammal, the method comprising the step of administering to the mammal in need thereof a therapeutically effective amount of a composition including a TVEMP comprising a targeting domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain, and an exogenous protease cleavage site, wherein administration of the composition reduces a symptom associated with cancer.
2. The method of Claim 1, wherein the TVEMP comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, the targeting domain, 2) the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the targeting domain, the Clostridial toxin translocation domain, 3) the targeting domain, the Clostridial toxin translocation domain, the exogenous protease cleavage site and the Clostridial toxin enzymatic domain, 4) the targeting domain, the Clostridial toxin enzymatic domain, the exogenous protease cleavage site, the Clostridial toxin translocation domain, 5) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the targeting domain, or 6) the Clostridial toxin translocation domain, the exogenous protease cleavage site, the targeting domain and the Clostridial toxin enzymatic domain.
3. The method of Claim 1, wherein the targeting domain is a neurotrophin peptide, a head activator peptide, a glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) peptide, a RF-amide related peptide, a neurohormone peptide, or a neuroregulatory cytokine peptide.
4. The method of Claim 3, wherein the neurotrophin peptide targeting domain is a nerve growth factor (NGF) peptide, a brain derived neurotrophic factor (BDNF) peptide, a neurotrophin-3 (NT-3) peptide, or a neurotrophin-4/5 (NT-4/5) peptide.
5. The method of Claim 4, wherein the neurotrophin peptide targeting domain comprises amino acids 139-257 of SEQ ID NO: 82, amino acids 133-240 or amino acids 129-247 of SEQ ID NO: 83, amino acids 144-249 or amino acids 19-257 of SEQ ID NO: 84, or amino acids 89-202 or amino acids 81-210 of SEQ ID NO: 85.
6. The method of Claim 4, wherein the cancer is a breast cancer, a neuroblastoma, a melanoma, a schwannoma, an insulinoma, a hepatoma, a medulloblastoma, a prolactinoma, a colon cancer, a leukemia, a chronic myelogenous leukemia, mast cell leukemia, an endometrial cancer, a prostate cancer, a thyroid cancer, a squamous-cell lung carcinoma, a lung cancer, an astrocytoma, a glioblastoma, a fibrosarcoma, or a pheochromocytoma.

7. The method of Claim 3, wherein the head activator peptide targeting domain is a head activator peptide.
8. The method of Claim 7, wherein the head activator peptide targeting domain comprises SEQ ID NO: 86.
9. The method of Claim 7, wherein the cancer is a breast cancer.
10. The method of Claim 3, wherein the glial cell line-derived neurotrophic factor family of ligands peptide targeting domain is a glial cell line-derived neurotrophic factor peptide, a Neurturin peptide, a Persephrin peptide, or an Artemin peptide.
11. The method of Claim 10, wherein the glial cell line-derived neurotrophic factor family of ligands peptide targeting domain comprises amino acids 118-211 of SEQ ID NO: 87, amino acids 107-196 or amino acids 96-197 of SEQ ID NO: 88, amino acids 66-155 of SEQ ID NO: 89, or amino acids 123-218 of SEQ ID NO: 90.
12. The method of Claim 10, wherein the cancer is a breast cancer, a pancreatic cancer, a thyroid cancer, a renal cancer, an adenocarcinoma, a melanoma, or a bladder cancer.
13. The method of Claim 3, wherein the RF-amide related peptide targeting domain a RF-amide related peptide-1, a RF-amide related peptide-2, a RF-amide related peptide-3, a neuropeptide AF, or a neuropeptide FF.
14. The method of Claim 13, wherein the RF-amide related peptide targeting domain comprises amino acids 81-92, amino acids 101-112, or amino acids 124-131 of SEQ ID NO: 91, amino acids 58-92 or amino acids 104-131 of SEQ ID NO: 92, amino acids 83-94 or amino acids 109-125 of SEQ ID NO: 93, or amino acids 65-77 or amino acids 92-111 of SEQ ID NO: 94.
15. The method of Claim 13, wherein the cancer is a breast cancer.
16. The method of Claim 3, wherein the neurohormone peptide targeting domain is a corticotropin-releasing hormone (CCRH), a parathyroid hormone (PTH), a parathyroid hormone-like hormone (PTH LH), a PHYH, a thyrotropin-releasing hormone (TRH), an urocortin-1 (UCN1), an urocortin-2 (UCN2), an urocortin-3 (UCN3), or an urotensin 2 (UTS2).
17. The method of Claim 16, wherein the neurohormone peptide targeting domain comprises amino acids 159-193 or amino acids 154-194 of SEQ ID NO: 95, amino acids 35-70 or amino acids 145-177 of SEQ ID NO: 96, or amino acids 39-206 of SEQ ID NO: 97.

