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(54) Title: BROAD-SPECTRUM PROTEOME EDITING WITH AN ENGINEERED BACTERIAL UBIQUITIN LIGASE MIMIC

(57) Abstract: The present application relates to an isolated chimeric molecule comprising a degradation domain comprising an E3 ubiquitin ligase (E3) motif and a targeting domain capable of specifically directing the degradation domain to a substrate, where the targeting domain is heterologous to the degradation domain. A linker couples the degradation domain to the targeting domain. Also disclosed are compositions as well as methods of treating a disease, substrate silencing, screening agents for therapeutic efficacy against a disease, and methods of screening for disease biomarkers.

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BROAD-SPECTRUM PROTEOME EDITING WITH AN ENGINEERED BACTERIAL UBIQUITIN LIGASE MIMIC

[0001] This application claims the priority benefit of U.S. Provisional Patent Application Serial No. 62/644,055 filed March 16, 2018, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present application relates generally to broad-spectrum proteome editing with an engineered bacterial ubiquitin ligase mimic.

BACKGROUND

[0003] Protein function has traditionally been investigated by disrupting the expression of a target gene encoding a protein and analyzing the resulting phenotypic consequences. Such loss-of-function experiments are now routinely performed using gene silencing and genome editing techniques such as antisense oligonucleotides (“ASOs”), RNA interference (“RNAi”), zinc finger nucleases (“ZFNs”), transcription activator-like effector nucleases (“TALENs”), and clustered, regularly interspaced, short palindromic repeat (“CRISPR”)-Cas systems. McManus et al., “Gene Silencing in Mammals by Small Interfering RNAs,” *Nat. Rev. Genet.* 3(10):737–47 (2002); Deleavey et al., “Designing Chemically Modified Oligonucleotides for Targeted Gene Silencing,” *Chemistry & Biology* 19(8):937–54 (2012); Boettcher et al., “Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR,” *Mol. Cell* 58(4):575–85 (2015); and Gaj et al., “TALEN, and CRISPR/Cas-Based Methods for Genome Engineering,” *Trends Biotechnol.* 31(7):397–405 (2013). These methods are widely used in basic research and hold promise for treating genetic disorders. Gaj et al., “TALEN, and CRISPR/Cas-Based Methods for Genome Engineering,” *Trends Biotechnol.* 31(7):397–405 (2013); Cox et al., “Therapeutic Genome Editing: Prospects and Challenges,” *Nat. Med.* 21(2):121–31 (2015); Soutschek et al., “Therapeutic Silencing of an Endogenous Gene by Systemic Administration of Modified siRNAs,” *Nature* 432(7014):173–78 (2004); Bumcrot et al., “RNAi Therapeutics: A Potential New Class of Pharmaceutical Drugs,” *Nat. Chem. Biol.* 2(12):711–19 (2006); and Wang et al., “Non-Viral Delivery of Genome-Editing Nucleases for Gene Therapy,” *Gene Ther.* 24(3):144–50 (2017). However, a number of challenges remain including: lack of temporal control, unpredictable off-target effects; the inability in the case of genome editing to remove essential genes and the irreversible nature of such knockouts, and the inability in the case of gene

silencing to decrease levels of proteins already present within cells, thereby leaving stable, long-lived proteins unaffected.

[0004] Proteome editing technology represents an orthogonal approach for studying protein function that operates at the post-translational level and has the potential to dissect complicated protein functions at higher resolution than methods targeting DNA or RNA and with post-translational precision. One of the most notable methods involves “inhibition-by-degradation” whereby the machinery of the cellular ubiquitin-proteasome pathway (“UPP”) is hijacked to specifically degrade proteins of interest. The canonical ubiquitination cascade requires the activities of three enzymes—ubiquitin activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3)—which act sequentially to tag proteins for degradation through the covalent attachment of a poly-ubiquitin chain to lysine residues in an energy-dependent manner.

[0005] E3s are the most heterogeneous class of enzymes in the UPP (there are >600 E3s in humans) and can be classified as HECT (homologous to E6AP C-terminus), RING (really interesting new gene), and RBR (RING-between-RING) depending on the presence of characteristic domains and on the mechanism of ubiquitin transfer to the substrate protein. Buetow et al., “Structural Insights into the Catalysis and Regulation of E3 Ubiquitin Ligases,” *Nat. Rev. Mol. Cell Biol.* 17(10):626–42 (2016). Because they mediate substrate specificity and generally exhibit remarkable plasticity, E3 ubiquitin ligases are the most frequently exploited component in proteome editing strategies described to date. For example, chemical knockdown has been achieved using small molecules called proteolysis targeting chimeras, or PROTACs (Neklesa et al., “Targeted Protein Degradation by PROTACs,” *Pharmacol. Ther.* 17:4138–144 (2017) and Deshaies, R. J., “Protein Degradation: Prime Time for PROTACs,” *Nat. Chem. Biol.* 11(9):634–35 (2015)), which are heterobifunctional molecules containing one ligand for an E3 ubiquitin ligase, another ligand for the protein to be degraded, and a linker connecting the two. These molecules bind to both the E3 and the target, promoting the formation of a ternary complex that triggers target polyubiquitination followed by its proteasomal degradation. A growing number of peptide- and small-molecule-based PROTACs have been reported that enable chemical knockout in cells and in mice. Schneekloth et al., “Chemical Genetic Control of Protein Levels: Selective *In Vivo* Targeted Degradation,” *J. Am. Chem. Soc.* 126(12):3748–54 (2004); Hines et al., “Posttranslational Protein Knockdown Coupled to Receptor Tyrosine Kinase Activation with PhosphoPROTACs,” *Proc. Natl. Acad. Sci. USA* 110(22):8942–47(2013); Schneekloth et al., “Targeted Intracellular Protein Degradation Induced by a Small Molecule: En Route to Chemical Proteomics,” *Bioorganic Med. Chem. Lett.* 18(22):5904–08 (2008);

Bondeson et al., “Catalytic *In Vivo* Protein Knockdown by Small-Molecule PROTACs,” *Nat. Chem. Biol.* 11(8):611–17 (2015); and Sakamoto et al., “Protacs: Chimeric Molecules that Target Proteins to the Skp1-Cullin-F Box Complex for Ubiquitination and Degradation,” *Proc. Natl. Acad. Sci. USA* 98(15):8554–59 (2001). An attractive feature of these compounds is their drug-
5 like properties including cell permeability; however, many peptide- and small-molecule-based PROTACs suffer from low potency—often requiring concentrations up to 25 μ M to induce sufficient degradation (Buckley et al., “Small-Molecule Control of Intracellular Protein Levels Through Modulation of the Ubiquitin Proteasome System,” *Angew Chem. Int. Ed. Engl.* 53(9):2312–30 (2014))—and the generation of custom PROTACs is limited by the relative lack
10 of available ligands for both E3 ubiquitin ligases and desired protein targets as well as the technical challenges associated with creating such ligands *de novo* (Osherovich, L., “Degradation From Within,” *Science-Business Exchange* 7:10–11 (2014)).

[0006] To circumvent these issues, protein-based chimeras have been developed where E3 ubiquitin ligases are genetically fused to a protein that binds the target of interest. Following
15 ectopic expression in cells, the engineered protein chimera recruits the E3 to the target protein, leading to its polyubiquitination and subsequent degradation by the proteasome. In the earliest example, protein knockout was achieved by creating an F-box chimera in which β -TrCP was fused to a peptide derived from the E7 protein encoded by human papillomavirus type 16 that is known to interact with retinoblastoma protein pRB (Zhou et al., “Harnessing the Ubiquitination
20 Machinery to Target the Degradation of Specific Cellular Proteins,” *Mol. Cell* 6(3):751–56 (2000) and Zhang et al., “Exploring the Functional Complexity of Cellular Proteins by Protein Knockout,” *Proc. Natl. Acad. Sci. USA* 100(24):14127–32 (2003)). Following ectopic expression, the engineered F-box recruited pRB to the Skp1-Cul1-F-box (“SCF”) machinery, a multi-protein E3 complex from the cullin-RING ligase (“CRL”) superfamily, for ubiquitination
25 and destruction. A handful of other studies have similarly leveraged natural protein-protein interactions, whereby fusion of one interacting protein to an E3 yielded a chimera that silenced the corresponding binding partner following expression in cells and mice. Hatakeyama et al., “Targeted Destruction of C-Myc by an Engineered Ubiquitin Ligase Suppresses Cell Transformation and Tumor Formation,” *Cancer Res.* 65(17):7874–79 (2005); Ma et al.,
30 “Targeted Degradation of KRAS by an Engineered Ubiquitin Ligase Suppresses Pancreatic Cancer Cell Growth *In Vitro* and *In Vivo*,” *Mol. Cancer Ther.* 12(3):286–94 (2013); and Kong et al., “Engineering a Single Ubiquitin Ligase for the Selective Degradation of all Activated ErbB Receptor Tyrosine Kinases,” *Oncogene* 33(8):986–95 (2014).

[0007] More recently, it was shown that a universal proteome editing technology could be extended beyond naturally occurring binding pairs. This approach involved fusing an E3 to a synthetic binding protein such as a single-chain antibody fragment (“scFv”), a designed ankyrin repeat protein (“DARPin”), or a fibronectin type III (“FN3”) monobody. Portnoff et al., “Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed With Unnatural Substrate Specificity for Targeted Protein Silencing,” *J. Biol. Chem.* 289(11):7844–55 (2014). These bifunctional chimeras, called “ubiquibodies” (“uAbs”), combined the flexible ubiquitin-tagging capacity of the human RING/U-box-type E3 CHIP (carboxyl terminus of Hsc70-interacting protein) with the engineerable affinity and specificity of synthetic binding proteins. The result is a customizable technology for efficiently directing otherwise stable proteins to the UPP for degradation independent of their biological function or interactions. Indeed, one of the greatest advantages of uAbs is their highly modular architecture – simply swapping synthetic binding proteins can generate a new uAb that specifically targets a different substrate protein (Caussinus et al., “Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody,” *Nat. Struct. Mol. Biol.* 19(1):117–21 (2011); Fulcher et al., “Targeting Endogenous Proteins For Degradation Through the Affinity-Directed Protein Missile System,” *Open Biol.* 7(5):170066 (2017); Fulcher et al., “An Affinity-Directed Protein Missile System for Targeted Proteolysis,” *Open Biol.* 6(10):160255 (2016); Shin et al., “Nanobody-Targeted E3-Ubiquitin Ligase Complex Degrades Nuclear Proteins,” *Sci. Rep.* 5:14269 (2015); and Kanner et al., “Sculpting Ion Channel Functional Expression with Engineered Ubiquitin Ligases,” *Elife* 6:e29744 (2017)) while swapping E3 domains can alter the kinetics or mechanism of ubiquitin transfer. Moreover, by incorporating synthetic binding proteins that recognize particular protein states (e.g., active vs. inactive conformation, mutant vs. wild-type, post-translationally modified), it becomes possible to deplete certain protein subpopulations while sparing others. Zhang et al., “Exploring the Functional Complexity of Cellular Proteins by Protein Knockout,” *Proc. Natl. Acad. Sci. USA* 100(24):14127–32 (2003) and Baltz et al., “Design and Functional Characterization of Synthetic E3 Ubiquitin Ligases for Targeted Protein Depletion,” *Curr. Prot. Chem. Biol.* 10(1):72–90 (2018). At present, however, the development of uAbs has centered around a relatively narrow set of mammalian E3s, most notably the “stand alone” E3 CHIP or members of the CRL superfamily of multi-protein E3 ligase complexes.

[0008] The present application unites expertise in protein-based chimeras whereby novel E3 ubiquitin ligase motifs are genetically fused to a protein that binds the target of interest to address the above challenges and overcome these and other deficiencies in the art.

SUMMARY

[0009] A first aspect of the present application relates to an isolated chimeric molecule. The isolated chimeric molecule comprises a degradation domain comprising an E3 ubiquitin ligase (E3) motif; a targeting domain capable of specifically directing the degradation domain to a substrate, wherein the targeting domain is heterologous to the degradation domain; and a linker coupling the degradation domain to the targeting domain.

[0010] A second aspect of the present application relates to a method of forming a ribonucleoprotein. The method includes providing a mRNA encoding the isolated chimeric molecule described herein; providing one or more polyadenosine binding proteins ("PABP"); and assembling a ribonucleoprotein complex from the mRNA and the one or more PABPs.

[0011] A third aspect of the present application relates to a composition comprising the chimeric molecule described herein and a pharmaceutically-acceptable carrier.

[0012] A fourth aspect of the present application relates to a method of treating a disease. The method includes selecting a subject having a disease and administering the composition described herein to the subject to give the subject an increased expression level of the substrate compared to a subject not afflicted with the disease.

[0013] A fifth aspect of the present application relates to a method for substrate silencing. The method includes selecting a substrate to be silenced; providing the chimeric molecule described herein; and contacting the substrate with the chimeric molecule under conditions effective to permit the formation of a substrate-molecule complex, wherein the complex mediates the degradation of the substrate to be silenced.

[0014] A sixth aspect of the present application relates to a method of screening agents for therapeutic efficacy against a disease. The method includes providing a biomolecule whose presence mediates a disease state; providing a test agent comprising (i) a degradation domain comprising an E3 ubiquitin ligase (E3) motif, (ii) a targeting domain capable of specifically directing the degradation domain to the biomolecule, wherein the targeting domain is heterologous to the degradation domain, and (iii) a linker coupling the degradation domain to the targeting domain; contacting the biomolecule with the test agent under conditions effective for the test agent to facilitate degradation of the biomolecule; determining the level of the biomolecule as a result of the contacting; and identifying the test agent which, based on the determining, decreases the level of the biomolecule as being a candidate for therapeutic efficacy against the disease.

[0015] A seventh aspect of the present application relates to a method of screening for disease biomarkers. The method includes providing a sample of diseased cells expressing one or

more ligands; providing a plurality of chimeric molecules comprising (i) a degradation domain comprising an E3 ubiquitin ligase (E3) motif, (ii) a targeting domain capable of specifically directing the degradation domain to the one or more ligands, wherein the targeting domain is heterologous to the degradation domain, and (iii) a linker coupling the degradation domain to the targeting domain; contacting the sample with the plurality of chimeric molecules under conditions effective for the diseased cells to fail to proliferate in the absence of the chimeric molecule; determining which of the chimeric molecules permit the diseased cells to proliferate; and identifying, as biomarkers for the disease, based on the determining the ligands which bind to the chimeric molecules and permit diseased cells to proliferate.

[0016] Manipulation of the ubiquitin-proteasome pathway to achieve targeted silencing of cellular proteins has emerged as a reliable and customizable strategy for remodeling the mammalian proteome. One such approach involves engineering bifunctional proteins called ubiquibodies that are comprised of a synthetic binding protein fused to an E3 ubiquitin ligase, thus enabling post-translational ubiquitination and degradation of a target protein independent of its function. Here, a panel of new ubiquibodies was designed based on E3 ubiquitin ligase mimics from bacterial pathogens that are capable of effectively interfacing with the mammalian proteasomal degradation machinery for selective removal of proteins of interest. One of these, the *Shigella flexneri* effector protein IpaH9.8 fused to a fibronectin type III (FN3) monobody that specifically recognizes green fluorescent protein (GFP), was observed to potentially eliminate GFP and its spectral derivatives as well as 15 different FP-tagged mammalian proteins that varied in size (27-179 kDa) and subcellular localization (cytoplasm, nucleus, membrane-associated, and transmembrane). To demonstrate therapeutically-relevant delivery of ubiquibodies, a bioinspired molecular assembly method was leveraged whereby synthetic mRNA encoding the GFP-specific ubiquibody was co-assembled with poly A binding proteins and packaged into nanosized complexes using biocompatible, structurally defined polypeptides bearing cationic amine side groups. The resulting nanoplexes delivered ubiquibody mRNA in a manner that caused efficient target depletion in cultured mammalian cells stably expressing GFP as well as in transgenic mice expressing GFP ubiquitously. Overall, the results presented here suggest that IpaH9.8-based ubiquibodies are a highly modular proteome editing technology with the potential for pharmacologically modulating disease-causing proteins.

[0017] The present application thus relates to chimeric molecules, compositions, treatments, pharmaceutical compositions, protein silencing techniques, the elucidation of therapeutic agents, and target screening technologies based on a novel class of chimeric molecules. Such chimeras, termed “ubiquibodies” herein, import the ligase function of an E3

ubiquitin enzyme to generate a molecule possessing target specificity. Such engineered chimeras facilitate the redirection and proteolytic degradation of specific substrate targets, which may not otherwise be bound for the proteasome.

[0018] In this respect, the targeted elimination of such specific substrates, *e.g.*,

5 intracellular proteins, ascribes a broad range of scientific and clinical indications to the chimeric molecules, compositions, treatments, pharmaceutical compositions, protein silencing techniques, elucidation of therapeutic agents, and screening technologies provided herein. The present application therefore imparts a variety of valuable tools for employing and developing specific prognostic and therapeutic applications based on the proteolytic degradation of aberrantly
10 expressed genes *via* ubiquitination.

[0019] Here, the range of E3s that can be functionally reprogrammed as bifunctional uAb chimeras was sought to be broadened. However, in a notable departure from previous efforts involving mammalian E3s, the focus was instead on a set of effector proteins from microbial pathogens that mimic host E3 ubiquitin ligases and hijack the UPP machinery to dampen the
15 innate immune response during infection. Maculins et al., “Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of Invasion. *Cell Res.* 26(4):499–510 (2016) and Lin et al., “Exploitation of the Host Cell Ubiquitin Machinery by Microbial Effector Proteins,” *J. Cell Sci.* 130(12):1985–96 (2017), which are hereby incorporated by reference in their entirety. The intrinsic plasticity of these enzymes led us to hypothesize that bacterial E3s could be
20 manipulated for targeted proteolysis just like their mammalian counterparts. Indeed, robust target silencing was achieved with a uAb comprised of the *Shigella flexneri* E3 ligase IpaH9.8, which exhibits similarities to eukaryotic HECT-type E3s but is classified as a novel E3 ligase (“NEL”) due to the absence of sequence and structural homology with any eukaryotic E3s. Maculins et al., “Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of Invasion. *Cell*
25 *Res.* 26(4):499–510 (2016); Lin et al., “Exploitation of the Host Cell Ubiquitin Machinery by Microbial Effector Proteins,” *J. Cell Sci.* 130(12):1985–96 (2017); Zhu et al., “Structure of a *Shigella* Effector Reveals a New Class of Ubiquitin Ligases,” *Nat. Struct. Mol. Biol.* 15(12):1302–08 (2008); Singer et al., “A Pathogen Type III Effector With a Novel E3 Ubiquitin
Ligase Architecture,” *PLoS Pathogens* 9(1):e1003121 (2013); and Rohde et al., “Type III
30 Secretion Effectors of the IpaH Family are E3 Ubiquitin Ligases,” *Cell Host Microbe* 1(1):77–83 (2007), which are hereby incorporated by reference in their entirety. When the C-terminal catalytic NEL domain of IpaH9.8 was fused to the GFP-specific FN3 monobody GS2 that specifically recognizes green fluorescent protein (“GFP”), potent degradation of EGFP following both transient and stable expression in cultured mammalian cells was observed. Moreover, the

GS2-IpaH9.8 chimera was also able to accelerate the degradation of spectral derivatives of EGFP including Emerald, Venus and Cerulean as well as 15 different FP-tagged mammalian proteins that ranged in size from 27 up to 179 kDa and localized in different subcellular compartments including the cytoplasm, nucleus, and cell membrane. For two of these targets, SHP2 and Ras, efficient silencing was also achieved when IpaH9.8 was fused to SHP2- or Ras-specific FN3 domains, highlighting the ease with which IpaH-based uAbs can be reconfigured.

[0020] As was noted previously, a major obstacle for the therapeutic development of uAbs is intracellular delivery. Osherovich, L., "Degradation From Within," *Science-Business Exchange* 7:10–11 (2014), which is hereby incorporated by reference in its entirety. Unlike smaller PROTACs, which can be designed for cell-permeability (Buckley et al., "Small-Molecule Control of Intracellular Protein Levels Through Modulation of the Ubiquitin Proteasome System," *Angew Chem. Int. Ed. Engl.* 53(9):2312–30 (2014), which is hereby incorporated by reference in its entirety), uAbs are relatively bulky proteins that do not effectively penetrate the cell membrane. To remedy this issue, a bioinspired mRNA delivery strategy was implemented whereby mRNA encoding GS2-IpaH with an additional 3'-terminal polyadenosine ("poly A") tail was stoichiometrically complexed with poly A binding proteins ("PABPs"), which served to improve mRNA stability and also stimulate mRNA translation in eukaryotic cells (Li et al., "Polyamine-Mediated Stoichiometric Assembly of Ribonucleoproteins for Enhanced mRNA Delivery," *Angew Chem. Int. Ed. Engl.* 56(44):13709–12 (2017), which is hereby incorporated by reference in its entirety). The resulting ribonucleoproteins ("RNPs") were stabilized with cationic polypeptides to protect the mRNA from degradation, enable its uptake by cells, and facilitate its endosomal escape. Importantly, these co-assembled nanoplexes delivered GS2-IpaH9.8 mRNA in a manner that caused efficient GFP silencing after introduction to cultured mammalian cells stably expressing GFP and after administration to transgenic mice expressing GFP ubiquitously. Collectively, the results described herein demonstrate that uAb-mediated proteome editing is an effective strategy for targeted degradation of proteins in cells and mice, thereby setting the stage for uAbs as tools for drug discovery and as therapeutic candidates with potential to pharmacologically hit so-called "undruggable" targets.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIGs. 1A–1C depict the engineering of bacterial E3 ligase IpaH9.8 as a GFP-specific ubiquibody. FIG. 1A shows linear representation of IpaH9.8, IpaH9.8 Δ LRR, and GS2-IpaH9.8. Numbers refer to amino acid positions from N terminus ("N") to C terminus ("C"). The proteins are aligned vertically with the LRR and NEL domains of IpaH9.8. IpaH9.8 Δ LRR

is a truncated version of IpaH9.8 lacking the LRR domain. FIG. 1B shows flow cytometric analysis of EGFP fluorescence activity in HEK293T cells transfected with plasmid pcDNA3-EGFP alone or co-transfected with pcDNA3-EGFP and a plasmid encoding one of the bacterial E3-based uAbs as indicated. FIG. 1C is the same as in 1C but with mammalian E3-based uAbs as indicated. Data are biological triplicates of the geometric mean fluorescence intensity (“MFI”) normalized to MFI measured for HEK283T cells expressing EGFP alone. Error bars represent standard deviation (“SD”) of the mean.

[0022] FIGs. 2A-2D illustrate that the catalytic domain of IpaH9.8 is essential for ubiquibody function. FIG. 2A shows representative fluorescence histograms obtained by flow cytometric analysis of EGFP fluorescence activity in HEK293T cells transfected with pcDNA3-EGFP alone or co-transfected with pcDNA3-EGFP and a plasmid encoding one of the following: GS2-IpaH9.8^{C337A}, AS15-IpaH9.8, or GS2-IpaH9.8. FIG. 2B shows flow cytometric quantification of EGFP fluorescence activity for cells described in FIG. 2A where data are biological triplicates of the geometric MFI normalized to MFI measured for HEK283T cells expressing EGFP alone. Error bars represent standard deviation (“SD”) of the mean. FIG. 2C depicts a western blot analysis of HEK293T cell lysates transfected as in FIGs. 2A and 2B. Blots were probed with antibodies specific for GFP, 6x-His (that detected tag on each uAb), and GAPDH as indicated. An equivalent amount of total protein was loaded in each lane as confirmed by immunoblotting with anti-GAPDH. Molecular weight (MW) markers are indicated on left. FIG. 2D depicts flow cytometric quantification of EGFP fluorescence activity for HEK293T cells co-transfected with pcDNA3-EGFP and a plasmid encoding GS2 fused to one of the IpaH9.8 homologs as indicated. Data are biological triplicates of the geometric MFI normalized to MFI measured for HEK283T cells expressing EGFP alone. Error bars represent standard deviation (“SD”) of the mean.

[0023] FIGs. 3A–3B show that GS2-IpaH9.8 degrades structurally diverse fluorescent protein fusions. FIG. 3A depicts flow cytometric quantification of fluorescence activity in HEK293T cells transfected with a plasmid encoding the indicated FP fusion alone (dark grey) or co-transfected with the FP fusion plasmid and either pcDNA3-GS2-IpaH9.8^{C337A} (white) or pcDNA3-GS2-IpaH9.8 (light grey). Data are biological triplicates of the geometric MFI normalized to MFI measured for HEK283T cells expressing the corresponding FP fusion protein alone. Error bars represent standard deviation (“SD”) of the mean. FIG. 3B shows confocal microscopy images corresponding to select FP targets expressed in HEK293T cells transfected/co-transfected as described in FIG. 3A. Hoescht stain (blue) denotes cell nuclei and

EGFP signal (green) denotes fluorescent proteins. For the EGFR-mEmerald fusion, immunostaining with an EGFR-specific antibody (red) is also depicted.

[0024] FIGs. 4A–4C depict IpaH9.8 ubiquibodies directed against disease-relevant targets. FIG. 4A illustrates flow cytometric quantification of EGFP fluorescence activity in HEK293T cells transfected with pcDNA3-SHP2-EGFP alone or co-transfected with pcDNA3-SHP2-EGFP and a plasmid encoding one of the following: GS2-IpaH9.8, GS2-IpaH9.8^{C337A}, NSa5-IpaH9.8, or NSa5-IpaH9.8^{C337A}. FIG. 4B is the same as in FIG. 4A but with pcDNA3-EGFP-HRas^{G12V} alone or co-transfected with pcDNA3-EGFP-HRas^{G12V} and a plasmid encoding one of the following: GS2-IpaH9.8, GS2-IpaH9.8^{C337A}, RasInII-IpaH9.8, or RasInII-IpaH9.8^{C337A}. Data are biological triplicates of the geometric MFI normalized to MFI measured for HEK293T cells expressing EGFP alone. Error bars represent standard deviation (“SD”) of the mean. FIG. 4C shows flow cytometric quantification of EGFP fluorescence activity in HEK293T cells co-transfected with pcDNA3-RasInII-IpaH9.8 and one of the following: pcDNA3-EGFP-KRas, pcDNA3-EGFP-KRas^{G12C}, pcDNA3-EGFP-KRas^{G12D}, or pcDNA3-EGFP-KRas^{G12V}. MFI ratio was determined by normalizing geometric MFI for cells expressing KRas mutant to geometric MFI for cells expressing wild-type (wt) KRas. Data are the average of biological triplicates and error bars represent standard deviation (SD) of the mean.

[0025] FIGs. 5A–5D depict proteome editing in mice via nanoplex delivery of ubiquibody mRNA. FIG. 5A is a schematic of polyamine (TEP (N4))-mediated stoichiometric assembly of mRNA/PABP ribonucleoproteins for enhanced mRNA delivery. Following internalization in cells (grey circle), nanoplex disassembly results in the release of mRNA/PABP that is either degraded or translated to produce uAb proteins. FIG. 5B depicts flow cytometric quantification of EGFP fluorescence activity in HEK293Td2EGFP cells incubated with: mRNA encoding GS2-IpaH9.8, GS2-IpaH9.8^{C337A}, or AS15-IpaH9.8; or with nanoplexes comprised of the same mRNAs formulated with PABP and TEP (N4) polyamine (mRNA:PABP weight ratio = 1:5). Measurements were taken at 24, 48, and 72 h post-delivery. Data are expressed as the mean ± S.D. of biological triplicates. FIG. 5C shows epifluorescence imaging of UBC-GFP mice at 0 h (top) and 24 h (bottom) after ear injection of nanoplexes containing mRNA encoding GS2-IpaH9.8 (solid white circle), GS2-IpaH9.8^{C337A} (dashed white circle, top), or AS15-IpaH9.8 (dashed white circle, bottom). Numbers on the heat bar represent radiant efficiency (p/sec/cm²/sr)/(μW/cm²). FIG. 5D depicts quantification of GFP fluorescence in the ears of Ubi-GFP mice in FIG. 5C. Data are reported as the mean radiant efficiency for each individual ear region (black circle) and the mean radiant efficiency (red bar) of each sample group (*n* = 6 for

GS2-IpaH9.8, $n = 3$ for GS2-IpaH9.8^{C337A}, and $n = 3$ for AS15-IpaH9.8). p values were determined by paired sample t-test.

[0026] FIGs. 6A–6B depict GFP silencing by uAbs harboring bacterial and mammalian E3 ubiquitin ligase domains. Representative fluorescence histograms obtained by flow cytometric analysis of EGFP fluorescence activity in HEK293T cells transfected with pcDNA3-EGFP alone or co-transfected with pcDNA3-EGFP and a plasmid encoding a uAb comprised of GS2 fused to one of the (as shown in FIG. 6A) bacterial or (as shown in FIG. 6B) mammalian E3 ubiquitin ligases as indicated. Values for geometric mean fluorescence intensity (“MFI”) are shown.

[0027] FIGs. 7A–7B show characterization of GS2-IpaH9.8 binding activity and expression. In FIG. 7A, binding activity of GS2-IpaH9.8 is shown compared to GS2 alone, IpaH9.8 lacking the LRR domain (“IpaH9.8 LRR”), or catalytically inactive GS2-IpaH9.8^{C337A} as indicated. Activity was measured by ELISA using GFP as immobilized antigen and 15 mg/mL of each protein applied per well. Detection was performed using anti-FLAG antibody conjugated to horseradish peroxidase (HRP). The quenched plate was read at 450 nm (Abs₄₅₀). Data is the average of three biological replicates and error bars are the standard deviation (“SD”) of the mean. FIG. 7B shows confocal microscopy images corresponding to HEK293T cells transfected with plasmid DNA encoding EGFP or co-transfected with plasmid DNA encoding EGFP and either pcDNA3-GS2-IpaH9.8C337A or pcDNA3-GS2-IpaH9.8 as indicated. Non-transfected HEK293T control cells are also depicted. Hoescht stain (blue) denotes cell nuclei, EGFP signal (green) denotes FP target expression, and -His signal (red) corresponds to immunolabeling of expressed uAb in permeabilized cells.

[0028] FIGs. 8A–8B depict uAb-mediated silencing of FP variants and additional FP fusion protein targets. FIG. 8A shows flow cytometric quantification of fluorescence activity in HEK293T cells co-transfected with plasmids encoding the FP variant and either pcDNA3-GS2-IpaH9.8C337A (white) or pcDNA3-GS2-IpaH9.8 (grey) as indicated. mCherry served as negative control. Data are biological triplicates of the geometric MFI normalized to MFI measured for HEK283T cells expressing the corresponding FP alone. Error bars represent standard deviation (SD) of the mean. FIG. 8B shows flow cytometric quantification of fluorescence activity in HEK293T cells transfected with a plasmid encoding the indicated FP fusion alone (dark grey) or co-transfected with the FP fusion plasmid and either pcDNA3-GS2-IpaH9.8C337A (white) or pcDNA3-GS2-IpaH9.8 (light grey). Data are biological triplicates of the geometric MFI normalized to MFI measured for HEK283T cells expressing the corresponding FP alone. Error bars represent standard deviation (SD) of the mean.

[0029] FIGs. 9A–9C illustrate modularity of the uAb platform. FIG. 9A shows flow cytometric quantification of EGFP fluorescence activity in HEK293T cells transfected with plasmid DNA encoding EGFP or co-transfected with a plasmid encoding uAb chimeras comprised of IpaH9.8 fused to a different GFP-directed binding protein as indicated. FIG. 9B shows flow cytometric quantification of EGFP fluorescence activity in HEK293T cells that transiently or stably expressed EGFP, ERK2-EGFP, H2B-EGFP, or EGFPHRasG12V as indicated. In all cases, cells were transiently transfected with plasmid DNA encoding either pcDNA3-GS2-IpaH9.8C337A (white) or pcDNA3-GS2-IpaH9.8 (grey). FIG. 9C shows flow cytometric quantification of EGFP fluorescence activity in MCF10a cells stably integrated with DNA encoding only EGFP-HRasG12V, EGFP-HRasG12V and GS2-IpaH9.8, EGFPHRasG12V and GS2-IpaH9.8C337A, or GS2-IpaH9.8 alone. All data are biological triplicates of the geometric MFI normalized to MFI measured for HEK293T cells expressing the EGFP alone. Error bars represent standard deviation (SD) of the mean.

DETAILED DESCRIPTION

[0030] It is to be appreciated that certain aspects, modes, embodiments, variations and features of the present application are described below in various levels of detail in order to provide a substantial understanding of the present technology. The definitions of certain terms as used in this specification are provided below. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which the present application belongs.

[0031] In practicing the subject matter of the present application, many conventional techniques in molecular biology, protein biochemistry, cell biology, immunology, microbiology and recombinant DNA are used. These techniques are well-known and are explained in, *e.g.*, *Current Protocols in Molecular Biology*, Vols. I–III, Ausubel, Ed. (1997); Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)); *DNA Cloning: A Practical Approach*, Vols. I and II, Glover, Ed. (1985); *Oligonucleotide Synthesis*, Gait, Ed. (1984); *Nucleic Acid Hybridization*, Hames & Higgins, Eds. (1985); *Transcription and Translation*, Hames & Higgins, Eds. (1984); *Animal Cell Culture*, Freshney, Ed. (1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); Perbal, *A Practical Guide to Molecular Cloning*; the series, *Meth. Enzymol.*, (Academic Press, Inc., 1984); *Gene Transfer Vectors for Mammalian Cells*, Miller & Calos, Eds. (Cold Spring Harbor Laboratory, New York (1987)); and *Meth. Enzymol.*, Vols. 154 and 155, Wu & Grossman, and Wu, Eds., respectively, all of which are hereby incorporated by reference in their

entirety. Methods to detect and measure levels of polypeptide gene expression products, *i.e.*, gene translation level, are well-known in the art and include the use polypeptide detection methods such as antibody detection and quantification techniques. *See also*, Strachan & Read, *Human Molecular Genetics*, Second Edition. (John Wiley and Sons, Inc., New York (1999), which is hereby incorporated by reference in its entirety.

[0032] A first aspect of the present application relates to an isolated chimeric molecule. The isolated chimeric molecule comprises a degradation domain comprising an E3 ubiquitin ligase (E3) motif; a targeting domain capable of specifically directing the degradation domain to a substrate, wherein the targeting domain is heterologous to the degradation domain; and a linker coupling the degradation domain to the targeting domain.

[0033] As used herein, the terms “chimeric molecule”, “chimeric polypeptide”, and “chimeric protein” encompass a molecule having a sequence that includes at least a portion of a full-length sequence of first protein or polypeptide sequence and at least a portion of a full-length sequence of a second protein or polypeptide sequence, where the first and second proteins or polypeptides are different proteins or polypeptides. A chimeric molecule also encompasses proteins or polypeptides that include two or more non-contiguous portions derived from the same protein or polypeptide. A chimeric molecule also encompasses proteins or polypeptides having at least one substitution, wherein the chimeric molecule includes a first protein or polypeptide sequence in which a portion of the first protein or polypeptide sequence has been substituted by a portion of a second protein or polypeptide sequence. As used herein, the term “chimeric molecule” further refers to a molecule possessing a degradation domain and a targeting region, as exemplified herein. The degradation domain and targeting region may be attached in manner known in the art. For example, they may be linked *via* linker molecule as exemplified herein, fused, covalently attached, non-covalently attached, etc. Moreover, the degradation domain and a targeting region may not be directly attached and/or the attachment may be transient, *e.g.*, if a linker is used, the linker may be cleavable or non-cleavable.

[0034] As used herein, the term “ubiquitination” refers to the attachment of the protein ubiquitin to lysine residues of other molecules. Ubiquitination of a molecule, such as a peptide or protein, can act as a signal for its rapid cellular degradation, and for targeting to the proteasome complex.

[0035] As used herein, the terms “ubiquibodies” and “chimeric molecules” are used interchangeably and refer to molecules with at least a degradation domain and a target region, linked by a linker region, as exemplified herein.

[0036] As used herein the terms “target domain” or “targeting domain” or “targeting moiety” means a polypeptide region bound covalently or non-covalently to a second region within a chimeric molecule, which enhances the concentration of the chimeric molecule or composition in a target sub-cellular location, cell, or tissue relative, as compared to the surrounding locations, cells, and/or tissue.

[0037] In accord, the chimeric molecules of the present application possess novel E3 ligase motif (referenced herein, for example, as “E3 ligase (EL)”) ubiquitin regions attached to targeting domains, which are accessible for substrate binding. In some embodiments, the substrate is an intracellular substrate. In order to facilitate versatility, however, the targeting domain is derived from a monobody (for example, fibronectin type III domain (“FN3”)), antibody, polyclonal antibody, monoclonal antibody, recombinant antibody, antibody fragment, Fab', F(ab')₂, Fv, scFv, tascFvs, bis-scFvs, sdAb, V_H, V_L, V_{nar}, scFvD10, scFv13R4, scFvD10, humanized antibody, chimeric antibody, complementary determining region (CDR), IgA antibody, IgD antibody, IgE antibody, IgG antibody, IgM antibody, nanobody, intrabody, unibody, minibody, PROTACs, aptameric domains, a ubiquitin binding domain sequence, an E3 binding domain, a non-antibody protein scaffold, Adnectin, Affibody and their two-helix variants, Anticalin, camelid antibody (for example, V_HH), knottin, DARPin, and/or Sso7d.

[0038] The skilled artisan will readily appreciate that such targeting domains, in some embodiments, possess cell/tissue specificity in accord with the novel E3 ligase motif regions described herein. In one embodiment, the targeting domain binds to a non-native substrate.

[0039] As used herein the term monobody may include any binding portion of a non-immunoglobulin molecule including, for example, FN3 and DARPins, or a polypeptide that contains a binding site, which specifically binds to, or reacts with, a substrate and the like. Monobodies in accordance with the present application include synthetic binding proteins that are constructed using a fibronectin type III domain (“FN3”) as a molecular scaffold. Monobodies are a simple and robust alternative to antibodies for creating target-binding proteins. Monobodies belong to a class of molecules collectively called antibody mimics (or antibody mimetics) and alternative scaffolds that aim to overcome shortcomings of natural antibody molecules. A major advantage of monobodies over conventional antibodies is that monobodies can readily be used as genetically encoded intracellular inhibitors, that is a monobody inhibitor may be expressed in a cell of choice by transfecting the cell with a monobody expression vector. This is attributed to the underlying characteristics of the FN3 scaffold: small (~90 residues), stable, easy to produce, and its lack of disulfide bonds that makes it possible to produce functional monobodies regardless of the redox potential of the cellular environment, including

the reducing environment of the cytoplasm and nucleus. In one embodiment, the targeting domain is a monobody. The monobody may be a fibronectin type III domain (FN3) monobody selected from the group consisting of GS2, Nsa5, and RasInII. The GS2 monobody may, for example, recognize green fluorescent protein ("GFP"). The Nsa5 monobody may, for example, be specific for the Src-homology 2 (SH2) domain of SHP2 (Sha et al., "Dissection of the BCR-ABL Signaling Network Using Highly Specific Monobody Inhibitors to the SHP2 SH2 Domains," *Proc. Natl. Acad. Sci. USA* 110(37):14924–29 (2013), which is hereby incorporated by reference in its entirety) and RasInII, which is specific for HRas, KRas, and the G12V mutants of each (Cetin et al., "RasIns: Genetically Encoded Intrabodies of Activated Ras Proteins," *J. Mol. Biol.* 429(4):562–573 (2017), which is hereby incorporated by reference in its entirety).

[0040] A targeting domain that is a monobody may be, for example, a fibronectin type III domain (FN3) monobody. Examples of FN3 monobodies include but are not limited to (with target antigen in parenthesis): GS2 (GFP), Nsa5 (SHP2), RasInI (HRas/KRas), and RasInII (HRas/KRas), 1D10 (CDC34), 1D7 (COPS5), 1C4 (MAP2K5), 2C12 (MAP2K5), 1E2 (SF3A1), 1C2 (USP11), 1A9 (USP11), Ubi4 (ubiquitin), EI1.4.1 (EGFR), EI2.4.6 (EGFR), EI3.4.3 (EGFR), EI4.2.1 (EGFR), EI4.4.2 (EGFR), EI6.2.6 (EGFR), EI6.2.10 (EGFR), E246(EGFR), C743(CEA), IIIa8.2.6 (FcγIIa), IIIa6.2.6 (FcγIIIa), hA2.2.1 (hA33), hA2.2.2 (hA33), hA3.2.1 (hA33), hA3.2.3 (hA33), mA3.2.1 (mA33), mA3.2.2 (mA33), mA3.2.3 (mA33), mA3.2.4 (mA33), mA3.2.5 (mA33), Alb3.2.1 (hAlb), mI2.2.1 (mIgG), HA4 (AblSH2), HA10 (AblSH2), HA16 (AblSH2), HA18 (AblSH2), 159 (vEGFR), MUC16 (MSLN), E2#3 (ERα/EF), E2#4 (ERα/EF), E2#5 (ERα/EF), E2#6 (ERα/EF), E2#7 (ERα/EF), E2#8 (ERα/EF), E2#9 (ERα/EF), E2#10 (ERα/EF), E2#11 (ERα/EF), E2#23 (ERα/EF), E3#2 (ERα/EF), E3#6 (ERα/EF), OHT#31 (ERα/EF), OHT#32 (ERα/EF), OHT#33 (ERα/EF), AB7-A1 (ERα/EF), AB7-B1 (ERα/EF), MBP-74 (MBP), MBP-76 (MBP), MBP-79 (MBP), hSUMO4-33 (hSUMO4), hSUMO-39 (hSUMO4), ySUMO-53 (ySUMO), ySUMO-56 (ySUMO), ySUMO-57 (ySUMO), T14.25 (TNFα), T14.20 (TNFα), FNfn10-3JCL14 (αvβ3 integrin), 1C9 (Src SH3), 1F11 (Src SH3), 1F10 (Src SH3), 2G10 (Src SH3), 2B2 (Src SH3), 1E3 (Src SH3), E18 (VEGFR2), E19 (VEGFR2), E26 (VEGFR2), E29 (VEGFR2), FG4.2 (Lysozyme), FG4.1 (Lysozyme), 2L4.1 (Lysozyme), BF4.1 (Lysozyme), BF4.9 (Lysozyme), BF4.4 (Lysozyme), BFslc4.01 (Lysozyme), BFslc4.07 (Lysozyme), BFslc4.02 (Lysozyme), BFslc4.06 (Lysozyme), BFslc4.01 (Lysozyme), 10C17C25 (phospho-IκBα), Fn-N22 (SARS N), Fn-N17 (SARS N), FN-N10 (SARS N), gI2.5.3T88I (goat IgG), gI2.5.2 (goat IgG), gI2.5.4 (goat IgG), rI4.5.4 (rabbit IgG), rI4.3.1

(rabbit IgG), rI3.6.6 (rabbit IgG), rI4.3.4 (rabbit IgG), rI3.6.4 (rabbit IgG), and rI4.3.3 (rabbit IgG).

[0041] As used herein the term antibody may include an immunoglobulin and any antigen-binding portion of an immunoglobulin, *e.g.*, IgG, IgD, IgA, IgM and IgE, or a polypeptide that contains an antigen binding site, which specifically or “immunospecifically binds” to, or “immunoreacts with”, an immunogen, antigen, substrate, and the like. Antibodies can comprise at least one heavy (H) chain and at least one light (L) chain inter-connected by at least one disulfide bond. The term “V_H” refers to a heavy chain variable region of an antibody. The term “V_L” refers to a light chain variable region of an antibody. In some embodiments, the term “antibody” specifically covers monoclonal and polyclonal antibodies. A “polyclonal antibody” refers to an antibody which has been derived from the sera of animals immunized with an antigen or antigens. A “monoclonal antibody” refers to an antibody produced by a single clone of hybridoma cells.

[0042] Antibody-related molecules, domains, fragments, portions, *etc.*, useful as targeting domains of the present application include, *e.g.*, but are not limited to, Fab, Fab' and F(ab')₂, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Examples include: (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH₁ domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH₁ domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., “Binding Activities of a Repertoire of Single Immunoglobulin Variable Domains Secreted From *Escherichia coli*,” *Nature* 341:544–46 (1989), which is hereby incorporated by reference in its entirety), which consists of a V_H domain; and (vi) an isolated complementary determining region (CDR). As such “antibody fragments” can comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Single-chain antibody molecules may comprise a polymer with a number of individual molecules, for example, dimer, trimer or other polymers.

