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(71) Applicant: **SANGAMO BIOSCIENCES, INC.** [US/US]; Point Richmond Tech Center, 501 Canal Blvd., Suite A100, Richmond, CA 94804 (US).

(72) Inventors: **MILLER, Jeffrey, C.**; c/ Sangamo Biosciences, Inc., Point Richmond Tech Center, 501 Canal Blvd., Suite A100, Richmond, CA 94804 (US). **REBAR, Edward, J.**; c/ Sangamo Biosciences, Inc., Point Richmond Tech Center, 501 Canal Blvd., Suite A100, Richmond, CA 94804 (US). **ZHANG, H., Steve**; c/ Sangamo Biosciences, Inc., Point Richmond Tech Center, 501 Canal Blvd., Suite A100, Richmond, CA 94804 (US).

(74) Agent: **PASTERNAK, Dahna, S.**; Pasternak Patent Law, 1900 Embarcadero Rd., Suite 211, Palo Alto, California 94303 (US).

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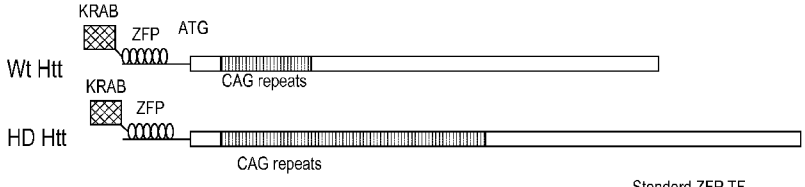

FIG. 1A

(57) Abstract: Disclosed herein are methods and compositions for treating or preventing Huntington's Disease.

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METHODS AND COMPOSITIONS FOR TREATING HUNTINGTON'S DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application No. 61/605,028, filed February 29, 2012, the disclosure of which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] The present disclosure is in the fields of gene expression and genome editing.

BACKGROUND

[0003] Huntington's Disease (HD), also known as Huntington's Chorea, is a progressive disorder of motor, cognitive and psychiatric disturbances. The mean age of onset for this disease is age 35-44 years, although in about 10% of cases, onset occurs prior to age 21, and the average lifespan post-diagnosis of the disease is 15-18 years. Prevalence is about 3 to 7 among 100,000 people of western European descent.

[0004] Huntington's Disease is an example of a trinucleotide repeat expansion disorders were first characterized in the early 1990s (*see* Di Prospero and Fischbeck (2005) *Nature Reviews Genetics* 6:756-765). These disorders involve the localized expansion of unstable repeats of sets of three nucleotides and can result in loss of function of the gene in which the expanded repeat resides, a gain of toxic function, or both. Trinucleotide repeats can be located in any part of the gene, including non-coding and coding gene regions. Repeats located within the coding regions typically involve either a repeated glutamine encoding triplet (CAG) or an alanine encoding triplet (CGA). Expanded repeat regions within non-coding sequences can lead to aberrant expression of the gene while expanded repeats within coding regions (also known as codon reiteration disorders) may cause mis-folding and protein aggregation. The exact cause of the pathophysiology associated with the aberrant proteins is often not known. Typically, in the wild-type genes that are subject to trinucleotide expansion, these regions contain a variable number of repeat sequences in the normal

population, but in the afflicted populations, the number of repeats can increase from a doubling to a log order increase in the number of repeats. In HD, repeats are inserted within the N terminal coding region of the large cytosolic protein Huntingtin (Htt). Normal Htt alleles contain 15-20 CAG repeats, while alleles containing 35 or more repeats can be considered potentially HD causing alleles and confer risk for developing the disease. Alleles containing 36-39 repeats are considered incompletely penetrant, and those individuals harboring those alleles may or may not develop the disease (or may develop symptoms later in life) while alleles containing 40 repeats or more are considered completely penetrant. In fact, no asymptomatic persons containing HD alleles with this many repeats have been reported. Those individuals with juvenile onset HD (<21 years of age) are often found to have 60 or more CAG repeats. In addition to an increase in CAG repeats, it has also been shown that HD can involve +1 and +2 frameshifts within the repeat sequences such that the region will encode a poly-serine polypeptide (encoded by AGC repeats in the case of a +1 frameshift) track rather than poly-glutamine (Davies and Rubinsztein (2006) *Journal of Medical Genetics* 43: 893-896).