18. The method of Claim 16, wherein the cancer is a breast cancer, an uterine cancer, a melanoma, a basal-cell skin carcinoma, a pituitary cancer, a neuroblastoma, a small cell lung carcinoma, an endometrial cancer, a pheochromocytoma, a squamous cell carcinoma, a colon cancer, an alveolar basal epithelial carcinoma, a testicular cancer, or an osteosarcoma.
19. The method of Claim 3, wherein the neuroregulatory cytokine peptide targeting domain is a ciliary neurotrophic factor peptide, a glycoporphin-A peptide, a leukemia inhibitory factor peptide, a cardiotrophin-1 peptide, a cardiotrophin-like cytokine peptide, a neuroleukin peptide, and an onostatin M peptide.
20. The method of Claim 19, wherein the neuroregulatory cytokine peptide targeting domain comprises SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 103.
21. The method Claim 19, wherein the cancer is a liver cancer, a stomach cancer, a lymphoma, a colon cancer, a gastric cancer.
22. The method of Claim 1, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.
23. The method of Claim 1, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.
24. The method of Claim 1, wherein the exogenous protease cleavage site is a plant papain cleavage site, an insect papain cleavage site, a crustacean papain cleavage site, an enterokinase cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a tobacco etch virus protease cleavage site, a Tobacco Vein Mottling Virus cleavage site, a subtilisin cleavage site, a hydroxylamine cleavage site, or a Caspase 3 cleavage site.

FIG. 1A.