[0043] The term “monoclonal antibody” as used herein may include an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Nevertheless, the monoclonal antibodies to be used in

accordance with the present application may be made by the hybridoma method first described by Kohler et al., "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity," *Nature* 256:495 (1975), which is hereby incorporated by reference in its entirety, or may be made by recombinant DNA methods. *See, e.g.*, U.S. Patent No. 4,816,567, which is hereby incorporated by reference in its entirety. The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., "Making Antibody Fragments Using Phage Display Libraries," *Nature* 352:624–28 (1991) and Marks et al., "By-Passing Immunization. Human Antibodies From V-Gene Libraries Displayed on Phage," *J. Mol. Biol.* 222:581–97 (1991), for example, which are hereby incorporated by reference in their entirety.

[0044] As used herein, the term "polyclonal antibody" includes, for example, a preparation of antibodies derived from at least two (2) different antibody-producing cell lines. The use of this term includes preparations of at least two (2) antibodies that contain antibodies that specifically bind to different epitopes or regions of an antigen.

[0045] As used herein, the terms "single chain antibodies" or "single chain Fv (scFv)" may refer to an antibody fusion molecule of the two domains of the Fv fragment, V_L and V_H. Although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv). *See, e.g.*, Bird et al., "Single-Chain Antigen-Binding Proteins," *Science* 242:423–26 (1988) and Huston et al., "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in *Escherichia coli*," *Proc. Natl. Acad. Sci. USA* 85:5879–83 (1988), which are hereby incorporated by reference in their entirety. Such single chain antibodies are included by reference to the term "antibody" fragments, and can be prepared by recombinant techniques or enzymatic or chemical cleavage of intact antibodies.

[0046] As used herein, the term "variable" may, for example, refer to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and defines specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15–30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" that are each 9–12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β -sheet

configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies. *See* Kabat et al.,
5 Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), which is hereby incorporated by reference in its entirety. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (“ADCC”).

10 **[0047]** The targeting domains of the present application can be, for example, monospecific, bispecific, trispecific or of greater multispecificity. Multispecific targeting domains can be specific for different epitopes of a substrate or can be specific for both a substrate polypeptide of the present application as well as for heterologous compositions, such as a heterologous polypeptide or solid support material. *See, e.g.*, WO 93/17715; WO 92/08802;
15 WO 91/00360; WO 92/05793; Tutt et al., “Trispecific F(ab')₃ Derivatives That Use Cooperative Signaling Via the TCR/CD3 Complex and CD2 to Activate and Redirect Resting Cytotoxic T Cells,” *J. Immunol.* 147:60–69 (1991); U.S. Pat. Nos. 5,573,920, 4,474,893, 5,601,819, 4,714,681, 4,925,648; 6,106,835; Kostelny et al., “Formation of a Bispecific Antibody by the Use of Leucine Zippers,” *J. Immunol.* 148:1547–53 (1992), which are hereby incorporated by
20 reference in their entirety. The targeting domains of the application can be from any animal origin, including birds and mammals. For example, the targeting domains may be from human, marine, rabbit, goat, guinea pig, camel, horse, or chicken.

[0048] Techniques for generating targeting domains directed to target substrates are well known to those skilled in the art. Examples of such techniques include, but are not limited to,
25 *e.g.*, those involving display libraries, xeno or humab mice, hybridomas, and the like. Target polypeptides—from which a targeting domain is derived—within the scope of the present application include any polypeptide or polypeptide derivative which is capable of exhibiting antigenicity. Examples include, but are not limited to, substrate and fragments thereof. In some embodiments, the targeting domain is a single-chain antibody.

30 **[0049]** Single chain antibodies (“scFv”) are genetically engineered antibodies that consist of the variable domain of a heavy chain at the amino terminus joined to the variable domain of a light chain by a flexible region. In some embodiments, scFv are generated by PCR from hybridoma cell lines that express monoclonal antibodies (mAbs) with known target specificity, or they are selected by phage display from libraries isolated from spleen cells or lymphocytes,

and preserve the affinity of the parent antibody. Employing a protocol to identify intracellular substrates, the yeast two-hybrid technology serves to identify candidate scFv–protein interactions. Such a system is useful to predict whether or not a scFv will be able to recognize its target substrate *in vivo*. See Pörtner-Taliana et al., “Identification of Protein Single chain

5 Antibody Interactions *In Vivo* Using Two-hybrid Protocols,” *Protein–Protein Interactions: A Molecular Cloning Manual*, Cold Spring Harbor Laboratory Press, Chapter 24 (© 2002), which is hereby incorporated by reference in its entirety.

[0050] Typically, scFv, hybrid antibodies or hybrid antibody fragments that are cloned into a display vector can be selected against the appropriate antigen in order to identify variants
10 that maintained good binding activity, because the antibody or antibody fragment will be present on the surface of the phage or phagemid particle. See e.g., Barbas III et al., *Phage Display, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001), which is hereby incorporated by reference in its entirety. However, other vector formats could be used for this process, such as cloning the antibody fragment library into a lytic phage vector
15 (modified T7 or Lambda Zap systems) for selection and/or screening.

[0051] In general, expression vectors useful in recombinant DNA techniques are often in the form of plasmids. However, the present application is intended to include such other forms of expression vectors that are not technically plasmids, such as viral vectors, e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses, which serve equivalent
20 functions. Such viral vectors permit infection of a subject and expression in that subject of a compound. The expression control sequences are typically eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences encoding the target domain, and the collection and
25 purification of the substrate binding agent, e.g., cross-reacting anti-substrate antibodies. See, generally, U.S. Patent Publication No. 2002/0199213, which is hereby incorporated by reference in its entirety. Vectors can also encode signal peptide, e.g., pectate lyase, useful to direct the secretion of extracellular antibody fragments. See U.S. Pat. No. 5,576,195, which is hereby incorporated by reference in its entirety.

30 [0052] As used herein, the terms “degradation domain” or “degradation region” includes a portion of a chimeric molecule that is capable of facilitating the ubiquitination of a substrate. The degradation domain may have a second “binding” region for interaction with a native binding protein. The binding region can be modified as to possess one or more mutations, substitutions, deletions, or may be deleted entirely.

[0053] The degradation domain may contain E3 mimics with folds similar to eukaryotic E3s such as HECT-type, RING or U-box (RING/U-box)-type, and F-box domains, as well as unconventional E3s with folds unlike any other eukaryotic E3s such as NEL, XL-box-containing, and SidC. The degradation domain relates to polypeptides or polypeptide regions capable of modifying substrates by attaching one or more ubiquitin molecules and/or ubiquitin-like molecules to the substrates. In this regard, such a region comports with the well-known ubiquitination cascade—involving the coordinated action of the E1, E2, and E3 enzymes—which functions to activate and concomitantly conjugate ubiquitin to a substrate. In some embodiments, the motif is a ubiquitin region composed of a novel E3 ligase, or fragment thereof, which catalyzes the transfer of ubiquitin in a substrate-specific manner. *See* Qian et al., “Engineering a Ubiquitin Ligase Reveals Conformational Flexibility Required for Ubiquitin Transfer,” *J. Biol. Chem.* 284(39):26797–802 (2009), which is hereby incorporated by reference in its entirety.

[0054] As used herein, the terms “modification(s)” or “amino acid modification” of a polypeptide, protein, region, domain, or the like, refers to a change in the native sequence such as a deletion, addition or substitution of a desired residue. Such modified polypeptides are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Any combination of deletion, insertion, and substitution is made to obtain the antibody of interest, as long as the obtained antibody possesses the desired properties. The modification also includes the change of the pattern of glycosylation of the protein. A useful method for identification of preferred locations for mutagenesis is called “alanine scanning mutagenesis” as described by Cunningham and Wells, “High-Resolution Epitope Mapping of hGH-Receptor Interactions by Alanine-Scanning Mutagenesis,” *Science* 244:1081–85 (1989), which is hereby incorporated by reference in its entirety. The mutated antibody is then screened for the desired activity.

[0055] The terms “polypeptide,” “protein,” and “peptide” are used herein interchangeably herein to refer to amino acid chains in which the amino acid residues are linked by peptide bonds or modified peptide bonds. The amino acid chains can be of any length of greater than two amino acids. Unless otherwise specified, the terms “polypeptide,” “protein,” and “peptide” also encompass various modified forms thereof. Such modified forms may be naturally occurring modified forms or chemically modified forms. Examples of modified forms include, but are not limited to, glycosylated forms, phosphorylated forms, myristoylated forms, palmitoylated forms, ribosylated forms, acetylated forms, ubiquitinated forms, etc. Modifications also include intra-molecular crosslinking and covalent attachment to various

moieties such as lipids, flavin, biotin, polyethylene glycol or derivatives thereof, etc. In addition, modifications may also include cyclization, branching and cross-linking. Further, amino acids other than the conventional twenty amino acids encoded by genes may also be included in a polypeptide. In one embodiment, the E3 ubiquitin ligase motif (E3), also referred to herein as
5 EL or NEL, comprises a modified binding region which inhibits or decreases binding to said substrate compared to said E3 motif without the modified binding region. In another embodiment, the modification is a mutation or deletion in the binding region.

[0056] As used herein, the terms “variant” or “mutant” are used to refer to a protein or peptide which differs from a naturally occurring protein or peptide, i.e., the “prototype” or “wild-
10 type” protein, by modifications to the naturally occurring protein or peptide, but which maintains the basic protein and side chain structure of the naturally occurring form. Such changes include, but are not limited to: changes in one, few, or even several amino acid side chains; changes in one, few or several amino acids, including deletions, *e.g.*, a truncated version of the protein or peptide, insertions and/or substitutions; changes in stereochemistry of one or a few atoms; and/or
15 minor derivatizations, including but not limited to: methylation, glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation and/or addition of glycosylphosphatidyl inositol. A “variant” or “mutant” can have enhanced, decreased, changed, or substantially similar properties as compared to the naturally occurring protein or peptide.

[0057] In some embodiments, the degradation domain of the chimeric molecule lacks an
20 endogenous substrate recognition region, *i.e.*, a portion of the polypeptide that interacts with a natural or native binding partner. The E3 motif of the degradation domain may possess a modified binding domain which inhibits or decreases binding to a substrate compared to the E3 motif without the modified binding region. Nevertheless, the E3 motif permits proteolysis of a substrate in some embodiments. In some embodiments, the modification is a mutation,
25 substitution, or deletion of the binding region. The substitution can be an amino acid substitution such as a conservative or a non-conservative amino acid substitution.

[0058] Non-conservative amino acid substitutions of the E3 motif, are substitutions in which an alkyl amino acid is substituted for an amino acid other than an alkyl amino acid in the sequence, an aromatic amino acid is substituted for an amino acid other than an aromatic amino
30 acid in the E3 motif, a sulfur-containing amino acid is substituted for an amino acid other than a sulfur-containing amino acid in the E3 motif, a hydroxy-containing amino acid is substituted for an amino acid other than a hydroxy-containing amino acid in the E3 motif, an acidic amino acid is substituted for an amino acid other than an acidic amino acid in the E3 motif, a basic amino acid is substituted for an amino acid other than a basic amino acid in the E3 motif, or a dibasic

monocarboxylic amino acid is substituted for an amino acid other than a dibasic monocarboxylic amino acid in the E3 motif.

[0059] Among the common amino acids, for example, “non-conservative amino acid substitutions” are illustrated by a substitution of an amino acids from one of the following groups with an amino acid that is not from the same group, as follows: (1) glycine, alanine, (2) valine, leucine, and isoleucine, (3) phenylalanine, tyrosine, and tryptophan, (4) cysteine and methionine, (5) serine and threonine, (6) aspartate and glutamate, (7) glutamine and asparagine, and (8) lysine, arginine and histidine.

[0060] Conservative or non-conservative amino acid changes in, *e.g.*, the E3 motif, can be introduced by substituting appropriate nucleotides for the nucleotides encoding such a region. These modifications can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like. Ausubel et al. (eds.), *Short Protocols in Molecular Biology*, 5th Edition, John Wiley & Sons, Inc. (2002); *see generally*, McPherson (ed.), *Directed Mutagenesis: A Practical Approach*, IRL Press (1991), which are hereby incorporated by reference in their entirety. A useful method for identification of locations for sequence variation is called “alanine scanning mutagenesis” as described by Cunningham and Wells “Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in *Escherichia coli*,” *Science* 244:1081–85 (1989), which is hereby incorporated by reference in its entirety.

[0061] Ubiquitin ligase families include, but are not limited to, the homologous to E6-associated protein C-terminus (“HECT”) domain ligases, which concerns the transfer of ubiquitin from the E2 conjugase to the substrate, the Really Interesting New Gene (“RING”) domain ligases, which bind E2, may mediate enzymatic activity in the E2-E3 complex, and the U-box ubiquitin family of ligases (“UULs”), which constitute a family of modified RING motif ligases without the full complement of Zn^{2+} -binding ligands. *See* Colas et al., “Targeted Modification and Transportation of Cellular Proteins.” *Proc. Natl. Acad. Sci. USA* 97(25):13720–25 (2005), which is hereby incorporated by reference in its entirety.

[0062] U-box ubiquitin ligases (“ULLs”) are characterized as having a protein domain, the U-box, which is structurally related to the RING finger, typical of many other ubiquitin ligases. In humans, the UUL-encoding genes include, but are not limited to, UBE4A and UBE4B genes (also respectively termed UFD2b and UFD2a), CHIP (also termed STUB1), UIP5 (also termed UBOX5), PRP19 (also termed PRPF19 or SNEV), CYC4 (also termed PPIL2 or Cyp-60), WDSUB1, and ACT1 (also termed TRAF3IP2). *See* Marin, I., “Ancient Origin of

Animal U-box Ubiquitin Ligases.” *BMC Evolutionary Biology* 10:331, pp. 1–15 (2010), which is hereby incorporated by reference in its entirety.

[0063] While HECT E3 ligases have a direct role in catalysis during ubiquitination, RING and U-box E3 proteins facilitate protein ubiquitination by acting as adaptor molecules that recruit E2 and substrate molecules to promote substrate ubiquitination. Although many RING-type E3 ligases, such as MDM2 (murine double minute clone 2 oncoprotein) and c-Cbl, may act alone, others are found as components of much larger multi-protein complexes, such as the anaphase-promoting complex (“APC”). Taken together, these multifaceted properties and interactions enable E3 enzymes to provide a powerful, and specific, mechanism for protein clearance within all cells of eukaryotic organisms. Ardley et al., “E3 Ubiquitin Ligases.” *Essays Biochem.* 41:15–30 (2005), which is hereby incorporated by reference in its entirety.

[0064] Functional information concerning the E3 gene products is variable, nonetheless. The U-box protein CHIP acts both as a co-chaperone, together with chaperones such as, *e.g.*, Hsc70, Hsp70 and Hsp90, and as a ubiquitin ligase, alone or as part of complexes that may include other E3 proteins. *See id.* The selectivity of the ubiquitin proteasome system for a particular substrate nevertheless relies on the interaction between a ubiquitin-conjugating enzyme, *e.g.*, E2, and a ubiquitin-protein ligase. Post-translational modifications of the protein substrate, such as, *e.g.*, phosphorylation or hydroxylation, are often required prior to ubiquitination. In this way, the precise spatio-temporal targeting and degradation for a particular substrate can be achieved.

[0065] The E3 motif of the degradation domain disclosed herein possesses a functional E3 ligase that is capable of ubiquitinating a substrate without steric disruption from native binding partners. In addition to the E3 motif, in some embodiments, the degradation domain possesses a ligase that is an E3 mimic with folds similar to eukaryotic E3s such as HECT-type, RING or U-box (RING/U-box)-type, and F-box domains, as well as unconventional E3s with folds unlike any other eukaryotic E3s such as NEL, XL-box-containing, and SidC. Such domains may possess cell or tissue specificity.

[0066] The E3 motif of the chimeric molecule may, in one embodiment, possess cell-type specific or tissue specific ligase function for, but not limited to, skin cells, muscle cells, epithelial cells, endothelial cells, stem cells, umbilical vessel cells, corneal cells, cardiomyocytes, aortic cells, corneal epithelial cells, somatic cells, fibroblasts, keratinocytes, melanocytes, adipose cells, bone cells, osteoblasts, airway cells, microvascular cells, mammary cells, vascular cells, chondrocytes, placental cells, hepatocytes, glial cells, epidermal cells, limbal stem cells, periodontal stem cells, bone marrow stromal cells, hybridoma cells, kidney cells, pancreatic

islets, articular chondrocytes, neuroblasts, lymphocytes, and erythrocytes, and/or any combination thereof.

[0067] In one embodiment, the degradation domain is from a bacterial pathogen, the pathogen being optionally selected from *Shigella*, *Salmonella*, *Bacillus*, *Bartonella*, *Bordetella*,
 5 *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia* and *Chlamydophila*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio*, and *Yersinia*. More particularly, in another embodiment, the bacterial pathogen is *Shigella flexneri*. The degradation
 10 domain, which may be derived from any bacteria may be from, for example, *Shigella flexneri* E3 ligase, SspH1, SspH2, SlrP, AvrPtoB, LubX, NLeG5-1, NleG5-1, NleG2-3, LegU1, LegAU13, NleL, SopA, SidC, XopL, GobX, VirF, GALA, AnkB, and/or SidE.

[0068] In one embodiment, the degradation domain is a member of the *Shigella* IpaH protein family and may be IpaH9.8, IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH0887, IpaH1389,
 15 IpaH2022, IpaH2202, IpaH2610, and/or IpaH0722. *Shigella* species are highly adapted human pathogens that cause bacillary dysentery (shigellosis). Via the type III secretion system (T3SS), *Shigella* deliver a subset of virulence proteins (effectors) that are responsible for pathogenesis, with functions including pyroptosis, invasion of the epithelial cells, intracellular survival, and evasion of host immune responses.

[0069] *Shigella* possesses 12 *ipaH* genes, which reside on both the large plasmid and the chromosome. See, e.g., Ashida & Sasakawa, “*Shigella* IpaH Family Effectors as a Versatile Model for Studying Pathogenic Bacteria,” *Front. Cell. Infect. Microbiol.* 5:100 (2016), which is hereby incorporated by reference in its entirety. IpaH family proteins contain N-terminal leucine-rich repeats (LRRs) and have E3 ubiquitin ligase activity in their conserved C-terminal
 20 regions (Rohde et al., “Type III Secretion Effectors of the IpaH Family are E3 Ubiquitin Ligase,” *Cell Host Microbe.* 1:77–83 (2007) and Ashida et al., “Exploitation of the Host Ubiquitin System by Human Bacterial Pathogens,” *Nat. Rev. Microbiol.* 12:399–413 (2014), both of which are hereby incorporated by reference in their entirety). Ubiquitination is accomplished via a series of reactions catalyzed by a multienzymatic cascade: E1 (ubiquitin-activating enzyme), E2
 25 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Ashida & Sasakawa, “*Shigella* IpaH Family Effectors as a Versatile Model for Studying Pathogenic Bacteria,” *Front. Cell. Infect. Microbiol.* 5:100 (2016), which is hereby incorporated by reference in its entirety. The ubiquitination cascade starts with ATP-dependent activation of ubiquitin via formation of a thioester linkage between the carboxyl-terminal Gly of ubiquitin and a Cys of E1. *Id.* Activated
 30

ubiquitin is transferred to the active-site Cys of E2, and finally E3 ligase mediates the transfer of ubiquitin from the E2 to specific substrate proteins (mainly via substrate Lys residues). *Id.* E3 ligases can be categorized into two groups based on their structures and functions: HECT (Homologous to the E6-AP Carboxyl Terminus)-type and RING (Really Interesting New Gene)/U-box-type. *Id.* HECT-type E3 ligases catalyze ubiquitin transfer by accepting ubiquitin from E2 via formation of a thioester bond with their catalytic cysteine residue, and then transfer ubiquitin to their target substrates. *Id.* On the other hand, RING/U-box-type E3 ligases catalyze direct ubiquitin transfer by acting as scaffold molecules to bind and recruit the E2-ubiquitin complex, and then directly transfer ubiquitin from E2 to E3-bound substrates. *Id.*

[0070] IpaH family proteins are widely conserved among animal and plant pathogens, including *Shigella* (IpaH), *Salmonella* (SspH1, SspH2, and SlrP), *Edwardsiella*, *Bradyrhizobium*, *Rhizobium*, and some *Pseudomonas* species, illustrating the importance of these effectors in bacterial infection. *Id.* Although IpaH family proteins have E3 ubiquitin ligase activity and their C-terminal domains contain a single conserved Cys that form a Cys-ubiquitin intermediate similar to that of HECT-type ligases, the catalytic domains of IpaH family members differ at the sequence and structural levels from eukaryotic E3 ubiquitin ligases. *Id.* Consequently, IpaH family proteins are now considered to constitute a new class of E3 ubiquitin ligases, NEL (Novel E3 ligase), distinct from typical RING-, and HECT-types of E3 ubiquitin ligases (Singer et al., “Structure of the *Shigella* T3SS effector IpaH Defines a New Class of E3 Ubiquitin Ligases,” *Nat. Struct. Mol. Biol.* 15:1293–1301 (2008); Zhu et al., “Structure of a *Shigella* Effector Reveals a New Class of Ubiquitin Ligases,” *Nat. Struct. Mol. Biol.* 15:1302–08 (2008); Quezada et al., “A Family of *Salmonella* Virulence Factors Functions as a Distinct Class of Autoregulated E3 Ubiquitin Ligases,” *Proc. Natl. Acad. Sci. USA* 106:4864–69 (2009), all of which are hereby incorporated by reference in their entirety). Although IpaH family proteins are highly similar to one another, the sequences of their LRR regions, regarded as substrate recognition sites, and subcellular localizations (e.g., nucleus, cytoplasm, or plasma membrane) are different. Ashida & Sasakawa, “*Shigella* IpaH Family Effectors as a Versatile Model for Studying Pathogenic Bacteria,” *Front. Cell. Infect. Microbiol.* 5:100 (2016), which is hereby incorporated by reference in its entirety.

[0071] Ubiquitin ligase families also include the “F-box” ligases—as in the Skp1-Cullin1-F-box (“SCF”) protein complex—which binds to a ubiquitinated substrate, such as, e.g., Cdc 4, which subsequently interacts with a target protein, such as, Sic1 or Grr1, which then binds Cln. See Bai et al., “SKP1 Connects Cell Cycle Regulators to the Ubiquitin Proteolysis Machinery

through a Novel Motif, the F-Box,” *Cell* 86 (2):263–74 (1997), which is hereby incorporated by reference in its entirety.

[0072] The F-box is a protein motif of approximately 50 amino acids that functions as a site of protein-protein interaction. *See, e.g.*, Kipreos et al., “The F-box Protein family.” *Genome Biol.* 1(5) (2000), which is hereby incorporated by reference in its entirety. F-box proteins were first characterized as components of SCF ubiquitin-ligase complexes, in which they bind substrates for ubiquitin-mediated proteolysis. The F-box motif links the F-box protein to other components of the SCF complex by binding the core SCF component Skp I. F-box proteins have more recently been discovered to function through non-SCF protein complexes in a variety of cellular functions. *See id.* F-box proteins often include additional carboxy-terminal motifs capable of protein-protein interaction; the most common secondary motifs in yeast and human F-box proteins are WD repeats and leucine-rich repeats, both of which have been found to bind phosphorylated substrates to the SCF complex. *See id.* The majority of F-box proteins have other associated motifs, and the functions of most of these proteins have not yet been defined. *See id.*

[0073] The least variant positions within the F-box motif include positions 8 (92% of the 234 F-box proteins used for the consensus have leucine or methionine), 9 (92% proline), 16 (86% isoleucine or valine), 20 (81% leucine or methionine), and 32 (92% serine or cysteine). *Id.* This lack of a strict consensus guides the skilled artisan to employ multiple search algorithms for detecting F-box sequences. Two algorithms, for example, can be found in the Prosite and Pfam databases. Occasionally, one database will give a significant score to an F-box in a given protein when the other does not detect it, so both databases should be searched. *Id.*

[0074] Expression of the chimeric molecules of the present application in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion polypeptides. Fusion vectors add a number of amino acids to a polypeptide encoded therein, usually to the amino terminus of the recombinant polypeptide. Such fusion vectors typically serve three purposes: (i) to increase expression; (ii) to increase the solubility; and (iii) to aid in purification by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant polypeptide to enable separation of the recombinant polypeptide from the fusion moiety subsequent to purification of the fusion polypeptide. Such enzymes, and their endogenous recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Smith and Johnson, “Single-Step Purification of Polypeptides Expressed in *Escherichia coli* as Fusions

With Glutathione S-Transferase,” *Gene* 67:31–40 (1988), which is hereby incorporated by reference in its entirety), pMAL (*New England Biolabs*, Beverly, Mass.) and pRIT5 (*Pharmacia*, Piscataway, N.J.) that fuse glutathione S-transferase (“GST”), maltose E binding polypeptide, or polypeptide A, respectively, to the target recombinant polypeptide.

5 [0075] Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann et al., “Tightly Regulated tac Promoter Vectors Useful for the Expression of Unfused and Fused Proteins in *Escherichia coli*,” *Gene* 69:301–15 (1988) and pET 11d (Studier et al., *Gene Expression Technology: Methods In Enzymology* 185, *Academic Press*, San Diego, Calif. 60–89 (1990)), which are hereby incorporated by reference in their entirety. Methods for
10 targeted assembly of distinct active peptide or protein domains to yield multifunctional polypeptides *via* polypeptide fusion has been described by Pack et al., U.S. Pat. Nos. 6,294,353; 6,692,935, which are hereby incorporated by reference in their entirety. One strategy to maximize recombinant polypeptide expression, *e.g.*, a chimeric molecule of the present application, in *E. coli* is to express the polypeptide in host bacteria with an impaired capacity to
15 proteolytically cleave the recombinant chimera. *See, e.g.*, Gottesman, *Gene Expression Technology: Methods In Enzymology* 185, *Academic Press*, San Diego, Calif. 119–128 (1990), which is hereby incorporated by reference in its entirety.

[0076] In some embodiments, a nucleic acid encoding a chimeric molecule of the present application—including a degradation domain and a targeting region—is expressed in mammalian
20 cells using a mammalian expression vector. Examples of mammalian expression vectors include, *e.g.*, but are not limited to, pcDNA3, pCDM8 (Seed, “An LFA-3 cDNA Encodes a Phospholipid-Linked Membrane Protein Homologous to its Receptor CD2,” *Nature* 329:840 (1987), which is hereby incorporated by reference in its entirety), and pMT2PC. When used in mammalian cells, the expression vector’s control functions are often provided by viral regulatory
25 elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, and simian virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells useful for expression of the targeting domains, degradation domains of the chimeric molecule. *See, e.g.*, Chapters 16 and 17 of Sambrook et al., *Molecular Cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor Laboratory, *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, N.Y., (1989), which are hereby incorporated by reference
30 in their entirety.

[0077] Notwithstanding chimeric molecule E3 degradation domains and targeting domain expression, the function of such domains/regions imparts the specificity of the present application. A known or unknown substrate is bound by the targeting domain for subsequent

ubiquitination *via* the degradation domain. In some embodiments, the substrates include, but are not limited to, intracellular substrates, extracellular substrates, modified substrates, glycosylated substrates, farnesylated substrates, post translationally modified substrates, phosphorylated substrates, and other modifications known in the art.

5 [0078] In some embodiments, the substrates include, but are not limited to, fluorescent protein, histone protein, nuclear localization signal (NLS), H-Ras protein, Src-homology 2 domain-containing phosphatase 2 (SHP2), β -galactosidase, gpD, Hsp70, MBP, CDC34, COPS5, MAP2K5, SF3A1, USP11, ubiquitin, EGFR, CEA, Fc γ IIa, Fc γ IIIa, hA33, mA33, hAlb, mIgG, AblSH2, vEGFR, MSLN, ER α /EF, hSUMO4, ySUMO, TNF α , α v β 3 integrin, Src SH3, 10 Lysozyme, phospho-I κ B α , SARS N, goat IgG, rabbit IgG, post-translationally modified proteins, fibrillin, huntingtin, tumorigenic proteins, p53, Rb, adhesion proteins, receptors, cell-cycle proteins, checkpoint proteins, HFE, ATP7B, prion proteins, viral proteins, bacterial proteins, parasitic proteins, fungal proteins, DNA binding proteins, metabolic proteins, regulatory proteins, structural proteins, enzymes, immunogenic proteins, autoimmunogenic proteins, 15 immunogens, antigens, pathogenic proteins, and the like. In one embodiment, the substrate is a fluorescent protein, for example, green fluorescent protein, emerald fluorescent protein, venus fluorescent protein, cerulean fluorescent protein, and enhanced cyan fluorescent protein.

[0079] As used herein, the term “amino acid” includes naturally-occurring amino acids, L-amino acids, D-amino acids, and synthetic amino acids, as well as amino acid analogs and 20 amino acid mimetics that function in a manner similar to the naturally-occurring amino acids. Naturally-occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally-occurring amino acid, *e.g.*, an α -carbon that is bound to a hydrogen, a carboxyl group, 25 an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R-groups, *e.g.*, norleucine, or modified peptide backbones, but retain the same basic chemical structure as a naturally-occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a 30 naturally-occurring amino acid. Amino acids can be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

[0080] An exemplary E3 ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase AvrPtoB, which is a U-box motif, from *Pseudomonas syringae* which has the amino acid sequence of SEQ ID NO: 1:

MAGINRAGPSGAYFVGHTDPEPVSGQAHGSGSGASSNSPQVQPRPSNTPPSNAPAPPPT
 5 GRERLSRSTALSRTREWLEQGMPTAEDASVRRRPQVTADAATPRAEARRTPEATADA
 SAPRRGAVAHANIVQQLVSEGADISHTRNMLRNAMNGDAVAFSRVEQNIFRQHFPNM
 PMHGISRDSELAIELRGALRRRAVHQQAASAPVRSPTPTPASPAASSSGSSQRSFLGRFARL
 MAPNQGRSSNTAASQTPVDRSPPRVNQRPIRVDRAMRNRGNDADAALRGLVQQGV
 NLEHLRTALERHVMQRLPIPLDIGSALQNVGINPSIDLGESLVQHPLLNLNVALNRMLGL
 10 RPSAERAPRPAVPVAPATASRRPDGTRATRLRVMPEREDYENNVAYGVRLNLNPGVG
 VRQAVAAAFVTDRAERPAAVANIRAALDPIASQFSQLRTISKADAEEELGFKDAADHHT
 DDVTHCLFGGELSLSNPDQQVIGLAGNPTDTSQPYSQEGNKDLAFMDMKKLAQFLAGK
 PEHPMTRETLNAENIAKYAFRIVP

[0081] The E3 ubiquitin ligase AvrPtoB from *Pseudomonas syringae* has the nucleotide sequence of SEQ ID NO: 2 as follows:

ATGGCGGGTATCAATAGAGCGGGACCATCGGGCGCTTATTTTGTGGCCACACAGA
 CCCCAGCCAGTATCGGGGCAAGCACACGGATCCGGCAGCGGCGCCAGCTCCTCGA
 ACAGTCCGCAGGTTACGCCGCGACCCTCGAATACTCCCCCGTCGAACGCGCCCGCAC
 CGCCGCCAACCAGGACGTGAGAGGCTTTCACGATCCACGGCGCTGTGCGGCCAAACC
 20 AGGGAGTGGCTGGAGCAGGGTATGCCTACAGCGGAGGATGCCAGCGTGCCTCGTAG
 GCCACAGGTGACTGCCGATGCCGCAACGCCGCGTGCAGAGGCAAGACGCACGCCGG
 AGGCAACTGCCGATGCCAGCGCACCGCGTAGAGGGGCGGTTGCACACGCCAACAGT
 ATCGTTTCAGCAATTGGTCAGTGAGGGCGCTGATATTTTCGCTACTCGTAACATGCTC
 CGCAATGCAATGAATGGCGACGCAGTCGCTTTTTCTCGAGTAGAACAGAACATATTT
 25 CGCCAGCATTTCGCCAACATGCCCATGCATGGAATCAGCCGAGATTCGGAACCTCGCT
 ATCGAGCTCCGTGGGGCGCTTCGTGAGCGGTTACCAACAGGCGGGCGTCAGCGCC
 AGTGAGGTCGCCCACGCCAACACCGGCCAGCCCTGCGGCATCATCATCGGGCAGCA
 GTCAGCGTTCTTTATTTGGACGGTTTGCCCGTTTGATGGCGCCAAACCAGGGACGGT
 CGTCGAACACTGCCGCCTCTCAGACGCCGGTCGACAGGAGCCCGCCACGCGTCAAC
 30 CAAAGACCCATACGCGTCGACAGGGCTGCGATGCGTAATCGTGGCAATGACGAGGC
 GGACGCCGCGCTGCGGGGGTATGATACAACAGGGGGTCAATTTAGAGCACCTGCGCA
 CGGCCCTTGAAAGACATGTAATGCAGCGCCTCCCTATCCCCCTCGATATAGGCAGCG
 CGTTGCAGAATGTGGGAATTAACCCAAAGTATCGACTTGGGGGAAAGCCTTGTGCAA
 CATCCCCTGCTGAATTTGAATGTAGCGTTGAATCGCATGCTGGGGCTGCGTCCAGC
 35 GCTGAAAGAGCGCCTCGTCCAGCCGTCCCCGTGGCTCCCGCGACCGCCTCCAGGCG
 ACCGGATGGTACGCGTGCAACACGATTGCGGGTGATGCCGGAGCGGGAGGATTACG
 AAAATAATGTGGCTTATGGAGTGCCTTGCTTAACCTGAACCCGGGGGTGGGGGTA
 AGGCAGGCTGTTGCGGCCTTTGTAACCGACCGGGCTGAGCGGCCAGCAGTGGTGGC
 TAATATCCGGGCAGCCCTGGACCCTATCGCGTCACAATTCAGTCAGCTGCGCACAAT
 40 TTCGAAGGCCGATGCTGAATCTGAAGAGCTGGGTTTTAAGGATGCGGCAGATCATC
 ACACGGATGACGTGACGCACTGTCTTTTTGGCGGAGAATTGTCGCTGAGTAATCCGG
 ATCAGCAGGTGATCGGTTTGGCGGGTAATCCGACGGACACGTGCGAGCCTTACAGC
 CAAGAGGGAAATAAGGACCTGGCGTTCATGGATATGAAAAAATTGCCCAATTCCT
 CGCAGGCAAGCCTGAGCATCCGATGACCAGAGAAACGCTTAACGCCGAAATATCG
 45 CCAAGTATGCTTTTAGAATAGTCCCC

[0082] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase IpaH0722, which is a

novel E3 ligase (also referred to herein as NEL or EL), from *Shigella flexneri* which has the amino acid sequence of SEQ ID NO: 3:

MKPAHNPSFFRSFCGLGCISRLSVEEQNITDYHRIWDNWAKEGAATEDRTQAVRLLKIC
LAFQEPA LNLSLLRLRSLPYLPPIHQELNISSNELRSLPELPPSLTVLKASDNRLSRLPALPP
5 HLVALDVSLNRVLTCLPSLPSSLQSL SALLNSLETLPDLPPALQKLSVGNQLTALPELPC
ELQELSAFDNRLQELPPLPQNLRLNLVGENQLHRLPELPQRLQSLYIPNNQLNTLPDSIM
NLHIYADVNIYNNPLSTRTLQALQRLTSSPDYHGPRIYFSMSDGQQNTLHRPLADAVTA
WFPENKQSDVSIWHA FEHEEHANTFSAFLDRLSDTV SARNTSGFREQVA AWLEKLSAS
AELRQQSF AVAADATESCEDRVALTWNNLRKTLLVHQASEGLFDNDTGALLSLGREMF
10 RLEILEDIARDKVRTLHFVDEIEVYLAFQTMLAEKLQLSTAVKEMRFYGVSGVTANDLR
TAEAMVRSRENEFTDWFSLWGPWHAVLKRTEADRWALAEQKYEMLENEY PQRVA
DRLKASGLSGDADAEREAGA QVMRETEQQIYRQLTDEV LALRLPENGSQLHHS

[0083] The E3 ubiquitin ligase IpaH0722, which is a novel E3 ligase, from *Shigella flexneri* has the nucleotide sequence of SEQ ID NO: 4 as follows:

15 ATGAAACCTGCCCCACAATCCTTCTTTTTTCCGCTCCTTTTGTGGTTTAGGATGTATAT
CCCGTTTATCCGTAGAAAGAGCAAAATATCACGGATTATCACCGCATCTGGGATAACT
GGGCCAAGGAAGGTGCTGCAACAGAAGACCGAACACAGGCAGTTCGATTACTGAA
AATATGTCTGGCTTTTCAAGAGCCAGCCCTCAATTTAAGTTTACTCAGATTACGCTCT
CTCCCATACCTGCCCCCGCACATACAAGAACTTAACATCTCTAGCAATGAGCTACGC
20 TCTCTGCCAGAACTCCCTCCGTCCTTAAGTGTACTTAAAGCCAGCGATAACAGACTG
AGCAGGCTCCCGGCTCTTCCGCCTCACCTGGTCGCTCTTGATGTTTCACTTAACAGA
GTTTTAACATGTTTGCCTTCTCTTCCATCTTCCCTTGCACTCACTCTCAGCCCTTCTCAA
TAGCCTGGAGACGCTACCTGATCTTCCCCCGGCTCTACAAAACTTTCTGTTGGCAA
CAACCAGCTTACTGCCTTACCAGAATTACCATGTGAACTACAGGAACCTAAGTGCTTT
25 TGATAACAGATTACAAGAGCTACCGCCCCCTTCTCAAAATCTGAGGCTTTTAAACGT
TGGGGAAAACCAACTACACAGACTGCCCCGAACCTCCACAACGTCTGCAATCACTAT
ATATCCCTAACAAATCAGCTGAACACATTGCCAGACAGTATCATGAATCTGCACATTT
ATGCAGATGTTAATATTTATAACAATCCATTGTGCGACTCGCACTCTGCAAGCCCTGC
AAAGATTAACCTCTTCGCCGACTACCACGGCCACGGATTTACTTCTCCATGAGTG
30 ACGGACAACAGAATACACTCCATCGCCCCCTGGCTGATGCCGTGACAGCATGGTTCC
CGGAAAACAAACAATCTGATGTATCACAGATATGGCATGCTTTTGAACATGAAGAG
CATGCCAACACCTTTTCCGCGTTTCTTGACCGCCTTCCGATACCGTCTCTGCACGCA
ATACCTCCGATTCCGTGAACAGGTGCTGCTGATGGCTGGAAAACTCAGTGCCTCTG
CGGAGCTTCGACAGCAGTCTTTGCTGTTGCTGCTGATGCCACTGAGAGCTGTGAGG
35 ACCGTGTCGCGCTCACATGGAACAATCTCCGGA AAAACCTCCTGGTCCATCAGGCAT
CAGAAGGCCTTTTTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGGGAAATGT
TCCGCCTCGAAATTCTGGAGGACATTGCCCGGGATAAAGTCAGAACTCTCCATTTTG
TGGATGAGATAGAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAACTTCAG
CTCTCTACTGCCGTGAAGGAAATGCGTTTCTATGGCGTGTGCGGAGTGACAGCAAAT
40 GACCTCCGCACTGCCGAAGCCATGGTCAGAAGCCGTGAAGAGAATGAATTTACGGA
CTGGTTCTCCCTCTGGGGACCATGGCATGCTGTACTGAAGCGTACGGAAGCTGACCG
CTGGGCGCTGGCAGAAGAGCAGAAATATGAGATGCTGGAGAATGAGTACCCTCAGA
GGGTGGCTGACCGGCTGAAAGCATCAGGTCTGAGCGGTGATGCGGATGCGGAGAGG
GAAGCCGGTGACAGGTGATGCGTGAGACTGAACAGCAGATTTACCGTCAGCTGAC
45 TGACGAGGTACTGGCCCTGCGATTGCCTGAAAACGGCTCACAACTGCACCATTTCATA
A

[0084] A further exemplary E3 ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase IpaH1.4, which is a novel E3 ligase, from *Shigella flexneri* has the amino acid sequence of SEQ ID NO: 5 as follows:

MIKSTNIQAIGSGIMHQINNIYSLTPFPLPMELTPSCNEFYLKAWSEWEKNGTPGEQRNIA
 5 FNRLKICLQNQEAEINLSELDLKTLPDLPPQITTTLEIRKNLLTHLPDLPPMLKVIHAQFNQ
 LESLPALPETLEELNAGDNKIKELPFLPENLTHLRVHNNRLHILPLLPELKLLVVSNGRL
 DSIPFPDKLEGLAMANNFIEQLPELPFSMNRAVLMNNNLTTLPESVRLAQNAFVNVA
 GNPLSGHTMRTLQQITTPDYSGPRIFFSMGNSATISAPEHSLADAVTAWFPENKQSDVS
 10 QIWHAFEHEEHANTFSAFLDRLSDTVSARNTSGFREQVAAWLEKLSASAELRQQSFAVA
 ADATESCEDRVALTWNRLRKTLVHQASEGLFDNDTGALLSLGREMFRLIEDIARDK
 VRTLHFVDEIEVYLAFQTMLAEKLQLSTAVKEMRFYGVSGVTANDLRTAEAMVRSREE
 NEFKDWFSWGPWHAVLKRTEADRWQAEEQKYEMLENEYSQRVADRLKASGLSGD
 TDAEREAGAQMRETEQQIYRQLTDEVLAALRLSENGSNHIA

[0085] The E3 ubiquitin ligase IpaH1.4, which is a novel E3 ligase, from *Shigella*

flexneri has the nucleotide sequence of SEQ ID NO: 6 as follows:

ATGATTAAATCAACCAATATACAGGCAATCGGTTCTGGTATTATGCATCAAATAAAC
 AATATATACTCGTTAACTCCATTTCTTTACCTATGGAAGTACTCCATCTTGTAATG
 AATTTTATTTAAAAGCCTGGAGTGAATGGGAAAAGAACGGTACCCAGGCGAGCAA
 CGCAATATCGCCTTCAATAGGCTGAAAATATGTTTACAAAATCAAGAGGCAGAATT
 20 AAATTTATCTGAGTTAGATTTAAAAACATTACCAGATTTACCGCCTCAGATAACAAC
 ACTGGAAATAAGAAAAAACCTATTAACACATCTCCCTGATTTACCACCAATGCTTAA
 GGTAATACATGCTCAATTTAATCAACTGGAAAGCTTACCTGCCTTACCCGAGACGTT
 AGAAGAGCTTAATGCGGGTGATAACAAGATAAAAGAATTACCATTTCTTCTGAAA
 ATCTAACTCATTTACGGGTTTATAATAACCGATTGCATATTCTGCCACTATTGCCACC
 25 GGAAGTAAAATTACTGGTAGTTTCTGGAAACAGATTAGACAGCATTCCCCCTTTCC
 AGATAAGCTTGAAGGGCTGGCTATGGCTAATAATTTTATAGAACAACCTACCGGAATT
 ACCTTTTAGTATGAACAGGGCTGTGCTAATGAATAATAATCTGACAACACTTCCGGA
 AAGTGTCTGAGATTAGCTCAGAAATGCCTTCGTAAATGTTGCAGGTAATCCACTGTC
 TGGCCATACCATGCGTACACTACAACAATAACCGACCGGACCAGATTATTCTGGTCC
 30 TCGAATATTTTCTCTATGGGAAATTCTGCCACAATTTCCGCTCCAGAACACTCCCTG
 GCTGATGCCGTGACAGCATGGTTCCCGGAAAACAACAATCTGATGTATCACAGAT
 ATGGCATGCTTTTGAACATGAAGAGCACGCCAACACCTTTCCGCGTTCTTGACCG
 CCTTCCGATACCGTCTCTGCACGCAATACCTCCGGATTCCGTGAACAGGTCGCTGC
 ATGGCTGGAAAACTCAGTGCCTCTGCGGAGCTTCGACAGCAGTCTTTCGCTGTTGC
 35 TGCTGATGCCACTGAGAGCTGTGAGGACCGTGTGCGCTCACATGGAACAATCTCCG
 GAAAACCTCCTGGTCCATCAGGCATCAGAAGGCCTTTTCGATAATGATACCGGCGC
 TCTGCTCTCCCTGGGCAGGGAAATGTTCCGCCTCGAAATTCTGGAGGACATTGCCCCG
 GGATAAAGTCAGAAGTCTCCATTTTGTGGATGAGATAGAAGTCTACCTGGCCTTCCA
 GACCATGCTCGCAGAGAACTTCAGCTCTCCACTGCCGTGAAGGAAATGCGTTTCTA
 40 TGGCGTGTGCGGAGTGACAGCAAATGACCTCCGCACTGCCGAAGCCATGGTCAGAA
 GCCGTGAAGAGAATGAATTTAAGGACTGGTTCTCCCTCTGGGGACCATGGCATGCTG
 TACTGAAGCGTACGGAAGCTGACCGCTGGGCGCAGGCAGAAGAGCAGAAGTATGA
 GATGCTGGAGAATGAGTACTCTCAGAGGGTGGCTGACCGGCTGAAAGCATCAGGTC
 TGAGCGGTGATACGGATGCGGAGAGGGAAGCCGGTGCACAGGTGATGCGTGAGACT
 45 GAACAGCAGATTTACCGTCAGTTGACTGACGAGGTACTGGCCCTGCGATTGTCTGAA
 AACGGCTCAAATCATATCGCATAA