[0005] In HD, the mutant Htt allele is usually inherited from one parent as a dominant trait. Any child born of a HD patient has a 50% chance of developing the disease if the other parent was not afflicted with the disorder. In some cases, a parent may have an intermediate HD allele and be asymptomatic while, due to repeat expansion, the child manifests the disease. In addition, the HD allele can also display a phenomenon known as anticipation wherein increasing severity or decreasing age of onset is observed over several generations due to the unstable nature of the repeat region during spermatogenesis.

[0006] Furthermore, trinucleotide expansion in Htt leads to neuronal loss in the medium spiny gamma-aminobutyric acid (GABA) projection neurons in the striatum, with neuronal loss also occurring in the neocortex. Medium spiny neurons that contain enkephalin and that project to the external globus pallidum are more involved than neurons that contain substance P and project to the internal globus pallidum. Other brain areas greatly affected in people with Huntington's disease include the substantia nigra, cortical layers 3, 5, and 6, the CA1 region of the hippocampus, the angular gyrus in the parietal lobe, Purkinje cells of the cerebellum,

lateral tuberal nuclei of the hypothalamus, and the centromedialparafascicular complex of the thalamus (Walker (2007) *Lancet* 369:218-228).

[0007] The role of the normal Htt protein is poorly understood, but it may be involved in neurogenesis, apoptotic cell death, and vesicle trafficking. In addition, there is evidence that wild-type Htt stimulates the production of brain-derived neurotrophic factor (BDNF), a pro-survival factor for the striatal neurons. It has been shown that progression of HD correlates with a decrease in BDNF expression in mouse models of HD (Zuccato *et al* (2005) *Pharmacological Research* 52(2): 133-139), and that delivery of either BDNF or glial cell line-derived neurotrophic factor (GDNF) via adeno-associated viral (AAV) vector-mediated gene delivery may protect straital neurons in mouse models of HD (Kells *et al*, (2004) *Molecular Therapy* 9(5): 682-688).

[0008] Treatment options for HD are currently very limited. Some potential methodologies designed to prevent the toxicities associated with protein aggregation that occurs through the extended poly-glutamine tract such as overexpression of chaperonins or induction of the heat shock response with the compound geldanamycin have shown a reduction in these toxicities in *in vitro* models. Other treatments target the role of apoptosis in the clinical manifestations of the disease. For example, slowing of disease symptoms has been shown via blockage of caspase activity in animal models in the offspring of a pairing of mice where one parent contained a HD allele and the other parent had a dominant negative allele for caspase 1. Additionally, cleavage of mutant HD Htt by caspase may play a role in the pathogenicity of the disease. Transgenic mice carrying caspase-6 resistant mutant Htt were found to maintain normal neuronal function and did not develop striatal neurodegeneration as compared to mice carrying a non-caspase resistant mutant Htt allele (see Graham *et al* (2006) *Cell* 125: 1179-1191). Molecules which target members of the apoptotic pathway have also been shown to have a slowing affect on symptomology. For example, the compounds zVAD-fmk and minocycline, both of which inhibit caspase activity, have been shown to slow disease manifestation in mice. The drug remacemide has also been used in small HD human trials because the compound was thought to prevent the binding of the mutant Htt to the NDMA receptor to prevent the exertion of toxic effects on the nerve cell. However, no statistically significant improvements were observed in neuron function in these trials. In addition, the

Huntington Study Group conducted a randomized, double-blind study using Co-enzyme Q. Although a trend towards slower disease progression among patients that were treated with coenzyme Q10 was observed, there was no significant change in the rate of decline of total functional capacity. (Di Prospero and Fischbeck, *ibid*). U.S. Patent Publication 2011/0082093 discloses specific zinc finger proteins targeted to Htt.

[0009] Thus, there remains a need for compositions and methods for the treatment and prevention of Huntington's Disease.