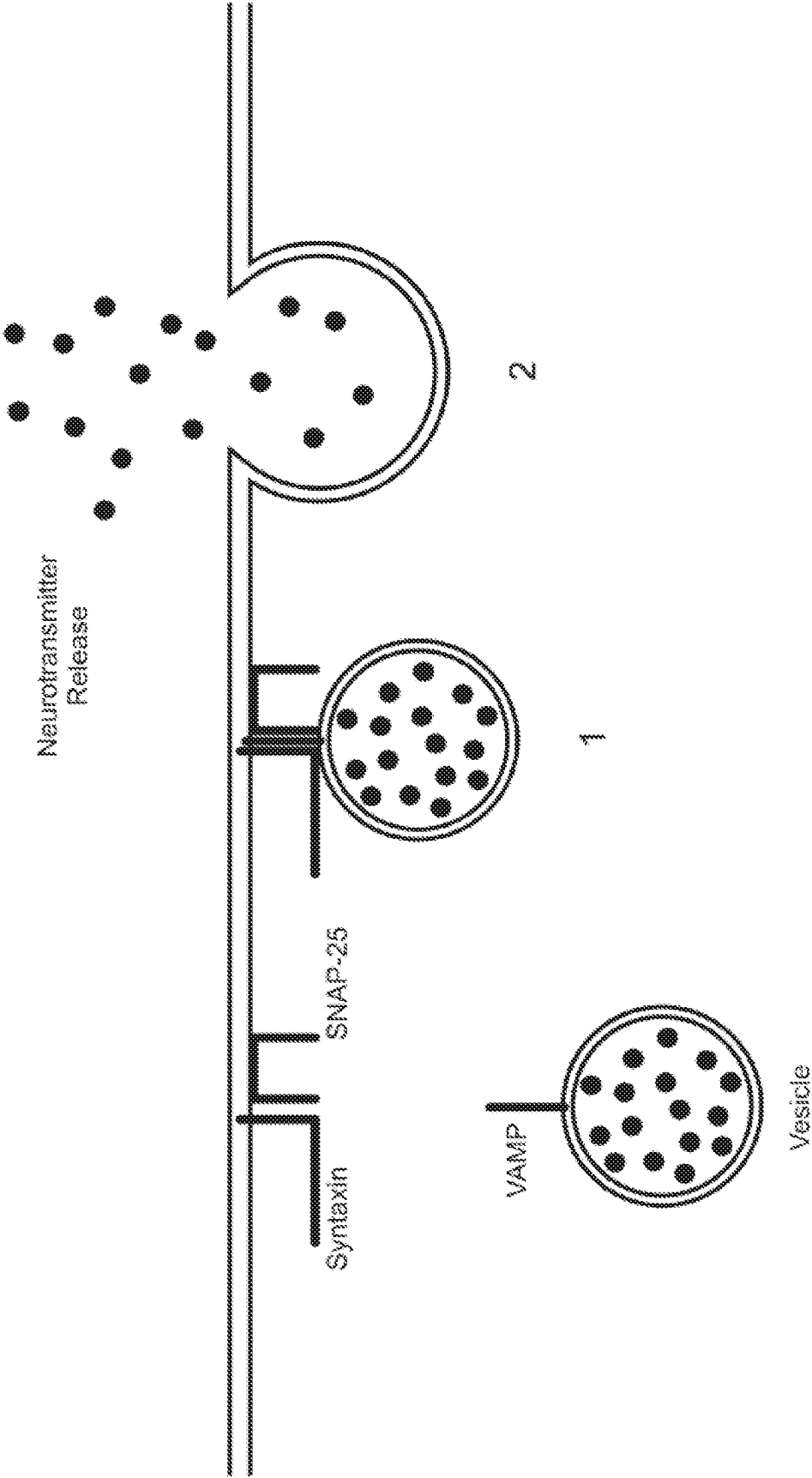


FIG. 1B.