[0086] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase IpaH2.5, which is a novel E3 ligase, from *Shigella flexneri* which has the amino acid sequence of SEQ ID NO: 7:

MLIRILVIMIKSTNIQAIGSGIMHQINNVYSLTPLSLPMELTPSCNEFYLKTWSEWEKNGT
 5 PGEQRNIAFNRLKICLQNQEALNLSLDLKTLPDLPPQITTLERKNLLTHLPDLPPMLK
 VIHAQFNQLESPLALPETLEELNAGDNKIKELPFLPENLTHLRVHNNRLHILPLLPELKL
 LVVSGNRLDSIPFPDKLEGLALANNFIEQLPELPFSMNRAVLMNNNLTTLPESVLRQAQ
 NAFVNVAGNPLSGHTMRTLQQITTPDYSGPRIFFSMGNSATISAPESHSLADAVTAWFPE
 10 NKQSDVSQIWHAFEHEEHANTFSAFLDRLSDTVSARNTSGFREQVAAWLEKLSASAELR
 QQSFVAADATESCEDRVALTWNLRKTLLVHQASEGLFDNDTGALLSLGREMFRLEI
 LEDIARDKVRTLHFVDEIEVYLAFQTMLAEKLQLSTAVKEMRFYGVSGVTANDLRTAE
 AMVRSREENEFTDWFSLWGPWHAFLKRTEADRWAAEQKYEMLENEYSQRVADRL
 KASGLSGDADAEREAGAQMRETEQQIYRQLTDEVLA

[0087] The E3 ubiquitin ligase IpaH2.5, which is a novel E3 ligase, from *Shigella*

15 *flexneri* has the nucleotide sequence of SEQ ID NO: 8 as follows:

atgTTGATAAGAATTCTAGTTATAATGATTAAATCAACCAATATACAGGCAATCGGTT
 CTGGCATTATGCATCAAATAACAATGTATACTCGTTAACTCCATTATCTTTACCTAT
 GGAACCTGACTCCATCTTGTAATGAATTTTATTTAAAAACCTGGAGCGAATGGGAAAA
 GAACGGTACCCAGGCGAGCAACGCAATATCGCCTTCAATAGGCTGAAAATATGTT
 20 TACAAAATCAAGAGGCAGAATTAAATTTATCTGAGTTAGATTTAAAAACATTACCA
 GATTTACCGCCTCAGATAACAACACTGGAAATAAGAAAAAACCTATTAACACATCT
 CCCTGATTTACCACCAATGCTTAAGGTAATACATGCTCAATTTAATCAACTGGAAAG
 CTTACCTGCCTTACCCGAGACGTTAGAAGAGCTTAATGCGGGTGATAACAAGATAA
 AAGAATTACCATTTCTTCCTGAAAATCTAACTCATTTACGGGTTCATAATAACCGAT
 25 TGCATATTCTGCCACTATTGCCACCGGAACTAAAATTACTGGTAGTTTCTGGAAACA
 GATTAGACAGCATTCCCCCTTTCCAGATAAGCTTGAAGGGCTGGCTCTGGCTAATA
 ATTTTATAGAACAACACTACCGGAATTACCTTTTAGTATGAACAGGGCTGTGCTAATGA
 ATAATAATCTGACAACACTTCCGGAAGTGTCCTGAGATTAGCTCAGAATGCCTTCG
 TAAATGTTGCAGGTAATCCATTGTCTGGCCATACCATGCGTACACTACAACAAATAA
 30 CCACCGGACCAGATTATTCTGGTCCTCGAATATTTTTCTCTATGGGAAATTCTGCCAC
 AATTTCCGCTCCAGAACACTCCCTGGCTGATGCCGTGACAGCATGGTTCCCGGAAAA
 CAAACAATCTGATGTATCACAGATATGGCATGCTTTTGAACATGAAGAGCATGCCA
 ACACCTTTTCCGCGTTCCCTGACCGCCTTTCCGATACCGTCTCTGCACGCAATACCTC
 CGGATTCCGTGAACAGGTGCTGATGGCTGGAAAACTCAGTGCCTCTGCGGAGC
 35 TTCGACAGCAGTCTTTGCTGTTGCTGCTGATGCCACTGAGAGCTGTGAGGACCGTG
 TCGCGCTCACATGGAACAATCTCCGGAACCCCTCCTGGTCCATCAGGCATCAGAA
 GGCCTTTTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGGGAAATGTTCCGC
 CTCGAAATTCTGGAGGATATTGCCCGGGATAAAGTCAGAACTCTCCATTTTGTGGAT
 GAGATAGAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAACTTCAGCTCTCT
 40 ACTGCCGTGAAGGAAATGCGTTTCTATGGCGTGTGCGGAGTGACAGCAAATGACCT
 CCGCACTGCCGAAGCCATGGTCAGAAGCCGTGAAGAGAATGAATTTACGGACTGGT
 TCTCCCTCTGGGGACCATGGCATGCTGTACTGAAGCGTACGGAAGCTGACCGCTGGG
 CGCAGGCAGAAGAGCAGAAGTATGAGATGCTGGAGAATGAGTACTCTCAGAGGGT
 GGCTGACCGGCTGAAAGCATCAGGTCTGAGCGGTGATGCGGATGCGGAGAGGGAA
 45 GCCGGTGACAGGTGATGCGTGAGACTGAACAGCAGATTTACCGTCAGTTGACTGA
 CGAGGTACTGGCCTGA

[0088] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase IpaH4.5, which is a novel E3 ligase, from *Shigella flexneri* and has the amino acid sequence of SEQ ID NO: 9:

5 MKPINNHSFFRSLCGLSCISRLSVEEQCTRDYHRIWDDWAREGTTTENRIQAVRLLKICL
DTREPVLNLSLLKLRLSLPPLPHIRELNISNNELISLPENSPLLTELHVNGNNLNILPTLPSQ
LIKLNISFNRLNSCLPSLPYLQSL SARFNSLETLPSTLTILRIEGRNLTVLPPELPHRLQE
LFVSGNRLQELPEFPQSLKYLVGENQLRRLSRLPQELLALDVSNNLLTSLPENIITLPIC
10 NVNISGNPLSTRVLQSLQRLTSSPDYHGPQIYFMSDGGQNTLHRPLADAVTAWFPENK
QSDVSIWHFAFEHEEHANTFSAFLDRLSDTVSARNTSGFREQVA AWLEKLSASAELRQQ
SFAVAADATESCEDRVALTWNRLRK TLLVHQASEGLFDNDTGALLSLGREMFRLEILED
IARDKVR TLHFVDEIEVYLAFQTMLAEKLQLSTAVKEMRFYGVSGVTANDLRTAEAMV
RSREENEFTDWFSLWGPWHAVLKRTEADRW AQAEQKYEMLENEYSQRVADRLKAS
GLSGDADAQREAGA QVMRETEQQIYRQLTDEVLA

[0089] The E3 ubiquitin ligase IpaH4.5, which is a novel E3 ligase, from *Shigella*

15 *flexneri* has the nucleotide sequence of SEQ ID NO: 10 as follows:

ATGAAACCGATCAACAATCATTCTTTTTTCGTTCCCTTTGTGGCTTATCATGTATAT
CTCGTTTATCGGTAGAAGAACAGTGTACCAGAGATTACCACCGCATCTGGGATGACT
GGGCTAGGGAAGGAACAACAACAGAAAATCGCATCCAGGCGGTTTCGATTATTGAAA
ATATGTCTGGATACCCGGGAGCCTGTTCTCAATTTAAGCTTACTGAACTACGTTCTT
20 TACCACCACTCCCTTTGCATATACGTGAACCTAATATTTCCAACAATGAGTTAATCTC
CCTACCTGAAAATTCTCCGCTTTTGACAGAACTTCATGTAAATGGTAACAACCTGAA
TATACTCCCGACACTTCCATCTCAACTGATTAAGCTTAATATTTTATTCAATCGAAAT
TTGTCATGTCTGCCATCATTACCACCATATTTACAATCACTCTCGGCACGTTTAAATA
GTCTGGAGACGTTACCAGAGCTTCCATCAACGCTAACAATATTACGTATTGAAGGTA
25 ATCGCCTTACTGTCTTGCCCTGAATTGCCTCATAGACTACAAGAACTCTTTGTTTCCGG
CAACAGACTACAGGAACCTACCAGAATTTCTCAGAGCTTAAAATATTTGAAGGTAG
GTGAAAATCAACTACGCAGATTATCCAGATTACCGCAAGAACTATTGGCTCTGGATG
TTTCCAATAACCTACTAACTTCATTACCCGAAAATATAATCACATTGCCCATTTGTAC
GAATGTTAACATTTTCAGGGAATCCATTGTCTGACTCGCGTTCTGCAATCCCTGCAAAG
30 ATTAACCTCTTCGCCGACTACCACGGCCCGCAGATTTACTTCTCCATGAGTGACGG
ACAACAGAATACACTCCATCGCCCCCTGGCTGATGCCGTGACAGCATGGTTCCCGGA
AAACAAACAATCTGATGTATCACAGATATGGCATGCTTTTGAACATGAAGAGCATG
CCAACACCTTTTCCGCGTTCTTGACCGCCTTTCCGATACCGTCTCTGCACGCAATAC
CTCCGGATTCCGTGAACAGGTGCTGCTGATGGCTGGAAAACTCAGTGCCTCTGCGGA
35 GCTTCGACAGCAGTCTTTTCGCTGTTGCTGCTGATGCCACTGAGAGCTGTGAGGACCG
TGTCGCGCTCACATGGAACAATCTCCGGAAAACCTCCTGGTCCATCAGGCATCAGA
AGGCCTTTTTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGGGAAATGTTCCG
CCTCGAAATTCTGGAGGACATTGCCCGGGATAAAGTCAGAACTCTCCATTTTGTGGA
TGAGATAGAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAACTTCAGCTCTC
40 CACTGCCGTGAAGGAAATGCGTTTCTATGGCGTGTGCGGAGTGACAGCAAATGACC
TCCGCACTGCCGAAGCTATGGTCAGAAAGCCGTGAAGAGAATGAATTTACGGACTGG
TTCTCCCTCTGGGGACCATGGCATGCTGTACTGAAGCGTACGGAAGCTGACCGCTGG
GCGCAGGCAGAAGAGCAGAAGTATGAGATGCTGGAGAATGAGTACTCTCAGAGGG
TGGCTGACCGGCTGAAAGCATCAGGTCTGAGCGGTGATGCGGATGCGCAGAGGGAA
45 GCCGGTGACAGGTGATGCGTGAGACTGAACAGCAGATTTACCGTCAGCTGACTGA
CGAGGTACTGGCCTGA

[0090] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase IpaH7.8, which is a novel E3 ligase, from *Shigella flexneri* and has the amino acid sequence of SEQ ID NO: 11:

MFSVNNTHSSVSCSPSINSNSTSNEHYLRILTEWEKNSSPGEERGIAFNRLSQCFQNQEAV
 5 LNLSDLNLTSLPELPKHISALIVENNKLTSLPKLPAFLKELNADNNRLSVIPELPESLTTL
 VRSNQLENLPVLPNHLTSLFVENNRLYNLPALPEKLFHLYVYNNRLTTLPLDPDKLEILC
 AQRNNLVTFPQFSDRNNIRQKEYYFHFNQITTLPEFSQLDSSYRINISGNPLSTRVLQSLQ
 RLTSPPDYHGPQIYFMSDGGQNTLHRPLADAVTAWFPENKQSDVSQIWHAFEHEEHA
 NTFSAFLDRLSDTVSARNTSGFREQVAAWLEKLSASAELRQQSFAVAADATESCEDRVA
 10 LTWNNLRKTLVHQASEGLFDNDTGALLSLGREMFRLEILEDIARDKVRTLHFVDEIEV
 YLAFQTMLAEKLQLSTAVKEMRFYGVSGVTANDLRTAEAMVRSRENEFTDWFSLWG
 PWHA VLKRTEADRWAAEQKYEMLENEYSQRVADRLKASGLSGDADAEREAGA QV
 MRETEQQIYRQLTDEV LALRLSENGSRLHHS

[0091] The E3 ubiquitin ligase IpaH7.8, which is a novel E3 ubiquitin ligase, from

Shigella flexneri has the nucleotide sequence of SEQ ID NO: 12 as follows:

ATGTTCTCTGTAAATAATACACACTCATCAGTTTCTTGCTCCCCCTCTATTA ACTCAA
 ACTCAACCAGTAATGAACATTATCTGAGAATCCTGACTGAATGGGAAAAGAACTCT
 TCTCCCGGGGAAGAGCGAGGCATTGCTTTTAAACAGACTCTCCAGTGCTTTCAGAAT
 CAAGAAGCAGTATTAAATTTATCAGACCTAAATTTGACGTCTCTTCCCGAATTACCA
 20 AAGCATATTTCTGCTTTGATTGTAGAAAATAATAAATTAACATCATTGCCAAAGCTG
 CCTGCATTTCTTAAAGAACTTAATGCTGATAATAACAGGCTTTCTGTGATACCAGAA
 CTTCTGAGTCATTAACAACCTTTAAGTGTTCTGTTCTAATCAACTGGAAAACCTTCCTG
 TTTTGCCAAACCATTTAACATCATTATTTGTTGAAAATAACAGGCTATATAACTTACC
 GGCTCTTCCCGAAAAATTGAAATTTTACATGTTTATTATAACAGGCTGACAACATT
 25 ACCCGACTTACCGGATAAACTGGAAATTTCTCTGTGCTCAGCGCAATAATCTGGTTAC
 TTTTCCTCAATTTTCTGATAGAAACAATATCAGACAAAAGGAATATTATTTTCATTTT
 AATCAGATAACCACTCTTCCGGAGAGTTTTTCACAATTAGATTCAAGTTACAGGATT
 AATATTTTCAAGGAATCCATTGTCGACTCGCGTTCTGCAATCCCTGCAAAGATTAACC
 TCTTCGCCGGACTACCACGGCCACAGATTTACTTCTCCATGAGTGACGGACAACAG
 30 AATACTACTCCATCGCCCCCTGGCTGATGCCGTGACAGCATGGTTCCCGGAAAACAA
 ACAATCTGATGTATCACAGATATGGCATGCTTTTGAACATGAAGAGCATGCCAACAC
 CTTTTCCGCGTTCCTTGACCGCCTTTCCGATACCGTCTCTGCACGCAATACCTCCGGA
 TTCCGTGAACAGGTCGCTGCATGGCTGGAAAACTCAGTGCTCTGCGGAGCTTCGA
 CAGCAGTCTTTCGCTGTTGCTGCTGATGCCACTGAGAGCTGTGAGGACCGTGTCGCG
 35 CTCACATGGAACAATCTCCGGA AAAACCTCCTGGTCCATCAGGCATCAGAAGGCCTT
 TTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGGGAAATGTTCCGCCTCGAA
 ATTCTGGAGGACATTGCCCGGGATAAAGTCAGAACTCTCCATTTTGTGGATGAGATA
 GAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAACTTCAGCTCTCTACTGCC
 GTGAAGGAAATGCGTTTCTATGGCGTGTGCGGAGTGACAGCAAATGACCTCCGCAC
 40 TGCCGAAGCCATGGTCAGAAGCCGTGAAGAGAATGAATTTACGGACTGGTTCTCCC
 TCTGGGGACCATGGCATGCTGTACTGAAGCGTACGGAAGCTGACCGCTGGGCGCAG
 GCAGAAGAGCAGAAGTATGAGATGCTGGAGAATGAGTACTCTCAGAGGGTGGCTGA
 CCGGCTGAAAGCATCAGGTCTGAGCGGTGATGCGGATGCGGAGAGGGAAGCCGGTG
 CACAGGTGATGCGTGAGACTGAACAGCAGATTTACCGTCAGTTGACTGACGAGGTA
 45 CTGGCCCTGCGATTGTCTGAAAACGGCTCACGACTGCACCATTTCATAA

[0092] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase IpaH9.8, which is a novel E3 ligase, from *Shigella flexneri* and has the amino acid sequence of SEQ ID NO: 13:

MLPINNNFSLPQNSFYNTISGTYADYFSAWDKWEKQALPGEERDEAVSRLKECLINNSD
 5 ELRLDRLNLSSLPDNLPAQITLLNVSYNQLTNLPELPVTLKKLYSASNKLSSELPVLPPALE
 SLQVQHNELENLPALPDSLLTMNISYNEIVSLPSLPQALKNLRA TRNFL TELPAFSEGN NP
 VVREYFFDRNQISHIPESILNLRNECSIHISDNPLSSHALQALQRLTSSPDYHGPRIYFSMS
 DGQQNTLHRPLADAVTAWFPENKQSDVSIWHA FEHEEHANTFSAFLDRLSDTVSARN
 TSGFREQVA AWLEKLSASAELRQQSFAVAADATESCEDRVALTWNNLRKTLLVHQASE
 10 GLFDNDTGALLSLGREMFRLEILEDIARDKVRTLHFVDEIEVYLA FQTMLAEKLQLSTAV
 KEMRFYGVSGVTANDLRTAEAMVRSRENEFTDWFSLWGPWHAVLKRTEADRWAQA
 EEQKYEMLENEY PQRVADRLKASGLSGDADAEREAGA QVMRETEQQIYRQLTDEVLA
 LRLSENGSQLHHS

[0093] The E3 ubiquitin ligase IpaH9.8, which is a novel E3 ligase, from *Shigella*

flexneri has the nucleotide sequence of SEQ ID NO: 14 as follows:

ATGTTACCGATAAATAATAACTTTTCATTGCCCCAAAATTCTTTTATAACACTATTT
 CCGGTACATATGCTGATTACTTTTCAGCATGGGATAAATGGGAAAAACAAGCGCTCC
 CCGGTGAAGAGCGTGATGAGGCTGTCTCCCGACTTAAAGAATGTCTTATCAATAATT
 CCGATGAACTTCGACTGGACCGTTTAAATCTGTCTCGCTACCTGACAACTTACCAG
 20 CTCAGATAACGCTGCTCAATGTATCATATAATCAATTAACCTACCTGAACTGC
 CTGTTACGCTAAAAAAATTATATTCGCCAGCAATAAATTATCAGAATTGCCCGTGC
 TACCTCCTGCGCTGGAGTCACTTCAGGTACAACACAATGAGCTGGAAAACCTGCCA
 GCTTTACCCGATTCTGTTATTGACTATGAATATCAGCTATAACGAAATAGTCTCCTTAC
 CATCGCTCCCACAGGCTCTTAAAAATCTCAGAGCGACCCGTAATTTCTCACTGAGC
 25 TACCAGCATTTTCTGAGGGGAAATAATCCCGTTGTGAGAGAGTATTTTTTTGATAGAA
 ATCAGATAAGTCATATCCCGGAAAGCATTCTTAATCTGAGGAATGAATGTTCAATAC
 ATATTAGTGATAACCCATTATCATCCCATGCTCTGCAAGCCCTGCAAAGATTAACTT
 CTTCCGCCGACTACCACGGCCCACGGATTTACTTCTCCATGAGTGACGGACAACAGA
 ATACACTCCATCGCCCCCTGGCTGATGCCGTGACAGCATGGTTCCCGGAAAACAAAC
 30 AATCTGATGTATCACAGATATGGCATGCTTTTGAACATGAAGAGCATGCCAACACCT
 TTCCGCGTTCTTTGACCGCCTTTCCGATACCGTCTCTGACGCAATACCTCCGGATT
 CCGTGAACAGGTCGCTGCATGGCTGGAAAACTCAGTGCCTCTGCGGAGCTTCGAC
 AGCAGTCTTTTCGCTGTTGCTGCTGATGCCACTGAGAGCTGTGAGGACCGTGTGCGC
 TCACATGGAACAATCTCCGGAAAACCTCCTGGTCCATCAGGCATCAGAAGGCCTTT
 35 TCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGGGAAATGTTCCGCCTCGAAA
 TTCTGGAGGATATTGCCCCGGGATAAAGTCAGAACTCTCCATTTTGTGGATGAGATAG
 AAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAACTTCAGCTCTCCACTGCCG
 TGAAGGAAATGCGTTTCTATGGCGTGTGCGGAGTGACAGCAAATGACCTCCGCACT
 GCCGAAGCCATGGTCAGAAGCCGTGAAGAGAATGAATTTACGGACTGGTTCTCCCT
 40 CTGGGGACCATGGCATGCTGTACTGAAGCGTACGGAAGCTGACCGCTGGGCGCAGG
 CAGAAGAGCAGAAATATGAGATGCTGGAGAATGAGTACCCTCAGAGGGTGGCTGAC
 CGGCTGAAAGCATCAGGTCTGAGCGGTGATGCGGATGCGGAGAGGGAAGCCGGTGC
 ACAGGTGATGCGTGAGACTGAACAGCAGATTTACCGTCAGCTGACTGACGAGGTAC
 TGGCCCTGCGATTGTCTGAAAACGGCTCACAACTGCACCATTTCATAA

[0094] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase LegAU13, which is a F-box motif, from *Legionella pneumophila* and has the amino acid sequence of SEQ ID NO: 15:

MKKNFFSDLPEETIVNTLSFLKANTLARIAQTCQFFNRLANDKHLELHQLRQQHIKREL
WGNLMVAARSNNLEEVKKILKKGIDPTQTNSYHLNRTPLLAIEGKAYQTANYLWRKY
TFDPNFKDNYGDSPISSLKKQLANPAFKDKEKKQIRALIRGMQEEKIAQSKCLVC

[0095] The E3 ubiquitin ligase LegAU13, which is a F-box motif, from *Legionella*

5 *pneumophila* has the nucleotide sequence of SEQ ID NO: 16 as follows:

ATGAAAAAGAATTTTTTTCTGATCTTCCTGAGGAAACAATTGTCAATACATTGAGT
TTCTTAAAAGCAAACACACTAGCTCGTATAGCTCAGACATGTCAATTTTTTAATCGC
TTGGCTAATGATAAACATCTGGAGCTGCATCAACTAAGACAACAGCATATAAAGCG
AGAGCTATGGGGAAATCTTATGGTGGCGGCAAGAAGCAATAACCTGGAAGAGGTCA
10 AAAAGATTCTAAAAAAGGAATCGATCCAACCCAGACCAATAGCTACCACTTAAAT
AGAACGCCTTTACTTGCAGCTATCGAAGGAAAAGCATATCAAAGTCAAATTACCTC
TGGAGAAAATACACTTTCGATCCCAATTTTAAAGATAACTATGGTGATTACCTATC
TCTCTTCTTAAAAAGCAACTGGCAAATCCAGCCTTCAAGGATAAGGAAAAAACA
AATACGCGCCTTAATTAGGGGAATGCAAGAAGAAAAAATAGCACAGAGCAAGTGC
15 CTTGTTTGTTAA

[0096] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase LegU1, which is a F-box motif, from *Legionella pneumophila* and has the amino acid sequence of SEQ ID NO: 17:

20 MKAKYDPTKPGQLKLPPEIKVMILEFLDAKSKLALSQTNYGWRDLILDRPEYTKEITNTL
FRLDKKRHRQAIAQMMSGRTVASSMAKLFEELLCSIPSSYVFLIFFASQKSVALIEVLTV
ILVFAAITSLAHDLDYFIESDTKAQKQHAHRRAFQFFAQPSQSAQQNLEENLSADPK
ACQCEPL

[0097] The E3 ubiquitin ligase LegU1, which is a F-box motif, from *Legionella pneumophila* has the nucleotide sequence of SEQ ID NO: 18 as follows:

25 ATGAAAGCAAATACGACCCACAAAGCCTGGACTCCAAAAGTTACCTCCTGAAAT
CAAGGTAATGATTCTTGAGTTTCTTGATGCCAAATCAAAGTCTTTTCACAGAC
AAATTATGGTTGGCGTGATTAAATTCTAGACCGGCCAGAATATACCAAAGAAATAA
CGAATACATTATTTTCGTCTTGATAAAAAACGCCATCGTCAAGCAATAGCACAAATGA
TGTCAGGAAGAGTTACAGCAAGTTCTATGGCTAAGCTATTTGAAGAATTACTATGTT
30 TTAGCATACCTTCGTCTCTATGTGTTTTTAATCTTTTTCGCATCGCAAAAATCTGTGGC
GCTTATAGAAGTCTTAACCGTAATCCTTGTTGTTTGCTGCAATAACCTCTCTCGCCCAT
GATCTGGTGGATTATTTTATTGAAAGTGATACAAAAGCTGAGAAACAGCATGCACA
TCGCCGTGCTTTTCAATTCTTTGCCCAACCCAGTCAAAGCGCTGCACAACAAACTT
GGAGGAAGAGAATTTAAGTGCTGATCCCAAGGCCTGCCAATGTGAGCCATTGTAG

35 **[0098]** A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase LubX, which is a U-box motif, from *Legionella pneumophila* and has the amino acid sequence of SEQ ID NO: 19:

40 MATRNPFDIDHKSKYLREAALEANLSHPETPTMLTCPIDSGFLKDPVITPEGFVYNKSSI
LKWLETKKEDPQSRKPLTAKDLQFPPELLIIVNRFVETQTNYEKLKNRLVQNARVAARQ
KEYTEIPDIFLCPISKTLIKTPVITAQGVYDQEALSNFLIATGNKDETGKKLSIDDVVVF
ELYQQIKVYNFYRKREVQKNQIQPSVSNGFGFFSLNFLTSWLWGTEEKKEKTSSDMTY

[0099] The E3 ubiquitin ligase LubX, which is a U-box motif, from *Legionella pneumophila* has the nucleotide sequence of SEQ ID NO: 20 as follows:

ATGGCGACGCGAAATCCTTTTGATATTGATCATAAAAGTAAATACTTAAGAGAAGC
 AGCATTAGAAGCCAATTTATCTCATCCAGAAACAACACCAACAATGCTGACTTGCCC
 TATTGACAGCGGATTTCTAAAAGATCCCGTGATCACACCTGAAGGTTTTGTTTATAA
 TAAATCCTCTATTTTAAAATGGTTAGAAACGAAAAAAGAAGACCCACAAAGCCGTA
 5 AACCCTTAACGGCTAAAGATTTGCAACCATTCCCCGAGTTATTGATTATAGTCAATA
 GATTTGTTGAGACACAAACGAACCTATGAAAAATTAAAAAACAGATTAGTGCAAAAT
 GCTCGGGTTGCTGCACGCCAAAAAGAATACACTGAAATTCCGGATATATTTCTTTGC
 CCAATAAGTAAAACGCTTATCAAAACACCTGTCATTACTGCCCAAGGGAAAGTATA
 TGATCAAGAAGCATTAAGTAACTTTCTTATCGCAACGGGTAATAAAGATGAAACAG
 10 GCAAAAAATTATCCATTGATGATGTAGTGGTGTGTTGATGAACTCTATCAACAGATAA
 AAGTTTATAATTTTTACCGCAAACGCGAAGTGCAAAAAAATCAAATTCAACCTTCAG
 TAAGTAATGGTTTTGGCTTTTTTAGCTTGAATTTTCTCACCTCATGGTTATGGGGAAC
 TGAGGAGAAAAAAGAAAAGACATCATCTGATATGACGTACTAA

[0100] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in
 15 accordance with the present application includes the E3 ubiquitin ligase NleG2-3, which is a U-
 box motif, Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and has the amino acid
 sequence of SEQ ID NO: 21:

MPLTSDIRSHSFNLGVEVVRARIVANGRGDITVGGGETVSIVYDSTNGRFSSSGGNGGLLS
 ELLLLGFNSGPRALGERMLSMLSDSGEAQSQESIQNKISQCKFSVCPERLQCPLEAIQCPI
 20 TLEQPEKGIFVKNSDGSVDCTLFDAAAF SRLVGEGLPHPLTREPI TASIIVKHEECIYDDTR
 GNFIKGN

[0101] The E3 ubiquitin ligase NleG2-3, which is a U-box motif, from
 Enterohemorrhagic *Escherichia coli* ("EHEC") O157:H7, has the nucleotide sequence of SEQ
 ID NO: 22 as follows:

ATGCCATTAACCTCAGATATTAGATCACATTCATTTAATCTTGGGGTGGAGGTTGTT
 CGTGCCCGAATTGTAGCCAATGGGCGCGGAGATATTACAGTCGGTGGTGAAACTGT
 CAGTATTGTGTATGATTCTACTAATGGGCGCTTTTCATCCAGTGGCGGTAATGGCGG
 ATTGCTTTCTGAGTTATTGCTTTTGGGATTTAATAGTGGTCCTCGAGCCCTTGGTGAG
 AGAATGCTAAGTATGCTTTCGGACTCAGGTGAAGCACAAATCGCAAGAGAGTATTCA
 25 GAACAAAATATCTCAATGTAAGTTTTCTGTTTGTCCAGAGAGACTTCAGTGCCCGCT
 TGAGGCTATTCAGTGTCCAATTACACTGGAGCAGCCTGAAAAAGGTATTTTTGTGAA
 GAATTCAGATGGTTCAGATGTATGTACTTTATTTGATGCCGCTGCATTTTCTCGTTTG
 30 GTTGGTGAAGGCTTACCCACCCACTGACCCGGAACCAATAACGGGCATCAATAAT
 TGTAACACATGAAGAATGCATTTATGACGATACCAGAGGAACTTCATTATAAAGG
 35 GTAATTGA

[0102] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in
 accordance with the present application includes the E3 Ubiquitin Ligase NleG5-1, which is a U-
 box motif, from Enterohemorrhagic *Escherichia coli* ("EHEC") O157:H7, and has the amino
 acid sequence of SEQ ID NO: 23:

MPVDLTPYILPGVSFLSDIPQETLSEIRNQTIRGEAQIRLGELMVSIRPMQVNGYFMGSLN
 QDGLSNDNIQIGLQYIEHIERTLNHGSLTSREVTVLREIEMLENMDLLSNYQLEELDKIE
 40 VCAFNVHAQLQVPESLRTCPTLCEPEDGVFMRNSMNSNVCMLYDKMALIHLVKTR
 AAHPLSRESIAVSMIVGRDNCAFDPRGNFVLKN

[0103] The E3 ubiquitin ligase NleG5-1, which is a U-box motif, from Enterohemorrhagic *Escherichia coli* ("EHEC") O157:H7, has the nucleotide sequence of SEQ ID NO: 24 as follows:

5 ATGCCTGTAGATTTAACGCCTTATATTTTACCTGGGGTTAGTTTTTTGTCTGACATTC
CTCAAGAAACCTTGTCTGAGATACGTAATCAGACTATTTCGTGGAGAAGCTCAAATA
AGACTGGGTGAGTTGATGGTGTCAATACGACCTATGCAGGTAAATGGATATTTTATG
GGAAGTCTTAACCAGGATGGTTTATCGAATGATAATATCCAGATTGGCCTTCAATAT
ATAGAACATATTGAACGTACACTTAATCATGGTAGTTTGACAAGCCGTGAAGTTACA
GTACTGCGTGAAATTGAGATGCTCGAAAATATGGATTTGCTTTCTAACTACCAGTTA
10 GAGGAGTTGTTAGATAAAATTGAAGTATGTGCATTTAATGTGGAGCATGCACAATTG
CAAGTGCCAGAGAGCTTACGAACATGCCCTGTTACATTATGTGAACCAGAAGATGG
GGTATTTATGAGGAATTCAATGAATTCAAATGTTTGTATGTTGTATGATAAAATGGC
ATTAATACATCTTGTTAAAACAAGGGCGGCTCATCCTTTGAGCAGGGAATCAATCGC
AGTTTCAATGATTGTAGGAAGAGATAATTGTGCTTTTGACCCTGACAGAGGTAACCT
15 CGTTTTAAAAAATTAA

[0104] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase NleL, which is a HECT motif, from Enterohemorrhagic *Escherichia coli* ("EHEC") O157:H7, and has the amino acid sequence of SEQ ID NO: 25:

20 MLPTTNISVNSGVISFESPVDSNEDVEVALEKWCAEGEFSEN RHEVASKILDVISTNGE
TLSISEPITTLPLDLLPGSLKELVLNGCTELKSINCLPPNLSSLMSVGCSSLEVINC SIPENVIN
LSLCHCSSLKHIIEGSFPEALRNSVYLN GCNSLNESQCQFLAYDVSQGRACLSKAELTADL
IWLSANRTGEESA EELNYS GCDLSGLSLVGLNLSSVNFSGAVLDDTDLRMSDLSQAVLE
NCSFKNSILNECNFCYANLSNCIIRALFENS NFNSNLKNASFKGSSYIQYPILNEADLTG
25 AIIPGMVLSGAILGDVKELFSEKSN TINLGGCYIDLSDIQENILSVLDNYTKSNKSILLTM
NTSDDKYNHDKVRAAEELIKKISLDELA AFRPYVKMSLADSF SIHPYLN NANIQQWLEPI
CDDFFDTIMSWFNNSIMMYMENGSL LQAGMYFERHPGAMVSYNSSFIQIVMNGSRRDG
MQERFREL YEYVLKNEKVYPVTQQSD FGLCDGSGKPDWDDSDLAYNWVLLSSQDDG
MAMMCSLSHMVDMLSPNTSTNWMSFFLYKDGEVQNTFGYSLSNLFSSEFPIFSIPYHKA
30 FSQNFVSGILDILISDNELKERFIEALNSNKSDYKMIADDQQRKLACVWNPF LDGWELNA
QHVDMMIMGSHVLKDMPLRKQAEILFCLGGVFCKYSSSDMFGTEYDSPEILRRYANGLIE
QAYKTDPQVFGSVYYYNDILDR LQGRNNVFTCTAVLTDMLTEHAKESFPEIFSLYYPVA
WR

[0105] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase NleL, which is a HECT motif, from Enterohemorrhagic *Escherichia coli* ("EHEC") O157:H7, and has the nucleotide sequence of SEQ ID NO: 26:

40 ATGCTGCCCCACTACAAATATCTCTGTAAATTCTGGAGTAATATCTTTTGAAAGTCCT
GTAGATTACCATCTAACGAGGATGTTGAAGTTGCCCTCGAAAAGTGGTGTGCTGAG
GGAGAATTTAGCGAAAATCGTCATGAGGTTGCATCAAAAATACTTGATGTTATAAGT
ACTAATGGAGAGACTTTATCAATCAGTGAGCCAATAACAACATTACCAGACTTGCTT
CCAGGTTCTCTGAAAGAACTGGTTTTGAATGGATGTACAGAGCTTAAATCAATAAAC
TGCTTACCCCCCAACTTATCTTCATTAAGTATGGTTGGATGCTCATCATTAGAGGTTA
TAAATTGCAGCATACCTGAAAATGTCATTAATTTATCTTTATGCCATTGTAGTTCTTT
45 GAAACATATAGAAGGTTCTTTCTGAGGCACTCAGAAATTCGTATATTTAATGG

CTGTAATTCATTAAATGAATCGCAATGTCAATTCCTTGCATATGATGTCAGTCAAGG
 CCGTGCCTGCCTGAGCAAAGCTGAGCTTACTGCTGACTTAATTTGGTTGTCAGCTAA
 CCGAACGGGTGAAGAGTCTGCTGAAGAATTGAATTACTCTGGATGTGACTTGTGAG
 GTCTAAGTCTTGTAGGGCTGAATTTATCATCAGTAAATTTTCTGGAGCAGTGCTTG
 5 ATGATACAGATCTCAGGATGAGTGATTTGTCTCAGGCTGTATTGGAAAACGTGTTCTT
 TTA AAAA ACTCGATTTTGAATGAATGTAATTTTGTATGCTAATTTATCTAATTGTAT
 TATTAGGGCTTTGTTTGAAAACCTAATTTTCAGCAATTCCAATCTTAAAAATGCATC
 ATTTAAAGGATCTTCATATATACAATATCCTCCAATTTGAACGAGGCTGATTTAAC
 AGGAGCTATTATAATTCCTGGAATGGTTTTAAGTGGTGCTATCTTAGGTGATGTAAA
 10 GGAGCTCTTTAGTGAAAAAAGTAATACCATTAATCTAGGAGGGTGTTACATAGATCT
 ATCTGACATACAGGAAAATATATTATCTGTGTTGGATAACTATACAAAATCAAATAA
 ATCAATTTTATTGACTATGAATACATCTGATGATAAGTATAACCATGATAAAGTAAG
 GGCCGCTGAAGAACTTATCAAAAAAATATCTCTTGACGAATTAGCGGCGTTCCGGCC
 CTATGTAAAGATGTCTTTGGCTGATTCATTTAGTATTCATCCTTATTTGAACAACGCA
 15 AATATACAGCAATGGCTCGAGCCTATATGTGATGACTTTTTTGATACTATAATGTCTT
 GGTTTAATAATTCAATAATGATGTATATGGAGAATGGTAGTTTATTGCAGGCAGGGA
 TGTATTTTGAGCGACATCCAGGTGCGATGGTATCTTATAATAGTTCCTTTATACAAAT
 TGTAATGAATGGTTCACGGCGTGATGGAATGCAGGAACGATTTAGGGAACCTCTATG
 AAGTATATTTAAAAAATGAAAAAGTTTATCCTGTACACAGCAGAGTGATTTTGGAT
 20 TGTGCGATGGCTCTGGGAAGCCTGACTGGGATGATGATTCCGATTTGGCTTATAACT
 GGGTTTTGTTATCATCACAGGATGATGGTATGGCAATGATGTGTTCTTTGAGTCATA
 TGGTTGATATGTTATCTCCTAATACATCAACTAATTGGATGTCCTTTTTTTTATATAA
 GGATGGAGAAGTTCAAAATACATTTGGGTATTCATTGAGCAATCTTTTTTCTGAATC
 ATTTCCAATTTTCAGTATTCCTTATCATAAAGCTTTTTCCCAGAATTCGTTTCTGGTA
 25 TTCTGGATATACTCATTCTGATAATGAACTCAAAGAGAGATTTATTGAGGCACTTA
 ATTCCAATAAATCAGATTATAAAATGATTGCTGATGATCAGCAAAGGAACTTGCCT
 GTGTCTGGAATCCCTTTCTTGATGGTTGGGAACCTGAACGCTCAGCATGTAGATATGA
 TTATGGGGAGCCATGTATTGAAAGATATGCCACTAAGAAAACAGGCTGAAATATTA
 TTTTGTTTAGGGGGGGTTTTCTGTAAATACTCATCGAGTGATATGTTTGGTACAGAGT
 30 ATGATTCTCCTGAGATTCTACGGAGATATGCAAATGGATTGATTGAACAAGCTTATA
 AAACAGATCCTCAGGTATTTGGCTCAGTTTATTATTACAATGATATTTTAGACAGGC
 TACAAGGAAGAAATAATGTTTTTACTTGTACCGCTGTGCTGACTGATATGCTAACGG
 AGCATGCAAAAGAATCTTTTCCTGAAATATTTTCATTGTATTATCCTGTTGCGTGGCG
 TTGA

35 **[0106]** A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in
 accordance with the present application includes the E3 ubiquitin ligase SidC, which is an
 unconventional motif, from *L. pneumophila*, and has the amino acid sequence of SEQ ID NO:
 27:

40 MVINMVDVIKFKEPERCDYLYVDENNKVHILLPIVGGDEIGLDNTCQTAVELITFFYGSA
 HSGVTKYSAEHLSEYKRQLEEDIKAINSQKKISPHAYDDLKEKIERLQQIEKYIELIQV
 LKKQYDEQNDIRQLRTGGIPQLPSGVKEIKSSENAFAVRLSPYDNDKFTRFDDPLFNVK
 RNISKYDTPSRQAPIPIYEGGLGYRLRSTLFPEDKTPTPINKKSLRDKVKSTVLSHYKDEDRI
 DGEKKDEKLNELITNLQNELVKELVKSDPQYSKLSLKDPRGKEINYDYLVNSLMLVDN
 DSEIGDWIDTILDATVDSTVWVAQASSPFYDGAKEISSDRDADKISIRVQYLLAEANIYC
 45 KTNKLS DANFGEFFDKEPHATEIAKRVKEGFTQGADIEPIIYDYINSNHAELGLKSPLTGK
 QQQEITDKFTKHNTIKESPHFDEFFVADPDKKGNIFSHQGRISCHFLDFFTRQTKGKHPL
 GDLASHQEALQEGTSNRLHHKNEVVAQGYEKLDQFKKEVVKLLAENKPKELLDYLVA
 TSPTGVPNYSMLSKETQNYIAYNRNWPAIQKELEKATSIPESQKQDLSRLLSRDNLQHD
 NLSAITWSKYSSKPLLDVELNKAIEGLELTAKIYNEKRGREWWFKGSRNEARKTQCEEL

Q R V S K E I N T L L Q S E S L T K S Q V L E K V L N S I E T L D K I D R D I S A E S N W F Q S T L Q K E V R L F R D Q L
K D I C Q L D K Y A F K S T K L D E I I S L E M E E Q F Q K I Q D P A V Q Q I V R D L P S H C H N D E A I E F F K T L N P
E E A A K V A S Y L S L E Y R E I N K S T D K K T L L E Q D I P R L F K E V N T Q L L S K L K E E K A I D E Q V H E K L
S Q L A D K I A P E H F T R N N I I K W S T N P E K L E E S N L N E P I K S V Q S P T T K Q T S K Q F R E A M G E I T G R
5 N E P P T D T L Y T G I I K K

[0107] The E3 ubiquitin ligase SidC, which is an unconventional motif, from *L. pneumophila*, has the nucleotide sequence of SEQ ID NO: 28 as follows:

ATGGTGATAAACATGGTTGACGTAATCAAATTCAAAGAGCCGGAACGTTGTGATTA
TCTATATGTTGATGAAAACAACAAAGTTCATATCCTTTTACCGATTGTAGGAGGAGA
10 TGAAATAGGCCTGGATAATACCTGTCAAACAGCAGTTGAGTTGATCACATTTTCTA
TGGTAGTGCGCACAGTGGTGTGACTAAATATTCTGCTGAACACCAACTCAGTGAATA
CAAAAGGCAATTGGAAGAAGACATCAAAGCCATCAATAGTCAAAAGAAAATTTTAC
CTCATGCTTATGACGATTTATTAAGAGAGAAAATAGAACGCTTACAGCAAATTGAA
AAATACATTGAATTAATTCAAGTACTAAAAACAATATGATGAACAAAATGATAT
15 CAGGCAACTTCGTACTGGAGGGATTCCGCAATTACCCTCTGGGGTAAAGGAAATCA
TTAAATCCTCTGAAAATGCTTTCGCTGTGAGACTTTCTCCATATGACAACGATAAAT
TCACTCGCTTTGATGACCCTTTATTCAATGTCAAAGAAACATCTCAAAATATGACA
CGCCCTCAAGACAAGCTCCTATTCCAATATACGAGGGATTAGGTTATCGCCTGCGTT
CAACACTGTTCCCGGAAGATAAAACACCAACTCCAATTAATAAAAAATCACTTAGG
20 GATAAAGTTAAAAGCACTGTTCTTAGTCATTATAAAGATGAAGATAGAATTGATGG
AGAAAAAAGATGAAAAATTAAACGAACATAACTACTAATCTTCAAAACGAACCTG
TAAAAGAGTTAGTAAAAGTGATCCTCAATATTCGAAACTATCTTTATCTAAAGATC
CAAGAGGAAAAGAAATAAATTACGATTATTTAGTAAATAGTTTGTATGCTTGTAGAT
AACGACTCTGAAATTGGTGATTGGATTGATACTATTCTCGACGCTACAGTAGATTCC
25 ACTGTCTGGGTAGCTCAGGCATCCAGCCCTTTCTATGATGGTGCTAAAGAAATATCA
TCAGACCGCGATGCGGACAAGATATCCATCAGAGTTCAGTACCTGTTGGCCGAAGC
CAATATTTACTGTAAAACAAACAAATTATCGGATGCTAACTTTGGAGAATTTTTTCGA
CAAAGAGCCTCATGCTACTGAAATTGCGAAAAGAGTAAAGGAAGGATTACGCAAG
GTGCAGATATAGAACCAATTATATACGACTATATTAACAGCAACCATGCCGAGCTG
30 GGATTAAATCTCCGTTAACCGGCAACAACAAGAAATCACTGATAAATTTAC
AAAACATTATAATACGATTAAAGAATCTCCACATTTTGATGAGTTTTTGTGCTGTA
TCCGGATAAAAAAGGCAATATCTTTTCTCATCAAGGCAGAATCAGTTGTCATTTTCT
GGATTTCTTTACTCGACAAACCAAGGCAAACATCCTCTTGGTGATCTTGCAAGTCA
TCAGGAAGCTCTCCAGGAAGGAACCTCCAATCGCTTACATCACAAGAATGAGGTAG
35 TAGCCAGGGGTACGAAAACTGGATCAATTCAAGAAAGAGGTTGTCAAACCTGCTG
GCTGAGAATAAACCAAAAGAATTATTGGATTATTTGGTTGCTACCTCACCTACAGGT
GTTCCAAATTACTCCATGCTTTCGAAGGAACTCAAATTAACATTGCTTATAATCGT
AACTGGCCAGCCATTCAAAAAGAGCTGGAAGAGGCTACCAGCATCCCGGAGAGTCA
AAAACAAGATCTTTCAAGATTGCTTTCGTGATAATTTACAACACGATAATCTAAG
40 CGCAATTACCTGGTCAAAATATTCCTCCAAGCCATTATTGGATGTGGAATTAATAA
AATCGCTGAAGGATTAGAATCACTGCAAAAATTTACAATGAAAAGAGAGGACGCG
AATGGTGGTTTAAAGGTTCAAGAAATGAAGCTCGTAAGACCCAATGTGAAGAATTG
CAAAGAGTATCCAAAGAAATCAATACTCTTCTGCAAAGTGAATCTTTAACGAAAAG
CCAGGTACTTGAAAAGGTTTTAAATTCTATAGAAACATTAGATAAAATTGACAGAG
45 ACATTTCTGCCGAATCCAATTGGTTTCAAAGTACTCTGCAAAAGGAAGTCAGGTTAT
TTCGAGATCAATTGAAAGATATTTGCCAATTGGACAAGTATGCCTTTAAATCAACAA
AACTTGATGAAATCATCTCTCTGGAAATGGAAGAACAATTTCAAAGATACAAGAT
CCTGCTGTTCAACAAATTGTCAGGGACTTGCCTTCTCATTGCCACAATGATGAAGCA
ATTGAATCTTTAAGACATTGAACCCTGAAGAGGCAGCAAAAGTAGCTAGCTATTTA
50 AGCCTGGAATACAGGGAAATTAATAAATCAACCGATAAGAAAACTCTCCTAGAACAA

AGATATTCCCAGACTGTTTAAAGAAGTCAATACGCAGTTACTCTCCAAACTCAAAGA
 AGAAAAAGCTATTGATGAGCAAGTTCATGAAAACTCAGTCAACTGGCTGACAAAA
 TTGCCCCCTGAGCATTTTACAAGAAATAACATTATAAAATGGTCTACCAACCCTGAAA
 AGCTTGAGGAATCAAATCTTAATGAGCCAATCAAATCAGTCCAAAGCCCTACTACTA
 5 AACAAACATCAAAACAATTTCAGGGAAGCGATGGGTGAAATCACTGGAAGAAATGA
 GCCTCCTACAGACACTTTGTACACGGGAATTATAAAGAAATAG

[0108] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase SlrP, which is a NEL motif, from EHEC O157:H7, and has the amino acid sequence of SEQ ID NO: 29:

10 MFNITNIQSTARHQSISNEASTEVLKEEIWNKISAFFSSEHQVEAQNCIAYLCHPPETASP
 EEIKSKFECLRMLAFPAYADNIQYSRGGADQYCILSENSQEILSIVFNTEGYTVEGGGKSV
 TYTRVTESEQASSASGSKDAVNIELIWSEWVKEAPAKEAANREEPVQRMRDCLKNNKT
 ELRLKILGLTTIPAYIPEQITTLILDNNELKSLPENLQGNIKTLYANSNQLTSIPATLPDTIQE
 MELSRITELPERLPSALQSLDLFHNKISCLPENLPEELRYLSVYDNSIRTLPAHLPSSEITH
 15 LNVQSNLSLTALPETLPPGLKTLEAGENALTSLPASLPPELQVLDVSKNQITVLPETLPPTIT
 TLDVSRNALTNLPENLPAALQIMQASRNNLVRLPESLPHFRGEGPQPTRIIVEYNPF SERT
 IQNMQRLMSSVDYQGPVRLVAMGDFSIVRVTRPLHQAVQGWLTSLLEEDVNQWRAFE
 AEANAAAFSGFLDYLGDTQNRHPDFKEQVSAWLMRLAEDSALRETVFIAMNATISCE
 DRVTLAYHQMQEATLVHDAERGAFDShLAELIMAGREIFRLEQIESLAREKVKRLFFIDE
 20 VEVFLGFQNLRESLSLTMTDRMRFYNVSGITESDLDEAEIRIKMAENRDFHKWFALW
 GPWHKVLERIAPEEWREMMAKRDECIEYQSRVNAELEDLRIADDSDAERTTEVQM
 DAERAIGIKIMEEINQTLFTEIMENILLKKEVSSLMSAYWR

[0109] The E3 ubiquitin ligase SlrP, which is a NEL motif, from EHEC O157:H7, has the nucleotide sequence of SEQ ID NO: 30 as follows:

25 ATGTTTAATATTACTAATATACAATCTACGGCAAGGCATCAAAGTATTAGCAATGAG
 GCCTCAACAGAGGTGCCTTTAAAAGAAGAGATATGGAATAAAATAAGTGCCTTTTT
 CTCTTCAGAACATCAGGTTGAAGCACAAAACCTGCATCGCTTATCTTTGTCATCCACC
 TGAAACCGCCTCGCCAGAAGAGATCAAAAGCAAGTTTGAATGTTTAAGGATGTTAG
 CTTTCCCGGCGTATGCGGATAATATTCAGTATAGTAGAGGAGGGGCAGACCAATAC
 30 TGTATTTTGAGTGAAAATAGTCAGGAAATCTGTCTATAGTTTTTAATACAGAGGGC
 TATACCGTTGAGGGAGGGGGAAAGTCAGTCACCTATACCCGTGTGACAGAAAGCGA
 GCAGGCGAGTAGCGCTTCCGGCTCCAAAGATGCTGTGAATTATGAGTTAATCTGGTC
 TGAGTGGGTAAAAGAGGCGCCAGCGAAAGAGGCAGCAAATCGTGAAGAACCCGTA
 CAACGGATGCGTGACTGCCTGAAAAATAATAAGACGGAACCTTCGTCTGAAAATATT
 35 AGGACTTACCACTATACCTGCCTATATTCCTGAGCAGATAACTACTCTGATACTCGA
 TAACAATGAACTGAAAAGTTTGCCGGAAAATTTACAGGGAAATATAAAGACCCTGT
 ATGCCAACAGTAATCAGCTAACCAGTATCCCTGCCACGTTACCGGATACCATAACAGG
 AAATGGAGCTGAGCATTAAACCGTATTACTGAATTGCCGGAACGTTTGCCTTCAGCGC
 TTCAATCGCTGGATCTTTCCATAATAAAATTAGTTGCTTACCTGAAAATCTACCTGA
 40 GGAACCTTCGGTACCTGAGCGTTTATGATAACAGCATAAGGACACTGCCAGCACATCT
 TCCGTCAGAGATTACCCATTTGAATGTGCAGAGTAATTCGTAAACCGCTTTGCCTGA
 AACATTGCCGCCGGGCTGAAGACTCTGGAGGCCGGCGAAAATGCCTTAACCAGTC
 TGCCCGCATCGTTACCACCAGAATTACAGGTCCTGGATGTAAGTAAAAATCAGATTA
 CGGTTCTGCCTGAAACACTTCCTCCACGATAACAACGCTGGATGTTTCCCGTAACG
 45 CATTGACTAATCTACCGGAAAACCTCCCGGCGGCATTACAAATAATGCAGGCCTCTC
 GCAATAACCTGGTCCGTCTCCCGGAGTCGTTACCCCATTTTCGTGGTGAAGGACCTC
 AACCTACAAGAATAATCGTAGAATATAATCCTTTTTTCAGAACGAACAATACAGAAT
 ATGCAGCGGCTAATGTCCTCTGTAGATTATCAGGGACCCCGGTATTGGTTGCCATG

GGCGACTTTTCAATTGTTTCGGGTAACCTCGACCACTGCATCAAGCTGTCCAGGGGTGG
 CTAACCAGTCTCGAGGAGGAAGACGTCAACCAATGGCGGGCGTTTGAGGCAGAGGC
 AAACGCGGCGGCTTTCAGCGGATTCTTGGACTATCTTGGTGATACGCAGAATACCCG
 ACACCCGGATTTTAAGGAACAAGTCTCCGCCTGGCTAATGCGCCTGGCTGAAGATA
 5 GCGCACTAAGAGAAACCGTATTTATTATAGCGATGAATGCAACGATAAGCTGTGAA
 GATCGGGTCACACTGGCATAACCACCAATGCAGGAAGCGACGTTGGTTCATGATGC
 TGAAAGAGGCGCCTTTGATAGCCACTTAGCGGAAGTATTATGGCGGGGCGTGAAA
 TCTTTCGGCTGGAGCAAATAGAATCGCTCGCCAGAGAAAAGGTAAAACGGCTGTTT
 TTTATTGACGAAGTCGAAGTATTTCTGGGGTTTCAGAATCAGTTACGAGAGTCGCTG
 10 TCGCTGACAACAATGACCCGGGATATGCGATTTTATAACGTTTCGGGTATCACTGAG
 TCTGACCTGGACGAGGCGGAAATAAGGATAAAAAATGGCTGAAAATAGGGATTTTCA
 CAAATGGTTTTCGCTGTGGGGGCGGTGGCATAAAGTGCTGGAGCGCATAGCGCCAG
 AAGAGTGGCGTGAAATGATGGCTAAAAGGGATGAGTGTATTGAAACGGATGAGTAT
 CAGAGCCGGGTCAATGCTGAACTGGAAGATTTAAGAATAGCAGACGACTCTGACGC
 15 AGAGCGTACTACTGAGGTACAGATGGATGCAGAGCGTGCTATTGGGATAAAAAATAA
 TGGAAGAGATCAATCAGACCCTCTTTACTGAGATCATGGAGAATATATTGCTGAAA
 AAAGAGGTGAGCTCGCTCATGAGCGCCTACTGGCGATAG

[0110] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in
 accordance with the present application includes the E3 ubiquitin ligase SopA, which is a HECT
 20 motif, from *Salmonella typhimurium*, and has the amino acid sequence of SEQ ID NO: 31:

MKISSGAINFSTIPNQVKKLITSIREHTKNGLTSKITSVKNTHTSLENEFKTGKDSPIEFALP
 QKIKDFFQPKDKNTLNKTLITVKNIKDTNAGKKNISAEDVSKMNAAFMRKHIANQTC
 DYNYRMTGAAPLPGGVSVSANNRPTVSEGRTPPVSPSLSLQATSSPSSPADWAKKLTD
 VLRQKAGETLTAADRDFSADFRNITFSKILPPSFMERDGDIIKGFNFNSKFTYSDISHL
 25 HFDECRFTYSTLSDVVCSNTKFSNSDMNEVFLQYSITTQQQPSFIDTTLKNTLIRHKANLS
 GVILNEPDNSSPPSVSGGGNFIRLGDIDLQMLLWTENAVDGFNLHEHNNGKSILMTIDS
 LPDKYSQEKVQAMEDLVKSLRGGRLTEACIRPVESLVSVAHPPYTQSALISEWLGPV
 QERFFAHQCQTYNDVPLPAPDTYYQQRILPVLLDSFDRNSAAMTTHSGLFNQVILHCMT
 GVDCTDGTQKAAALYEQYLAHPAVSPHIHNGLFNGNYDGSPDWTTRAADNFFLLSSQD
 30 SDTAMMLSTDITLLTMLNPTPDATWDNFYLLRAGENVSTAQISPVELFRHDFPVFLAAFN
 QQATQRRFGEIDILSTEEHGELNQQLAATNQKHSTVKLIDDAVSRLATIFDPLLPEG
 KLSPAHYQHILSAYHLTDATPQKQAETLFCLSTAFARYSSSAIFGTEHDSPPALRGYAEA
 LMQKAWELSPAIFPSSEQFTEWSDRFHGLHGAFCTSVVADSMQRHARKYFPSVLSSILP
 LAW

[0111] The E3 ubiquitin ligase SopA, which is a HECT motif, from *Salmonella*
typhimurium, has the nucleotide sequence of SEQ ID NO: 32 as follows:

ATGAAGATATCATCAGGCGCAATTAATTTTTCTACTATTCCTAACCAGGTAAAAA
 TTAATTACCTCTATTCGTGAACATACGAAAAACGGGCTCACCTCAAAAATAACCAGT
 GTTAAAAACACGCATACATCTTTAAATGAAAAATTTAAAACAGGAAAGGACTCACC
 40 GATTGAGTTCGCGTTACCACAAAAAATAAAAGACTTCTTTCAGCCGAAAGATAAAA
 ACACCTTAAACAAAACATTGATTACTGTAAAAAATATTAAAGATACAAATAATGCA
 GGCAAGAAAAATATTTACGACAGAAGATGTCTCAAAAATGAATGCAGCATTCATGCG
 TAAGCATATTGCAAATCAAACATGTGATTATAATTACAGAATGACAGGTGCGGCCC
 CGCTCCCCGGTGGAGTCTCTGTATCAGCCAATAACAGGCCCACGGTTTCTGAAGGTA
 45 GAACACCACCAGTATCCCCCTCCCTCTCACTTCAGGCTACCTCTCCCCGTCATCACC
 TGCCGACTGGGCTAAGAACTCACGGATGCAGTTTTACGACAGAAAGCCGGAGAAA
 CCCTTACGGCCGCAGATCGCGATTTTTCAAACGCAGATTTCCGTAATATTACATTCA
 GCAAAATATTGCCCCCAGCTTCATGGAGCGAGACGGCGATATTATTAAGGGGTTT

AACTTTTCAAATTCAAAATTTACTTATTCTGATATATCTCATTACATTTTGACGAAT
 GCCGATTCACTTATTCGACACTGAGTGATGTAGTCTGCAGTAATACGAAATTTAGTA
 ATTCAGACATGAATGAAGTGTTTTTACAGTATTCAATTACTACACAACAGCCCT
 CGTTTATTGATACAACATTAAAAAATACGCTTATACGTCACAAAGCCAACCTCTCTG
 5 GCGTTATTTTAAATGAACCGGATAATTCATCACCTCCGTCAGTGTCAGGGGGCGGAA
 ATTTTATTCGTCTAGGTGATATCTGGCTGCAAATGCCACTCCTTTGGACTGAGAACG
 CTGTGGATGGATTTTTAAATCATGAGCACAATAATGGTAAAAGTATTCTGATGACCA
 TTGACAGCCTGCCCGATAAATACAGTCAGGAAAAAGTCCAGGCAATGGAAGACCTG
 GTTAAGTCATTGCGGGGTGGCCGCTTAACAGAGGCATGTATCCGGCCAGTTGAAAG
 10 TTCGCTGGTAAGCGTACTGGCCACCCCCCTATACGCAAAGTGCGCTTATCAGCGA
 GTGGCTCGGGCCTGTTTACGGAACGTTTTTTTGGCCACCAGTGCCAGACCTATAATGA
 CGTCCCCCTGCCGGCTCCTGACACATATTATCAGCAGCGCATACTGCCTGTGTTGCT
 GGATTCGTTTGACAGGAACAGCGCCGCCATGACCACTCACAGCGGACTCTTTAATCA
 GGTGATTTTACACTGTATGACAGGCGTGGACTGCACTGATGGCACCCGCCAGAAAG
 15 CTGCAGCGCTTTATGAACAGTATCTTGCTCACCCGGCGGTGTCTCCCCACATCCATA
 ATGGGCTCTTCGGCAATTATGATGGCAGCCCGGACTGGACAACCCGCGCTGCAGAT
 AATTTCTGCTGCTTTCCTCCCAAGATTACAGACACGGCGATGATGCTCTCCACTGAC
 ACGCTGTAAACAATGCTAAACCCTACTCCTGACACTGCATGGGACAACCTTTTACCTG
 CTGCGAGCCGGAGAGAACGTTTCCACCGCGCAAATCTCTCCGGTAGAGTTATTCCGT
 20 CATGACTTTCCGGTGTTTCTCGCCGCATTTAATCAGCAGGCCACGCAGCGACGCTTT
 GGGGAGCTGATTGATATCATCCTCAGCACTGAAGAGCACGGGGAGCTGAACCAGCA
 GTTTCTTGCCGCCACGAACCAGAAACATTCCACCGTGAAGTTGATTGATGATGCCTC
 AGTGTCGCGTCTGGCCACCATTTTTGACCCCTTGCTTCCTGAAGGCAAACCTCAGCCC
 GGCACACTACCAGCACATCCTCAGTGCTTATCACCTGACGGACGCCACCCACAGA
 25 AGCAGGCGGAAACCCTGTTCTGTCTCAGTACCGCATTCGCACGCTATTCTCCAGCG
 CCATTTTCGGCACTGAGCATGACTCTCCGCCGGCCCTGAGAGGCTATGCGGAGGCGC
 TGATGCAGAAAGCCTGGGAGCTGTCTCCGGCGATATTCCCATCCAGCGAACAGTTTA
 CCGAGTGGTCCGACCGTTTTACAGGCCTCCATGGCGCCTTTACCTGTACCAGCGTTG
 TGGCGGATAGTATGCAACGTATGCCAGAAAATATTTCCCGAGTGTTCTGTATCCA
 30 TCCTGCCACTGGCCTGGGCGTAA

[0112] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase SspH1, which is a novel E3 ligase motif, from *Salmonella typhimurium*, and has the amino acid sequence of SEQ ID NO: 33:

35 MFNIRNTQPSVSMQAIAGAAPEASPEEIVWEKIQVFFPQENYEEAQQCLAELCHPARG
 MLPDHISSQFARLKALTFPAWEENIQCNRDGINQFCILDAGSKEILSITLDDAGNYTVNC
 QGYSEAHDFIMDTEPGEECTEFAEGASGTSLRPATTVSQKAAEYDAVWSKWERDAPAG
 ESPGRAAVVQEMRDCLNNGNPVLNVGASGLTTLPDRLPPHITTLVIPDNNLTSLPELPEG
 LRELEVSGNLQLTSLPSLPQGLQKLWAYNNWLASLPTLPPGLGDLAVSNNQLTSLPEMP
 40 PALRELRVSGNNLTSLPALPSGLQKLWAYNNRLTSLPEMSPGLQELDVSHNQLTRL PQS
 LTGLSSAARVYLDGNPLSVRTLQALRDIIGHSGIRIHFD MAGPSVPREARALHLAVADW
 LTSAREGEAAQADRWQAFGLDNAAAFSLVLDRLRETFENFKKDAGFKAQISSWLTQLA
 EDAAALRAKTFAMATEATSTCEDRVTHALHQMNNVQLVHNAEKGEYDNNLQGLVSTG
 REMFRLATLEQIAREKAGTLALVDDVEVYLAFQNKLELTSVTSEMRFFDVSGVTV
 45 SDLQAAELQVKTAENSGFSKWILQWGPLHSVLERK VPERFNALREKQISDYEDTYRKLY
 DEVLKSSGLVDDTDAERTIGVSAMDSAKKEFLDGLRALVDEVLGSYLTARWRLN

[0113] The E3 ubiquitin ligase SspH1, which is a novel E3 ligase motif, from *Salmonella typhimurium*, has the nucleotide sequence of SEQ ID NO: 34 as follows:

ATGTTTAATATCCGCAATACACAACCTTCTGTAAGTATGCAGGCTATTGCTGGTGCA
 GCGGCACCAGAGGCATCTCCGGAAGAAATTGTATGGGAAAAAATTCAGGTTTTTTTC
 CCGCAGGAAAAATTACGAAGAAGCGCAACAGTGTCTCGCTGAACTTTGCCATCCGGC
 CCGGGGAATGTTGCCTGATCATATCAGCAGCCAGTTTGCGCGTTTAAAAGCGCTTAC
 5 CTTCCCCGCGTGGGAGGAGAATATTCAGTGTAAACAGGGATGGTATAAATCAGTTTTG
 TATTCTGGATGCAGGCAGCAAGGAGATATTGTCAATCACTCTTGATGATGCCGGGAA
 CTATACCGTGAATTGTCAGGGGTACAGTGAAGCACATGACTTCATCATGGACACAG
 AACCGGGAGAGGAATGCACAGAATTCGCGGAGGGGGCATCCGGGACATCCCTCCGC
 CCTGCCACAACGTTTTACAGAAGGCAGCAGAGTATGATGCTGTCTGGTCAAAATG
 10 GGAAAGGGATGCACCAGCAGGAGAGTACCCGGCCGCGCAGCAGTGGTACAGGAA
 ATGCGTGATTGCCTGAATAACGGCAATCCAGTGCTTAACGTGGGAGCGTCAGGTCTT
 ACCACCTTACCAGACCGTTTACCACCGCATATTACAACACTGGTTATTCCTGATAAT
 AATCTGACCAGCCTGCCGGAGTTGCCGGAAGGACTACGGGAGCTGGAGGTCTCTGG
 TAACCTACAACCTGACCAGCCTGCCATCGCTGCCGCAGGGACTACAGAAGCTGTGGG
 15 CCTATAATAATTGGCTGGCCAGCCTGCCGACGTTGCCGCCAGGACTAGGGGATCTGG
 CGGTCTCTAATAACCAGCTGACCAGCCTGCCGGAGATGCCGCCAGCACTACGGGAG
 CTGAGGGTCTCTGGTAACAACCTGACCAGCCTGCCGGCGCTGCCGTCAGGACTACA
 GAAGCTGTGGGCCTATAATAATCGGCTGACCAGCCTGCCGGAGATGTCGCCAGGAC
 TACAGGAGCTGGATGTCTCTCATAACCAGCTGACCCGCCTGCCGCAAAGCCTCACGG
 20 GTCTGTCTTCAGCGGCACGCGTATATCTGGACGGGAATCCACTGTCTGTACGCACTC
 TGCAGGCTCTGCGGGACATCATTGGCCATTACAGGCATCAGGATACACTTCGATATGG
 CGGGGCCTTCCGTCCCCCGGGAAGCCCGGGCACTGCACCTGGCGGTCTGCTGACTGG
 CTGACGTCTGCACGGGAGGGGGAAGCGGCCAGGCAGACAGATGGCAGGCGTTTCG
 GACTGGAAGATAACGCCGCCGCCTTCAGCCTGGTCTTGACAGACTGCGTGAGACG
 25 GAAAACCTTCAAAAAAGACGCGGGCTTTAAGGCACAGATATCATCCTGGCTGACACA
 ACTGGCTGAAGATGCTGCGCTGAGAGCAAAAACCTTTGCCATGGCAACAGAGGCAA
 CATCAACCTGCGAGGACCGGGTACACATGCCCTGCACCAGATGAATAACGTACAA
 CTGGTACATAATGCAGAAAAAGGGGAATACGACAACAATCTCCAGGGGCTGGTTTC
 CACGGGGCGTGAGATGTTCCGCCTGGCAACACTGGAACAGATTGCCCGGGAAAAAG
 30 CCGGAACACTGGCTTTAGTCGATGACGTTGAGGTCTATCTGGCGTTCCAGAATAAGC
 TGAAGGAATCACTTGAGCTGACCAGCGTGACGTCAGAAATGCGTTTCTTTGACGTTT
 CCGGCGTGACGGTTTCAGACCTTCAGGCTGCGGAGCTTCAGGTGAAAACCGCTGAA
 AACAGCGGGTTTCAGTAAATGGATACTGCAGTGGGGGCCGTTACACAGCGTGCTGGA
 ACGCAAAGTGCCGGAACGCTTTAACGCGCTTCGTGAAAAGCAAATATCGGATTATG
 35 AAGACACGTACCGGAAGCTGTATGACGAAGTGCTGAAATCGTCCGGGCTGGTCGAC
 GATACCGATGCAGAACGTACTATCGGAGTAAGTGCGATGGATAGTGCGAAAAAAGA
 ATTTCTGGATGGCCTGCGCGCTCTTGTGGATGAGGTGCTGGGTAGCTATCTGACAGC
 CCGGTGGCGTCTTAACTAA

[0114] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in
 40 accordance with the present application includes the E3 ubiquitin ligase SspH2, which is a novel
 E3 ligase motif, from *Salmonella typhimurium*, and has the amino acid sequence of SEQ ID NO:
 35:

MPFHIGSGCLPATISNRRIYRIAWS DTPPEMSSWEKMKEFFCSTHQTEALECIWTICHPPA
 GTTREDVINRFELLRTLAYAGWEESIHSQGHGENYFCILDEDSQEILSVTLDDAGNYTVN
 45 CQGYSETHRLTLDTAQGEEGTGHAEGASGTFRTSFLPATTAPQTPAEYDAVWSAWRRA
 APAEESRGRAAVVQKMRACLNNNGNAVLNVGESGLTTLPDCLPAHITTLVIPDNNLTSLP
 ALPPELRTLTVSGNQLTSLPVLPPGLLELSIFSNPLTHLPALPSGLCKLWIFGNQLTSLPVL
 PPGLQELSVSDNQLASLPALPSELCKLWAYNNQLTSLPMLPSGLQELSVSDNQLASLPTL
 PSELYKLWAYNNRLTSLPALPSGLKELIVSGNRLTSLPVLPSSELKELMVSGNRLTSLPML

PSGLLSLSVYRNQLTRLPESLIHLSSSETTVNLEGNPLSERTLQALREITSAPGYSGPIIRFD
 MAGASAPRETRALHLAAADWLVPAREGEPAPADRWHMFGQEDNADAFSLFLDRLSET
 ENFIKDAGFKAQISSWLAQLAEDEALRANTFAMATEATSSCEDRVTFLLHQMKNVQLV
 HNAEKGYDNDLAALVATGREMFRLGKLEQIAREKVRTLALVDEIEVWLAYQNKLLK
 5 SLGLTSVTSEMRFFDVSGVTVTDLQDAELQVKA AEKSEFREWILQWGPLHRVLERKAPE
 RVNALREKQISDYEETYRMLSDTELRPGLVGNTDAERTIGARAMESA KKTFLDGLRPL
 VEEMLGSYLNVQWRRN

[0115] The E3 ubiquitin ligase SspH2, which is a novel E3 ligase motif, from *Salmonella typhimurium*, has the nucleotide sequence of SEQ ID NO: 36 as follows:

10 ATGCCCTTTCATATTGGAAGCGGATGTCTTCCC GCCACCATCAGTAATCGCCGCATT
 TATCGTATTGCCTGGTCTGATACCCCCCTGAAATGAGTTCCTGGGAAAAAATGAAG
 GAATTTTTTTTGCTCAACGCACCAGACTGAAGCGCTGGAGTGCATCTGGACGATTTGT
 CACCCGCCGGCCGGAACGACGCGGGAGGATGTGATCAACAGATTTGAACTGCTCAG
 GACGCTCGCGTATGCCGGATGGGAGGAAAGCATTCATTCCGCCAGCACGGGGAAA
 15 ATTACTTCTGTATTCTGGATGAAGACAGTCAGGAGATATTGTCAGTCACCCTTGATG
 ATGCCGGGAACATAACCGTAAATTGCCAGGGGTACAGTGAAACACATCGCCTCACC
 CTGGACACAGCACAGGGTGAGGAGGGCACAGGACACGCGGAAGGGGCATCCGGGA
 CATTACAGGACATCCTTCTCCCTGCCACAACGGCTCCACAGACGCCAGCAGAGTATG
 ATGCTGTCTGGTCAGCGTGGAGAAGGGCTGCACCCGCAGAAGAGTCACGCGGCCGT
 20 GCAGCAGTGGTACAGAAAATGCGTGCCTGCCTGAATAATGGCAATGCAGTGCTTAA
 CGTGGGAGAATCAGGTCTTACCACCTTGCCAGACTGTTTACCCGCGCATATTACCAC
 ACTGGTTATTCCTGATAATAATCTGACCAGCCTGCCGGCGCTGCCGCCAGAACTGCG
 GACGCTGGAGGTCTCTGGTAACCAGCTGACTAGCCTGCCGGTGCTGCCGCCAGGACT
 ACTGGAACGTGTCGATCTTTAGTAACCCGCTGACCCACCTGCCGGCGCTGCCGTCAGG
 25 ACTATGTAAGCTGTGGATCTTTGGTAATCAACTGACCAGCCTGCCGGTGTTGCCGCC
 AGGGCTACAGGAGCTGTCGGTATCTGATAACCAACTGGCCAGCCTGCCGGCGCTGC
 CGTCAGAATTATGTAAGCTGTGGGCCTATAATAACCAGCTGACCAGCCTGCCGATGT
 TGCCGTCAGGGCTACAGGAGCTGTCCGTATCTGATAACCAACTGGCCAGCCTGCCG
 ACGCTGCCGTCAGAATTATATAAGCTGTGGGCCTATAATAATCGGCTGACCAGCCTG
 30 CCGGCGTTGCCGTCAGGACTGAAGGAGCTGATTGTATCTGGTAACCGGCTGACCAGT
 CTGCCGGTGCTGCCGTCAGAACTGAAGGAGCTGATGGTATCTGGTAACCGGCTGAC
 CAGCCTGCCGATGCTGCCGTCAGGACTACTGTCGCTGTCCGTCTATCGTAACCAGCT
 GACCCGCTGCCGGAAAGTCTCATTCTGTCTTCAGAGACAACCGTAAATCTGGA
 AGGGAACCCACTGTCTGAACGTACTTTGCAGGCGCTGCGGGAGATCACCAGCGCGC
 35 CTGGCTATTACAGGCCCCATAATACGATTCGATATGGCGGGAGCCTCCGCCCCCGGG
 AAACCTCGGGCACTGCACCTGGCGGCCGCTGACTGGCTGGTGCCTGCCCGGGAGGGG
 GAACCGGCTCCTGCAGACAGATGGCATATGTTCCGGACAGGAAGATAACGCCGACGC
 ATTCAGCCTCTTCCTGGACAGACTGAGTGAGACGGAAACTTCATAAAGGACGCGG
 GGTTTAAGGCACAGATATCGTCCTGGCTGGCACAACCTGGCTGAAGATGAGGCGTTA
 40 AGAGCAAACACCTTTGCTATGGCAACAGAGGCAACCTCAAGCTGCGAGGACCGGGT
 CACATTTTTTTTGACACAGATGAAGAACGTACAGCTGGTACATAATGCAGAAAAAG
 GGCAATACGATAACGATCTCGCGGCGCTGGTTGCCACGGGGCGTGAGATGTTCCGT
 CTGGGAAAACCTGGAACAGATTGCCCGGGAAAAGGTCAGAACGCTGGCTCTCGTTGA
 TGAAATTGAGGTCTGGCTGGCGTATCAGAATAAGCTGAAGAAATCACTCGGGCTGA
 45 CCAGCGTGACGTCAGAAATGCGTTTCTTTGACGTATCCGGCGTGACGGTTACAGACC
 TTCAGGACGCGGAGCTTCAGGTGAAAGCCGCTGAAAAAAGCGAGTTCAGGGAGTGG
 TAACTGCAGTGGGGGGCGTTACACAGAGTGCTGGAGCGCAAAGCGCCGGAACGCGT
 TAACGCGCTTCGTGAAAAGCAAATATCGGATTATGAGGAAACGTACCGGATGCTGT
 CTGACACAGAGCTGAGACCGTCTGGGCTGGTCGGTAATACCGATGCAGAGCGCACT

ATCGGAGCAAGAGCGATGGAGAGCGCGAAAAAGACATTTTTGGATGGCCTGCGACC
TCTTGTGGAGGAGATGCTGGGGAGCTATCTGAACGTTCAAGTGGCGTCGTAACCTGA

[0116] A further exemplary E3 ubiquitin ligase that is useful as a degradation domain in accordance with the present application includes the E3 ubiquitin ligase XopL, which is an
5 unconventional motif, from *Xanthomonas campestris*, and has the amino acid sequence of SEQ ID NO: 37:

MRRVDQPRPPGTPFGLREQTTSNADAPARTAPPAHPAPERPTGMLGGLTRYVPGDRSGR
PPAMPAAEASRRPTTSARPLPYGGSGSAARMNEAAGHPLRMPQLPQLSDIERARFHSV
TTDSQHLRPVRPRMPPVVGASPLRRSTALRPYHDVLSQWQRHYNADRNRWHSARQA
10 NSNNPQIETRTGRALKATADLLEDATQPGRVALELRVPLPQFPDQAFRLSHLQHMTID
AAGLMELPD TMQQFAGLET LTLARNPLRALPASIASLNRLRELSIRACPELTELPEPLAST
DASGEHQGLVNLQSLRLEWTGIRSLPASIANLQNLKSLKIRNSPLSALGPAIHHLPKLEEL
DLRGCTALRNYPPIFGGRAPLKRLILKDCSNLLTLPLDIHRLTQLEKLDLRGCVNLSRLPS
LIAQLPANCILVPPHLQAQLDQHRPVARPAEPGRTGPTTPALSPSAAGDRAGPSSSATAS
15 ELLLTAALERIEDTAQAMLSTVIDEERNPFLEGAPSYLPGKRPTDVTTFGQVPALRDMLA
ESRDLEFLQRVSDMAGPSPIEDPSEEGLARHYTNVSNWKAQKSAHLGIVDHLGQFVYH
EGSPLDVATLAKAVQMWKTRRELIVHAHPQDRARFPELAVHIPEQVSDSDSEQQTSPEP
SGHQ

[0117] The E3 ubiquitin ligase XopL, which is an unconventional motif, from

20 *Xanthomonas campestris*, has the nucleotide sequence of SEQ ID NO: 38 as follows:

ATGCGACGCGTCGATCAACCACGCCCCGCCGGGCACGCCTTTCGGACTGCGGGAGCA
GACTACGTCCAATGCGGATGCGCCCCGCGCGCACTGCCCCACCCGCACACCCCGCGC
CCGAGCGCCCTACCGGCATGCTCGGCGGACTGACCAGATATGTGCCTGGCGATCGG
TCCGGGCGACCGCCAGCAATGCCTGCCGCTGCCGAGACCTCTCGCCGGCCAACCAC
25 CTCCGCCCCGCCGCTTCCCTACGGCGGATCCGGCAGCGCCGCGCGGATGAACGAGG
CGGCTGGACATCCTTTGCGGATGCCGCAATTGCCACAGCTCAGCGACATAGAACGC
GCTCGCTTCCACTCCGTCACCACCGACTCGCAACACTTGCGGGCCGGTGCGCCCCCGT
ATGCCACCGCCCCGTGGGCGCTTACCCTTACGGCGCTCCACAGCGCTGCGCCCCGTAC
CACGACGTGCTGTCGCAATGGCAACGCCACTACAACGCAGATCGCAATCGCTGGCA
30 CAGCGCATGGCGCCAGGCCAACAGCAACAACCCGCAGATCGAGACTCGCACAGGCC
GGGCGCTGAAGGCGACAGCCGACCTGCTGGAGGACGCAACCCAACCGGGCCGGGTC
GCGCTGGAGCTGCGCTCAGTTCCGCTGCCGCAATTTCCCGACCAAGGCATTCCGTCTT
TCGCATCTGCAGCACATGACGATCGACGCGGCAGGGTTGATGGAGCTCCCGGACAC
CATGCAGCAATTTGCGGGCCTGGAAACACTCACGCTCGCACGCAATCCGCTTCGCGC
35 GCTACCGGCATCCATCGCAAGCCTCAACCGATTACGCGAGCTCTCCATCCGCGCCTG
CCCGGAATTGACGGAACCTTCCCGAACCCCTGGCAAGCACCGATGCATCCGGCGAGC
ACCAGGGCTTGGTCAACCTGCAGAGCCTACGGCTGGAATGGACCGGGATCAGATCG
CTTCCGGCGTCCATCGCCAACTGCAAAATCTGAAAAGCCTGAAGATACGCAACTC
GCCGCTGTCCGCCCTTGGCCCCGGCCATCCATCACCTGCCAAAGTTGGAGGAGCTTGA
40 TTTGCGGGGCTGTACCGCGCTGCGCAACTATCCGCCGATTTTCGGCGGGCGTGCGCC
ACTGAAGCGACTGATTCTGAAAGACTGCAGCAACCTGCTCACGCTGCCACTGGACA
TTCACCGCCTGACGCAGCTGGAAAACTCGATCTGCGAGGTTGCGTCAACCTTTCCA
GACTGCCCTCGTTGATCGCCCAATTACCTGCCAATTGCATCATCCTGGTGCCGCCGC
ATCTCCAAGCGCAGCTCGACCAGCATCGTCCAGTTGCGCGCCCCGCCGAACCAGGG
45 CGGACCGGACCGACCAACCCAGCTCTCTCGCCCTCTGCTGCCGGCGACCGCGCCGGG
CCATCCTCTTCGGCGACCGCCAGCGAACTGCTTCTTACCGCTGCGCTCGAACGCATC
GAAGACACCGCACAGGCCATGCTGAGCACGGTCATCGATGAAGAAAGAAATCCCTT

TCTGGAAGGTGCTCCATCCTATCTCCCAGGAAAACGCCCTACCGATGTCACCACTT
 CGGCCAAGTTCCGGCATTGCGGGACATGCTGGCAGAAAGCAGGGATCTTGAGTTCC
 TGCAACGGGTAAAGCGACATGGCAGGCCCATCCCCAGAATCGAAGACCCGAGCGAG
 GAAGGCCTCGCCCGCCACTACACGAACGTCAGCAACTGGAAGGCGCAGAAGAGCGC
 5 ACACCTGGGCATCGTCGATCATCTCGGGCAGTTCGTTTATCACGAAGGAAGCCCGCT
 CGACGTAGCGACATTGGCCAAGGCAGTGCAGATGTGGAAGACCCGTGAGCTGATCG
 TCCACGCACACCCGCAAGACCGCGCGCGCTTTCCCGAGCTCGCTGTGCACATTCCCG
 AGCAGGTCAGCGACGACTCTGATAGCGAACAGCAGACAAGCCCGGAACCTTCAGGC
 CATCAGTAG

10

[0118] Although targeting domains possess intrinsic binding interactions, *e.g.*, secondary, tertiary or quaternary flexibility, there must still be flexibility with respect to the association with the E3 motif ubiquitin region. In this regard, absence adequate spacing, it is possible for the E3 motif to sterically hinder the substrate-target domain interaction. As such, the present
 15 application employs polypeptide linkers of sufficient length to prevent the steric disruption of binding between the targeting domain and the substrate, in some embodiments.

[0119] In some embodiments, the targeting domain is covalently attached to the ubiquitin region *via* a linker that may be cleavable or non-cleavable under physiological conditions. The linker can entail an organic moiety comprising a nucleophilic or electrophilic reacting group
 20 which allows covalent attachment to the targeting domain to the ubiquitin region agent. In some embodiments, the linker is an enol ether, ketal, imine, oxime, hydrazone, semicarbazone, acylimide, or methylene radical. The linker may be an acid-cleavable linker, a hydrolytically cleavable linker, or enzymatically-cleavable linker, in some embodiments.

[0120] Peptide-based linking groups are cleaved by enzymes such as peptidases and
 25 proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides, *e.g.*, dipeptides, tripeptides, and poly-peptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynylene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based
 30 cleavage group is generally limited to the peptide bond, *i.e.*, the amide bond, formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide cleavable linking groups have the general formula –

NHCHR1C(O)NHCHR2C(O)-, where R1 and R2 are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

[0121] For *in vitro* applications, appropriate linkers, which can be cross-linking agents for use for conjugating a polypeptide to a solid support, include a variety of agents that can react with a functional group present on a surface of the support, or with the polypeptide, or both.

Reagents useful as cross-linking agents include homo-bi-functional and, in particular, hetero-bi-functional reagents. Useful bi-functional cross-linking agents include, but are not limited to, *N*-SIAB, dimaleimide, DTNB, *N*-SATA, *N*-SPDP, SMCC and 6-HYNIC. A cross-linking agent can be selected to provide a selectively cleavable bond between a polypeptide and the solid support. For example, a photolabile cross-linker, such as 3-amino-(2-nitrophenyl)propionic acid can be employed as a means for cleaving a polypeptide from a solid support. *See* Brown et al., “A Single-Bead Decode Strategy Using Electrospray Ionization Mass Spectrometry and a New Photolabile Linker: 3-amino-3-(2-nitrophenyl)propionic Acid,” *Mol. Divers* 4–12 (1995) and US. Pat. No. 5,643,722, which are hereby incorporated by reference in their entirety.

[0122] An antibody, polypeptide, or fragment thereof, such as a targeting domain, can be immobilized on a solid support, such as a bead, through a covalent amide bond formed between a carboxyl group functionalized bead and the amino terminus of the polypeptide or, conversely, through a covalent amide bond formed between an amino group functionalized bead and the carboxyl terminus of the polypeptide. In addition, a bi-functional trityl linker can be attached to the support, *e.g.*, to the 4-nitrophenyl active ester on a resin, such as a Wang resin, through an amino group or a carboxyl group on the resin *via* an amino resin. Using a bi-functional trityl approach, the solid support can require treatment with a volatile acid, such as formic acid or trifluoroacetic acid to ensure that the polypeptide is cleaved and can be removed. In such a case, the polypeptide can be deposited as a beadless patch at the bottom of a well of a solid support or on the flat surface of a solid support. After addition of a matrix solution, the polypeptide can be desorbed into a MS.

[0123] It will be readily apparent to the skilled artisan that the methods and techniques described above can be employed for the chimeric molecule of the present application, including its constituent parts, *e.g.*, degradation domains, ubiquitin regions, target regions, and modifications thereof, as well as the linker molecules, as described above.

[0124] A second aspect of the present application relates to a method of forming a ribonucleoprotein. The method includes providing a mRNA encoding the isolated chimeric molecule described herein; providing one or more polyadenosine binding proteins (“PABP”); and assembling a ribonucleoprotein complex from the mRNA and the one or more PABPs. In one embodiment, the mRNA comprises a 3'-terminal polyadenosine (poly A) tail.

[0125] The chimeric molecule described in this aspect is carried out in accordance with the previously described aspect of the application.

[0126] The polyadenosine binding proteins (“PABP”) (also referred to as poly(A)-binding proteins) as described herein refer to a RNA-binding protein which binds to the poly(A)

tail of mRNA. The poly(A) tail is located on the 3' end of mRNA and is 200-250 nucleotides long. The binding protein is also involved in mRNA precursors by helping polyadenylate polymerase add the poly(A) nucleotide tail to the pre-mRNA before translation. The nuclear isoform selectively binds to around 50 nucleotides and stimulates the activity of polyadenylate polymerase by increasing its affinity towards RNA. Poly(A)-binding protein is also present during stages of mRNA metabolism including nonsense-mediated decay and nucleocytoplasmic trafficking. The poly(A)-binding protein may also protect the tail from degradation and regulate mRNA production.