SUMMARY

[0010] Disclosed herein are methods and compositions for treating Huntington's Disease. In particular, provided herein are methods and compositions for modifying (*e.g.,* modulating expression of) an HD Htt allele so as to treat Huntington Disease. Also provided are methods and compositions for generating animal models of Huntington's Disease.

[0011] Thus, in one aspect, engineered DNA binding domains (*e.g.*, zinc finger proteins or TAL effector (TALE) proteins) that modulate expression of a HD allele (*e.g.,* Htt) are provided. Engineered zinc finger proteins or TALEs are non-naturally occurring zinc finger or TALE proteins whose DNA binding domains (*e.g.*, recognition helices or RVDs) have been altered (*e.g.*, by selection and/or rational design) to bind to a pre-selected target site. Any of the zinc finger proteins described herein may include 1, 2, 3, 4, 5, 6 or more zinc fingers, each zinc finger having a recognition helix that binds to a target subsite in the selected sequence(s) (*e.g.,* gene(s)). Similarly, any of the TALE proteins described herein may include any number of TALE RVDs. In some embodiments, at least one RVD has non-specific DNA binding. In some embodiments, at least one recognition helix (or RVD) is non-naturally occurring. In certain embodiments, the zinc finger proteins have the recognition helices shown in Tables 1A and 1B. In other embodiments, the zinc finger proteins bind to the target sequences shown in Tables 2A and 2B. In some embodiments, the zinc finger proteins comprise the recognition helices in Table 2C. In certain embodiments, the zinc finger proteins are formulated into a pharmaceutical composition, for example, for administration to a subject.

[0012] In one aspect, repressors (ZFP-TFs or TALE-TFs) are provided that bind to sequences entirely or partially outside the CAG repeat region of Htt. In another aspect, ZFP or TALE repressors (ZFP-TFs or TALE-TFs) are provided that bind to sequences within CAG repeat region of Htt. In some embodiments, these ZFP-TFs or TALE-TFs preferentially bind to expanded trinucleotide tracts relative to repeat tracts of a wild-type length, thereby achieving preferential repression of the expanded allele. In some embodiments these ZFP-TFs or TALE-TFs include protein interaction domains (or "dimerization domains") that allow multimerization when bound to DNA. In some embodiments, these ZFP-TFs or TALE TFs achieve cooperative DNA binding to the repeat sequence so that the expanded allele is bound more efficiently by a larger number of ZFPs or TALE proteins than the wild-type allele, allowing preferential repression of the mutant allele. These cooperative binding ZFP-TFs or TALE TFs may or may not further contain protein interaction domains that allow multimerization when bound to DNA. In some embodiments, ZFP TFs or TALE TFs form a stable complex of multimers of a given size, and thus are capable of preferentially interacting with a CAG tract above a certain minimum size, wherein that minimum size is greater than the length of a wild-type CAG tract.

[0013] In certain embodiments, the ZFPs or TALE proteins as described herein (*e.g.*, two-handed, multimerizing, etc.) preferentially modify expression of a mutant Htt allele. In some embodiments, the ZFP or TALE binds specifically to mutant Htt alleles wherein the expanded tract encodes poly-glutamine, while in other embodiments, the ZFP or TALE binds specifically to a mutant Htt allele wherein the expansion tract encodes poly-serine. Thus, in some embodiments, the ZFP-TF or TALE-TF modulates both the wild type and mutant forms of the Htt allele. In certain embodiments, the ZFP or TALE modulates only the wild type Htt allele. In other embodiments, the ZFP or TALE modulates only the mutant form of Htt.

[0014] In other embodiments, repressing ZFP-TFs or TALE-TFs are provided which preferentially bind to known SNPs associated with the expanded HD Htt alleles. In this way, the ZFP-TFs or TALE-TFs are specific for mutant Htt alleles which contain the SNP, allowing for specific repression of the mutant Htt allele. In another aspect, ZFP-TFs or TALE-TFs that specifically activate the wild-type Htt allele by interacting with SNPs associated with wild-type alleles are provided. In this way, only the wild-type Htt allele is activated.