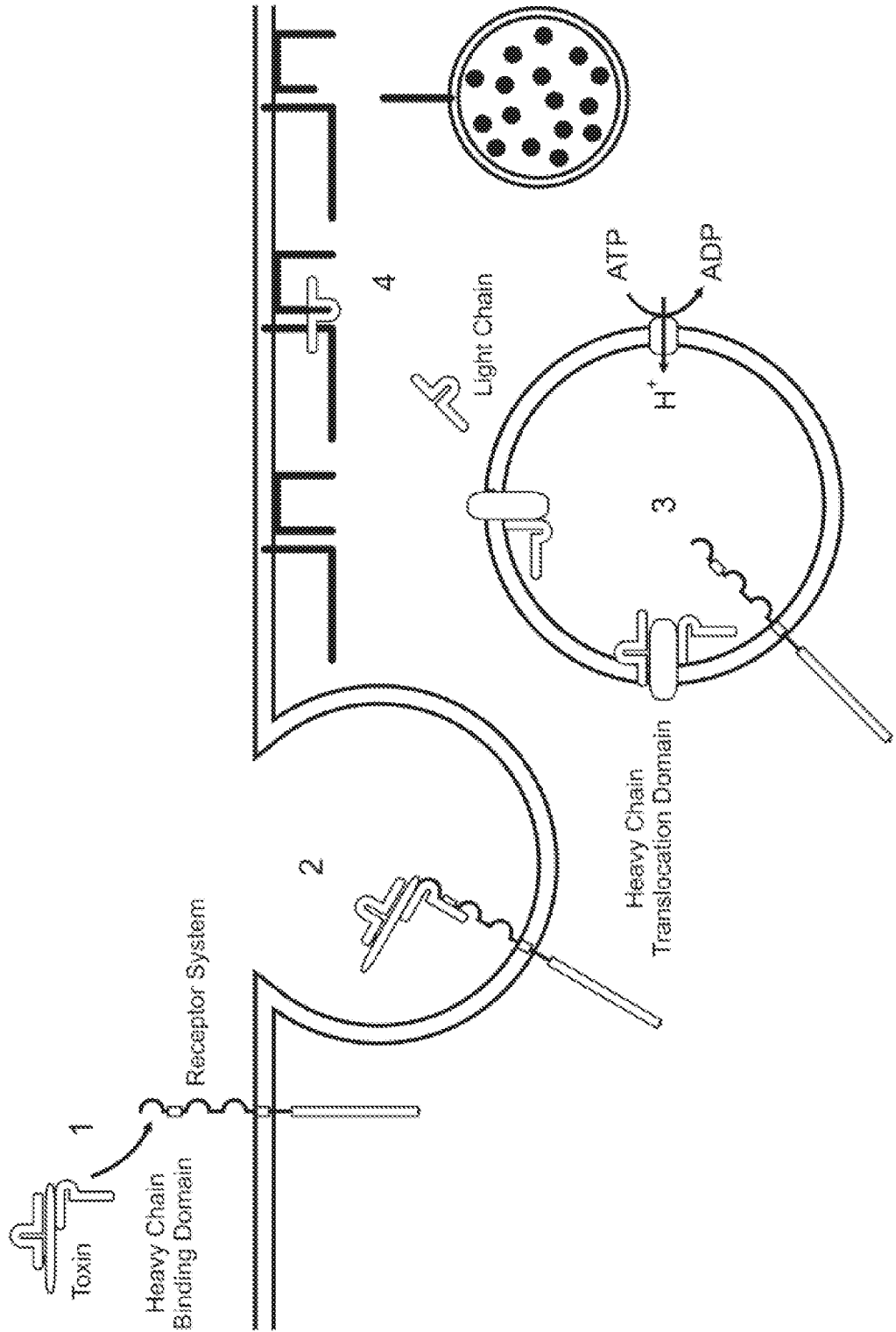


FIG. 2.

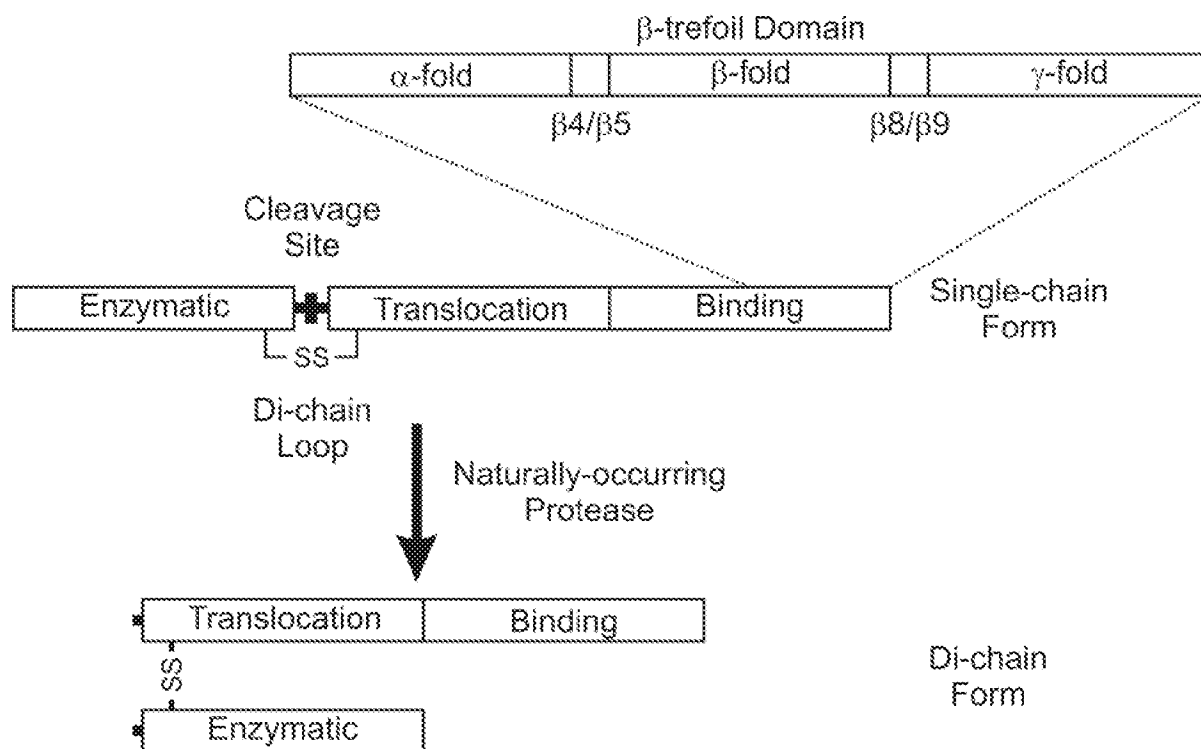


FIG. 3A.