[0127] The ribonucleoprotein, which is also referred to herein as a nanoplex, may, in one embodiment, be a nanoparticle. In a preferred embodiment, the nanoplex includes a nanoparticle. The ribonucleoprotein or nanoplex is a complex formed by a drug nanoparticle with an oppositely charged polyelectrolyte. Both cationic and anionic drugs form complexes with oppositely charged polyelectrolytes. Compared with other nanostructures, the yield of Nanoplex is generally greater and the complexation efficiency is generally better. Nanoplexes are also easier to prepare as compared to other nanostructures. Ribonucleoprotein or nanoplex formulation according to the present application is characterized through the production yield, complexation efficiency, drug loading, particle size and zeta potential using scanning electron microscopy, differential scanning calorimetry, X-ray diffraction and dialysis studies. Nanoplexes have wide-ranging applications in different fields such as cancer therapy, gene drug delivery, drug delivery to the brain and protein and peptide drug delivery.

[0128] The ribonucleoprotein or nanoplex can have any suitable size. In at least one embodiment, ribonucleoprotein or nanoplex is less than about 200 nm in diameter, less than about 100 nm in diameter, less than about 95 nm, less than about 90 nm, less than about 85 nm, less than about 80 nm, less than about 75 nm, less than about 70 nm, less than about 65 nm, less than about 60 nm, less than about 55 nm, less than about 50 nm, less than about 45 nm, less than about 40 nm, less than about 35 nm, less than about 30 nm, less than about 25 nm, less than about 20 nm, less than about 15 nm, less than about 10 nm, less than about 9 nm, less than about 8 nm, less than about 7 nm, less than about 6 nm, less than about 5 nm, less than about 4 nm, less than about 3 nm, less than about 2 nm, or less than about 1 nm. Nanoparticles having a diameter in a range having an upper limit of about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, about 50 nm, about 45 nm, about 40 nm, about 35 nm, about 30 nm, about 25 nm, about 20 nm, about 15 nm, about 10 nm, about 9 nm, about 8 nm, about 7 nm, about 6 nm, about 5 nm, about 4 nm, about 3 nm, or about 2 nm and a lower limit of about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75

nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, about 50 nm, about 45 nm, about 40 nm, about 35 nm, about 30 nm, about 25 nm, about 20 nm, about 15 nm, about 10 nm, about 9 nm, about 8 nm, about 7 nm, about 6 nm, about 5 nm, about 4 nm, about 3 nm, about 2 nm, or about 1 nm, and any combination thereof, are also contemplated. The present application further provides that in certain embodiments the nanoplex ranges in size from about 50 nm to about 200 nm or from about 10 nm to about 100 nm. In certain embodiments, the size of the nanoplex is about 50 nm to about 150 nm. In other embodiments, the size of the nanoplex can be about 50 nm, about 75 nm, or about 100 nm.

[0129] The size of the ribonucleoprotein and/or nanoplex may be optimized to localize at a particular location within a subject. For example, the ribonucleoprotein and/or nanoplex may be optimized so that it can move into various organs of a subject.

[0130] In one embodiment, the ribonucleoprotein and/or nanoplex contains an organic gel.

[0131] The ribonucleoprotein and/or nanoplex of the present application can have any suitable shape. For example, the present ribonucleoprotein and/or nanoplex can have a shape of a mesh, sphere, square, rectangle, triangle, circular disc, cube-like shape, cube, rectangular parallelepiped (cuboid), cone, cylinder, prism, polyhedral, pyramid, right-angled circular cylinder, rod, branched cylindrical, and other regular or irregular shape. The polymer matrix of the ribonucleoprotein and/or nanoplex may, in one embodiment, contain entangled and covalently bound polymers. In one embodiment, the matrix is a hydrogel.

[0132] In one embodiment, the number of polymeric units in the ribonucleoprotein and/or nanoplex matrix ranges from 10 to 5000, for instance from 20 to 400, for each particle formed from the polymeric units. In another embodiment, the number of polymeric units ranges from 10,000 to 200,000, for instance from 15,000 to 200,000 polymeric units.

[0133] The ribonucleoprotein and/or nanoplex according to the present application may include a functionalized surface. In one embodiment, the ribonucleoprotein and/or nanoplex is negatively functionalized. Alternatively, the ribonucleoprotein and/or nanoplex may be positively functionalized. In other embodiments, the ribonucleoprotein and/or nanoplex has a no charge or is neutral.

[0134] In one embodiment, the ribonucleoprotein and/or nanoplex is biodegradable. In another embodiment, the ribonucleoprotein and/or nanoplex is non-toxic.

[0135] In one embodiment, the ribonucleoprotein and/or nanoplex may further include at least one stabilizer. The stabilizer may be adsorbed on the surfaces of the ribonucleoprotein and/or nanoplex. The ribonucleoprotein and/or nanoplex may be dispersed into a liquid medium,

and the stabilizer may be employed as an adjuvant to aid in the separation of the individual ribonucleoprotein and/or nanoplex during a dispersion process. The ability of a stabilizer to aid in the separation of the individual ribonucleoprotein and/or nanoplex may be determined by comparing the dispersion processes for a composition containing the stabilizer and a control composition without the stabilizer. The ability of a stabilizer to aid in the separation of individual nanoparticles may be indicated by shorter dispersion times. Alternatively, the stabilizer may be employed to promote stability of the dispersed nanoplex in the liquid medium, preferably an aqueous medium.

[0136] A third aspect of the present application relates to a composition comprising the chimeric molecule described herein and a pharmaceutically-acceptable carrier.

[0137] According to the methods of the present application, the chimeric molecule can be incorporated into pharmaceutical compositions suitable for administration. As used herein, the term “pharmaceutically-acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal compounds, isotonic and absorption delaying compounds, and the like, compatible with pharmaceutical administration.

[0138] The chimeric molecule described in this aspect is carried out in accordance with the previously described aspects of the present application.

[0139] The pharmaceutical compositions generally entail recombinant or substantially purified chimeric molecules and a pharmaceutically-acceptable carrier in a form suitable for administration to a subject. Pharmaceutically-acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions for administering the protein compositions. *See, e.g., Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA 18th ed. (1990), which is hereby incorporated by reference in its entirety. The pharmaceutical compositions are generally formulated as sterile, substantially isotonic and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

[0140] As used herein, the term pharmaceutically- acceptable carrier includes, for example, a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. *Remington's Pharmaceutical Sciences* Ed. by Gennaro, Mack Publishing, Easton, Pa., 1995, which is hereby incorporated by reference in its entirety, discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose, and sucrose;

starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as TWEEN™ 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate. Coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and/or antioxidants can also be present in the composition, according to the judgment of the formulator.

[0141] The compounds of the present application can be administered orally, parenterally, for example, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal instillation, or by application to mucous membranes, such as, that of the nose, throat, and bronchial tubes. They may be administered alone or with suitable pharmaceutical carriers, and can be in solid or liquid form such as, tablets, capsules, powders, solutions, suspensions, or emulsions.

[0142] The compositions of the present application may be orally administered, for example, with an inert diluent, or with an assimilable edible carrier, or they may be enclosed in hard or soft shell capsules, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, these compositions may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the composition in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. Preferred compositions according to the present application are prepared so that an oral dosage unit contains between about 1 and 250 mg of active compound.

[0143] The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, or saccharin. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a fatty oil.

[0144] These compositions may also be administered parenterally. Solutions or suspensions of the present compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols such as, propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0145] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

[0146] The composition of the present application may also be administered directly to the airways in the form of an aerosol. For use as aerosols, the compositions of the present application in solution or suspension may be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. The materials of the present application also may be administered in a non-pressurized form such as in a nebulizer or atomizer.

[0147] The compositions of the present application further contain, in some embodiments, a second agent or pharmaceutical composition selected from the non-limiting group of anti-inflammatory agents, antidiabetic agents, hypolipidemic agents, chemotherapeutic agents, antiviral agents, antibiotics, metabolic agents, small molecule inhibitors, protein kinase inhibitors, adjuvants, apoptotic agents, proliferative agents, organotropic targeting agents, immunological agents, antigens from pathogens, such as viruses, bacteria, fungi and parasites, optionally in the form of whole inactivated organisms, peptides, proteins, glycoproteins, carbohydrates, or combinations thereof, any examples of pharmacological or immunological agents that fall within the above-mentioned categories and that have been approved for human use that may be found in the published literature, any other bioactive component, or any combination of any of these.

[0148] In some embodiments, a second agent or pharmaceutical composition selected from the non-limiting group of anti-inflammatory agents, antidiabetic agents, hpyolipidemic agents, chemotherapeutic agents, antiviral agents, antibiotics, metabolic agents, small molecule inhibitors, protein kinase inhibitors, adjuvants, apoptotic agents, proliferative agents, organotropic targeting agents.

[0149] The importance of E3 ubiquitin ligases, and functional domains thereof, is highlighted by the number of normal cellular processes they regulate, and underlies the attendant diseases associated with loss of function or inappropriate targeting. See Ardley et al., "E3 Ubiquitin Ligases. *Essays Biochem.*" 41:15–30 (2005), which is hereby incorporated by reference in its entirety.

[0150] A fourth aspect of the present application relates to a method of treating a disease. The method includes selecting a subject having a disease and administering the composition described herein to the subject to give the subject an increased expression level of the substrate compared to a subject not afflicted with the disease.

[0151] The chimeric molecule described in this aspect is carried out in accordance with the previously described aspects of the present application.

[0152] As used herein, the term "subject" refers to a mammal, such as a human, but can also be another animal such as a domestic animal (e.g., a dog, cat, or the like), a farm animal (e.g., a cow, a sheep, a pig, a horse, or the like) or a laboratory animal (e.g., a monkey, a rat, a mouse, a rabbit, a guinea pig, or the like). The term "patient" refers to a "subject" who is, or is suspected to be, afflicted with a disease or condition.

[0153] The methods may involve administering compositions to a subject, where the disease possesses a measurable phenotype. The phenotype of the disease involves an increased expression level of a substrate compared to the phenotype from a subject not afflicted with the disease, in some embodiments. In this respect, chimeric molecules contained in the pharmaceutical compositions of the present application are efficacious against treating or alleviating the symptoms from a disease characterized by a phenotypic increase in the expression level of one or more substrates compared to the phenotype from a subject not afflicted with the disease.

[0154] Non-limiting examples of diseases that can be treated or prevented in the context of the present application, include, cancer, metastatic cancer, solid cancers, invasive cancers, disseminated cancers, breast cancer, lung cancer, NSCLC cancer, liver cancer, prostate cancer, brain cancer, pancreatic cancer, lymphatic cancer, ovarian cancer, endometrial cancer, cervical cancer, and other solid cancers known in the art, blood cell malignancies, lymphomas,

leukemias, myelomas, stroke, ischemia, myocardial infarction, congestive heart failure, stroke, ischemia, peripheral vascular disease, alcoholic liver disease, cirrhosis, Parkinson's disease, Alzheimer's disease, diabetes, cancer, arthritis, ALS, pathogenic diseases, idiopathic diseases, viral diseases, bacterial, diseases, prionic diseases, fungal diseases, parasitic diseases, arthritis, wound healing, immunodeficiency, inflammatory disease, aplastic anemia, anemia, genetic disorders, congenital disorders, type 1 diabetes, type 2 diabetes, gestational diabetes, high blood glucose, metabolic syndrome, lipodystrophy syndrome, dyslipidemia, insulin resistance, leptin resistance, atherosclerosis, vascular disease, hypercholesterolemia, hypertriglyceridemia, non-alcoholic fatty liver disease, septic shock, multiple organ dysfunction syndrome, rheumatoid arthritis, trauma, stroke, heart infarction, systemic autoimmune disease, chronic hepatitis, overweight, and/or obesity, or any combination thereof.

[0155] In some embodiments, the disease is cancer, metastatic cancer, stroke, ischemia, peripheral vascular disease, alcoholic liver disease, hepatitis, cirrhosis, Parkinson's disease, Alzheimer's disease, cystic fibrosis diabetes, ALS, pathogenic diseases, idiopathic diseases, viral diseases, bacterial, diseases, prionic diseases, fungal diseases, parasitic diseases, arthritis, wound healing, immunodeficiency, inflammatory disease, aplastic anemia, anemia, genetic disorders, congenital disorders, type 1 diabetes, type 2 diabetes, gestational diabetes, high blood glucose, metabolic syndrome, lipodystrophy syndrome, dyslipidemia, insulin resistance, leptin resistance, atherosclerosis, vascular disease, hypercholesterolemia, hypertriglyceridemia, non-alcoholic fatty liver disease, overweight, or obesity, and any combination thereof.

[0156] When used *in vivo* for therapy, the compositions are administered to the subject in effective amounts, *i.e.*, amounts that have desired therapeutic effect. The dose and dosage regimen will depend upon the degree of the disease in the subject, the characteristics of the particular peptide used, *e.g.*, its therapeutic index, the subject, and the subject's history. The effective amount may be determined during pre-clinical trials and clinical trials by methods familiar to physicians and clinicians. An effective amount of a peptide useful in the methods may be administered to a mammal in need thereof by any of a number of well-known methods for administering pharmaceutical compounds.

[0157] Dosage, toxicity and therapeutic efficacy of the compositions can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit high therapeutic indices may be desirable. While compositions that exhibit toxic

side effects may be used, care should be taken to design a delivery system that targets such compositions to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0158] In some embodiments, the compositions of the present application are administered orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal instillation, by implantation, by intracavitary or intravesical instillation, intraocularly, intraarterially, intralesionally, transdermally, or by application to mucous membranes.

[0159] A fifth aspect of the present application relates to a method for substrate silencing. The method includes selecting a substrate to be silenced; providing the chimeric molecule described herein; and contacting the substrate with the chimeric molecule under conditions effective to permit the formation of a substrate-molecule complex, wherein the complex mediates the degradation of the substrate to be silenced.

[0160] The chimeric molecule described in this aspect is carried out in accordance with the previously described aspects of the present application.

[0161] A sixth aspect of the present application relates to a method of screening agents for therapeutic efficacy against a disease. The method includes providing a biomolecule whose presence mediates a disease state; providing a test agent comprising (i) a degradation domain comprising an E3 ubiquitin ligase (E3) motif, (ii) a targeting domain capable of specifically directing the degradation domain to the biomolecule, wherein the targeting domain is heterologous to the degradation domain, and (iii) a linker coupling the degradation domain to the targeting domain; contacting the biomolecule with the test agent under conditions effective for the test agent to facilitate degradation of the biomolecule; determining the level of the biomolecule as a result of the contacting; and identifying the test agent which, based on the determining, decreases the level of the biomolecule as being a candidate for therapeutic efficacy against the disease.

[0162] The chimeric molecule described in this aspect is carried out in accordance with the previously described aspects of the present application.

[0163] As used herein, the terms “reference level” or “control level” refer to an amount or concentration of biomarker (or biomolecule, ligand, substrate and the like) which may be of interest for comparative purposes. In some embodiments, a reference level may be the level of at least one biomarker expressed as an average of the level of at least one biomarker taken from a control population of healthy subjects or from a diseased population possessing aberrant expression of a protein or substrate. In another embodiment, the reference level may be the level

of at least one biomarker in the same subject at an earlier time, *i.e.*, before the present assay. In even another embodiment, the reference level may be the level of at least one biomarker in the subject prior to receiving a treatment regime.

[0164] As used herein, the term “sample” may include, but is not limited to, bodily tissue or a bodily fluid such as blood (or a fraction of blood such as plasma or serum), lymph, mucus, tears, saliva, sputum, urine, semen, stool, CSF, ascities fluid, or whole blood, and including biopsy samples of body tissue. A sample may also include an *in vitro* culture of microorganisms grown from a sample from a subject. A sample may be obtained from any subject, *e.g.*, a subject/patient having or suspected to have a disease or condition characterized by a disease.

[0165] As used herein, the term “screening” means determining whether a chimeric molecule or composition has capabilities or characteristics of preventing or slowing down (lessening) the targeted pathologic condition stated herein, namely a disease or condition characterized by defects in specified disease.

[0166] As used herein, the terms “effective amount” or “therapeutically effective amount” of a chimeric molecule or composition is a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, for example, an amount which results in the prevention of or a decrease in the symptoms associated with a disease that is being treated. The amount of compound administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity or stage of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

[0167] The term “enzyme linked immunosorbent assay” (ELISA) as used herein includes an antibody-based assay in which detection of the antigen of interest is accomplished via an enzymatic reaction producing a detectable signal. An ELISA can be run as a competitive or non-competitive format. ELISA also includes a 2-site or “sandwich” assay in which two antibodies to the antigen are used, one antibody to capture the antigen and one labeled with an enzyme or other detectable label to detect captured antibody-antigen complex. In a typical 2-site ELISA, the antigen has at least one epitope to which unlabeled antibody and an enzyme-linked antibody can bind with high affinity. An antigen can thus be affinity captured and detected using an enzyme-linked antibody. Typical enzymes of choice include alkaline phosphatase or horseradish peroxidase, both of which generate a detectable product when contacted by appropriate substrates.

[0168] As used herein, the term “epitope” includes a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings

of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Typically, an epitope will be a determinant region form a substrate, which can be recognized by one or more target domains.

[0169] To screen for targeting domains or substrates which possess an epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), which is hereby incorporated by reference in its entirety, can be performed. This assay can be used to determine if a target domain binds the same site or epitope of a substrate as a different targeting domain, antibody, antibody fragment and the like. Alternatively, or additionally, epitope mapping can be performed by methods known in the art. For example, the antibody sequence can be mutagenized such as by alanine scanning, to identify contact residues. In a different method, peptides corresponding to different regions of substrate can be used in competition assays with a test target domain or with a test antibody and a target domain or an antibody with a characterized epitope.

[0170] As used herein, the term “hypervariable region” refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a “complementarity determining region” or “CDR”, *e.g.*, around about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the V_L, and around about 31-35B (H1), 50-65 (H2) and 95-102 (H3) in the V_H (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), which is hereby incorporated by reference in its entirety), and/or those residues from a “hypervariable loop” (*e.g.*, residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the V_L, and 26-32 (H1), 52A-55 (H2) and 96-101 (H3) in the V_H (Chothia and Lesk, “Canonical Structures for the Hypervariable Regions of Immunoglobulins,” *J. Mol. Biol.* 196:901–17 (1987)), which is hereby incorporated by reference in its entirety).

[0171] As used herein, the terms “isolated” or “purified” polypeptide, peptide, molecule, or chimeric molecule, is substantially free of cellular material or other contaminating polypeptides from the cell or tissue source from which the agent is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. For example, a chimeric molecule would be free of materials that would interfere with such a molecules intended function, diagnostic or therapeutic uses. Such interfering materials may include

proteins or fragments other than the materials encompassed by the chimeric molecule, enzymes, hormones and other proteinaceous and nonproteinaceous solutes.

[0172] In one embodiment, the identifying is carried out with respect to a standard biomolecule level in a subject not afflicted with the disease. The identifying may also be carried out with respect to the biomolecule level absent the contacting, in some embodiments. A control level, in the regard, can be employed to compare to the level of the biomolecule in a sample. In some embodiments, the control level is the level of the biomolecule from a subject not afflicted with the disease. An overabundance of the biomolecule in the sample obtained from the subject suspected of having the disease or condition affecting substrate levels compared with the sample obtained from the healthy subject is indicative of the biomolecule-associated disease or condition in the subject being tested.

[0173] There are a myriad of diseases in which the degree of overabundance of certain substrate biomolecules are known to be indicative of whether a subject is afflicted with a disease or is likely to develop a disease. *See, e.g.,* Anderson et al., “Discovering Robust Protein Biomarkers for Disease from Relative Expression Reversals in 2-D DIGE Data,” *Proteomics* 7:1197–1207 (2007), which is hereby incorporated by reference in its entirety. Examples of conditions in which biomolecules are increased compared to control subjects include the diseases described above.

[0174] Accordingly, the chimeric molecules and compositions of the present application are administered to a subject in need of treatment. E3 ubiquitin ligases, such as the E3 gene products encoding a E3 motif, are described above, and can be used in the present screening methods for determining the efficacy of the chimeric molecules disclosed herein. In some embodiments, suitable *in vitro* or *in vivo* assays are performed to determine the effect of the chimeric molecules and compositions of the present application and whether administration is indicated for treatment. Compositions for use in therapy can be tested in suitable animal model systems including, but not limited to rats, mice, chicken, cows, monkeys, rabbits, and the like, prior to testing in human subjects. Similarly, for *in vivo* testing, any of the animal model system known in the art can be used prior to administration to human subjects.

[0175] Any method known to those in the art for contacting a cell, organ or tissue with a composition may be employed. *In vivo* methods typically include the administration of a chimeric molecule or composition, such as those described above, to a mammal, suitably a human. When used *in vivo* for therapy, the chimeric molecules or compositions are administered to the subject in effective amounts, as described herein. Results can be ascertained as per the empirical variables set forth at the outset of the methods described herein.

[0176] *In vitro* methods typically include the assaying the effect of chimeric molecule or composition, such as those described above, on a sample or extract. In some embodiments, chimeric molecule efficacy can be determined by assessing the affect on substrate degradation, *i.e.*, the ability of the chimeric molecules and compositions to exert a phenotypic change in a sample. Such methods include, but are not limited to, immunohistochemistry, immunofluorescence, ELISPOT, ELISA, or RIA. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Nakamura et al., Enzyme Immunoassays: Heterogeneous and Homogeneous Systems, Handbook of Experimental Immunology, Vol. 1: *Immunochemistry* 27.1–27.20 (1986), each of which is incorporated herein by reference in its entirety and specifically for its teaching regarding immunodetection methods.

[0177] Immunoassays, in their most simple and direct sense, are binding assays involving binding between antibodies and antigen. Many types and formats of immunoassays are known and all are suitable for detecting the disclosed biomarkers. Examples of immunoassays are enzyme linked immunosorbent assays (ELISAs), enzyme linked immunospot assay (ELISPOT), radioimmunoassays (RIA), radioimmune precipitation assays (RIPA), immunobead capture assays, Western blotting, dot blotting, gel-shift assays, Flow cytometry, immunohistochemistry, fluorescence microscopy, protein arrays, multiplexed bead arrays, magnetic capture, *in vivo* imaging, fluorescence resonance energy transfer (FRET), and fluorescence recovery/localization after photobleaching (FRAP/ FLAP).

[0178] In general, immunoassays involve contacting a sample suspected of containing a molecule of interest (such as the disclosed biomolecule) with an antibody to the molecule of interest or contacting an antibody to a molecule of interest (such as antibodies to the disclosed biomolecule) with a molecule that can be bound by the antibody, as the case may be, under conditions effective to allow the formation of immunocomplexes. In this regard, the skilled artisan will be able to assess the presence and or level of specific biomolecules in a given sample. Subsequently, the chimeric molecule compositions of the present application are added to the assay. Thereafter, the level of biomolecule can be assessed, *i.e.*, the presence or level thereof, using the immunoassays described herein to determine the post-treatment phenotypic effect.

[0179] Immunoassays can include methods for detecting or quantifying the amount of a biomolecule of interest in a sample, which methods generally involve the detection or quantitation of any immune complexes formed during the binding process. In general, the detection of immunocomplex formation is well known in the art and can be achieved through the application of numerous approaches. These methods are generally based upon the detection of a

label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or any other known label. *See, for example*, U.S. Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each of which is incorporated herein by reference in its entirety and specifically for teachings regarding immunodetection methods and labels.

5 **[0180]** The treatment methods of the present application possess ubiquitin regions attached to targeting domains, as described above. In some embodiments, the targeting domain binds an intracellular biomolecule, as described above. Likewise, the treatment methods of the present application employ polypeptide linkers of sufficient length to prevent the steric disruption of binding between the targeting domain and the substrate. In some embodiments, the
10 biomolecule is associated with a disease as described above.

[0181] In one embodiment, the method is carried out with a plurality of test agents.

[0182] A seventh aspect of the present application relates to a method of screening for disease biomarkers. The method includes providing a sample of diseased cells expressing one or more ligands; providing a plurality of chimeric molecules comprising (i) a degradation domain
15 comprising an E3 ubiquitin ligase (E3) motif, (ii) a targeting domain capable of specifically directing the degradation domain to the one or more ligands, wherein the targeting domain is heterologous to the degradation domain, and (iii) a linker coupling the degradation domain to the targeting domain; contacting the sample with the plurality of chimeric molecules under conditions effective for the diseased cells to fail to proliferate in the absence of the chimeric
20 molecule; determining which of the chimeric molecules permit the diseased cells to proliferate; and identifying, as biomarkers for the disease, based on the determining the ligands which bind to the chimeric molecules and permit diseased cells to proliferate.

[0183] The chimeric molecule described in this aspect is carried out in accordance with the previously described aspects of the present application.

25 **[0184]** As used herein, the terms “biomarker” or “biomolecule” or “molecule” refer to a polypeptide (of a particular expression level) which is differentially present in a sample taken from patients having a disease as compared to a comparable sample taken from a control subject or a population of control subjects.

[0185] As used herein, the terms “ligand” or “substrate” refer to substance that are able
30 to bind to and form transient or stable complexes with a protein, molecule, chimeric molecule, ligand (dimer), substrate (dimer), a second substrate, a second ligand, target domain, regions, portions, and fragments thereof, ubiquitin or E3 motif regions, domains, or portions thereof, biomolecules, biomarkers, and the like, to serve a biological purpose, for example a substrate which interacts with an enzyme in the process of an enzymatic reaction. Ligands also include

signal triggering molecules which bind to sites on a target protein, by intermolecular forces such as ionic bonds, hydrogen bonds and Van der Waals forces. In some embodiments, substrates bind ligands and/or ligands bind substrates.

[0186] Many, if not all diseases, are complex and multifactorial. When considering neurodegeneration, for example, substantial neuronal cell loss occurs before pathologic presentation. Screening for and developing such drugs—to treat neurodegenerative diseases—is further stymied by ancillary therapies which ameliorate the symptoms. Thus, target detection is obfuscated by prior therapeutic administration, which, may in turn, slow disease progression and further confound treatment regimes. In this way, the present application provides new, inventive, screening methods for elucidation of disease biomarkers by employing phenotypic screening analyses. *See, e.g.,* Pruss, R.M., “Phenotypic Screening Strategies for Neurodegenerative Diseases: A Pathway to Discover Novel Drug Candidates and Potential Disease Targets or Mechanisms.” *CNS & Neurological Disorders - Drug Targets*, 9, 693–700 (2010), which is hereby incorporated by reference in its entirety.

[0187] Phenotypic screening involves using an appropriate sample, *e.g.*, class of cells, cell extract, neurons, tissue, and the like, from a patient afflicted with a disease and subjecting the sample to one or more chimeric molecules as described herein. Subsequently, the sample is screened for viability, proliferation, cell processes and/or phenotypic characteristic of the diseased cell, *e.g.*, shrinking, loss of membrane potential, morphological changes, and the like. Image analysis software allows for cell bodies or other objects to empirically assess the results. Hits coming from the screen may maintain cell survival by stimulating survival pathways, mimicking trophic factors, or inhibiting death signaling. Higher content screening and profiling in target-directed secondary assays can then be used to identify targets and mechanisms of action of promising hits.

[0188] Examples of diseases conditions from which a biomarker screening analysis can be performed include the diseases described above. In some embodiments, the method of screening for disease biomarkers includes a plurality of molecules, where the molecules possess a E3 motif as described above. In some embodiments, the biomarker screening methods include molecules possessing a targeting domain as described above. The screening methods of the present application employ polypeptide linkers of sufficient length to prevent the steric disruption of binding between the targeting domain and the biomolecule and/or ligand.

[0189] Once a chimeric molecule is determined to provide a therapeutic indication, the biomarker is isolated using the targeting domain region (or the entire chimeric molecule) to immunoprecipitate the biomarker, from a sample, which is subsequently identified using

methods well known in the art. Biomarker isolation and purification methods include, but are not limited to, for example, HPLC or FPLC chromatography using size-exclusion or affinity-based column resins. *See, e.g.,* Sambrook et al. 1989, Cold Spring Harbor Laboratory Press, which is hereby incorporated by reference in its entirety.

5 **[0190]** Active fragments, derivatives, or variants of the polypeptides of the present application may be recognized by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure, and biological activity of the polypeptide. For example, a polypeptide may be joined to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs sub-cellular or
10 extracellular localization of the protein.

[0191] The biomarker can then be elucidated using techniques known in the art. In some embodiments, determining the identity of the biomarker is performed using MALDI-TOF, mass spectrometry, mass spectroscopy, protein sequencing, antibody interactions, western blot, immunoassay, ELISA, chromatographic techniques, reverse proteomics, immunoprecipitations,
15 radioimmunoassay, and immunofluorescence, or any combinations thereof.

[0192] Suitable mass spectrometric techniques for the study and identification of proteins include, laser desorption ionization mass spectrometry and electrospray ionization mass spectrometry. Within the category of laser desorption ionization (LDI) mass spectrometry (MS), both matrix assisted LDI (MALDI) and surface assisted LDI (SELDI) time-of-flight (TOF) MS
20 may be employed. SELDI TOF-MS is particularly well-suited for use in the present methods because it provides attomole sensitivity for analysis, quantification of low abundant proteins (pg-ng/ml) and highly reproducible results.

[0193] The methods described herein can be performed, *e.g.,* by utilizing pre-packaged kits comprising at least one reagent, *e.g.,* a chimeric molecule or composition described herein,
25 which can be conveniently used, *e.g.,* in clinical settings to treat subjects exhibiting symptoms of a disease or illness involving an overexpressed substrate, biomolecule, or biomarker. Furthermore, any cell type or tissue in which the chimeric molecule of the present application can be expressed is suitable for use in the kits described herein.

[0194] In another aspect of the present application, a kit or reagent system for using the
30 chimeric molecules and compositions of the present application. Such kits will contain a reagent combination including the particular elements required to conduct an assay according to the methods disclosed herein. The reagent system is presented in a commercially packaged form, as a composition or admixture where the compatibility of the reagents will allow, in a test device configuration, or more typically as a test kit, *i.e.,* a packaged combination of one or more

containers, devices, or the like holding the necessary reagents, and preferably including written instructions for the performance of assays. The kit may be adapted for any configuration of an assay and may include compositions for performing any of the various assay formats described herein.

- 5 **[0195]** Reagents useful for the disclosed methods can be stored in solution or can be lyophilized. When lyophilized, some or all of the reagents can be readily stored in microtiter plate wells for easy use after reconstitution. It is contemplated that any method for lyophilizing reagents known in the art would be suitable for preparing dried down reagents useful for the disclosed methods.
- 10 **[0196]** Also within the scope of the present application are kits comprising the chimeric molecules/compositions and second agents of the application and instructions for use. The kits are useful for detecting the presence of a substrate in a biological sample *e.g.*, any body fluid including, but not limited to, *e.g.*, serum, plasma, lymph, cystic fluid, urine, stool, cerebrospinal fluid, acitic fluid or blood and including biopsy samples of body tissue. For example, the kit can
- 15 comprise one or more chimeric molecules composed of a E3 motif ubiquitin region linked to a targeting domain capable of binding a substrate in a biological sample.

EXAMPLES

- 20 **[0197]** The following examples are provided to illustrate embodiments of the present application but they are by no means intended to limit its scope.

- [0198]** The following examples are included to demonstrate illustrative embodiments of the present application. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to
- 25 function well in the practice of the present application, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present application, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the present application.

30 **Experimental Methods**

- [0199]** *Plasmids.* All plasmids used in this study are provided in **Table 1**.

Table 1. Bacterial strains, cell lines and plasmids used in this study.

Bacterial strain		
DH5α	F- (Φ80Δ <i>lacZ</i> Δ <i>M15</i> ,) Δ(<i>lacZ</i> <i>Y</i> <i>A</i> - <i>argF</i>)U169 <i>recA1 endA1 hsdR17</i> (<i>r_k</i> ⁻ , <i>m_k</i> ⁺) <i>phoA</i> <i>supE44 λ- thi-1 gyrA96 relA1</i>	Laboratory stock
BL21(DE3)	F- <i>ompT gal dcm lon hsdS_B</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) λ(DE3)	Laboratory stock
Rosetta(DE3)	F- <i>ompT gal dcm lon hsdS_B</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) λ(DE3) pRARE (Cm ^R)	Laboratory stock
Cell line		
HEK293T		Laboratory stock
HEK293T-EGFP	HEK293T cells stably expressing EGFP	This study
HEK293T-ERK2-EGFP	HEK293T cells stably expressing ERK2-EGFP	This study
	HEK293T cells stably expressing EGFP-HRas ^{G12V}	This study
HEK293T-EGFP-HRas ^{G12V}		
HEK293T-d2EGFP	HEK293T cells stably expressing d2EGFP	This study
HeLa H2B-EGFP	HeLa cells stably expressing H2B-EGFP fusion	[1]
MCF-10A		Laboratory stock
MCF-10A rTA		Laboratory stock
MCF-10A rTA EGFP- HRAS ^{G12V}	MCF-10A cells stably expressing EGFP- HRAS ^{G12V} fusion and rTA	This study
MCF-10A rTA GS2-lpaH9.8	MCF-10A cells stably expressing GS2- lpaH9.8 fusion and rTA	This study
MCF-10A rTA EGFP- HRAS ^{G12V} + GS2-lpaH9.8	MCF-10A cells stably expressing EGFP- HRAS ^{G12V} fusion, GS2-lpaH9.8, and rTA	This study
MCF-10A rTA EGFP HRAS ^{G12V} + GS2-lpaH9.8 ^{C337A}	MCF-10A cells stably expressing EGFP- HRAS ^{G12V} fusion, GS2-lpaH9.8 ^{C337A} , and rTA	This study
Plasmid	Relevant features	Source
pcDNA3	CMV promoter, Amp ^R	Laboratory stock
pCDH1-CMV-MCS-EF1α- Puro	CMV promoter; Pur ^R , Amp ^R	Laboratory stock
pET28a(+)	T7lac promoter; Kan ^R	Novagen
pET24d(+)	T7lac promoter; Kan ^R	Novagen
pTriEx-3	CMV promoter; T7 promoter; Amp ^R	Novagen
psPAX2	Lentiviral packaging vector; CMV promoter; Amp ^R	Laboratory stock
pMD2.G	Lentiviral packaging vector; CMV promoter; Amp ^R	Laboratory stock
pPB TetOn Hygro	Transposon vector; SV40 promoter; Amp ^R	Laboratory stock
pLV rTA-NeoR	Tetracycline inducible reverse transcriptional transactivator; CMV promoter; EF1α promoter; Neo ^R ; Amp ^R	Laboratory stock
pCMV-hyPBase	PiggyBac transposase; CMV promoter; Amp ^R	[2]

¹ Caussinus et al., “Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody,” *Nat. Struct. Mol. Biol.* 19:117–21 (2011), which is hereby incorporated by reference in its entirety.

² Yusa et al., “A Hyperactive PiggyBac Transposase for Mammalian Applications,” *Proc. Natl. Acad. Sci. USA* 108:1531–6 (2011), which is hereby incorporated by reference in its entirety.

pHIV-d2EGFP	Lentiviral vector expressing d2EGFP EF-1 ^α promoter, IRES; Amp ^R	[3]
pGEM4Z/GFP/A64	In vitro transcription of GFP with 3' 64 residue poly A tail; T7 promoter; Amp ^R	[4]
pHFT2-GS2	GS2 monobody in pHFT2 plasmid; T7 promoter, Kan ^R	[5]
pHFT2-GS5	GS5 monobody in pHFT2 plasmid; T7 promoter, Kan ^R	[5]
pHFT2-GL4	GL4 monobody in pHFT2 plasmid; T7 promoter, Kan ^R	[5]
pHFT2-GL6	GL6 monobody in pHFT2 plasmid; T7 promoter, Kan ^R	[5]
pGalAga-GL8	GL8 monobody in pGalAgaCamR; Cam ^R	[5]
pHFT2-AbISH2MB#AS15	AS15 monobody in pHFT2 plasmid; T7	[4]
pHFT2-SHP2NSa5	NSa5 monobody in pHFT2 plasmid; T7	[6]
pET24a(+)-RasInII	RasInII with C-terminal GS linker, 6xHis, Avi, and Flag tags; T7lac promoter; Kan ^R	[7]
pcDNA3-R4-CHIPΔTPR	scFv13-R4 fused to CHIP lacking TPR domain with C- terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter;	[8]
pcDNA3_NSimb-vhhGFP4	vhhGFP4 fused to F-box domain from <i>Drosophila melanogaster</i> Simb cloned in	[1]; Addgene #35579
pETHis6MEK1 R4F+ERK2	pET-based expression of two proteins: constitutively active MEK1 and wild-type,	[9]
pHFT2-SHP2	Full-length SHP2 in pHFT2 plasmid; T7	[6]
pcDNA3-EGFP	EGFP cloned in pcDNA3; CMV promoter, Amp ^R ;	This study
pcDNA3-HF-GS2	GS2 with N-terminal Kozak, Flag and 6xHis tags, and BamHI and EcoRI sites; CMV	This study
pcDNA3-GS2-FH	GS2 with C-terminal NheI and SbfI sites, Flag tag, and 6xHis tag; CMV promoter; Amp ^R .	This study
pcDNA3-GS2-AvrPtoB	GS2 fused to <i>Pseudomonas syringae</i> AvrPtoB lacking N- terminal domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV	This study

³ Li et al., "Structurally Modulated Codelivery of siRNA and Argonaute 2 for Enhanced RNA interference," *Proc. Natl. Acad. Sci. USA* 115:E2696–e2705 (2018), which is hereby incorporated by reference in its entirety.

⁴ Boczkowski et al., "Induction of Tumor Immunity and Cytotoxic T Lymphocyte Responses Using Dendritic Cells Transfected With Messenger RNA Amplified From Tumor Cells," *Cancer Res.* 60:1028–34 (2000), which is hereby incorporated by reference in its entirety.

⁵ Koide et al., "Teaching an Old Scaffold New Tricks: Monobodies Constructed Using Alternative Surfaces of the FN3 Scaffold," *J. Mol. Biol.* 415:393–405 (2012), which is hereby incorporated by reference in its entirety.

⁶ Sha et al., "Dissection of the BCR-ABL Signaling Network Using Highly Specific Monobody Inhibitors to the SHP2 Domains," *Proc. Natl. Acad. Sci. USA* 110:14924–29 (2013), which is hereby incorporated by reference in its entirety.

⁷ Cetin et al., "RasIns: Genetically Encoded Intrabodies of Activated Ras Proteins," *J. Mol. Biol.* 429:562–73 (2017), which is hereby incorporated by reference in its entirety.

⁸ Portnoff et al., "Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed With Unnatural Substrate Specificity for Targeted Protein Silencing," *J. Biol. Chem.* 289:7844–55 (2014), which is hereby incorporated by reference in its entirety.

⁹ Khokhlatchev et al., "Reconstitution of Mitogenactivated Protein Kinase Phosphorylation Cascades in Bacteria. Efficient Synthesis of Active Protein Kinases," *J. Biol. Chem.* 272:11057–62 (1997), which is hereby incorporated by reference in its entirety.

pcDNA3-GS2-IpaH9.8	GS2 fused to <i>Shigella flexneri</i> IpaH9.8 lacking LRR domain (IpaH9.8ΔLRR) with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV	This study
pcDNA3-GS2-IpaH9.8 ^{C337A}	pcDNA3-GS2-IpaH9.8 containing Cys337Ala	This study
pcDNA3-AS15-IpaH9.8	AS15 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-GS2-IpaH1.4	GS2 fused to <i>S. flexneri</i> IpaH1.4 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-GS2-IpaH2.5	GS2 fused to <i>S. flexneri</i> IpaH2.5 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-GS2-IpaH4.5	GS2 fused to <i>S. flexneri</i> IpaH4.5 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-GS2-IpaH7.8	GS2 fused to <i>S. flexneri</i> IpaH7.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-GS2-IpaH0722	GS2 fused to <i>S. flexneri</i> IpaH0722 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter;	This study
pcDNA3-LegAU13-GS2	GS2 fused to <i>L. pneumophila</i> LegAU13 F-box with N-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-LegU1-GS2	GS2 fused to <i>L. pneumophila</i> LegU1 F-box with N-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-LubX-GS2	GS2 fused to <i>Legionella pneumophila</i> LubX lacking CTD domain with N-terminal Flag and 6x-His tags cloned in pcDNA3;	This study
pcDNA3-GS2-NleG2-3	GS2 fused to EHEC O157:H7 NleG2-3 lacking N-terminal domain with C-terminal Flag and 6x-His tags cloned in pcDNA3;	This study
pcDNA3-GS2-NleG5-1	GS2 fused to EHEC O157:H7 NleG5-1 lacking N-terminal domain with C-terminal Flag and 6x-His tags cloned in pcDNA3;	This study
pcDNA3-GS2-NleL	GS2 fused to EHEC O157:H7 NleL lacking β-helix domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-SidC-GS2	GS2 fused to <i>L. pneumophila</i> SidC lacking N-terminal domain with N-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter;	This study
pcDNA3-GS2-SlrP	GS2 fused to Enterohemorrhagic <i>Escherichia coli</i> (EHEC) O157:H7 SlrP lacking LRR domain with C-terminal Flag and 6x-His tags	This study
pcDNA3-GS2-SopA	GS2 fused to <i>S. typhimurium</i> SopA lacking β-helix domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-GS2-SspH1	GS2 fused to <i>Salmonella typhimurium</i> SspH1 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3;	This study
pcDNA3-GS2-SspH2	GS2 fused to <i>S. typhimurium</i> SspH2 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-GS2-XopL	GS2 fused to <i>Xanthomonas campestris</i> XopL lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3;	This study
pcDNA-βTrCP-GS2	GS2 fused to full length <i>H. sapiens</i> βTrCP with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study

pcDNA3-GS2-CHIPΔTPR	GS2 cloned in place of scFvR4 in pcDNA3-R4-CHIPΔTPR; CMV promoter; Kozak;	This study
pcDNA3-Slmb-GS2	GS2 cloned in place of vhhGFP4 in pcDNA3-NSlmb- vhhGFP4; CMV	This study
pcDNA3-GS2-SPOP	GS2 fused to <i>Homo sapiens</i> SPOP F-box with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak;	This study
pcDNA3-VHL-GS2	GS2 fused to <i>H. sapiens</i> VHL F-box with N-terminal Flag and 6x-His tags cloned in	This study
pcDNA3-GS5-IpaH9.8	GS5 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-GL4-IpaH9.8	GL4 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-GL6-IpaH9.8	GL6 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-GL8-IpaH9.8	GL8 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-vhhGFP4-IpaH9.8	vhhGFP4 fused to IpaH9.8 lacking LRR domain with C- terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter;	This study
pcDNA3-NSa5-IpaH9.8	NSa5 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-NSa5-IpaH9.8 ^{C337A}	NSa5 fused to IpaH9.8 ^{C337A} lacking LRR domain with C- terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter;	This study
pcDNA3-RasInII-IpaH9.8	RasInII fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pcDNA3-RasInII-IpaH9.8 ^{C337A}	RasInII fused to IpaH9.8 ^{C337A} lacking LRR domain with C- terminal Flag and 6x-His tags cloned in pcDNA3; CMV promoter;	This study
mEmerald-C1	CMV promoter; Kan ^R	Laboratory stock
mVenus-N1	CMV promoter; Kan ^R	Laboratory stock
pcDNA3-mCerulean	CMV promoter; Kozak; Amp ^R	This study
pcDNA3-CFP	CMV promoter; Kozak; Amp ^R	This study
pcDNA3-sfGFP	CMV promoter; Kozak; Amp ^R	This study
pCDH--mCherry	CMV promoter; EF1α promoter; Puro ^R ; Amp ^R	Laboratory stock
pPB-GS2-IpaH9.8	GS2-IpaH9.8 with C-terminal Flag and 6x-His tags cloned in pPB TetOn Hygro; CMV	This study
pPB-GS2-IpaH9.8 ^{C337A}	GS2-IpaH9.8 ^{C337A} with C-terminal Flag and 6x-His tags cloned in pPB TetOn Hygro; CMV promoter; SV40 promoter; Amp ^R	This study
pCDH1-ERK2-EGFP	ERK2 fused to N-terminus of EGFP cloned in pCDH1-CMV- MCS-EF1α-Puro; CMV	This study
pCDH1-EGFP	EGFP cloned in pCDH1-CMV-MCS-EF1α-Puro; CMV promoter; Pur ^R ;	This study
pCDH1-EGFP-HRas ^{G12V}	HRas ^{G12V} fused to C-terminus of EGFP cloned in pCDH1- CMV-MCS-EF1α-Puro;	This study

mEmerald- α -actinin-19	α -actinin fused to N-terminus of mEmerald; CMV promoter; Kan ^R	Laboratory stock
pcDNA3.1(-)- α -synuclein-EGFP	α -synuclein fused to the N-terminus of EGFP cloned in pcDNA3.1; CMV	[10]
mEmerald-FAK-5	FAK fused to C-terminus of mEmerald; CMV	Laboratory stock
pEGFP-C1 F-tractin-EGFP	F-tractin fused to N-terminus of EGFP; CMV	Addgene #58473
EGFR-mEmerald	EGFR fused to N-terminus of mEmerald; CMV promoter; Amp ^R	Laboratory stock
pErbB2-EGFP	ErbB2 fused to N-terminus of EGFP; CMV	Addgene #39321
pcDNA3-ERK2-EGFP	ERK2 fused to EGFP in pcDNA3; CMV promoter; Kozak; Amp ^R	This study
pLV pGK-H2B-EGFP	H2B fused to C-terminus of EGFP; pGK	Laboratory stock
mEGFP-HRas ^{G12V}	HRas ^{G12V} (constitutively active) fused to mEGFP cloned in pCI; CMV promoter;	[11]; Addgene #18666
mEmerald-Muc1-FL	Muc1 fused to N-terminus of mEmerald; CMV	Laboratory stock
pcDNA3-EGFP-NLS	NLS sequence derived from C-terminus of SV40 fused to C- terminus of EGFP and cloned in pcDNA3; CMV promoter; Amp ^R	This study
mEmerald-Paxillin-22	Paxillin fused to N-terminus of mEmerald; CMV promoter; Kan ^R	Laboratory stock
pcDNA3-SHP2-EGFP	SHP2 fused to C-terminus of EGFP; CMV	This study
mEmerald-Vinculin-23	Vinculin fused to C-terminus of mEmerald; CMV promoter; Kan ^R	Laboratory stock
pET28-EGFP	EGFP with C-terminal 6x-His tag in pET28a(+); T7lac promoter; Kan ^R	This study
pET28(+)-GS2	GS2 with C-terminal Flag and 6x-His tags in pET28a(+); T7lac promoter; Kan ^R	This study
pET24d(+)-GS2-IpaH9.8	GS2 fused to IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags in	This study
pET24d(+)-IpaH9.8 Δ LRR	IpaH9.8 lacking LRR domain with C-terminal Flag and 6x-His tags in pET28a(+); T7lac	This study
pTriEX-3-GS2-IpaH9.8 ^{C337A}	GS2 fused to IpaH9.8 lacking LRR domain and containing Cys337Ala mutation in pTriEx-3; CMV promoter; T7 promoter;	This study
pGEM4Z/GS2-IpaH9.8/A64	<i>In vitro</i> transcription of GS2-IpaH9.8 with 3' human globin UTR and 64 residue poly A	This study
pGEM4Z/GS2-IpaH9.8 ^{C337A} /A64	<i>In vitro</i> transcription of GS2-IpaH9.8 ^{C337A} with 3' human globin UTR and 64 residue	This study
pGEM4Z/AS15-IpaH9.8/A64	<i>In vitro</i> transcription of AS15-IpaH9.8 with 3' human globin UTR and 64 residue poly A	This study

E. coli strain DH5 α was used for the construction and propagation of all plasmids. To construct pcDNA3-EGFP, EGFP was PCR amplified using primers that introduced a 5' Kozak sequence and the resulting PCR product was ligated into pcDNA3. Plasmid pCDH1-ERK2-EGFP was created by gene assembly of ERK2 and EGFP using overlap extension PCR with primers that introduced a 5' Kozak sequence followed by ligation into pCDH1. Plasmid pcDNA3-EGFP-NLS was created by PCR amplification of EGFP with primers that added a 5' Kozak sequence

¹⁰ Butler et al., "Bifunctional Anti-Nonamyloid Component α -synuclein Nanobodies are Protective *In Situ*," *PLoS ONE* 11:e0165964 (2016), which is hereby incorporated by reference in its entirety.