[0015] In certain embodiments, the zinc finger proteins (ZFPs) or TALE proteins as described herein can be placed in operative linkage with a regulatory domain (or functional domain) as part of a fusion protein. The functional domain can be, for example, a transcriptional activation domain, a transcriptional repression domain and/or a nuclease (cleavage) domain. By selecting either an activation domain or repression domain for fusion with the ZFP or TALE, such fusion proteins can be used either to activate or to repress gene expression. In some embodiments, a fusion protein comprising a ZFP or TALE targeted to a mutant Htt as described herein fused to a transcriptional repression domain that can be used to down-regulate mutant Htt expression is provided. In some embodiments, a fusion protein comprising a ZFP or TALE targeted to a wild-type Htt allele fused to a transcription activation domain that can up-regulate the wild type Htt allele is provided. In certain embodiments, the activity of the regulatory domain is regulated by an exogenous small molecule or ligand such that interaction with the cell's transcription machinery will not take place in the absence of the exogenous ligand. Such external ligands control the degree of interaction of the ZFP-TF or TALE-TF with the transcription machinery. The regulatory domain(s) may be operatively linked to any portion(s) of one or more of the ZFPs or TALEs, including between one or more ZFPs or TALEs, exterior to one or more ZFPs or TALEs and any combination thereof. Any of the fusion proteins described herein may be formulated into a pharmaceutical composition.

[0016] In some embodiments, the engineered DNA binding domains as described herein can be placed in operative linkage with nuclease (cleavage) domains as part of a fusion protein. In other embodiments, nuclease systems such as the CRISPR/Cas system may be utilized with a specific single guide RNA to target the nuclease to a target location in the DNA. In certain embodiments, such nucleases and nuclease fusions may be utilized for targeting mutant Htt alleles in stem cells such as induced pluripotent stem cells (iPSC), human embryonic stem cells (hESC), mesenchymal stem cells (MSC) or neuronal stem cells wherein the activity of the nuclease fusion will result in an Htt allele containing a wild type number of CAG repeats. In certain embodiments, pharmaceutical compositions comprising the modified stem cells are provided.

[0017] In yet another aspect, a polynucleotide encoding any of the DNA binding proteins described herein is provided. In another aspect, polynucleotides

encoding a CRIPSR/Cas nuclease and a single guide RHA are provided. Such polynucleotides can be administered to a subject in which it is desirable to treat Huntington's Disease.

[0018] In still further aspects, the invention provides methods and compositions for the generation of specific model systems for the study of Huntington's Disease. In certain embodiments, provided herein are models in which mutant Htt alleles are generated using embryonic stem cells to generate cell and animal lines in which trinucleotide expansion tracts of specific lengths (50, 80, 109 and 180 CAG repeats, for example) are inserted into a wild-type Htt allele using zinc finger nuclease (ZFN), TALE-nuclease (TALEN), or CRISPR/Cas nuclease driven targeted integration. In certain embodiments, the model systems comprise *in vitro* cell lines, while in other embodiments, the model systems comprise transgenic animals. In any of the animal models described herein, the animal may be, for example, a rodent (*e.g.,* rat, mouse), a primate (*e.g.,* non-human primate) or a rabbit.

[0019] In yet another aspect, a gene delivery vector comprising any of the polynucleotides described herein is provided. In certain embodiments, the vector is an adenovirus vector (*e.g.*, an Ad5/F35 vector), a lentiviral vector (LV) including integration competent or integration-defective lentiviral vectors, or an adenovirus associated viral vector (AAV). Thus, also provided herein are adenovirus (Ad) vectors, LV or adenovirus associate viral vectors (AAV) comprising a sequence encoding at least one nuclease (ZFN or TALEN) and/or a donor sequence for targeted integration into a target gene. In certain embodiments, the Ad vector is a chimeric Ad vector, for example an Ad5/F35 vector. In certain embodiments, the lentiviral vector is an integrase-defective lentiviral vector (IDLV) or an integration competent lentiviral vector. In certain embodiments the vector is pseudo-typed with a VSV-G envelope, or with other envelopes.

[0020] In some embodiments, model systems are provided for Huntington's disease wherein the target alleles (*e.g.,* mutant Htt) are tagged with expression markers. In certain embodiments, the mutant alleles (*e.g.,* mutant Htt) are tagged. In some embodiments, the wild type allele (*e.g.,* wild-type Htt) is tagged, and in additional embodiments, both wild type and mutant alleles are tagged with separate expression markers. In certain embodiments, the model systems comprise *in vitro* cell lines, while in other embodiments, the model systems comprise transgenic animals.