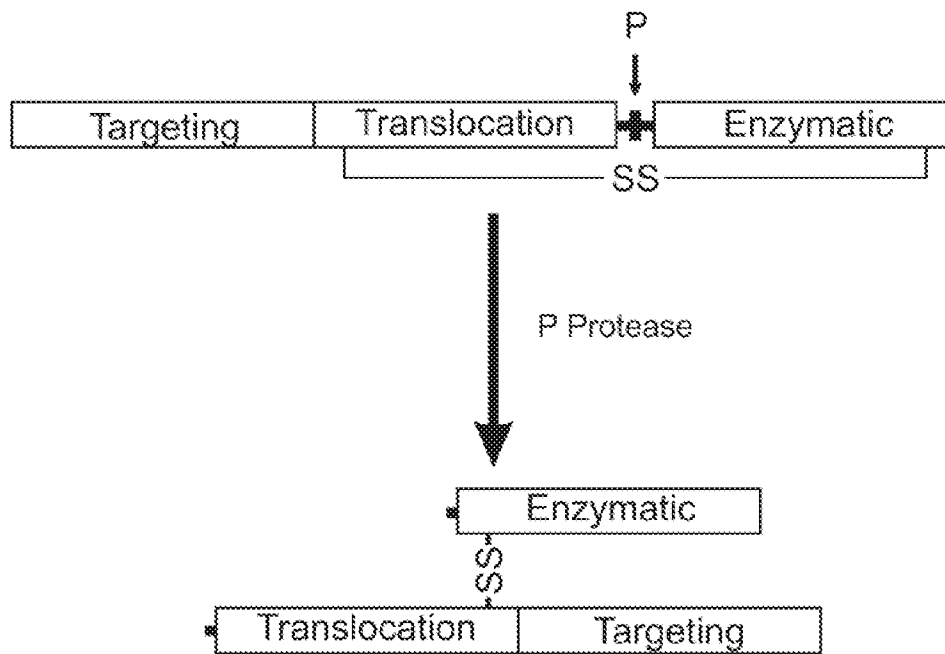


FIG. 3B.

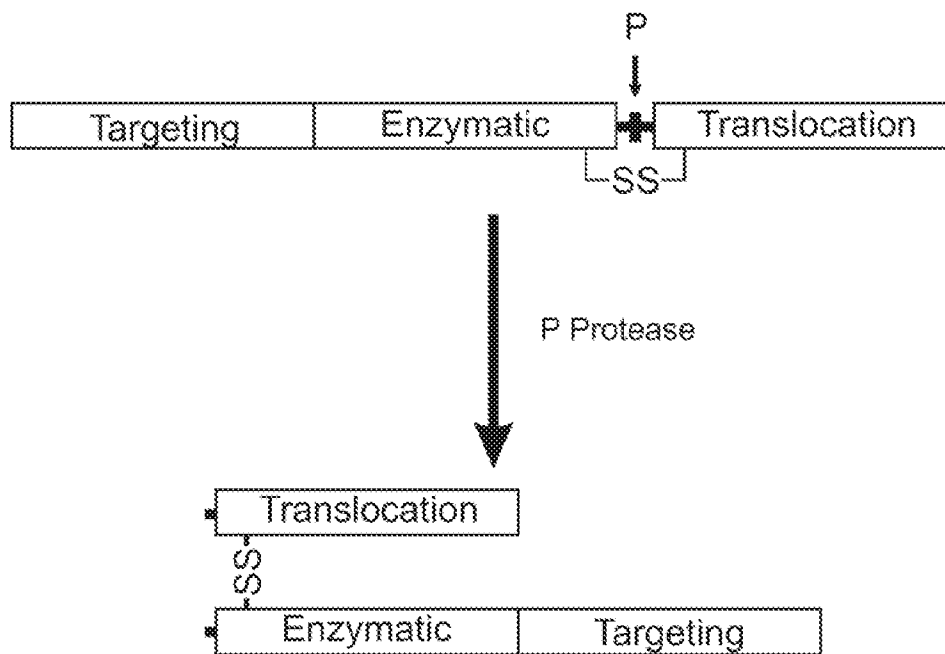


FIG. 4A.