¹¹ Yasuda et al., "Supersensitive Ras Activation in Dendrites and Spines Revealed by Two-Photon Fluorescence Lifetime Imaging," *Nat. Neurosci.* 9:283–91 (2006), which is hereby incorporated by reference in its entirety.

and 3' SV40 NLS sequence and then ligation of the PCR product into pcDNA3. Plasmid pcDNA3-SHP2-EGFP was created by PCR amplification of SHP2 with a 5' Kozak sequence followed by ligation into pcDNA3-EGFP. Plasmid pcDNA3-EGFP-HRas^{G12V} was generated by PCR amplification of EGFP-HRas^{G12V} from plasmid mEGFP-HRas G12V and the PCR product was subsequently ligated into pCDH1.

[0200] For creation of GFP-directed uAbs, plasmid pcDNA3-HF-GS2 was created by PCR amplification of GS2 from pHFT2-GS2 (Koide et al., "Teaching an Old Scaffold New Tricks: Monobodies Constructed Using Alternative Surfaces of the FN3 Scaffold," *J. Mol. Biol.* 415(2):393–405 (2012), which is hereby incorporated by reference in its entirety) using primers that introduced upstream Kozak, 6x-His, and FLAG sequences followed by ligation into pcDNA3 such that BamHI and EcoRI restriction sites were available upstream of GS2 for generating N-terminal fusions. For C-terminal fusions, plasmid pcDNA3-GS2-FH was created by PCR amplifying GS2 with primers that introduced an upstream Kozak sequence and downstream NheI and SbfI restriction sites followed by ligation into pcDNA3. The genes encoding AvrPtoB, IpaH9.8, NleG2-3, NleG5-1, NleL, SlrP, SopA, SPOP, SspH1, SspH2, and XopL were PCR amplified with primers introducing NheI and SbfI sites, after which the resulting PCR products were ligated in pcDNA3-GS2-FH. The genes encoding LegAU13, LegU1, and SidC were PCR amplified with primers that introduced BamHI and EcoRI sites, after which the resulting PCR products were ligated in pcDNA3-HF-GS2. Plasmid pcDNA3-GS2-CHIP was created by PCR amplification of GS2 from pHFT2-GS2 using primers that introduced an upstream HindIII and Kozak sequence and downstream NheI site, followed by ligation into pcDNA3-R4-CHIPΔTPR in place of scFvR4. Plasmids pcDNA3-VHL-GS2 and pcDNA3-βTrCP-GS2 were created by PCR amplification of genes encoding VHL and βTrCP with primers that introduced HindII and XhoI (VHL) or BamHI and XhoI (βTrCP) sites after which the resulting PCR products were ligated in place of NSlmb in pcDNA3-NSlmb-GS2. Plasmids pcDNA3-GS2-IpaH9.8^{C337A}, pcDNA3-GS2-IpaH0722, pcDNA3-GS2-IpaH1.4, pcDNA3-GS2-IpaH2.5, pcDNA3-GS2-IpaH4.5, and pcDNA3-GS2-IpaH7.8 were created by site-directed mutagenesis of pcDNA3-GS2-IpaH9.8. The following genes were purchased: SspH1 (Twist Biosciences), IpaH9.8 (Twist Biosciences), VHL (GenScript, Ohu23297D), LubX (Twist Biosciences), LegU1(IDT), and LegAU13 (IDT). All others were amplified from existing plasmids in laboratory stocks or from genomic DNA.

[0201] Plasmid pET24d-GS2-IpaH9.8 and pET24d-IpaH9.8ΔLRR were created by PCR amplification of full-length GS2-IpaH9.8 and truncated IpaH9.8ΔLRR, respectively, with primers that introduced NcoI and NotI (GS2-IpaH9.8) or NheI and NotI (IpaH9.8 ΔLRR) sites,

after which the resulting PCR products were ligated into pET24d(+). Plasmid pET28a-GS2 was created by PCR amplification of GS2 from pHFT2-GS2 using primers that introduced an upstream NcoI site and downstream FLAG, 6x-His, and HindIII sequences, after which the resulting PCR product was ligated into pET28a(+). Plasmid pTriEx-3-GS2-IpaH9.8^{C337A} was created by PCR amplification of GS2-IpaH9.8^{C337A} from pcDNA3-GS2-IpaH9.8^{C337A} with primers that introduced EcoRV and HindIII sites, after which the resulting PCR product was ligated into pTriEx-3. Plasmid pET28a-EGFP was created by PCR amplification of GFP with primers adding C-terminal 6x-His tag, after which the resulting PCR product was ligated in pET28a(+). All plasmids were verified by DNA sequencing at the Cornell Biotechnology Resource Center (BRC).

[0202] *Cell Lines, Culture and Transfection.* All cell lines used in this study are provided in **Table 1**. Briefly, HEK293T and HeLa cells were obtained from ATCC, HeLa H2B-EGFP cells were kindly provided by Elena Nigg, and MCF10A rtTA cells were kindly provided by Matthew Paszek, HEK293T, HeLa, and HeLa H2B-EGFP cells were cultured in DMEM with 4.5 g/L glucose and L-glutamine (VWR) supplemented with 10% FetalCloneI (VWR) and 1% penicillin-streptomycin-amphotericin B (ThermoFisher) MCF-10a cells were grown in DMEM/F12 media (ThermoFisher) supplemented with 5% horse serum (ThermoFisher), 20 ng/mL EGF (Peprotech), 0.5 mg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), 10 µg/mL insulin (Sigma), and 1% penicillin-streptomycin-amphotericin B (ThermoFisher). All cells were maintained at 37°C, 5% CO₂ and and 90% relative humidity (RH).

[0203] Stable MCF10A rtTA cell lines were generated using Nucleofection Kit V (Lonza) and HyPBBase, an expression plasmid for the hyperactive version of the PiggyBac transposase. Transposition of MCF10A rtTA cells was performed to generate the following stable lines: MCF10A EGFP-HRas^{G12V}; MCF10A EGFP-HRas^{G12V}:GS2-IpaH9.8; MCF10A EGFP- HRas^{G12V}:GS2-IpaH9.8^{C337A}; and MCF10A GS2-IpaH9.8. Stable cell lines were selected using 200 µg/mL hygromycin B (ThermoFisher).

[0204] Stable HEK293T cell lines expressing EGFP, EGFP-HRas^{G12V}, ERK2-EGFP, d2EGFP were generated by lentiviral transformation. Specifically, pLV IRES eGFP, pcDH1 eGFP-HRas^{G12V}, pcDH1 ERK2-EGFP, or pHIV-d2EGFP were transfected into HEK293T cells along with psPAX2 and pMD2.G by calcium phosphate transfection. Media was replaced after ~16 h, followed by a 48-h incubation to allow virus production. Viral supernatant was removed and Polybrene (Sigma-Aldrich) added to a final concentration of 8 µg/mL, followed by clearance of cell debris by centrifugation at 2,000 rpm for 5 min. Resultant supernatant was diluted 1:6 with cell media and added to previously plated HEK293T cells for stable integration. HEK293T

EGFP and HEK293T ERK2-EGFP cell lines were selected by fluorescence activated cell sorting (BD FACS Aria). The HEK293T EGFP-HRas^{G12V} cell line was selected using 1 µg/mL puromycin (Sigma-Aldrich).

[0205] *Western blot analysis.* HEK293T cells were plated at 10,000 cells/cm² and transfected as described above before lysis with RIPA lysis buffer (Thermo Fisher). MCF10A cells were plated at 20,000 cells/cm² and induced with 0.2 µg/mL doxycycline for 24 h before lysis with cell lysis buffer. Lysates were separated on Any kD polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes. α-HIS-HRP (Abcam), α-GFP (Krackeler) and α-GAPDH (Millipore) antibodies were diluted 1:5,000 and in TBST + 1% milk and incubated for 1 h at room temperature. Secondary antibody goat anti-mouse IgG with HRP conjugation (Promega) was diluted at 1:2,500 and used as needed.

[0206] *Flow cytometric analysis.* Cells were passed into 12-well plates at 10,000 cells/cm². 16-24 h after seeding, cells were transiently transfected with 1 µg total DNA at a 1:2 ratio of DNA:jetPrime (Polyplus Transfection). Cells were transfected with 0.05 µg of target, 0.25 µg of ubiquibody or control, and balanced with empty pcDNA3 vector. Culture media was replaced 4-6 h post-transfection. Then, 24 h post-transfection, cells were harvested and resuspended in phosphate buffered saline (PBS) for analysis using a FACSCalibur or FACS Aria Fusion (BD Biosciences). FlowJo Version 10 was used to analyze samples by geometric mean fluorescence determined from 10,000 events.

[0207] *Microscopy.* Cells were plated at 10,000 cells/cm² on a glass bottom 12-well plate pre-treated with poly-L-lysine (Sigma-Aldrich). After seeding for 16-24 h, cells were transfected with 1 µg total DNA at a 1:2 ratio of DNA:jetPrime (Polyplus Transfection). Cells were transfected with 0.05 µg of target, 0.25 µg of ubiquibody or control, and balanced with empty pcDNA3 vector. Culture media was replaced 4-6 h post-transfection. Then, 24 h post-transfection, cells were fixed with 4% paraformaldehyde. For EGFR-EGFP samples, cells were blocked with 5% normal goat serum in PBS for 2 h at room temperature. The anti-EGFR antibody (Cell Signalling #4267) was diluted 1:200 in 5% normal goat serum in PBS and incubated overnight at 4°C. Cells were washed three times with PBS, then incubated for 1 h at room temperature with anti-rabbit-AF647 diluted 1:200 in 5% normal goat serum in PBS. Cells were washed three times with PBS. Cell nuclei were stained with Hoechst diluted 1:10,000 in PBS for 10 min, then washed three times in PBS. Samples were imaged on an inverted Zeiss LSM88-confocal/multiphoton microscope (i880) using a 40x water immersion objective. Images were analyzed with FIJI.

[0208] *Protein expression and purification.* Purified proteins were obtained by growing *E. coli* BL21(DE3) cells containing a pET28a-based plasmid encoding the desired protein or Rosetta(DE3) cells containing a pTriEx-3-based plasmid in 200 mL of Luria-Bertani (LB) medium at 37°C. Expression was induced with 0.1 mM IPTG when the culture density (Abs₆₀₀) reached 0.6-0.8 and growth continued for 6 h at 30°C. Cultures were harvested by centrifugation at 4,000 x g for 30 min at 4°C. Cell pellets were stored at -20°C overnight. Thawed pellets were resuspended in 10 mL equilibration buffer (25 mM Tris-HCl, pH 7.4, 500 mM NaCl and 20 mM imidazole) and lysed with a high-pressure homogenizer (Avestin Emulsi-Flex C5). The insoluble fraction was cleared by centrifugation at 12,000 x g for 30 min at 4°C. His-tagged protein was purified by gravity flow using 500 µL HisPur Ni-NTA resin (ThermoFisher). The soluble fraction was passed through the resin, after which the resin was washed with 3 mL of wash buffer (25 mM Tris-HCl, pH 7.4, 500 mM NaCl and 50 mM imidazole). Protein was eluted with 1.5 mL elution buffer (25 mM Tris-HCl, pH 7.4, 500 mM NaCl and 250 mM imidazole). Purified fractions were desalted and concentrated (Pierce PES Protein Concentrators).

[0209] *ELISA.* A 96-well enzyme immunoassay plate was coated with 100 µL of EGFP at 10 µg/mL in 0.05 M NaCO₃ buffer, pH 9.6 at 4°C overnight. The plate was then washed three times 200 µL PBST (1x PBS + 0.1% Tween 20) per well and blocked with 250 µL PBS with 3% milk per well at room temperature for 3 h, slowly mixing. The plate was washed three more times, followed by the addition of serial dilutions of purified proteins in blocking buffer at 60 µL per well. Plate was incubated at room temperature, slowly mixing for 1 h. The plate was washed three times to remove un-bound protein and then incubated with 50 µL/well of anti-FLAG (DDDYK) antibody conjugated to horseradish peroxidase (HRP) diluted 1:10,000 in PBST + 1% milk for 1 h with slow mixing. The plate was washed three times before the addition of 50 µL/well 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine) (ThermoFisher). The reaction was allowed to incubate with slow mixing and then quenched with 50 µL/well of 3N H₂SO₄. The quenched plate was then read at 450 nm.

[0210] *Synthesis and characterization of cationic polypeptides.* N4 (TEP) polyamines were synthesized as the researchers of the present group described recently (Li et al., “Polyamine-Mediated Stoichiometric Assembly of Ribonucleoproteins for Enhanced mRNA Delivery,” *Angew Chem. Int. Ed. Engl.* 56(44):13709–12 (2017), which is hereby incorporated by reference in its entirety) according to a modified procedure of Uchida and coworkers. Uchida et al., “Modulated Protonation of Side Chain Aminoethylene Repeats in N-Substituted Polyaspartamides Promotes mRNA Transfection,” *J. Amer. Chem. Soc.* 136(35):12396–405

(2014), which is hereby incorporated by reference in its entirety. Briefly, to a chilled solution of poly(β -benzyl-L-aspartate) in *N*-methyl-2-pyrrolidone (NMP) (Sigma) (2 mL) was added dropwise with stirring 50 equivalents of tetraethylenepentamine (Sigma) diluted two-fold with NMP. After stirring for 2 h at 0°C, the pH was adjusted to 1 with dropwise addition while stirring of cold 6 N HCl. The resulting solution was dialyzed from a regenerated cellulose membrane bag (Spectrum Laboratories, 1 kDa MWCO) against 0.01 N HCl followed by distilled water, frozen, and lyophilized to give a white powder. Polyamines used in this study were characterized by ¹H NMR spectra in deuterium oxide (Cambridge Isotope Laboratories) using a Bruker Avance 400 MHz NMR spectrometer at 25°C: ¹H NMR (400 MHz, D₂O) δ 4.72 (s, 1H), 3.64 – 3.39 (m, 9H), 3.37 – 3.05 (m, 5H), 3.00 – 2.62 (m, 4H).

[0211] *Preparation of mRNA by in vitro transcription.* cDNA encoding uAbs was cloned into pGEM4Z/GFP/A64 by replacing the GFP fragment with XbaI and NotI sites. Additionally, the human α -globin 3' UTR sequence was placed between the cDNA and the poly A tail using NotI and EcoRI to improve mRNA translation. Linearization with SpeI, followed by *in vitro* transcription (IVT) with HiScribe™ T7 High Yield RNA Synthesis Kit (NEB), yielded a transcript containing 64 nucleotides of vector-derived sequence, the coding sequence, α -globin 3' UTR, and 64 A residues. In a typical 20 μ l reaction, the following nucleotides were prepared: ATP (10 mM), pseudo-UTP (10 mM), methyl-CTP (10 mM), GTP (2 mM), anti-reverse Cap analog (8 mM, NEB). RNA was purified by RNeasy purification kit (Qiagen, Hilden, Germany). RNA quality was confirmed by running a 1% agarose gel. Concentration was determined by Abs₂₆₀.

[0212] *Nanoplex transfection.* Polyamines were dissolved in 10 mM HEPES buffer (pH 7.4). For each well of a 96-well plate, 200 ng mRNA diluted in 5 μ l OptiMEM (Thermo Fisher) was mixed with 5 μ l OptiMEM containing PABP (mRNA:PABP weight ratio = 1:5) at room temperature for 10 min. Afterwards, 5 μ l OptiMEM containing polyamines was added and incubated at room temperature for 15 min prior to transfection into HEK293T stably expressing d2EGFP. Polyamines were adjusted to achieve 50 to 1 (N/P) ratio for transfection. EGFP expression was measured by BD FACSCelesta (Becton Dickinson) at different time points after transfection.

[0213] *Animal experiments.* Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidelines and approved protocols from the MIT Division of Comparative Medicine. C57BL/6-Tg(UBC-GFP)30Scha/J mice were purchased from Jackson Laboratory. 8-10 week-old mice were injected subcutaneously in ears with 5 μ g mRNA and 25 μ g PABP packaged with N4 (TEP)

polyamines in a volume of 25 µl OptiMEM under anesthesia. Fluorescent imaging was performed with a CCD camera mounted in a light-tight specimen box (Xenogen). The exposure time was 1 s. Imaging and quantification of signals were controlled by Living Image acquisition and analysis software (Xenogen).

5

Example 1 – Engineered IpaH9.8 Potently Silences GFP in Mammalian Cells

[0214] To determine whether E3 ubiquitin ligase mimics from pathogenic bacteria could be redesigned for silencing of non-native targets, the focus was on a panel of 14 candidate enzymes representing the major classes of E3s found in bacteria to date (**Table 2, *infra***).

- 10 Maculins et al., “Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of Invasion. *Cell Res.* 26(4):499–510 (2016) and Lin et al., “Exploitation of the Host Cell Ubiquitin Machinery by Microbial Effector Proteins,” *J. Cell Sci.* 130(12):1985–96 (2017), which are hereby incorporated by reference in their entirety.

Table 2. Bacterial E3 ubiquitin ligases evaluated in this study.

E3 ubiquitin ligase	Classification	Organism	Construction	Ref. ¹
AvrPtoB	U-box	<i>Pseudomonas syringae</i>	AvrPtoB ₁₋₄₃₆ fused to N-terminus of GS2	[12]
βTrCP	F-box	<i>Homo sapiens</i>	βTrCP ₁₋₅₆₈ fused to N-terminus of GS2 with (GlvSer) ₁₀ linker	[13]
CHIP	U-box	<i>H. sapiens</i>	CHIP ₁₂₈₋₃₀₃ fused to C-terminus of GS2	[14]
IpaH0722	NEL	<i>Shigella flexneri</i>	IpaH0722 ₂₉₅₋₅₈₇ fused to C-terminus of GS2	[15]
IpaH1.4	NEL	<i>S. flexneri</i>	IpaH1.4 ₂₈₅₋₅₇₅ fused to C-terminus of GS2	[15]
IpaH2.5	NEL	<i>S. flexneri</i>	IpaH2.5 ₂₉₂₋₅₇₀ fused to C-terminus of GS2	[15]
IpaH4.5	NEL	<i>S. flexneri</i>	IpaH4.5 ₂₉₆₋₅₇₄ fused to C-terminus of GS2	[15]
IpaH7.8	NEL	<i>S. flexneri</i>	IpaH7.8 ₂₇₄₋₅₆₅ fused to C-terminus of GS2	[15]
IpaH9.8	NEL	<i>S. flexneri</i>	IpaH9.8 ₂₅₄₋₅₄₅ fused to C-terminus of GS2	[16]
LegAU13	F-box	<i>Legionella pneumophila</i>	LegAU13 ₁₋₅₀ fused to N-terminus of GS2	[17]
LegU1	F-box	<i>L. pneumophila</i>	LegU1 ₁₋₅₆ fused to N-terminus of GS2	[17]
LubX	U-box	<i>L. pneumophila</i>	LubX ₁₋₂₁₅ fused to N-terminus of GS2	[18]
NleG2-3	U-box	Enterohemorrhagic <i>Escherichia coli</i> (EHEC) O157:H7	NleG2-3 ₉₀₋₁₉₁ fused to C-terminus of GS2	[19]
NleG5-1	U-box	EHEC O157:H7	NleG5-1 ₁₁₃₋₂₁₃ fused to C-terminus of GS2	[19]
NleL	HECT	EHEC O157:H7	NleL ₃₇₁₋₇₈₂ fused to C-terminus of GS2	[20]
SidC	Unconventional	<i>L. pneumophila</i>	SidC ₁₋₅₄₂ fused to N-terminus of GS2	[21]
SlrP	NEL	EHEC O157:H7	SlrP ₄₆₅₋₇₆₅ fused to N-terminus of GS2	[22]
SopA	HECT	<i>Salmonella typhimurium</i>	SopA ₃₇₀₋₇₈₂ fused to C-terminus of GS2	[23]
SPOP	F-box	<i>H. sapiens</i>	SPOP ₁₆₇₋₃₇₄ fused to C-terminus of GS2	[24]
SspH1	NEL	<i>S. typhimurium</i>	SspH1 ₄₀₄₋₇₀₁ fused to N-terminus of GS2	[25]
SspH2	NEL	<i>S. typhimurium</i>	SspH2 ₄₉₂₋₇₈₈ fused to C-terminus of GS2	[26]
VHL	SCF-like ECV	<i>H. sapiens</i>	VHL ₁₋₂₁₃ fused to N-terminus of GS2	[27]
XopL	Unconventional	<i>Xanthomonas campestris</i>	XopL ₄₇₄₋₆₆₀ fused to C-terminus of GS2	[28]

¹References listed in Table 2 provide clear proof or annotation of the catalytic domains of each E3 ubiquitin ligase.

5 Abbreviations in Table 2: **NEL**, novel E3 ligase; **HECT**, homologous to E6AP C terminus; **SCF**, Skp1/Cdc53 or Cullen-1/F-box protein; **SPOP**, speckle-type POZ protein; **VHL**, von Hippel–Lindau; **ECV**, Elongin B/C, Cullen-2, VHL

10 [12] Janjusevic et al., “A Bacterial Inhibitor of Host Programmed Cell Death Defenses is an E3 ubiquitin ligase,” *Science* 311:222–26 (2006), which is hereby incorporated by reference in its entirety

15 [13] Zhou et al., “Harnessing the Ubiquitination Machinery to Target the Degradation of Specific Cellular Proteins,” *Mol. Cell* 6:751–56 (2000), which is hereby incorporated by reference in its entirety

20 [14] Portnoff et al., “Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed With Unnatural Substrate Specificity for Targeted Protein Silencing,” *J. Biol. Chem.* 289:7844–55 (2014), which is hereby incorporated by reference in its entirety

[15] Ashida et al., “*Shigella* Chromosomal IpaH Proteins are Secreted Via the Type III Secretion System and act as effectors,” *Mol. Microbiol.* 63:680–93 (2007), which is hereby incorporated by reference in its entirety

25 [16] Okuda et al. *Shigella* Effector IpaH9.8 Binds to a Splicing Factor U2AF(35) to Modulate Host Immune Responses,” *Biochem. Biophys. Res. Commun.* 333:531–39 (2005), which is hereby incorporated by reference in its entirety

30 [17] Ensminger, A.W., “RR E3 Ubiquitin Ligase Activity and Targeting of BAT3 by Multiple *Legionella pneumophila* Translocated Substrates,” *Infect. Immun.* 78:3905–19 (2010), which is hereby incorporated by reference in its entirety

35 [18] Quaille et al., “Molecular Characterization of LubX: Functional Divergence of the U-Box Fold by *Legionella pneumophila*,” *Structure* 23:1459–69 (2015), which is hereby incorporated by reference in its entirety

40 [19] Wu et al., “NleG Type 3 Effectors From Enterohaemorrhagic *Escherichia coli* are U-Box E3 Ubiquitin Ligases,” *PLoS Pathog.* 6:e1000960 (2010), which is hereby incorporated by reference in its entirety

[20] Lin et al., “Biochemical and Structural Studies of a HECT-like Ubiquitin Ligase From *Escherichia coli* O157:H7,” *J. Biol. Chem.* 286:441–49 (2011), which is hereby incorporated by reference in its entirety

45 [21] Hsu et al., “The *Legionella* Effector SidC Defines a Unique Family of Ubiquitin Ligases Important for Bacterial Phagosomal Remodeling,” *Proc. Natl. Acad. Sci. USA* 111:10538–43 (2014), which is hereby incorporated by reference in its entirety

50 [22] Zouhir et al., “The Structure of the Slrp-Trx1 Complex Sheds Light on the Autoinhibition Mechanism of the Type III Secretion System Effectors of the NEL family,” *Biochem. J.* 464:135–44 (2014), which is hereby incorporated by reference in its entirety

[23] Diao et al., "Crystal Structure of SopA, a *Salmonella* Effector Protein Mimicking a Eukaryotic Ubiquitin Ligase," *Nat. Struct. Mol. Biol.* 15:65–70 (2008), which is hereby incorporated by reference in its entirety

[24] Shin et al., "Nanobody-Targeted E3-Ubiquitin Ligase Complex Degrades Nuclear Proteins," *Sci. Rep.* 5:14269 (2015), which is hereby incorporated by reference in its entirety

[25] Keszei et al., "Structure of an SspH1-PKN1 Complex Reveals the Basis for Host Substrate Recognition and Mechanism of Activation for a Bacterial E3 Ubiquitin Ligase," *Mol. Cell Biol.* 34:362–73 (2014), which is hereby incorporated by reference in its entirety

[26] Bhavsar et al., "The *Salmonella* Type III Effector SspH2 Specifically Exploits the NLR Co-Chaperone Activity of SGT1 to Subvert Immunity," *PLoS Pathog.* 9:e1003518 (2013), which is hereby incorporated by reference in its entirety

[27] Maniaci et al., "Homo-PROTACs: Bivalent Small-Molecule Dimerizers of the VHL E3 Ubiquitin Ligase to Induce Self-Degradation," *Nat. Commun.* 8:830 (2017), which is hereby incorporated by reference in its entirety

[28] Singer et al., "A Pathogen Type III Effector With a Novel E3 Ubiquitin Ligase Architecture," *PLoS Pathog.* 9:e1003121 (2013), which is hereby incorporated by reference in its entirety

This panel included E3 mimics with folds similar to eukaryotic E3s such as HECT-type, RING or U-box (RING/U-box)-type, and F-box domains, as well as unconventional E3s with folds unlike any other eukaryotic E3s such as novel E3 ligase (NEL), XL-box-containing, and SidC. In general, uAbs were engineered by removing the native substrate-binding domain from each E3 mimic and replacing it with a synthetic binding protein (FIG. 1A), akin to the previously designed uAbs based on human CHIP. Portnoff et al., "Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed With Unnatural Substrate Specificity for Targeted Protein Silencing," *J. Biol. Chem.* 289(11):7844–55 (2014), which is hereby incorporated by reference in its entirety. For example, *S. flexneri* IpaH9.8 consists of an N-terminal domain with eight 20-residue leucine-rich repeats (LRRs) that mediate binding and specificity to native substrate proteins such as NF- κ B essential modulator (NEMO) (Ashida et al., "A Bacterial E3 Ubiquitin Ligase IpaH9.8 Targets NEMO/IKKgamma to Dampen the Host NF-KapPab-Mediated Inflammatory Response," *Nat Cell Biol.* 12(1):66–73, sup. pp. 1–9 (2010), which is hereby incorporated by reference in its entirety) and guanylate-binding proteins (GBPs) (Li et al., "Ubiquitination and Degradation of GBPs by a *Shigella* Effector to Suppress Host Defence," *Nature* 551(7680):378–83 (2017), which is hereby incorporated by reference in its entirety), while the C-terminal domain adopts a novel E3 ubiquitin ligase architecture. Zhu et al., "Structure of a *Shigella* Effector Reveals a New Class of Ubiquitin Ligases," *Nat. Struct. Mol. Biol.* 15(12):1302–08 (2008) and Singer et

al., “A Pathogen Type III Effector With a Novel E3 Ubiquitin Ligase Architecture,” *PLoS Pathogens* 9(1):e1003121 (2013), which are hereby incorporated by reference in their entirety. Hence, the N-terminal LRR domain of IpaH9.8 was replaced with GS2, an FN3 monobody that binds GFP with nanomolar affinity ($K_d = 31$ nM). Koide et al., “Teaching an Old Scaffold New Tricks: Monobodies Constructed Using Alternative Surfaces of the FN3 Scaffold,” *J. Mol. Biol.* 415(2):393–405 (2012), which is hereby incorporated by reference in its entirety. By swapping the natural substrate recognition function of these enzymes with the GS2 monobody, synthetic E3 ligases were created that was hypothesized to target GFP and promote its proteasomal degradation. To test this hypothesis, the different GS2-E3 chimeras were transiently co-expressed along with enhanced GFP (EGFP) in mammalian cells and fluorescence activity was monitored by flow cytometric analysis. By far, the most striking depletion of EGFP was achieved with GS2-IpaH9.8, which reduced EGFP fluorescence to near background levels (FIG. 1B and FIGS. 6A–6B). All of the other uAbs showed relatively weak silencing activity under the conditions tested here. GS2-NleG5-1, GS2-SspH1, SidC-GS2, and GS2-SopA were the most active among these, reducing EGFP fluorescence by ~20–40% (FIG. 1B and FIGS. 6A–6B).

[0215] In light of this robust silencing activity, the attention was focused on the GS2-IpaH9.8. In cells expressing this chimera, the elimination of EGFP was efficient, with removal of up to 90% of the fluorescence activity (FIGs 2A and 2B) and no detectable EGFP protein in cell lysates (FIG. 2C). Importantly, silencing activity was completely abrogated when the catalytic cysteine of IpaH9.8 (Rohde et al., “Type III Secretion Effectors of the IpaH Family are E3 Ubiquitin Ligases,” *Cell Host Microbe* 1(1):77–83 (2007), which is hereby incorporated by reference in its entirety) was mutated to alanine (GS2-IpaH9.8^{C337A}) and when the non-cognate FN3 monobody AS15, which is specific for the Abl SH2 domain (Koide et al., “High-Affinity Single-Domain Binding Proteins with a Binary-Code Interface,” *Proc. Natl. Acad. Sci. USA* 104(16):6632–37 (2007), which is hereby incorporated by reference in its entirety), was substituted for GS2 (FIGs. 2A–2C), indicating that target degradation was dependent on cooperation of both uAb domains. In the case of GS2-IpaH9.8^{C337A}, expression in mammalian cells and EGFP binding activity *in vitro* were unaffected by the alanine substitution (FIGs. 7A–7B), confirming that loss of silencing activity was due to catalytic inactivation. It should also be noted that removal of the LRR domain was essential for knockdown activity, as direct fusion of GS2 to full-length IpaH9.8 that had not been truncated resulted in no measurable silencing activity. Interestingly, the genome sequences of *S. flexneri* strains indicate that several IpaH family members, namely IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8 and IpaH9.8, are encoded on the 220-kb virulence plasmid pWR100 while seven additional *ipaH* cognate genes are present on the chromosome. Maculins et al., “Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of

Invasion. *Cell Res.* 26(4):499–510 (2016), which is hereby incorporated by reference in its entirety. To determine whether these family members were as proficient as IpaH9.8 at degrading EGFP in the uAb context, chimeras were generated between GS2 and the catalytic domains derived from each of the pWR100-encoded IpaH family members as well as one chromosomally encoded member, IpaH0722. When expressed ectopically in cultured cells, all of the IpaH-based uAbs were capable of efficient (~90% or greater) EGFP knockdown in mammalian cells (FIG. 2D). This result was not entirely surprising in light of the high homology shared by the different catalytic domains. Indeed, whereas the different IpaH family members were only ~70% similar to IpaH9.8 overall, the catalytic domains were much more similar (>99%) with just 1-3 amino acid substitutions and, in the case of IpaH1.4 and IpaH4.5, minor C-terminal truncations (Table 2).

[0216] To benchmark the potency of the present engineered bacterial ligase, the GFP silencing activity catalyzed by GS2-IpaH9.8 was compared with that of other synthetic ligases based on eukaryotic E3 machinery that have previously been reconfigured for targeted proteolysis. Zhou et al., “Harnessing the Ubiquitination Machinery to Target the Degradation of Specific Cellular Proteins,” *Mol. Cell* 6(3):751–56 (2000); Zhang et al., “Exploring the Functional Complexity of Cellular Proteins by Protein Knockout,” *Proc. Natl. Acad. Sci. USA* 100(24):14127–32 (2003); Hatakeyama et al., “Targeted Destruction of C-Myc by an Engineered Ubiquitin Ligase Suppresses Cell Transformation and Tumor Formation,” *Cancer Res.* 65(17):7874–79 (2005); Ma et al., “Targeted Degradation of KRAS by an Engineered Ubiquitin Ligase Suppresses Pancreatic Cancer Cell Growth *In Vitro* and *In Vivo*,” *Mol. Cancer Ther.* 12(3):286–94 (2013); Kong et al., “Engineering a Single Ubiquitin Ligase for the Selective Degradation of all Activated ErbB Receptor Tyrosine Kinases,” *Oncogene* 33(8):986–95 (2014); Caussinus et al., “Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody,” *Nat. Struct. Mol. Biol.* 19(1):117–21 (2011); Fulcher et al., “Targeting Endogenous Proteins For Degradation Through the Affinity-Directed Protein Missile System,” *Open Biol.* 7(5):170066 (2017); Fulcher et al., “An Affinity-Directed Protein Missile System for Targeted Proteolysis,” *Open Biol* 6(10):160255 (2016); Shin et al., “Nanobody-Targeted E3-Ubiquitin Ligase Complex Degrades Nuclear Proteins,” *Sci. Rep.* 5:14269 (2015); and Kanner et al., “Sculpting Ion Channel Functional Expression with Engineered Ubiquitin Ligases,” *Elife* 6:e29744 (2017), which are hereby incorporated by reference in their entirety. Specifically, the natural substrate-binding domains for several eukaryotic E3 ubiquitin ligases from humans including carboxyl terminus of Hsc70-interacting protein (CHIP), speckle-type POZ protein (SPOP), β -transducing repeat-containing protein (β TrCP), and von Hippel-Lindau protein (VHL), as well as the *Drosophila*

melanogaster supernumerary limbs (Slmb) protein were replaced with the GS2 monobody, resulting in a panel of synthetic ligases analogous to GS2-IpaH9.8. When the resulting panel of GFP-specific uAbs was transiently co-expressed with EGFP in mammalian cells, all were capable of measurably reducing EGFP levels, but silencing activity for each was relatively inefficient (~25-45%) under the conditions tested here (FIG. 1C and FIGs. 6A–6B), reminiscent of previous results with a Slmb-nanobody chimera that was similarly ineffective at reducing unfused GFP levels. Caussinus et al., “Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody,” *Nat. Struct. Mol. Biol.* 19(1):117–21 (2011), which is hereby incorporated by reference in its entirety. The weak EGFP knockdown observed here for Slmb-GS2 was actually an improvement over previous results obtained with a chimera between Slmb and a GFP-specific VHH nanobody, cAbGFP4, that was incapable of promoting degradation of unfused GFP. Caussinus et al., “Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody,” *Nat. Struct. Mol. Biol.* 19(1):117–21 (2011), which is hereby incorporated by reference in its entirety. It should be noted, however, that the Slmb-cAbGFP4 fusion eliminated the fluorescence associated with larger GFP fusion proteins, suggesting that the data reported here are not necessarily indicative of uAb dysfunction but instead may reflect differences in substrate preference/compatibility or extent of ubiquitin decoration. Regardless, none of the engineered chimeras involving eukaryotic E3s displayed the potency and robustness of GS2-IpaH9.8, which reproducibly degraded 90-95% of cellular fluorescence.

Example 2 - A Broad Range of Substrate Proteins is Degraded by GS2-IpaH9.8.

[0217] To more deeply explore the substrate compatibility issue, the ability of GS2-IpaH9.8 to degrade a range of different substrates was tested. A growing number of GFP-derived fluorescent proteins (FPs) have been developed and optimized over the years, providing a diverse collection of new tools for biological imaging. Tsien, R. Y., “The Green Fluorescent Protein,” *Ann. Rev. Biochem.* 67:509–44 (1998) and Shaner et al., “A Guide to Choosing Fluorescent Proteins,” *Nat. Methods* 2(12):905–09 (2005), which are hereby incorporated by reference in their entirety. To determine the extent to which different FP targets could be degraded, GS2-IpaH9.8 was transiently co-expressed in mammalian cells with monomeric versions of Emerald, Venus and Cerulean, as well as enhanced cyan fluorescent protein (ECFP). Approximately 65-85% of the cellular fluorescence activity associated with each of the FPs was ablated by GS2-IpaH9.8, whereas the structurally unrelated mCherry protein was not targeted by GS2-IpaH9.8, which was expected given the specificity of GS2 for the GFP fold (FIG. 8A). Interestingly, the fluorescence activity of superfolder GFP (sfGFP), a rapidly folding and robustly stable mutant of EGFP, was unaffected by GS2-IpaH9.8, consistent with recent findings

that sfGFP is resistant to proteasomal degradation. Khmelinskii et al., “Incomplete Proteasomal Degradation of Green Fluorescent Proteins in the Context of Tandem Fluorescent Protein Timers,” *Mol. Biol. Cell* 27(2):360–70 (2016), which is hereby incorporated by reference in its entirety.

5 **[0218]** Encouraged by the ability of GS2-IpaH9.8 to degrade different FPs, the ability of GS2-IpaH9.8 to degrade structurally diverse, FP-tagged substrate proteins was next evaluated. GS2-IpaH9.8 proficiently degraded 15 unique target proteins that varied in terms of their molecular weight (27-179 kDa) and subcellular localization (*i.e.*, cytoplasm, nucleus, membrane-associated, and transmembrane) (FIG. 3A and FIG. 8B). For example, GS2-IpaH9.8 triggered
10 degradation of 80-92% of the fluorescence activity associated with FP fusions involving the cytoplasmic proteins α -actinin, α -synuclein (α -syn), extracellular signal-regulated kinase 2 (ERK2), focal adhesion kinase (FAK), F-tractin, paxillin (PXN), and vinculin (VCL) as determined by flow cytometric analysis (FIG. 3A and FIG. 8B). Similarly robust silencing was observed for: nuclear-targeted FP fusions involving histone H2B and the nuclear localization
15 signal (NLS) derived from SV40 Large T-antigen; membrane-associated FP fusions involving Harvey rat sarcoma virus oncogene homolog carrying the oncogenic G12V mutation (HRas^{G12V}), Src-homology 2 domain-containing phosphatase 2 (SHP2), and the farnesyl sequence derived from HRas; and transmembrane FP fusions involving epidermal growth factor receptor (EGFR), avian erythroblastic leukemia viral oncogene homolog 2 (ErbB2), and mucin 1 (MUC1) (FIG.
20 3A and FIG. 8B). Microscopy analysis of representative substrate proteins α -actinin-mEmerald, EGFP-NLS, farnesyl-mEmerald, and EGFR-mEmerald confirmed the expected subcellular localization of each fusion and corroborated the efficient degradation activity measured by flow cytometric analysis (FIG. 3B). The transmembrane protein EGFR-mEmerald was examined by immunolabeling with an antibody specific to the extracellular domain of EGFR. Importantly, the
25 α -EGFR signal decreased concomitantly with GFP disappearance (FIG. 3B), indicating that degradation of the entire transmembrane protein was achieved. Taken together, these results establish GS2-IpaH9.8 as a robust proteome editing tool that is capable of silencing a broad spectrum of substrates that span several distinct subcellular locations.

30 **Example 3 - GS2-IpaH9.8-Mediated Proteome Editing is Flexible and Modular.**

[0219] An attractive feature of uAbs is their highly modular architecture – the E3 catalytic domain and synthetic binding protein domain can be interchanged to reprogram the activity and specificity. Indeed, the results above revealed the ease with which different bacterial and eukaryotic E3 domains can be chimerized to form functional uAbs. To investigate the
35 interchangeability of the synthetic binding protein domain in IpaH9.8-based uAbs, GS2 was first

replaced with other high-affinity GFP-binding proteins such as the FN3 monobody GS5 ($K_d = 62$ nM) (Koide et al., "Teaching an Old Scaffold New Tricks: Monobodies Constructed Using Alternative Surfaces of the FN3 Scaffold," *J. Mol. Biol.* 415(2):393–405 (2012), which is hereby incorporated by reference in its entirety) or cAbGFP4 ($K_d = 0.32$ nM) (Saerens et al.,
5 "Identification of a Universal VHH Framework to Graft Non-Canonical Antigen-Binding Loops of Camel Single-Domain Antibodies," *J. Mol. Biol.* 352(3):597–607 (2005), which is hereby incorporated by reference in its entirety). For these constructs, efficient EGFP silencing activity was observed that rivaled that seen with the GS2 monobody (FIG. 9A). Interestingly, introduction of lower affinity (~200–500 nM) FN3 monobodies (Koide et al., "Teaching an Old
10 Scaffold New Tricks: Monobodies Constructed Using Alternative Surfaces of the FN3 Scaffold," *J. Mol. Biol.* 415(2):393–405 (2012), which is hereby incorporated by reference in its entirety) resulted in less efficient EGFP elimination (FIG. 9A), suggesting that silencing activity may be a function of the affinity for the target protein. Although, because spatial arrangements and surface complementarity prioritize lysine sites for ubiquitination (Buetow et al., "Structural
15 Insights into the Catalysis and Regulation of E3 Ubiquitin Ligases," *Nat. Rev. Mol. Cell Biol.* 17(10):626–42 (2016), which is hereby incorporated by reference in its entirety), an equally plausible explanation for these findings is that the various FN3 domains may differentially orient the uAb with respect to GFP in a manner that affects how the substrate is ubiquitinated.