[0021] Additionally, pharmaceutical compositions comprising the nucleic acids and/or proteins (*e.g.,* ZFPs or TALEs or fusion proteins comprising the ZFPs or TALEs) are also provided. For example, certain compositions include a nucleic acid comprising a sequence that encodes one of the ZFPs or TALEs described herein operably linked to a regulatory sequence, combined with a pharmaceutically acceptable carrier or diluent, wherein the regulatory sequence allows for expression of the nucleic acid in a cell. In certain embodiments, the ZFPs or TALEs encoded are specific for a HD Htt allele. In some embodiments, pharmaceutical compositions comprise ZFPs or TALEs that modulate a HD Htt allele and ZFPs or TALEs that modulate a neurotrophic factor. Protein based compositions include one of more ZFPs or TALEs as disclosed herein and a pharmaceutically acceptable carrier or diluent.

[0022] In yet another aspect also provided is an isolated cell comprising any of the proteins, polynucleotides and/or compositions as described herein.

[0023] In another aspect, provided herein are methods for treating and/or preventing Huntington's Disease using the methods and compositions described herein. In some embodiments, the methods involve compositions where the polynucleotides and/or proteins may be delivered using a viral vector, a non-viral vector (*e.g.,* plasmid) and/or combinations thereof. In some embodiments, the methods involve compositions comprising stem cell populations comprising a ZFP or TALE, or altered with the ZFNs, TALENs or the CRISPR/Cas nuclease system of the invention.

[0024] These and other aspects will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **Figure 1**, panels A to E, are schematics depicting wild type and mutant (Huntington's Disease, HD) Huntingtin (Htt) allele and various ZFP-TFs binding to those alleles. Figure 1A shows ZFP designs that bind outside of the CAG repeat, and therefore are predicted to bind equally to the wild type allele and the mutant (HD) allele. "KRAB" refers to the KRAB repression domain from the KOX1 gene and "ZFP" refers to the zinc finger DNA binding protein. "Standard ZFP TF" is a ZFP transcription factor fusion protein in which the zinc finger DNA binding domains are

linked to the KRAB repression domain. Figure 1B shows ZFP-TFs designed to bind within the CAG region. Figure 1C depicts a "two-handed ZFP TF," which is a ZFP transcription factor in which two clusters of zinc finger domains are separated by a rigid protein sequences. The functional (repression) domain is depicted exterior to one ZFP in this Figure, but it will be apparent that the functional domain may be between the ZFPs or exterior to the ZFPs on either end of the protein. Figure 1D depicts a "multimerizing ZFP TF," which is a ZFP TF that is capable of multimerizing through a multimerization domain (depicted as speckled boxes). Figure 1E depicts a ZFP-ZFP-KRAB configuration where two zinc finger DNA binding domains are linked by a flexible linker and are also fused to a KRAB domain. It will be apparent to the skilled artisan that in all fusion proteins, the functional domain can be on either end of the DNA binding domain, and that the DNA binding domain may comprise a wide number range of zinc fingers. Also depicted in Figure 1 as a box with black diamonds is a functional domain (*e.g.,* activation, repression, cleavage domain). It will be apparent to the skilled artisan that the exemplary models presented in the Figures may apply to TALE TFs as well.

[0026] **Figure 2, panels A through E,** depict the repression of both alleles of Htt by ZFPs as described in Figure 1A using ZFP TFs that do not bind to the CAG repeat sequences. The ZFP identification numbers as shown in Tables 1A and 1B are indicated below the bars. Figure 2A depicts repression of the human Htt alleles in HEK293 cells using ZFPs targeted to five loci in the human gene. A diagram of the human Htt gene is shown and the locations of ZFP binding sites are shown. For each ZFP group, each bar represents an independent transfection. Figure 2B depicts a Western blot showing Htt protein levels in HEK293 cells transfected with the GFP control or the 18856 ZFP TF repressor (comprising the KRAB repression domain of KOX1), where the NFκB p65 levels ("p65") were used to confirm equal protein loading. The Western blot confirms the repression of Htt expression by the ZFP-TF. Figure 2C depicts a similar set of data as Figure 2A for the mouse Htt specific ZFP in Neuro2A cells. As in Figure 2A, a diagram of the mouse Htt gene is shown and the locations of ZFP binding sites are indicated. Figure 2D and 2E demonstrate the repression of mouse Htt gene expression (RNA) in immortalized striatal cells, where different doses of ZFP-TF mRNA were used for transfection. In all cases except