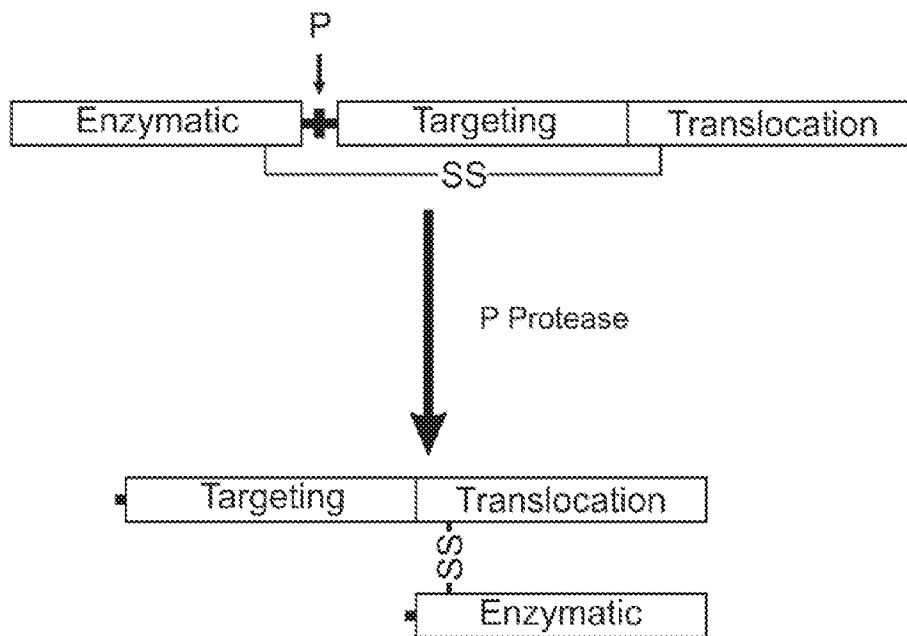


FIG. 4B.

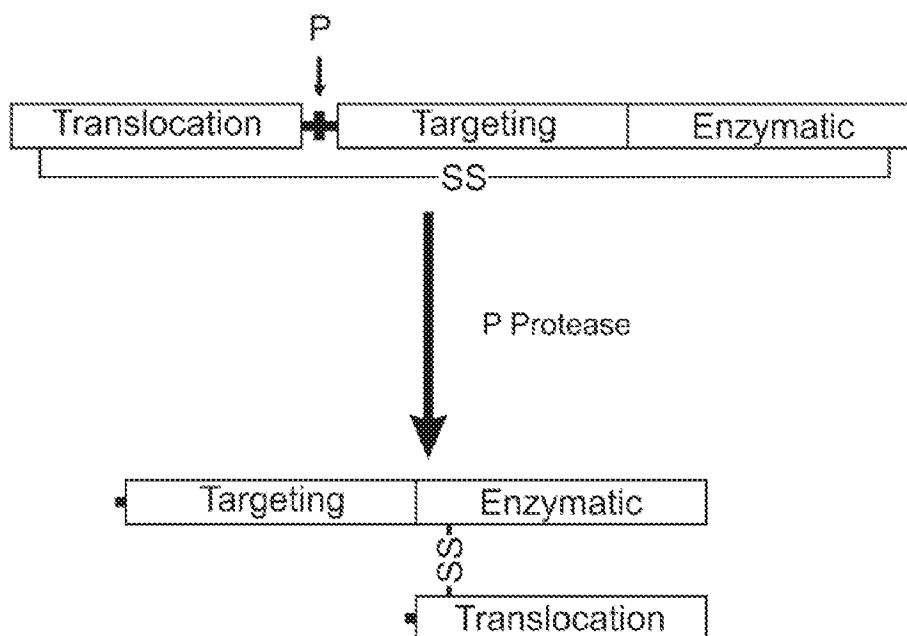


FIG. 4C.

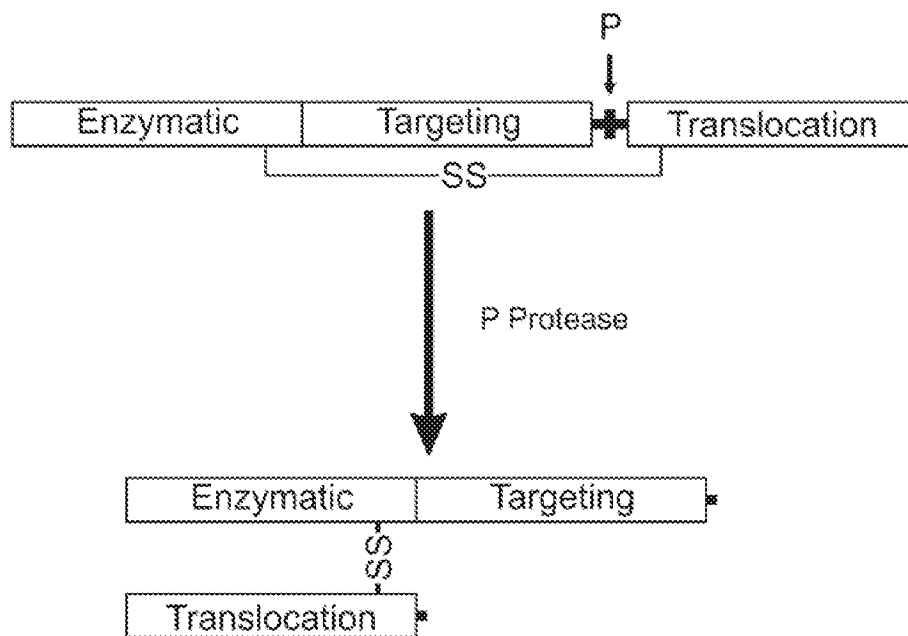


FIG. 4D.