[0220] Next, the compatibility of the IpaH9.8 catalytic domain with two different FN3
20 monobodies was investigated: NSa5 that is specific for the Src-homology 2 (SH2) domain of SHP2 (Sha et al., "Dissection of the BCR-ABL Signaling Network Using Highly Specific Monobody Inhibitors to the SHP2 SH2 Domains," *Proc. Natl. Acad. Sci. USA* 110(37):14924–29 (2013), which is hereby incorporated by reference in its entirety) and RasInII that is specific for HRas, KRas, and the G12V mutants of each (Cetin et al., "RasIns: Genetically Encoded
25 Intrabodies of Activated Ras Proteins," *J. Mol. Biol.* 429(4):562–573 (2017), which is hereby incorporated by reference in its entirety). The resulting NSa5-IpaH9.8 and RasInII-IpaH9.8 chimeras were tested for their ability to silence SHP2-EGFP and EGFP-HRas^{G12V}, respectively, by flow cytometric analysis. Both exhibited strong silencing activity, degrading their EGFP-tagged targets almost as efficiently as the GFP-directed GS2-IpaH9.8 (FIGs. 4A and 4B).
30 Interestingly, RasInII-IpaH9.8 degraded EGFP-KRas^{G12C} and other KRas mutants (e.g., G12C, G12D) more efficiently than EGFP-KRas (FIG. 4C), in line with its selectivity for the G12V mutant over wild-type Ras isoforms (Cetin et al., "RasIns: Genetically Encoded Intrabodies of Activated Ras Proteins," *J. Mol. Biol.* 429(4):562–573 (2017), which is hereby incorporated by reference in its entirety) and thus providing a potential route for mutant selective silencing of
35 Ras. Collectively, these results reveal a remarkable plasticity for IpaH9.8, enabling its use as a

“one-size fits all” degrader of diverse target proteins in transiently and stably transfected cell lines.

[0221] In all the experiments described above, efficient knockdown was achieved when GS2-IpaH9.8 and its corresponding target were transiently expressed. However, transient expression is not always an option, due to the experimental timescale, necessity for a precise expression profile, or the use of a recalcitrant mammalian cell line. Thus, to demonstrate the flexibility of GS2-IpaH9.8-mediated silencing, degradation activity was evaluated against target proteins that were expressed as stably integrated transgenes. Specifically, when GS2-IpaH9.8 was transiently expressed in cells that stably co-expressed EGFP, reduction of fluorescence activity was virtually identical to that observed for transiently expressed EGFP (FIG. 9B). Robust degradation was also observed for ERK2-EGFP, H2B-EGFP, and EGFP-HRas^{G12V}, regardless of their mode of expression (FIG. 9B). When the uAb and the target were both expressed as stable transgenes, thereby eliminating the need for transfection entirely, strong silencing activity was again observed for GS2-IpaH9.8 but not its inactive GS2-IpaH9.8^{C337A} counterpart (FIG. 9C).

Example 4 - Delivery of mRNA Encoding GS2-IpaH9.8 Enables Proteome Editing in Mice.

[0222] From a therapeutic standpoint, one of the biggest challenges facing protein-based technologies such as uAbs is intracellular delivery. Osherovich, L., “Degradation From Within,” *Science-Business Exchange* 7:10–11 (2014), which is hereby incorporated by reference in its entirety. The researchers of the present group previously showed that co-assembled nanoplexes comprised of synthetic mRNA containing a poly A tail, PABPs, and biocompatible cationic polypeptides (FIG. 5A) resulted in greatly enhanced mRNA expression *in vitro* and in mice. Li et al., “Polyamine-Mediated Stoichiometric Assembly of Ribonucleoproteins for Enhanced mRNA Delivery,” *Angew Chem. Int. Ed. Engl.* 56(44):13709–12 (2017), which is hereby incorporated by reference in its entirety. Here, it was hypothesized that delivery of GS2-IpaH9.8 mRNA/PABP nanoplexes to mammalian cells would result in significantly greater uAb expression relative to mRNA transfection alone by the same polyamine in HEK293T cells, thereby leading to potent protein degradation. To test this hypothesis, GS2-IpaH9.8 mRNA/PABP nanoplex delivery was first evaluated *in vitro* by quantifying the degradation of d2EGFP, a destabilized GFP variant that was expressed as a stable transgene in HEK293T cells. As expected, only when the cationic nanoplexes contained the active, target-specific GS2-IpaH9.8 mRNA and PABP was robust d2EGFP degradation achieved (FIG. 5B). All other controls including catalytically inactive GS2-IpaH9.8^{C337A} mRNA/PABP nanoplexes, non-

specific AS15-IpaH9.8 nanoplexes, and naked GS2-IpaH9.8 mRNA that was delivered without PABPs showed little to no silencing activity (FIG. 5B). At 24 hours post-treatment, HEK293Td2EGFP cells receiving GS2-IpaH9.8 mRNA/PABP nanoplexes exhibited an 85% decrease in fluorescence activity, which was directly comparable to the knockdown activity achieved following DNA transfection seen above.

[0223] Encouraged by these results, uAb nanoplex-mediated delivery and silencing activity *in vivo* was next evaluated. Transgenic UBI-GFP/BL6 mice, which constitutively express EGFP in all tissues (Schaefer et al., “Observation of Antigen-Dependent CD8⁺ T-Cell/Dendritic Cell Interactions *In Vivo*,” *Cell Immunol.* 214(2):110–22 (2001), which is hereby incorporated by reference in its entirety), were given subcutaneous injections of GS2-IpaH9.8 mRNA/PABP nanoplexes in ears. Note that although this mouse strain ubiquitously expresses EGFP, fluorescence is absorbed and undetectable in areas that are covered by hairs. Fluorescent imaging at 24 hours post-injection revealed that EGFP fluorescence in the left ears, which received GS2-IpaH9.8 mRNA/PABP nanoplex injections, was robustly ablated with a 70% decrease in ear fluorescence (FIGs. 5C and 5D). In stark contrast, fluorescence in the right ears, which received either catalytically inactive GS2-IpaH9.8^{C337A} or non-specific AS15-IpaH9.8 nanoplex injections, was unaffected (FIGs. 5C and 5D). Importantly, these results set the stage for therapeutic delivery of uAbs as a viable strategy to post-translationally silence aberrantly expressed proteins in cancer and other human diseases.

Example 5 - Discussion of Examples 1–4

[0224] Ubiquibodies are a relatively new proteome editing modality that enable selective removal of otherwise stable proteins in somatic cells (Portnoff et al., “Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed With Unnatural Substrate Specificity for Targeted Protein Silencing,” *J. Biol. Chem.* 289(11):7844–55 (2014), which is hereby incorporated by reference in its entirety), with potential applications in basic research, drug discovery, and therapy. In this study, a new class of uAbs that feature bacterial E3 ubiquitin ligases was created, thereby opening the door to a previously untapped source of ubiquitination activity for uAb development. Specifically, 14 bacterial E3 ligases belonging to a growing class of effector proteins that mimic host cell E3 ligases to exploit the ubiquitination pathway was evaluated. Maculins et al., “Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of Invasion. *Cell Res.* 26(4):499–510 (2016) and Lin et al., “Exploitation of the Host Cell Ubiquitin Machinery by Microbial Effector Proteins,” *J. Cell Sci.* 130(12):1985–96 (2017), which are hereby incorporated by reference in their entirety. Most notable among these was IpaH9.8 from *S. flexneri*, which proved to be a remarkable catalyst of protein turnover when directed to target substrates via a

genetically fused synthetic binding domain. This silencing activity was found to be independent of the substrate's subcellular localization (*i.e.*, cytoplasm, nucleus, plasma membrane) or expression modality (*i.e.*, transient versus stable). The only other E3 ligases that functioned comparably were homologs of IpaH9.8 found in *S. flexneri*, either on the pWR100 virulence plasmid or the chromosome. Maculins et al., "Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of Invasion. *Cell Res.* 26(4):499–510 (2016), which is hereby incorporated by reference in its entirety. The N-terminal catalytic NEL domains of these enzymes share striking homology (99-100%), which explains their similar performance in the uAb context.

Accordingly, the next best functioning bacterial E3 ubiquitin ligase was *S. typhimurium* SspH1, which is also a NEL type enzyme with 38% identity to IpaH9.8 overall and 42% identity within the NEL domain. Norkowski et al., "The Species-Spanning Family of LPX-Motif Harboring Effector Proteins," *Cell Microbiol.* 20(11):e12945 (2018), which is hereby incorporated by reference in its entirety. It should also be pointed out that none of the mammalian E3 ubiquitin ligases were able to reduce EGFP levels below 60% under the conditions tested here. While the reasons for this are not entirely clear, given the successful knockdown results reported previously for these different E3 ligases in the uAb format (Portnoff et al., "Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed With Unnatural Substrate Specificity for Targeted Protein Silencing," *J. Biol. Chem.* 289(11):7844–55 (2014); Caussinus et al., "Fluorescent Fusion Protein Knockout Mediated by Anti-GFP Nanobody," *Nat. Struct. Mol. Biol.* 19(1):117–21 (2011); Fulcher et al., "Targeting Endogenous Proteins For Degradation Through the Affinity-Directed Protein Missile System," *Open Biol.* 7(5):170066 (2017); Fulcher et al., "An Affinity-Directed Protein Missile System for Targeted Proteolysis," *Open Biol* 6(10):160255 (2016); Shin et al., "Nanobody-Targeted E3-Ubiquitin Ligase Complex Degrades Nuclear Proteins," *Sci. Rep.* 5:14269 (2015); and Kanner et al., "Sculpting Ion Channel Functional Expression with Engineered Ubiquitin Ligases," *Elife* 6:e29744 (2017), all of which are hereby incorporated by reference in their entirety), it is suspected that EGFP may represent a poor substrate for these engineered chimeras.

[0225] While the work here was predominantly focused on silencing FPs and FP-tagged substrates, IpaH9.8-based uAbs that potently degraded disease-related targets including HRas was designed, which together with KRas and NRas comprise the most commonly mutated oncoproteins in cancer, and SHP2, a regulator of the Ras/MAPK signaling pathway. Importantly, the ability to deplete these clinically important targets along with all of the other FP fusions serves to highlight the extraordinary modularity of the uAb technology. Simply swapping the native substrate-binding domain of the E3 ubiquitin ligase can generate a made-to-order uAb with specificity for a different substrate protein. Interestingly, *Shigella* have evolved

a similar strategy for subverting host defenses during infection whereby plasmid and chromosomally-encoded IpaH proteins play a key role in dampening the host inflammatory response by mediating proteasomal degradation of NF- κ B-related proteins. Ashida et al., “A Bacterial E3 Ubiquitin Ligase IpaH9.8 Targets NEMO/IKKgamma to Dampen the Host NF-KapPab-Mediated Inflammatory Response,” *Nat Cell Biol.* 12(1):66–73, sup. pp. 1–9 (2010) and Ashida et al., “*Shigella* IpaH Family Effectors as a Versatile Model for Studying Pathogenic Bacteria,” *Front Cell Infect. Microbiol.* 5:100 (2015), which are hereby incorporated by reference in their entirety. Specifically, by employing different LRR domains, which only share ~50% similarity (Norkowski et al., “The Species-Spanning Family of LPX-Motif Harboring Effector Proteins,” *Cell Microbiol.* e12945 (2018), which is hereby incorporated by reference in its entirety), *Shigella* are able to redirect virtually identical catalytic NEL domains to an array of host proteins (*e.g.*, NEMO, U2AF53 for IpaH9.8; Glomulin for IpaH7.8; p65 for IpaH4.5; HOIP for IpaH2.5 and IpaH1.4; TRAF2 for IpaH0722). Maculins et al., “Bacteria-Host Relationship: Ubiquitin Ligases as Weapons of Invasion. *Cell Res.* 26(4):499–510 (2016); Lin et al., “Exploitation of the Host Cell Ubiquitin Machinery by Microbial Effector Proteins,” *J. Cell Sci.* 130(12):1985–96 (2017); and Ashida et al., “*Shigella* IpaH Family Effectors as a Versatile Model for Studying Pathogenic Bacteria,” *Front Cell Infect. Microbiol.* 5:100 (2015), all of which are hereby incorporated by reference in their entirety. It is believed that the inherent conformational flexibility required to ubiquitinate these structurally diverse substrates helps to explain the NEL motif’s remarkable ability for customizable target degradation. It should also be pointed out that while the work here leveraged previously confirmed E3 ubiquitin ligases, an analogous swapping strategy could be used to create GS2-based uAbs for identifying novel E3 ligases. Such an approach could enable systematic identification of E3 ligases, which is an important objective given that the human genome encodes over 600 putative E3 ligases (Metzger et al., “HECT and RING Finger Families of E3 Ubiquitin Ligases at a Glance,” *J. Cell Sci.* 125(Pt 3):531–37 (2012), which is hereby incorporated by reference in its entirety) and bacterial genomes likely encode hundreds of others, many of which remain to be validated as catalysts of ubiquitin transfer.

[0226] From a drug development standpoint, pharmacological control of gene products has traditionally been achieved using small molecule inhibitors that target enzymes and receptors having well-defined hydrophobic pockets where the small molecules are tightly bound. Unfortunately, a majority (~80-85%) of the human proteome is comprised of intractable targets, such as transcription factors, scaffold proteins, and non-enzymatic proteins, that cannot be inhibited pharmacologically and thus have been deemed ‘undruggable’. Crews, C. M., “Targeting the Undruggable Proteome: The Small Molecules of My Dreams,” *Chem. Biol.*

17(6):551–55 (2010) and Arkin et al., “Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing Towards The Dream,” *Nat. Rev. Drug Discov.* 3(4):301–17 (2004), which are hereby incorporated by reference in its entirety. As an alternative, a number of techniques for silencing proteins at the DNA or RNA level are now available such as CRISPR, RNAi, TALENs, and ZFNs, with the first RNAi therapy, patisiran, gaining approval in 2018 for hereditary transthyretin amyloidosis. Adams et al., “Patisiran, An RNAi Therapeutic, For Hereditary Transthyretin Amyloidosis,” *N. Engl. J. Med.* 379(1):11–21 (2018), which is hereby incorporated by reference in its entirety. Nonetheless, new adaptable technologies, such as uAbs and the related PROTACs technology, that offer temporal and post-translational control over protein silencing are desirable especially because of their potential to overcome some of the limitations associated with nucleic acid targeting-based approaches such as irreversibility, lack of temporal control, and off-target effects. Deleavey et al., “Designing Chemically Modified Oligonucleotides for Targeted Gene Silencing,” *Chemistry & Biology* 19(8):937–54 (2012); Gaj et al., “TALEN, and CRISPR/Cas-Based Methods for Genome Engineering,” *Trends Biotechnol.* 31(7):397–405 (2013); Fu et al., “High-Frequency Off-Target Mutagenesis Induced by CRISPR-Cas Nucleases in Human Cells,” *Nat. Biotechnol.* 31(9):822–26 (2013); and Fedorov et al., “Off-Target Effects by siRNA Can Induce Toxic Phenotype,” *RNA* 12(7):1188–96 (2006), which are hereby incorporated by reference in its entirety. In principle, both uAbs and PROTACs can degrade proteins regardless of their function, including the currently undruggable proteome. Moreover, unlike conventional 'occupancy-based' therapeutics, uAbs and PROTACs act catalytically, making them substantially more potent than the target-binding antibody mimetics and small molecule inhibitors, respectively, from which they are built.

[0227] A major advantage of uAbs is the ease with which they can be rapidly adapted to hit a variety of intracellular targets due to their recombinant, modular design, which capitalizes on a large, preexisting repertoire of synthetic binding proteins as well as systematic, genome-wide efforts to generate and validate protein binders *de novo* against the human proteome. Colwill et al., “A Roadmap to Generate Renewable Protein Binders to the Human Proteome,” *Nat. Methods* 8(7):551–58 (2011), which is hereby incorporated by reference in its entirety. Because obtaining antibody mimetics that bind with high specificity and affinity to a target should be easier than obtaining small molecules with the same properties, making custom-designed PROTACs is likely to be a much more challenging task. Osherovich, L., “Degradation From Within,” *Science-Business Exchange* 7:10–11 (2014), which is hereby incorporated by reference in its entirety. Nonetheless, PROTACs holds great promise as a therapeutic approach because it is based on small molecules that have strong odds of getting into cells. Indeed, impressive preclinical *in vitro* and *in vivo* data are propelling the development of clinically viable

PROTACs as evidenced by the founding of Arvinas in 2013 and C4 Therapeutics in 2016. It should be pointed out, however, that traditional medicinal chemistry approaches will be needed to improve the oral bioavailability, pharmacokinetics, and absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of PROTACs. Neklesa et al.,

5 “Targeted Protein Degradation by PROTACs,” *Pharmacol. Ther.* 17:4138–144 (2017) and Deshaies, R. J., “Protein Degradation: Prime Time for PROTACs,” *Nat. Chem. Biol.* 11(9):634–35 (2015), which are hereby incorporated by reference in their entirety. Compared to PROTACs, intracellular delivery of uAb-based therapeutics is a much bigger hurdle as most globular protein drugs do not spontaneously cross plasma membranes due to their relatively large
10 size and biochemical properties. Osherovich, L., “Degradation From Within,” *Science-Business Exchange* 7:10–11 (2014), which is hereby incorporated by reference in its entirety. One possible solution that is investigated here is the use of mRNA as a source of therapeutic gene product *in vivo*. In recent years, impediments to the use of mRNA, including its instability and immunogenicity, have been largely overcome through structural modifications, while issues
15 related to delivery and protein expression profiles have been addressed through advances in nanotechnology and material science. Guan et al., “Nanotechnologies in Delivery of mRNA Therapeutics Using Nonviral Vector-Based Delivery Systems,” *Gene Ther.* 24(3):133–43 (2017), which is hereby incorporated by reference in its entirety. Here, this unique approach to create a first-in-kind therapeutic uAb delivery strategy is taken advantage of; this method involved a
20 recently demonstrated strategy of electrostatics to stabilize pre-formed protein-RNA complexes for delivery. Li et al., “Polyamine-Mediated Stoichiometric Assembly of Ribonucleoproteins for Enhanced mRNA Delivery,” *Angew Chem. Int. Ed. Engl.* 56(44):13709–12 (2017), which is hereby incorporated by reference in its entirety. Here, synthetic mRNA encoding the GFP-directed GS2-IpaH9.8 chimera was co-assembled with PABPs and the assembled
25 ribonucleoproteins were packaged into nanosized complexes using structurally defined polypeptides bearing cationic aminated side groups. The resulting nanoplexes achieved highly efficient silencing of GFP *in vitro* and *in vivo*, thereby demonstrating a new proteome editing paradigm and opening the door to clinical translation of uAb-based therapeutics.

[0228] Although preferred embodiments have been depicted and described in detail
30 herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the application and these are therefore considered to be within the scope of the present application as defined in the claims which follow.

WHAT IS CLAIMED:

1. An isolated chimeric molecule comprising:
a degradation domain comprising an E3 ubiquitin ligase (E3) motif;
a targeting domain capable of specifically directing said degradation domain to a
5 substrate, wherein said targeting domain is heterologous to said degradation domain; and
a linker coupling said degradation domain to said targeting domain.
2. The chimeric molecule of claim 1, wherein said E3 motif comprises a
modified binding region which inhibits or decreases binding to said substrate compared to said
E3 motif without the modified binding region.
- 10 3. The chimeric molecule of claim 2, wherein the modification is a mutation
or deletion in said binding region.
4. The chimeric molecule of claim 1, wherein said E3 motif permits
proteolysis of said substrate.
5. The chimeric molecule of claim 1, wherein said E3 motif possesses a cell-
15 type specific or tissue specific ligase function.
6. The chimeric molecule of claim 5, wherein said ligase function is cell-type
specific and the cell-type is selected from the group consisting of skin cells, muscle cells,
epithelial cells, endothelial cells, stem cells, umbilical vessel cells, corneal cells, cardiomyocytes,
aortic cells, corneal epithelial cells, somatic cells, fibroblasts, keratinocytes, melanocytes,
20 adipose cells, bone cells, osteoblasts, airway cells, microvascular cells, mammary cells, vascular
cells, chondrocytes, placental cells, hepatocytes, glial cells, epidermal cells, limbal stem cells,
periodontal stem cells, bone marrow stromal cells, hybridoma cells, kidney cells, pancreatic
islets, articular chondrocytes, neuroblasts, lymphocytes, and erythrocytes.
7. The chimeric molecule of claim 1, wherein said degradation domain is
25 from a bacterial pathogen.
8. The chimeric molecule of claim 7, wherein said bacterial pathogen is
selected from the group consisting of *Shigella*, *Salmonella*, *Bacillus*, *Bartonella*, *Bordetella*,
Borrelia, *Brucella*, *Campylobacter*, *Chlamydia* and *Chlamydophila*, *Clostridium*,
Corynebacterium, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*,

Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Staphylococcus, Streptococcus, Treponema, Ureaplasma, Vibrio, and Yersinia.

9. The chimeric molecule of claim 7, wherein said degradation domain is from a bacterial pathogen and comprises *Shigella flexneri* E3 ligase, SspH1, SspH2, SlrP,

5 AvrPtoB, LubX, NleG5-1, NleG2-3, LegU1, LegAU13, NleL, SopA, SidC, XopL, GobX, VirF, GALA, AnkB, or SidE.

10. The chimeric molecule of claim 1, wherein said degradation domain is a *Shigella* IpaH protein.

11. The chimeric molecule of claim 10, wherein said *Shigella* IpaH protein is
10 selected from the group consisting of IpaH9.8, IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH0887, IpaH1389, IpaH2022, IpaH2202, IpaH2610, and IpaH0722.

12. The chimeric molecule of claim 7, wherein when said bacterial pathogen is *Shigella flexneri*.

13. The chimeric molecule of claim 1, wherein said targeting domain is a
15 monobody, fibronectin type III domain (FN3), antibody, polyclonal antibody, monoclonal antibody, recombinant antibody, antibody fragment, Fab', F(ab')₂, Fv, scFv, tascFvs, bis-scFvs, sdAb, VH, VL, Vnar, scFvD10, scFv13R4, scFvD10, humanized antibody, chimeric antibody, complementary determining region (CDR), IgA antibody, IgD antibody, IgE antibody, IgG antibody, IgM antibody, nanobody, intrabody, unibody, minibody, non-antibody protein
20 scaffold, Adnectin, Affibody and their two-helix variants, Anticalin, camelid antibody, V_HH, knottin, DARPin, or Sso7d.

14. The chimeric molecule of claim 13, wherein said targeting domain is a monobody, said monobody being a fibronectin type III domain (FN3) monobody selected from the group consisting of (with target antigen in parenthesis): GS2 (GFP), Nsa5 (SHP2), RasInI
25 (HRas/KRas), and RasInII (HRas/KRas), 1D10 (CDC34), 1D7 (COPS5), 1C4 (MAP2K5), 2C12 (MAP2K5), 1E2 (SF3A1), 1C2 (USP11), 1A9 (USP11), Ubi4 (ubiquitin), EI1.4.1 (EGFR), EI2.4.6 (EGFR), EI3.4.3 (EGFR), EI4.2.1 (EGFR), EI4.4.2 (EGFR), EI6.2.6 (EGFR), EI6.2.10 (EGFR), E246(EGFR), C743(CEA), IIIa8.2.6 (FcγIIa), IIIa6.2.6 (FcγIIIa), hA2.2.1 (hA33), hA2.2.2 (hA33), hA3.2.1 (hA33), hA3.2.3 (hA33), mA3.2.1 (mA33), mA3.2.2 (mA33),
30 mA3.2.3 (mA33), mA3.2.4 (mA33), mA3.2.5 (mA33), Alb3.2.1 (hAlb), mI2.2.1 (mIgG), HA4 (AblSH2), HA10 (AblSH2), HA16 (AblSH2), HA18 (AblSH2), 159 (vEGFR), MUC16

(MSLN), E2#3 (ER α /EF), E2#4 (ER α /EF), E2#5 (ER α /EF), E2#6 (ER α /EF), E2#7 (ER α /EF), E2#8 (ER α /EF), E2#9 (ER α /EF), E2#10 (ER α /EF), E2#11 (ER α /EF), E2#23 (ER α /EF), E3#2 (ER α /EF), E3#6 (ER α /EF), OHT#31 (ER α /EF), OHT#32 (ER α /EF), OHT#33 (ER α /EF), AB7-A1 (ER α /EF), AB7-B1 (ER α /EF), MBP-74 (MBP), MBP-76 (MBP), MBP-79 (MBP),

- 5 hSUMO4-33 (hSUMO4), hSUMO-39 (hSUMO4), ySUMO-53 (ySUMO), ySUMO-56 (ySUMO), ySUMO-57 (ySUMO), T14.25 (TNF α), T14.20 (TNF α), FNfn10-3JCL14 (av β 3 integrin), 1C9 (Src SH3), 1F11 (Src SH3), 1F10 (Src SH3), 2G10 (Src SH3), 2B2 (Src SH3), 1E3 (Src SH3), E18 (VEGFR2), E19 (VEGFR2), E26 (VEGFR2), E29 (VEGFR2), FG4.2 (Lysozyme), FG4.1 (Lysozyme), 2L4.1 (Lysozyme), BF4.1 (Lysozyme), BF4.9 (Lysozyme),
10 BF4.4 (Lysozyme), BFslc4.01 (Lysozyme), BFslc4.07 (Lysozyme), BFslc4.02 (Lysozyme), BFslc4.06 (Lysozyme), BFslc4.01 (Lysozyme), 10C17C25 (phospho-I κ B α), Fn-N22 (SARS N), Fn-N17 (SARS N), FN-N10 (SARS N), gI2.5.3T88I (goat IgG), gI2.5.2 (goat IgG), gI2.5.4 (goat IgG), rI4.5.4 (rabbit IgG), rI4.3.1 (rabbit IgG), rI3.6.6 (rabbit IgG), rI4.3.4 (rabbit IgG), rI3.6.4 (rabbit IgG), and rI4.3.3 (rabbit IgG).

- 15 15. The chimeric molecule of claim 1, wherein said substrate is selected from the group consisting of fluorescent protein, histone protein, nuclear localization signal (NLS), H-Ras protein, Src-homology 2 domain-containing phosphatase 2 (SHP2), β -galactosidase, gpD, Hsp70, MBP, CDC34, COPS5, MAP2K5, SF3A1, USP11, ubiquitin, EGFR, CEA, Fc γ IIa, Fc γ IIIa, hA33, mA33, hAlb, mIgG, Ab1SH2, vEGFR, MSLN, ER α /EF, hSUMO4, ySUMO,
20 TNF α , av β 3 integrin, Src SH3, Lysozyme, phospho-I κ B α , SARS N, goat IgG, rabbit IgG, post-translationally modified proteins, fibrillin, huntingtin, tumorigenic proteins, p53, Rb, adhesion proteins, receptors, cell-cycle proteins, checkpoint proteins, HFE, ATP7B, prion proteins, viral proteins, bacterial proteins, parasitic proteins, fungal proteins, DNA binding proteins, metabolic proteins, regulatory proteins, structural proteins, enzymes, immunogenic proteins,
25 autoimmunogenic proteins, immunogens, antigens, and pathogenic proteins.

16. The chimeric molecule of claim 1, wherein the substrate is a fluorescent protein selected from the group consisting of green fluorescent protein, emerald fluorescent protein, venus fluorescent protein, cerulean fluorescent protein, and enhanced cyan fluorescent protein.

- 30 17. The chimeric molecule of claim 1, wherein said targeting domain is binds to a non-native substrate.

18. A method of forming a ribonucleoprotein comprising:

providing a mRNA encoding the isolated chimeric molecule of claim 1;
providing one or more polyadenosine binding proteins ("PABP"); and
assembling a ribonucleoprotein complex from the mRNA and the one or more
PABPs.

5 19. The method of claim 18, wherein said mRNA comprises a 3'-terminal
polyadenosine (poly A) tail.

20. A composition comprising:
the chimeric molecule of claim 1; and
a pharmaceutically-acceptable carrier.

10 21. The composition of claim 20 further comprising:
a second agent selected from the group consisting of an anti-inflammatory agent,
an antidiabetic agent, a hypolipidemic agent, a chemotherapeutic agent, an antiviral agent, an
antibiotic, a metabolic agent, a small molecule inhibitor, a protein kinase inhibitor, adjuvants,
apoptotic agents, a proliferative agent, and organotropic targeting agents, and any combination
15 thereof.

22. A method of treating a disease comprising:
selecting a subject having a disease and
administering the composition of claim 20 to the subject to give the subject an
increased expression level of said substrate compared to a subject not afflicted with said disease.

20 23. The method of claim 22, wherein said disease is selected from the group
consisting of cancer, metastatic cancer, stroke, ischemia, peripheral vascular disease, alcoholic
liver disease, hepatitis, cirrhosis, Parkinson's disease, Alzheimer's disease, cystic fibrosis
diabetes, ALS, pathogenic diseases, idiopathic diseases, viral diseases, bacterial, diseases,
prionic diseases, fungal diseases, parasitic diseases, arthritis, wound healing, immunodeficiency,
25 inflammatory disease, aplastic anemia, anemia, genetic disorders, congenital disorders, type 1
diabetes, type 2 diabetes, gestational diabetes, high blood glucose, metabolic syndrome,
lipodystrophy syndrome, dyslipidemia, insulin resistance, leptin resistance, atherosclerosis,
vascular disease, hypercholesterolemia, hypertriglyceridemia, non-alcoholic fatty liver disease,
overweight, and obesity.

30 24. The method of claim 22, wherein the administering is carried out orally,
parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal

instillation, by implantation, by intracavitary or intravesical instillation, intraocularly, intraarterially, intralesionally, transdermally, or by application to mucous membranes.

25. A method for substrate silencing, the method comprising:

selecting a substrate to be silenced;

5 providing said chimeric molecule of claim 1; and

contacting said substrate with said chimeric molecule under conditions effective to permit the formation of a substrate-molecule complex, wherein said complex mediates the degradation of said substrate to be silenced.

26. The method of claim 25, wherein said substrate is selected from the group

10 consisting of fluorescent protein, histone protein, nuclear localization signal (NLS), H-Ras protein, SHP2 protein, Src-homology 2 domain-containing phosphatase 2 (SHP2), β -galactosidase, gpD, Hsp70, MBP, CDC34, COPS5, MAP2K5, SF3A1, USP11, ubiquitin, EGFR, CEA, Fc γ IIa, Fc γ IIIa, hA33, mA33, hAlb, mIgG, AblSH2, vEGFR, MSLN, ER α /EF, hSUMO4, ySUMO, TNF α , α v β 3 integrin, Src SH3, Lysozyme, phospho-I κ B α , SARS N, goat IgG, rabbit
15 IgG, post-translationally modified proteins, fibrillin, huntingtin, tumorigenic proteins, p53, Rb, adhesion proteins, receptors, cell-cycle proteins, checkpoint proteins, HFE, ATP7B, prion proteins, viral proteins, bacterial proteins, parasitic proteins, fungal proteins, DNA binding proteins, metabolic proteins, regulatory proteins, structural proteins, enzymes, immunogenic proteins, autoimmunogenic proteins, immunogens, antigens, and pathogenic proteins.

27. The method of claim 25, wherein the substrate is a fluorescent protein

selected from the group consisting of green fluorescent protein, emerald fluorescent protein, venus fluorescent protein, cerulean fluorescent protein, and enhanced cyan fluorescent protein.

28. A method of screening agents for therapeutic efficacy against a disease, said method comprising:

25 providing a biomolecule whose presence mediates a disease state;

providing a test agent comprising (i) a degradation domain comprising an E3 ubiquitin ligase (E3) motif, (ii) a targeting domain capable of specifically directing said degradation domain to said biomolecule, wherein said targeting domain is heterologous to said degradation domain, and (iii) a linker coupling said degradation
30 domain to said targeting domain;

contacting said biomolecule with said test agent under conditions effective for the test agent to facilitate degradation of the biomolecule;

determining the level of said biomolecule as a result of said contacting; and
identifying said test agent which, based on said determining, decreases the level
of said biomolecule as being a candidate for therapeutic efficacy against said disease.

29. The method of claim 28, wherein said identifying is carried out with
5 respect to a standard biomolecule level in a subject not afflicted with said disease.

30. The method of claim 28, wherein said identifying is carried out with
respect to the biomolecule level absent said contacting.

31. The method of claim 28, wherein the method is carried out with a plurality
of test agents.

10 32. The method of claim 28, wherein said degradation domain is a bacterial
pathogen.

33. The method of claim 32, wherein said bacterial pathogen is selected from
the group consisting of *Shigella*, *Salmonella*, *Bacillus*, *Bartonella*, *Bordetella*, *Borrelia*,
Brucella, *Campylobacter*, *Chlamydia* and *Chlamydophila*, *Clostridium*, *Corynebacterium*,
15 *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*,
Listeria, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Staphylococcus*,
Streptococcus, *Treponema*, *Ureaplasma*, *Vibrio*, and *Yersinia*.

34. The method of claim 32, wherein said degradation domain is from a
bacterial pathogen and comprises *Shigella flexneri* E3 ligase, SspH1, SspH2, SlrP, AvrPtoB,
20 LubX, NleG5-1, NleG2-3, LegU1, LegAU13, NleL, SopA, SidC, XopL, GobX, VirF, GALA,
AnkB, or SidE.

35. The method of claim 28, wherein said degradation domain is a *Shigella*
IpaH protein.

36. The method of claim 35, wherein said *Shigella* IpaH protein is selected
25 from the group consisting of IpaH9.8, IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH0887,
IpaH1389, IpaH2022, IpaH2202, IpaH2610, and IpaH0722.

37. The method of claim 32, wherein when said bacterial pathogen is *Shigella*
flexneri.

38. The method of claim 28, wherein said targeting domain is a monobody, fibronectin type III domain (FN3), antibody, polyclonal antibody, monoclonal antibody, recombinant antibody, antibody fragment, Fab', F(ab')₂, Fv, scFv, tascFvs, bis-scFvs, sdAb, VH, VL, Vnar, scFvD10, scFv13R4, scFvD10, humanized antibody, chimeric antibody, complementary determining region (CDR), IgA antibody, IgD antibody, IgE antibody, IgG antibody, IgM antibody, nanobody, intrabody, unibody, minibody, non-antibody protein scaffold, Adnectin, Affibody and their two-helix variants, Anticalin, camelid antibody, V_HH, knottin, DARPin, or Sso7d.

39. The method of claim 38, wherein said targeting domain is a monobody, said monobody being a fibronectin type III domain (FN3) monobody selected from the group consisting of (with target antigen in parenthesis): GS2 (GFP), Nsa5 (SHP2), RasInI (HRas/KRas), and RasInII (HRas/KRas), 1D10 (CDC34), 1D7 (COP5), 1C4 (MAP2K5), 2C12 (MAP2K5), 1E2 (SF3A1), 1C2 (USP11), 1A9 (USP11), Ubi4 (ubiquitin), EI1.4.1 (EGFR), EI2.4.6 (EGFR), EI3.4.3 (EGFR), EI4.2.1 (EGFR), EI4.4.2 (EGFR), EI6.2.6 (EGFR), EI6.2.10 (EGFR), E246(EGFR), C743(CEA), IIIa8.2.6 (FcγIIa), IIIa6.2.6 (FcγIIIa), hA2.2.1 (hA33), hA2.2.2 (hA33), hA3.2.1 (hA33), hA3.2.3 (hA33), mA3.2.1 (mA33), mA3.2.2 (mA33), mA3.2.3 (mA33), mA3.2.4 (mA33), mA3.2.5 (mA33), Alb3.2.1 (hAlb), mI2.2.1 (mIgG), HA4 (AblSH2), HA10 (AblSH2), HA16 (AblSH2), HA18 (AblSH2), 159 (vEGFR), MUC16 (MSLN), E2#3 (ERα/EF), E2#4 (ERα/EF), E2#5 (ERα/EF), E2#6 (ERα/EF), E2#7 (ERα/EF), E2#8 (ERα/EF), E2#9 (ERα/EF), E2#10 (ERα/EF), E2#11 (ERα/EF), E2#23 (ERα/EF), E3#2 (ERα/EF), E3#6 (ERα/EF), OHT#31 (ERα/EF), OHT#32 (ERα/EF), OHT#33 (ERα/EF), AB7-A1 (ERα/EF), AB7-B1 (ERα/EF), MBP-74 (MBP), MBP-76 (MBP), MBP-79 (MBP), hSUMO4-33 (hSUMO4), hSUMO-39 (hSUMO4), ySUMO-53 (ySUMO), ySUMO-56 (ySUMO), ySUMO-57 (ySUMO), T14.25 (TNFα), T14.20 (TNFα), FNfn10-3JCL14 (avβ3 integrin), 1C9 (Src SH3), 1F11 (Src SH3), 1F10 (Src SH3), 2G10 (Src SH3), 2B2 (Src SH3), 1E3 (Src SH3), E18 (VEGFR2), E19 (VEGFR2), E26 (VEGFR2), E29 (VEGFR2), FG4.2 (Lysozyme), FG4.1 (Lysozyme), 2L4.1 (Lysozyme), BF4.1 (Lysozyme), BF4.9 (Lysozyme), BF4.4 (Lysozyme), BFs1c4.01 (Lysozyme), BFs1c4.07 (Lysozyme), BFs3_4.02 (Lysozyme), BFs3_4.06 (Lysozyme), BFs3_8.01 (Lysozyme), 10C17C25 (phospho-IκBα), Fn-N22 (SARS N), Fn-N17 (SARS N), FN-N10 (SARS N), gI2.5.3T88I (goat IgG), gI2.5.2 (goat IgG), gI2.5.4 (goat IgG), rI4.5.4 (rabbit IgG), rI4.3.1 (rabbit IgG), rI3.6.6 (rabbit IgG), rI4.3.4 (rabbit IgG), rI3.6.4 (rabbit IgG), and rI4.3.3 (rabbit IgG).

40. The method of claim 28, wherein said substrate is selected from the group consisting of fluorescent protein, histone protein, nuclear localization signal (NLS), H-Ras

protein, SHP2 protein, Src-homology 2 domain-containing phosphatase 2 (SHP2), β -galactosidase, gpD, Hsp70, MBP, CDC34, COPS5, MAP2K5, SF3A1, USP11, ubiquitin, EGFR, CEA, Fc γ IIa, Fc γ IIIa, hA33, mA33, hAlb, mIgG, AblSH2, vEGFR, MSLN, ER α /EF, hSUMO4, ySUMO, TNF α , α v β 3 integrin, Src SH3, Lysozyme, phospho-I κ B α , SARS N, goat IgG, rabbit IgG, post-translationally modified proteins, fibrillin, huntingtin, tumorigenic proteins, p53, Rb, adhesion proteins, receptors, cell-cycle proteins, checkpoint proteins, HFE, ATP7B, prion proteins, viral proteins, bacterial proteins, parasitic proteins, fungal proteins, DNA binding proteins, metabolic proteins, regulatory proteins, structural proteins, enzymes, immunogenic proteins, autoimmunogenic proteins, immunogens, antigens, and pathogenic proteins.

10 41. The method of claim 28, wherein the substrate is a fluorescent protein selected from the group consisting of green fluorescent protein, emerald fluorescent protein, venus fluorescent protein, cerulean fluorescent protein, and enhanced cyan fluorescent protein.

 42. The method of claim 28, wherein said linker is a polypeptide linker of sufficient length to prevent the steric disruption of binding between said targeting domain and
15 said biomolecule.

 43. The method of claim 28, wherein said biomolecule is associated with cancer, metastatic cancer, stroke, ischemia, peripheral vascular disease, alcoholic liver disease, hepatitis, cirrhosis, Parkinson's disease, Alzheimer's disease, cystic fibrosis diabetes, ALS, pathogenic diseases, idiopathic diseases, viral diseases, bacterial, diseases, prionic diseases,
20 fungal diseases, parasitic diseases, arthritis, wound healing, immunodeficiency, inflammatory disease, aplastic anemia, anemia, genetic disorders, congenital disorders, type 1 diabetes, type 2 diabetes, gestational diabetes, high blood glucose, metabolic syndrome, lipodystrophy syndrome, dyslipidemia, insulin resistance, leptin resistance, atherosclerosis, vascular disease, hypercholesterolemia, hypertriglyceridemia, non-alcoholic fatty liver disease, overweight, or
25 obesity, and any combination thereof.

 44. A method of screening for disease biomarkers, said method comprising:
 providing a sample of diseased cells expressing one or more ligands;
 providing a plurality of chimeric molecules comprising (i) a degradation domain comprising an E3 ubiquitin ligase (E3) motif, (ii) a targeting domain capable of specifically
30 directing said degradation domain to said one or more ligands, wherein said targeting domain is heterologous to said degradation domain, and (iii) a linker coupling said degradation domain to said targeting domain;

contacting said sample with said plurality of chimeric molecules under conditions effective for the diseased cells to fail to proliferate in the absence of the chimeric molecule;

determining which of said chimeric molecules permit the diseased cells to proliferate; and

5 identifying, as biomarkers for the disease, based on said determining the ligands which bind to the chimeric molecules and permit diseased cells to proliferate.

45. The method of claim 44, wherein said disease is selected from the group consisting of cancer, metastatic cancer, stroke, ischemia, peripheral vascular disease, alcoholic liver disease, hepatitis, cirrhosis, Parkinson's disease, Alzheimer's disease, cystic fibrosis
10 diabetes, ALS, pathogenic diseases, idiopathic diseases, viral diseases, bacterial, diseases, prionic diseases, fungal diseases, parasitic diseases, arthritis, wound healing, immunodeficiency, inflammatory disease, aplastic anemia, anemia, genetic disorders, congenital disorders, type 1 diabetes, type 2 diabetes, gestational diabetes, high blood glucose, metabolic syndrome, lipodystrophy syndrome, dyslipidemia, insulin resistance, leptin resistance, atherosclerosis,
15 vascular disease, hypercholesterolemia, hypertriglyceridemia, non-alcoholic fatty liver disease, overweight, and obesity.

46. The method of claim 44, wherein said degradation domain is a bacterial pathogen.

47. The method of claim 46, wherein said bacterial pathogen is selected from
20 the group consisting of *Shigella*, *Salmonella*, *Bacillus*, *Bartonella*, *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia* and *Chlamydophila*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio*, and *Yersinia*.

25 48. The method of claim 46, wherein said degradation domain is from a bacterial pathogen and comprises *Shigella flexneri* E3 ligase, SspH1, SspH2, SlrP, AvrPtoB, LubX, NleG5-1, NleG2-3, LegU1, LegAU13, NleL, SopA, SidC, XopL, GobX, VirF, GALA, AnkB, or SidE.

30 49. The method of claim 44, wherein said degradation domain is a *Shigella* IpaH protein.