Figure 2B, Htt mRNA levels were measured by real-time RT-PCR and normalized to those of Actin mRNA.

[0027] **Figure 3, panels A to G,** depict selective repression of mutant Htt by using ZFPs binding within the CAG repeat region, as illustrated in Figure 1B. This model illustrates that the longer CAG repeat region in the mutant allele allows for increased binding of CAG-targeted ZFP repressor molecules. Figure 3A depicts different repressor activities on the endogenous Htt gene (with normal CAG repeat length) by CAG-targeted ZFPs in HEK293 cells. Figure 3B shows repression of luciferase reporters controlled by Htt promoter/exon1 fragments containing CAG repeats of varying lengths, ranging from 10 to 47 CAG repeats. CAG10 (left-most bar for each of the two indicated conditions) shows results with 10 CAG repeats; CAG17 (bar second from the left for each of the two indicated conditions) shows results with 17 CAG repeats; CAG23 (bar second from the right for each of the two indicated conditions) shows results with 23 CAG repeats; and CAG47 (right-most bar for each of the two indicated conditions) shows results with 47 CAG repeats. The schematic above the graph depicts the arrangement of the Htt promoter, exon 1, the CAG repeats and the reporter luciferase gene used in this system. The data demonstrate that increasing the number of CAGs leads to a decreased expression from the Htt promoter by a CAG-targeted ZFP. Furthermore, Figure 3C demonstrates that, while a relatively weak CAG-targeted ZFP does not repress the luciferase reporter that contains a normal-length CAG repeat as well as a strong CAG repressor, it drives similar repression of a luciferase reporter that contains an expanded CAG repeat as the strong CAG-targeted ZFP.at all doses tested. "pRL-Htt-CAG23-intron 1" (left bar of each pair) corresponds to expression from the wild type allele while "pRL-HttCAG47-intron 1" (right bar of each pair) correlates with expression from the mutant expanded Htt allele (containing 47 CAG repeats). Figure 3D is a graph depicting repression of mutant Htt (111 CAG) by CAG-targeted ZFPs in immortalized mouse striatal cells derived from HdH(Q111/Q7) knock-in mice. Wild-type expression is shown in the left bar of each pair and knock-in expression in the right bar of each pair. ZFP-TFs comprising the specified ZFP fused to the KRAB repression domain were tested using three different concentrations of ZFP mRNA in the transfections. Figure 3E depicts mutant Htt repression by CAG-targeted ZFPs in a HD patient derived fibroblast line (CAG15/70). In this fibroblast line, the wild type

Htt allele comprises 15 CAG repeats ("099T(CAG15)", middle bar of each indicated condition) and the mutant expanded Htt allele comprises 70 CAG repeats ("099C(CAG70)", right bar of each indicated condition). Figure 3F shows selective repression of mutant Htt expression in 4 different HD patient derived fibroblast cell lines. The numbers above each grouping indicate the number of CAG repeats on the wildtype Htt allele (*e.g.* 15 or 18) and on the mutant allele (*e.g.* 70, 67, 45 and 44); where two different doses of ZFP mRNA were tested. The left-bar of each pair shows wild-type Htt expression and the right bar of each shows expression of mutant Htt. Figure 3G depicts Htt expression in HD derived patient fibroblasts as assayed by Western blot analysis in the presence of the ZFP-TFs 30640, 32528 and 30657. The slower migrating protein bands are those produced by the expanded mutant Htt alleles. 32528 binds to the transcription start site of Htt (TSS) and thus inhibits expression from both alleles, while 30640 and 30657 bind to the CAG repeats (CAG).