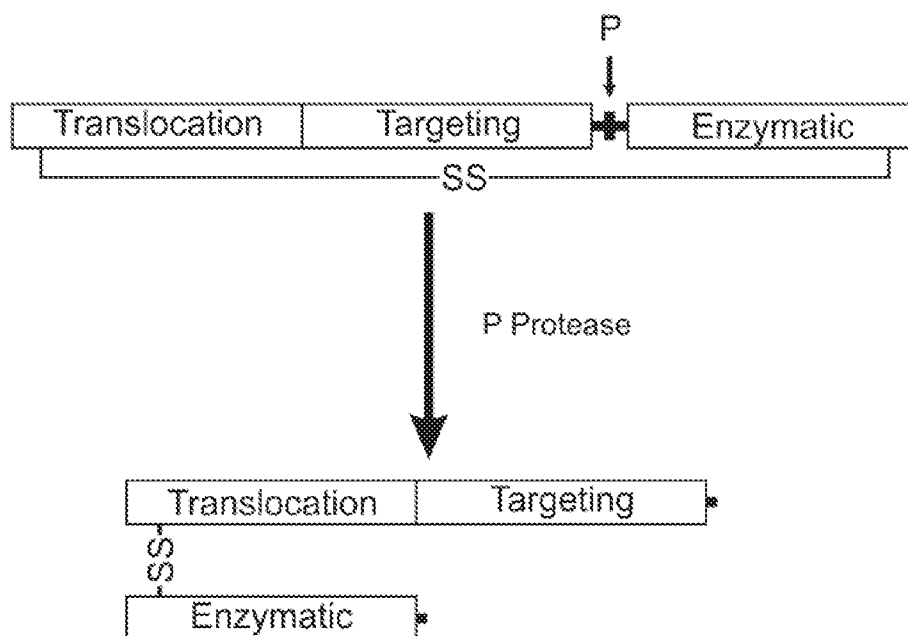


FIG. 5A.

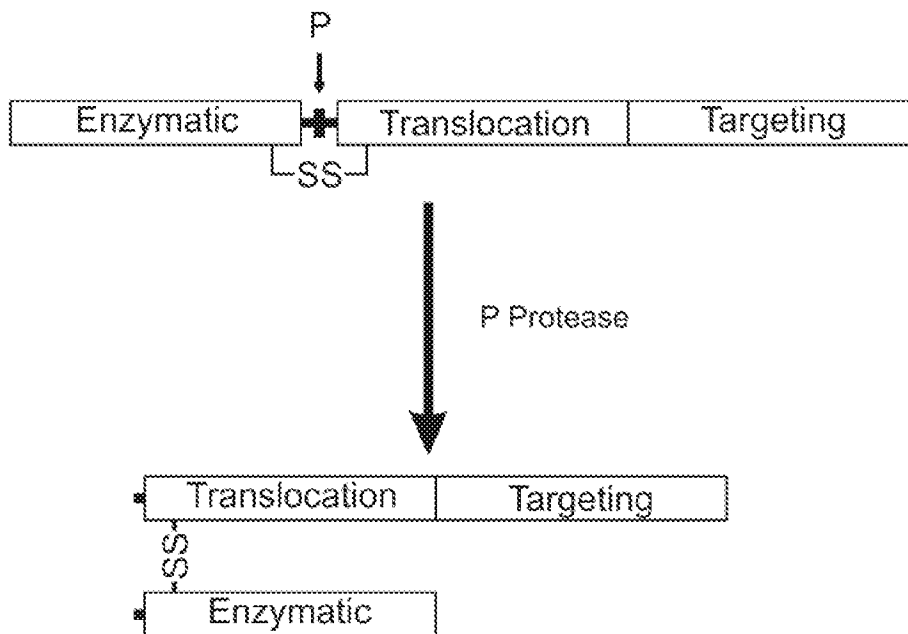


FIG. 5B.