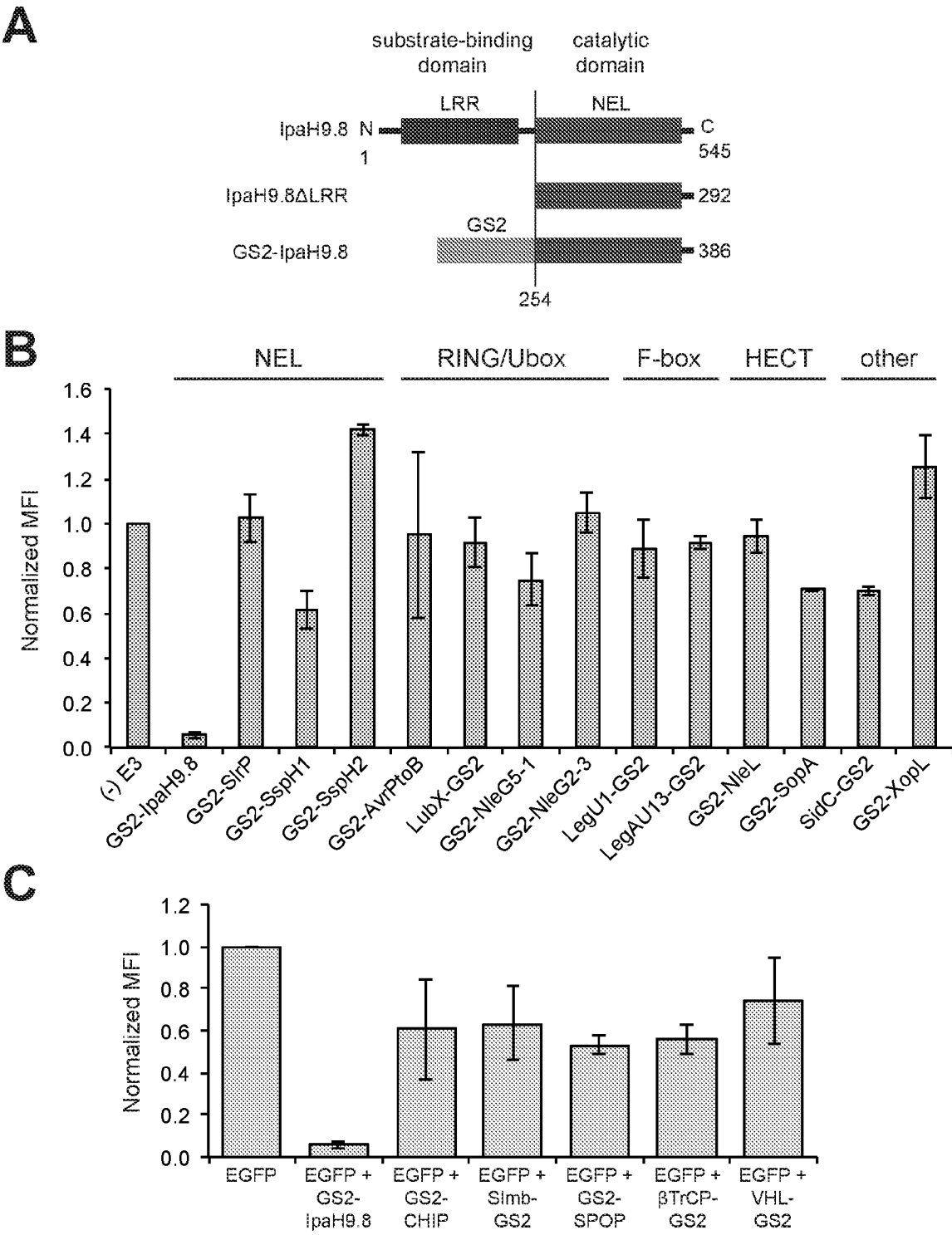
50. The method of claim 49, wherein said *Shigella* IpaH protein is selected from the group consisting of IpaH9.8, IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH0887, IpaH1389, IpaH2022, IpaH2202, IpaH2610, and IpaH0722.

51. The method of claim 46, wherein when said bacterial pathogen is *Shigella flexneri*.

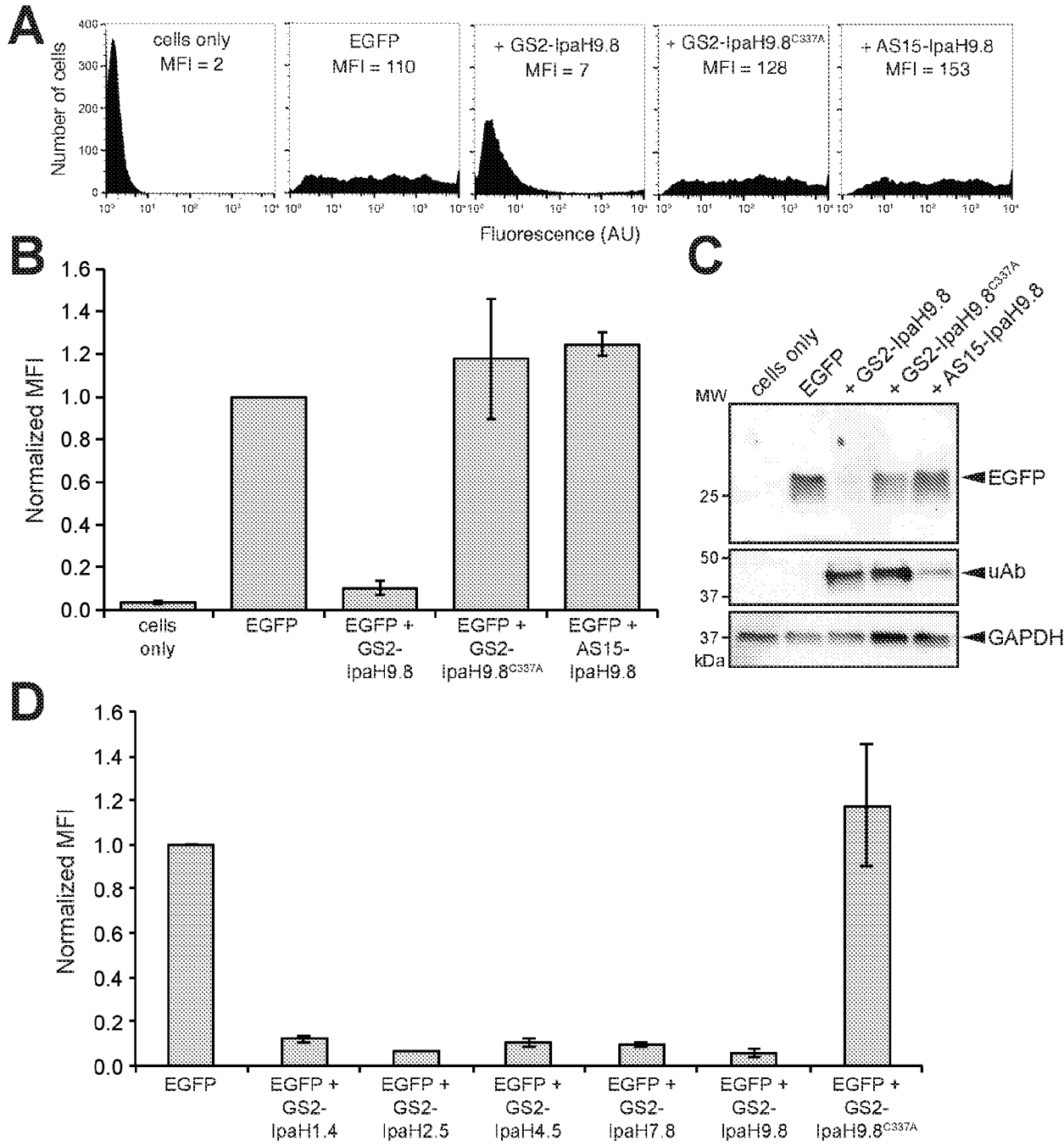
52. The method of claim 44, wherein said targeting domain is a monobody, fibronectin type III domain (FN3), antibody, polyclonal antibody, monoclonal antibody, recombinant antibody, antibody fragment, Fab', F(ab')₂, Fv, scFv, tascFvs, bis-scFvs, sdAb, VH, VL, Vnar, scFvD10, scFv13R4, scFvD10, humanized antibody, chimeric antibody, complementary determining region (CDR), IgA antibody, IgD antibody, IgE antibody, IgG antibody, IgM antibody, nanobody, intrabody, unibody, minibody, non-antibody protein scaffold, Adnectin, Affibody and their two-helix variants, Anticalin, camelid antibody, V_HH, knottin, DARPin, or Sso7d.

53. The method of claim 50, wherein said targeting domain is a monobody, said monobody being a fibronectin type III domain (FN3) monobody selected from the group consisting of (with target antigen in parenthesis): GS2 (GFP), Nsa5 (SHP2), RasInI (HRas/KRas), and RasInII (HRas/KRas), 1D10 (CDC34), 1D7 (COPS5), 1C4 (MAP2K5), 2C12 (MAP2K5), 1E2 (SF3A1), 1C2 (USP11), 1A9 (USP11), Ubi4 (ubiquitin), EI1.4.1 (EGFR), EI2.4.6 (EGFR), EI3.4.3 (EGFR), EI4.2.1 (EGFR), EI4.4.2 (EGFR), EI6.2.6 (EGFR), EI6.2.10 (EGFR), E246(EGFR), C743(CEA), IIIa8.2.6 (FcγIIa), IIIa6.2.6 (FcγIIIa), hA2.2.1 (hA33), hA2.2.2 (hA33), hA3.2.1 (hA33), hA3.2.3 (hA33), mA3.2.1 (mA33), mA3.2.2 (mA33), mA3.2.3 (mA33), mA3.2.4 (mA33), mA3.2.5 (mA33), Alb3.2.1 (hAlb), mI2.2.1 (mIgG), HA4 (AblSH2), HA10 (AblSH2), HA16 (AblSH2), HA18 (AblSH2), 159 (vEGFR), MUC16 (MSLN), E2#3 (ERα/EF), E2#4 (ERα/EF), E2#5 (ERα/EF), E2#6 (ERα/EF), E2#7 (ERα/EF), E2#8 (ERα/EF), E2#9 (ERα/EF), E2#10 (ERα/EF), E2#11 (ERα/EF), E2#23 (ERα/EF), E3#2 (ERα/EF), E3#6 (ERα/EF), OHT#31 (ERα/EF), OHT#32 (ERα/EF), OHT#33 (ERα/EF), AB7-A1 (ERα/EF), AB7-B1 (ERα/EF), MBP-74 (MBP), MBP-76 (MBP), MBP-79 (MBP), hSUMO4-33 (hSUMO4), hSUMO-39 (hSUMO4), ySUMO-53 (ySUMO), ySUMO-56 (ySUMO), ySUMO-57 (ySUMO), T14.25 (TNFα), T14.20 (TNFα), FNfn10-3JCL14 (αvβ3 integrin), 1C9 (Src SH3), 1F11 (Src SH3), 1F10 (Src SH3), 2G10 (Src SH3), 2B2 (Src SH3), 1E3 (Src SH3), E18 (VEGFR2), E19 (VEGFR2), E26 (VEGFR2), E29 (VEGFR2), FG4.2 (Lysozyme), FG4.1 (Lysozyme), 2L4.1 (Lysozyme), BF4.1 (Lysozyme), BF4.9 (Lysozyme), BF4.4 (Lysozyme), BFslc4.01 (Lysozyme), BFslc4.07 (Lysozyme), BFslc4.02 (Lysozyme),

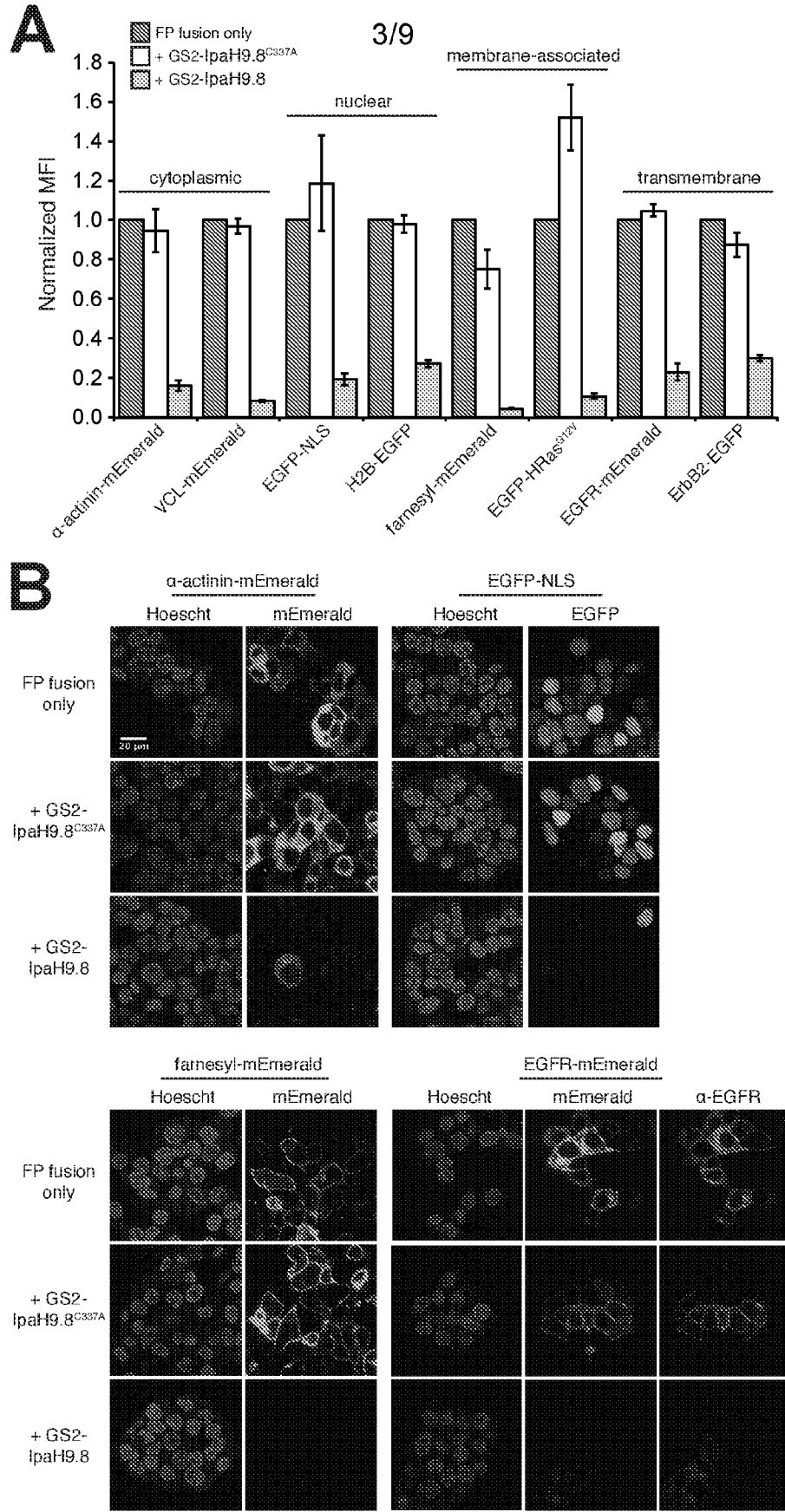
BFs3_4.06 (Lysozyme), BFs3_8.01 (Lysozyme), 10C17C25 (phospho-IkB α), Fn-N22 (SARS N), Fn-N17 (SARS N), FN-N10 (SARS N), gI2.5.3T88I (goat IgG), gI2.5.2 (goat IgG), gI2.5.4 (goat IgG), rI4.5.4 (rabbit IgG), rI4.3.1 (rabbit IgG), rI3.6.6 (rabbit IgG), rI4.3.4 (rabbit IgG), rI3.6.4 (rabbit IgG), and rI4.3.3 (rabbit IgG).



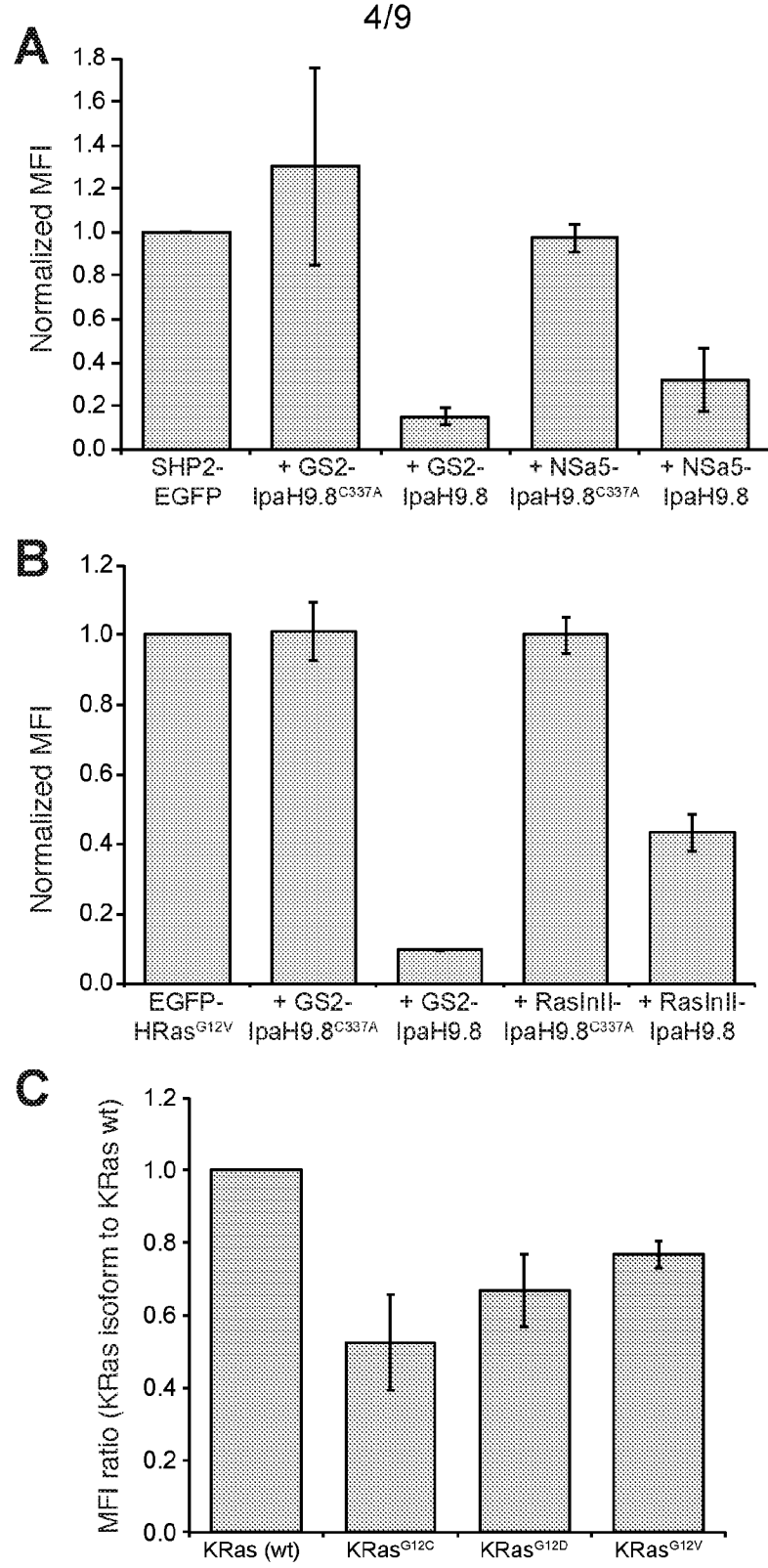
FIGs. 1A-1C



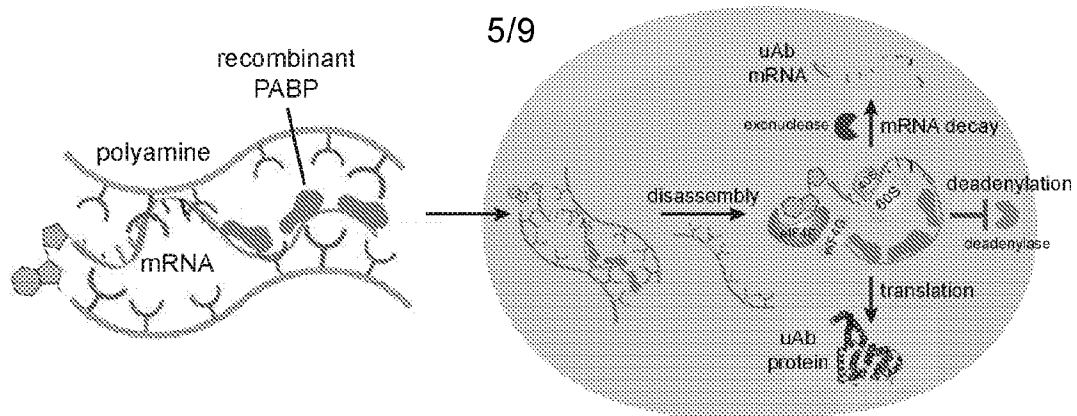
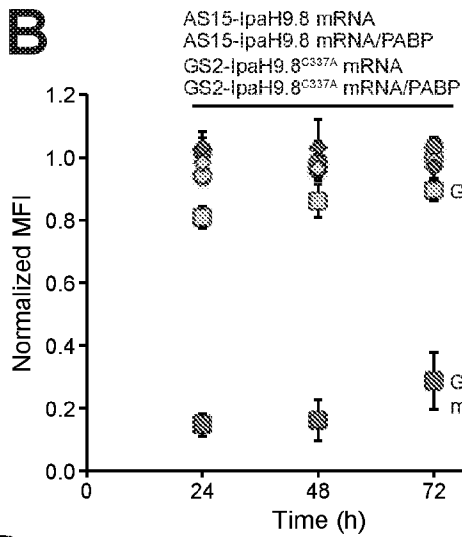
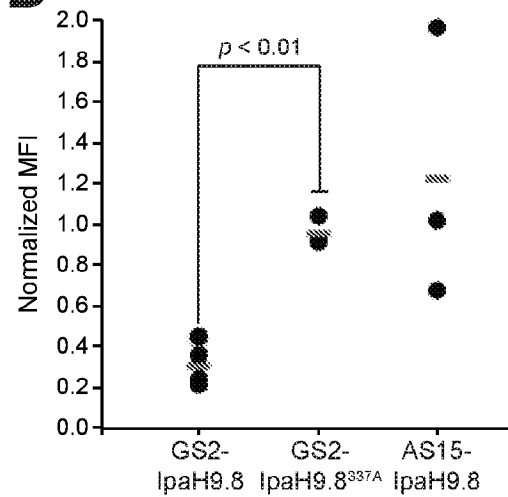
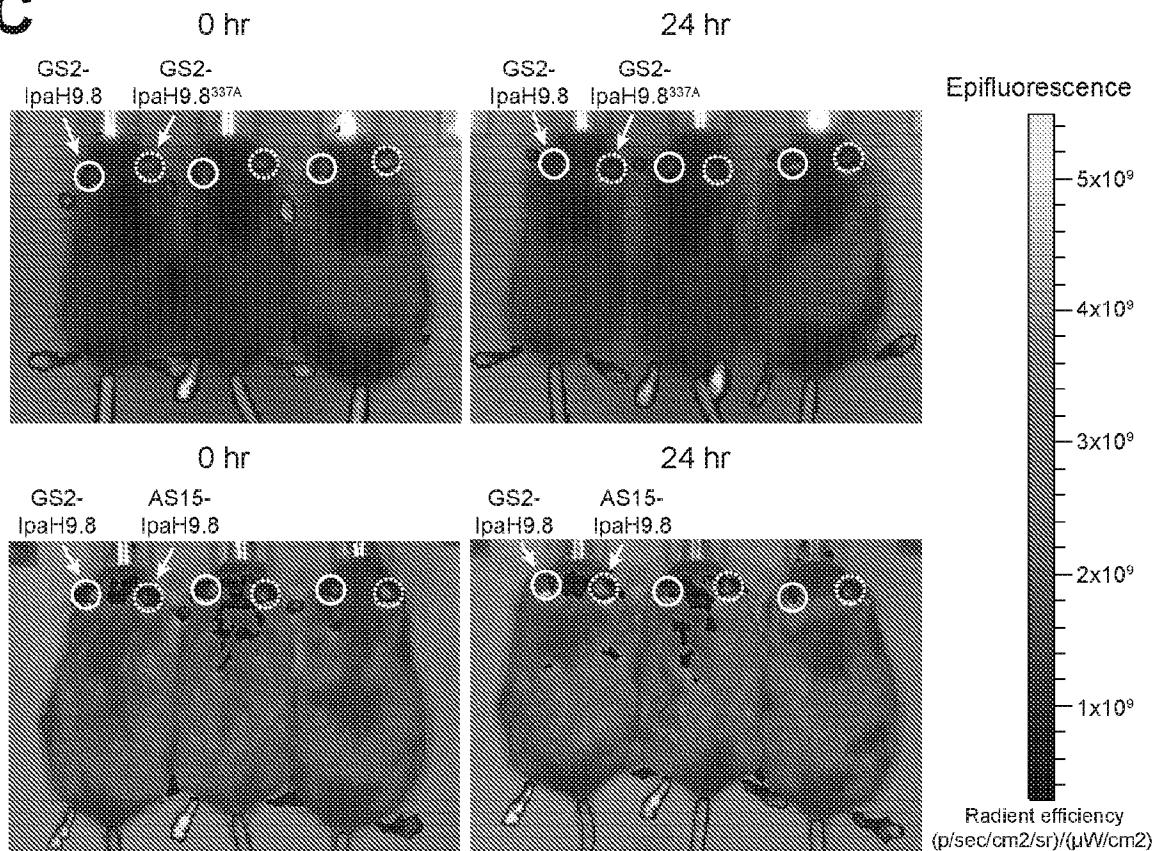
FIGs. 2A-2D

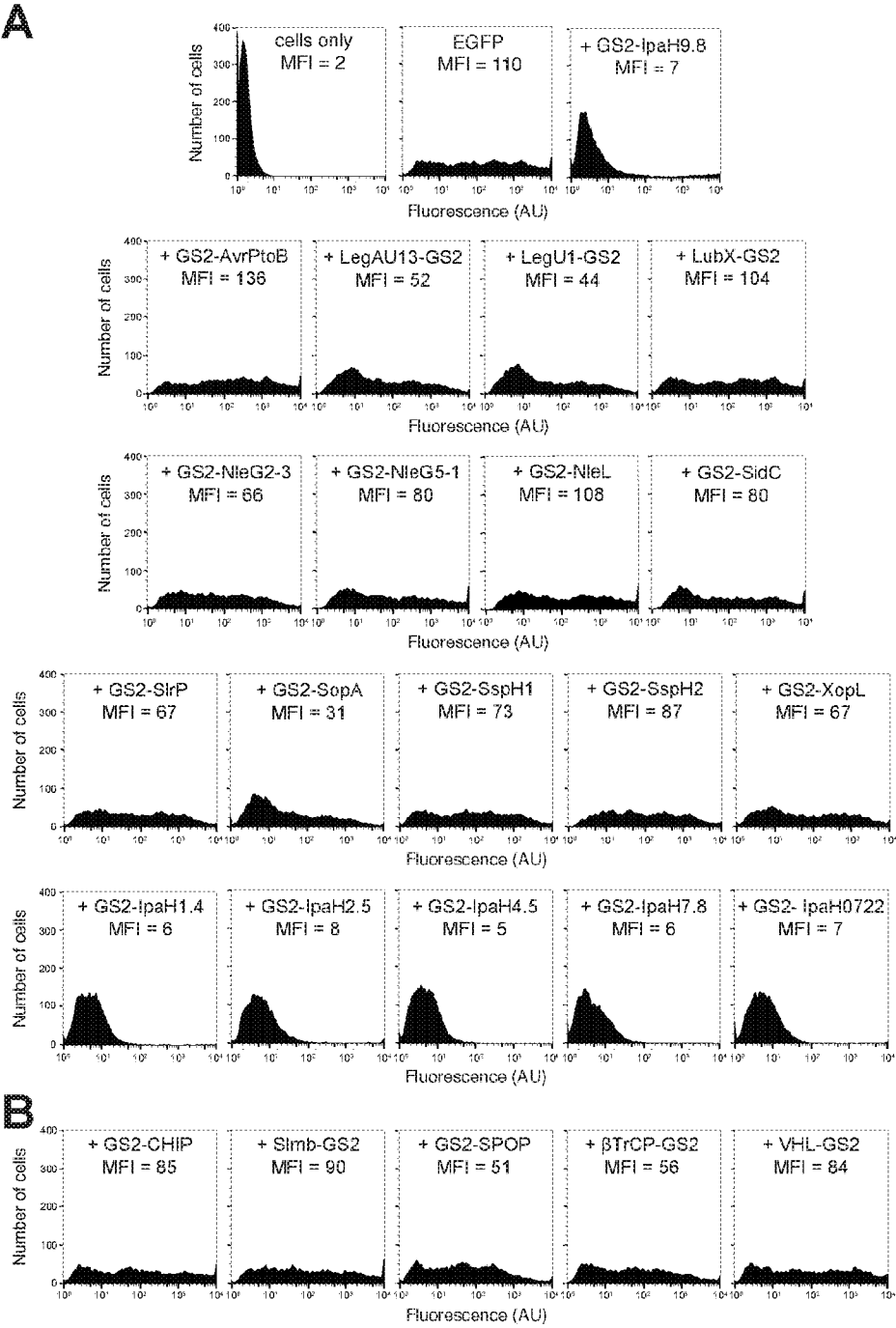


FIGs. 3A-3B

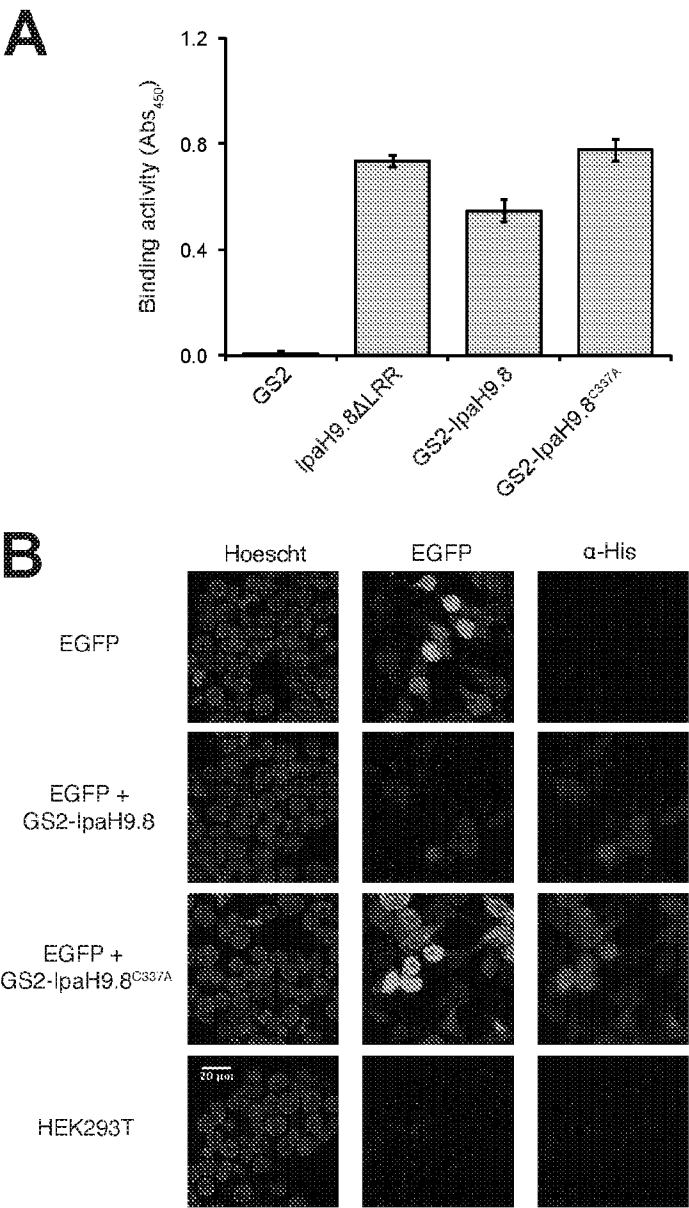


FIGs. 4A-4C

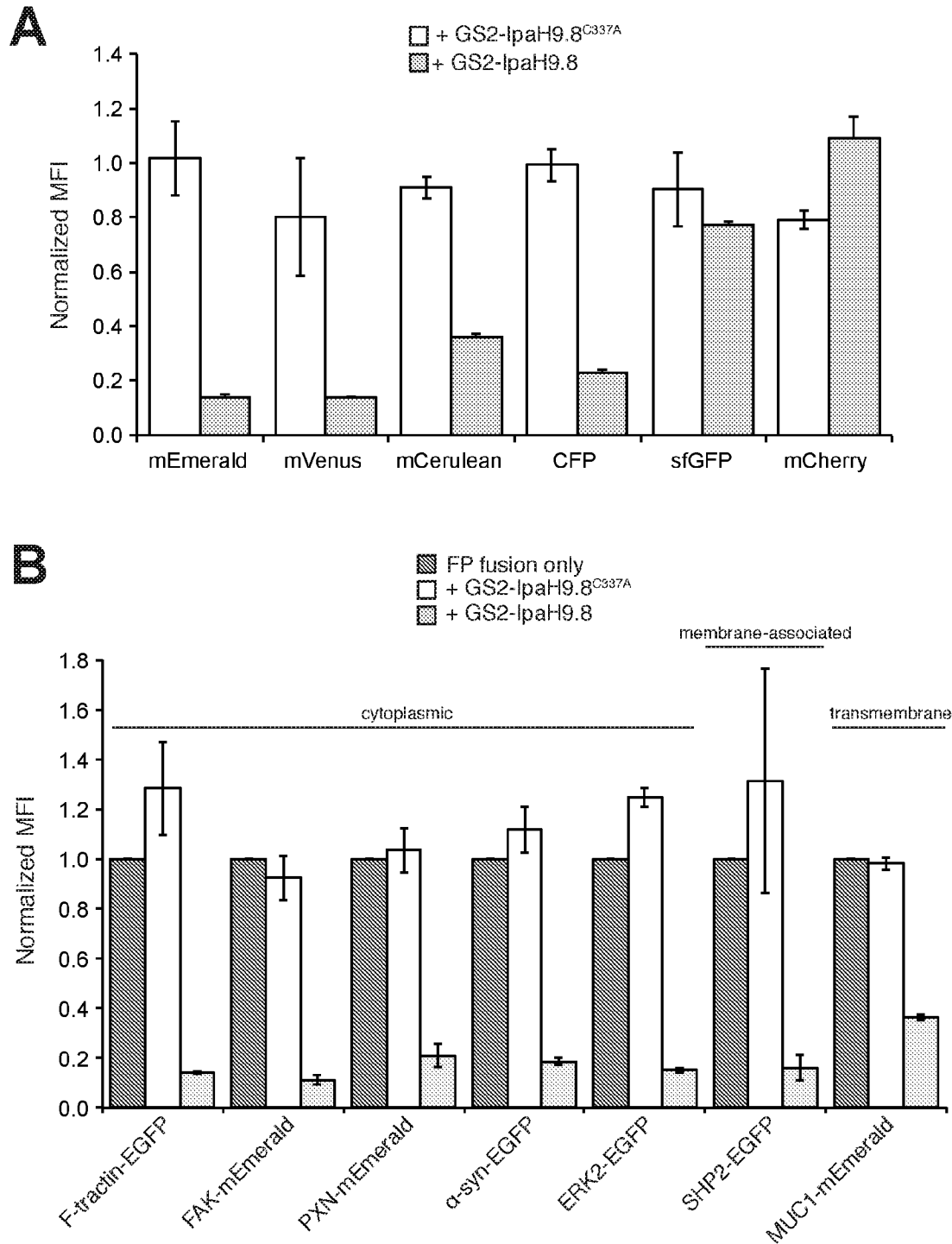
A**B****D****C****FIGs. 5A-5D**



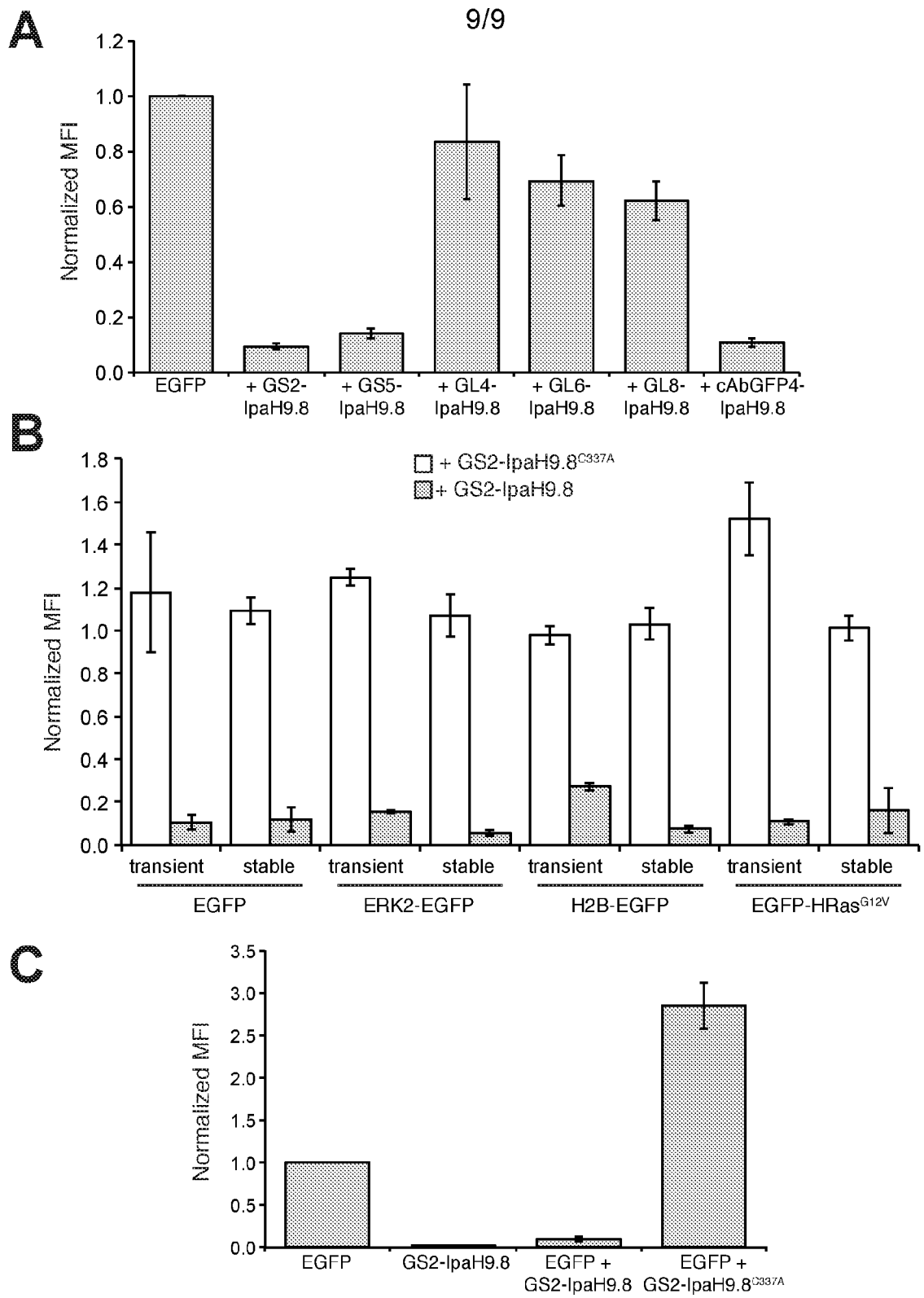
FIGS. 6A-6B



FIGs. 7A-7B



FIGs. 8A-8B



FIGs. 9A-9C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22783

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 9/00; C12N 15/11; C07K 19/00; C07K 14/78 (2019.01)

CPC - C12N 9/93; C07K 2319/00; C07K 2319/95; C07K 2318/20; C12Y603/02019

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2014/0112922 A1 (DeLisa et al.) 24 April 2014 (24.04.2014). Especially para [0010], [0056], claims 1, 3-8, 11, 12, 16, 17.	1-6, 20, 21 ----- 7-16
Y	Ashida et al. A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKK.gamma. to dampen the host NF-KB-mediated inflammatory response. Nat Cell Biol January 2010 Vol 12 No 1 Pages 66-69 [NIH Public Access Author's Manuscript utilized]. Especially abstract, pg 2 para 3.	7-12
Y	US 2014/0057807 A1 (Loew et al.) 27 February 2014 (27.02.2014). Especially para [0063], [0075], sheet 63 fig 31A.	13-16
T	Ludwicki et al. Broad-Spectrum Proteome Editing with an Engineered Bacterial Ubiquitin Ligase Mimic. ACS Cent Sci ePub 10 May 2019 Vol 5 No 5 Pages 852-866. Especially entire article.	1-16

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 June 2019

Date of mailing of the international search report

22 AUG 2019

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22783

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
-----Go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-16, 20, 21, limited to degradation domain lpaH9.8, (claim 11), targeting domain GS2 (claim 14), substrate GFP (claim 16)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22783

Continuation of Box III: Observations where Unity of Invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+: Claims 1-17, 20, 21, drawn to a chimeric molecule comprising a degradation domain, a targeting domain, and a linker coupling the degradation and targeting domains.

The chimeric molecules will be searched to the extent that the degradation domain is the first named, *Shigella flexneri* E3 ligase (claim 9), IpaH9.8 (claims 10, 11) [note that IpaH9.8 is a *Shigella* E3 ligase], the targeting domain is the first named targeting domain, monobody fibronectin type III domain GS2 (claims 13, 14), and the substrate is the first named substrate, fluorescent protein, GFP (claims 15, 16). It is believed that claims 1-16, 20, 21 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass IpaH9.8, GS2, and GFP. Additional degradation domains, targeting domains and substrates will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected degradation domains, targeting domains and substrates. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: degradation domain from *Salmonella* SspH1 (claim 9), targeting domain ScFv (claim 13), and substrate EGFR (claim 15) (claims 1-9, 13, 15-17, 20, 21).

Group II: Claims 18-19, drawn to a method of forming a ribonucleoprotein.

Group III: Claims 22-24, drawn to a method of treating a disease.

Group IV: Claims 25-27, drawn to a method for substrate silencing.

Group V: Claims 28-43, drawn to a method of screening agents for therapeutic efficacy against a disease.

Group VI: Claims 44-53, drawn to a method of screening for disease biomarkers.

The inventions listed as Groups I+, II-VI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ has the special technical feature of a composition comprising a chimeric molecule comprising a degradation domain, a targeting domain, and a linker coupling the degradation and targeting domains, not required by Groups II-VI.

Group II has the special technical feature of a method of forming a ribonucleoprotein, not required by Groups I+, III-VI.

Group III has the special technical feature of treating a disease by administering a chimeric molecule, not required by Groups I+, II, IV-VI.

Group IV has the special technical feature of substrate silencing, not required by Groups I+, II, III, V, VI.

Group V has the special technical feature of a method of screening agents for therapeutic efficacy, by providing a biomolecule whose presence mediates a disease and a test agent, and contacting the biomolecule with the test agent under conditions effective for the test agent to facilitate degradation of the biomolecule, not required by Groups I+, II, III, IV, VI.

Group VI has the special technical feature of a method for disease biomarkers, by providing a sample of diseased cells, providing a plurality of chimeric molecules, and contacting the sample with the plurality of chimeric molecules, and determining which of the chimeric molecules permit the diseased cells to proliferate, not required by Groups I+, II-V.

Among the inventions listed as Groups I+ are the specific degradation domains, targeting domains and substrates, recited therein. Each invention requires a specific degradation domain, targeting domain, and substrate not required by any other inventions.

Common Technical Feature:

1. Group I+ inventions share claim 1.

2. Group II claim 18, Group III claim 22 and Group IV claim 25 all depend from Group I claim 1.

3. Groups I+, II-VI share the common technical feature of chimeric molecule comprising a degradation domain comprising an E3 ubiquitin ligase (E3) motif and a targeting domain capable of specifically directing the degradation domain to a substrate, where the targeting domain is heterologous to the degradation domain and a linker coupling the degradation domain to the targeting domain.

4. Groups III, V and VI share the common technical feature of a disease.

5. Groups I+ and III share the common technical feature of a pharmaceutically acceptable carrier.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22783

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However, said common technical features do not represent a contribution over the prior art, and are anticipated by US 2014/0112922 A1 to DeLisa et al. (hereinafter "DeLisa").

As to common technical features #1-#3, DeLisa discloses (claim 1), an isolated chimeric molecule comprising: a degradation domain comprising an E3 ubiquitin ligase (E3) motif; a targeting domain capable of specifically directing said degradation domain to a substrate, wherein said targeting domain is heterologous to said degradation domain; and a linker coupling said degradation domain to said targeting domain (claim 1; "An isolated chimeric molecule comprising: a degradation domain comprising a eukaryotic U-box motif; a targeting domain capable of immunospecifically directing said degradation domain to a substrate, wherein said targeting domain is heterologous to said degradation domain; and a linker coupling said degradation domain to said targeting domain"; para [0056]; "In some embodiments, the U-box motif is a ubiquitin region composed of an E3 ligase, or fragment thereof").

As to common technical feature #4, a disease, DeLisa discloses (para [0010]; "In some embodiments, a method of treating a disease involves administering the composition to a subject having a disease").

As to common technical feature #5, a pharmaceutically acceptable carrier, DeLisa discloses (para [0010]; "A further aspect of the present invention relates to a composition comprising the chimeric molecule and a pharmaceutically-acceptable carrier").

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+, II-VI lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.