[0028] **Figure 4, panels A and B,** depict repression of the mutant Htt in a HD patient derived fibroblast line by a panel of ZFPs targeted to the CAG repeat. A range of RNA concentrations were used from 0.1 ng to 3 μg. In Figures 4A and 4B, the left bar of each indicated conditions shows total Htt expression, the middle bar shows expression of Htt in fibroblasts in which the Htt allele comprises 18 CAG repeats ("099T(CAG18)" and the mutant expanded Htt allele comprises 45 CAG repeats 099T (CAG45

[0029] **Figure 5** shows the effect of CAG-targeted ZFP repressors on the expression of Htt and other CAG-containing genes in HD patient-derived fibroblasts. The left bar under each indicated condition shows results with 30640; the middle bar under each indicated condition shows results with 30675; and the right bar shows mock transfections.

[0030] **Figure 6, panels A and B,** depicts an experiment that examines the genome-wide specificity of three CAG-targeted ZFPs. Figure 6A depicts qPCR analysis of Htt repression performed on six biological replicates (six separate transfections) of HD fibroblasts (CAG18 (middle bars)/ CAG45, right bars) using 30640, 30645, or 33074. The four most similar replicates by qPCR were then selected for microarray analysis, and the data is presented in Figure 6B.

[0031] **Figure 7** depicts Htt repression in CAG17/69 neuronal stem cells (NSC). The cells were transfected with ZFP mRNA at indicated doses. Left bars

under each of the indicated doses show results in CAG17 cells, middle bars show results in wild-type cells, right bars show results in CAG69 cells.

[0032] **Figure 8** depicts Htt expression in neurons differentiated from HD embryonic stem cells (ESC) (CAG 17/48) treated with ZFP TFs. The cells were transfected with ZFP mRNA at indicated doses.

[0033] **Figure 9** depicts repression of a mutant Htt transgene expression in R6/2 mice following treatment with the ZFP TF 30640.

[0034] **Figure 10, panels A through D,** depicts ZFPs with multimerization domains that specifically targets expanded CAG repeats, as illustrated in Figure 1D. Figure 10A shows a single ZFP that have four components: (i) a KOX repressor domain (oval labeled "repressor"); (ii) an array of 2-6 fingers (two shown, small ovals marked "Z") that binds to $(CAG)_N$ or a permutation of this sequence; and (iii) two dimerization domains (rectangles labeled "d1" and "d2") that interact in an antiparallel configuration. These domains allow the ZFP to polymerize within the major groove of a CAG tract. Figure 10B shows a sketch of the binding event with a multimer of 3 ZFPs. It will be apparent that any number of multimers can be used and that the functional domain may be positioned anywhere on one or more of the individual ZFPs and that these diagrams are applicable to TALE-TFs as well. Figure 10C shows protein sequences of the four ZFP monomer scaffolds that are designed to multimerize via interactions between dimerizing zinc fingers (DZ). Scaffolds are named DZ1 (SEQ ID NO:180), DZ2 (SEQ ID NO:181), DZ3 (SEQ ID NO:182) and DZ4 (SEQ ID NO:183). Dimerizing zinc finger domains are underlined, while the repression domain and nuclear localization sequence are indicated by bold underline and italic text (respectively). Figure 10D shows protein sequences of the seven ZFP monomer scaffolds that are designed to multimerize via interactions between coiled-coils (CC). Scaffolds are named CC1 (SEQ ID NO:184), CC2 (SEQ ID NO:185), CC3 (SEQ ID NO:186), CC4 (SEQ ID NO:187), CC5 (SEQ ID NO:188), CC6 (SEQ ID NO:189) and CC7 (SEQ ID NO:190). Coiled-coil sequences are underlined, while the repression domain and nuclear localization sequence are indicated by bold underline and italic text (respectively). The location of the ZFP region of each scaffold, which will vary between designs, is indicated by "[ZFP]." The location of the (DNA-binding) ZFP region of each scaffold, which will vary between designs, is indicated by "[ZFP]."

[0035] **Figure 11, panels A and B,** depict activity of ZFP-TFs with dimerization domains. In Figure 11A, ZFP-TFs with "coiled coil" (CC) domains were tested with luciferase reporters. pRL-Htt CAG17 (left bar of each pair) stands for renilla luciferase reporter controlled by human Htt promoter/exon1 fragment with 17 CAG; pGL3-Htt-CAG47 (right bar of each pair) stands for firefly luciferase reporter controlled by human Htt promoter/exon1 fragment with 47 CAG repeats. See text in Example 10 for description of the various dimerization domains. In Figure 11B, ZFPs with the dimerizing zinc finger "DZ" domains were tested with the same luciferase reporters, and demonstrates increased repression with some ZFP-TF dimerization domains. The left bar in each doublet indicates the expression from the 17CAG repeat Htt allele while the right bar indicates expression from 47 CAG repeat Htt allele.

[0036] **Figure 12, panels A and B,** depict repression of Htt by ZFP-ZFP-KOX proteins. Figure 12A depicts Htt repression by the single 33088 and 33084 ZFP-TFs, and repression by the 33088-33088 and 33088-33084 ZFP-ZFP-KOX proteins in wild-type (left bar), CAG18 (middle bar) and CAG45 (right bar) (Figure 12A) HD fibroblasts; Figure 12B depicts Htt repression by 33088-33088 and 33088-33084 ZFP-ZFP-KOX in wild type (left bar), CAG 20 (middle bar) and CAG41 (left bar) HD fibroblasts.

[0037] **Figure 13, panels A to E,** depict activation of mouse Htt. Figure 13A demonstrates ZFP-TF-driven up-regulation of the mouse Htt genes at the RNA level in Neuro2A cells using a ZFP fused to the p65 activation domain. Double bars indicate duplicate transfections. Figure 13B depicts a Western blot demonstrating increased Htt protein production driven by the ZFP. Figure 13C depicts a wild type mouse Htt allele and a "knock in" Htt allele where mouse sequence (most of exon1 and part of intron 1, line above wild-type allele schematic) has been replaced with corresponding human sequence with CAG expansion (line over knock-in allele schematic). Figure 13D depicts the alignment between mouse sequence (SEQ ID NO:191) that was replaced with the corresponding human sequence (SEQ ID NO:192) such that the knock-in allele has sufficient sequence divergence to allow ZFPs (shown in A and B) to be designed to bind specifically the mouse sequence. Figure 13E depicts specific activation of the wild type mouse Htt allele in immortalized striatal cells derived from the HdhQ111/Q7 knock-in mice. The left-bar

shows results in wild-type cells and right bar shows results in knock-in mutant allele cells.

[0038] **Figure 14, panels A and B,** depicts the results of Cel-I mismatch assays (Surveyor™, Transgenomics) following treatment of K562 cells with Htt specific ZFN pairs. The percent NHEJ activity (in-del) for an active ZFN is shown at the bottom of the corresponding lane. "GFP" indicates cells that have been transfected with a GFP encoding plasmid. Figure 14A depicts results from ZFNs that cleave early Htt exons while Figure 14B depicts results from ZFNs that cleave near the stop codon. Inactive ZFN pairs were also observed (lanes not annotated with in-del percentages).

[0039] **Figure 15** depicts graphs of the Htt repression results for several candidate TALE-TF proteins. The TALE-TFs were tested in HD patient derived fibroblasts (CAG 20/41). The results demonstrate that some of the TALE TFs were active in repressing overall Htt expression, while others exhibited mutant Htt-preferential repression.

DETAILED DESCRIPTION

[0040] Disclosed herein are compositions and methods for treating Huntington's disease (HD). In particular, Htt-modulating transcription factors comprising zinc finger proteins (ZFP TFs) or TALEs (TALE-TF) and methods utilizing such proteins are provided for use in treating or preventing Huntington's disease. For example, ZFP-TFs or TALE-TFs which repress expression of a mutant Htt allele or activate expression of a wild-type Htt allele are provided. In addition, zinc finger nucleases (ZFNs), TALE nucleases (TALENs) or CRISPR/Cas nuclease systems that modify the genomic structure of the genes associated with HD are provided. For example, ZFNs, TALENs or CRISPR/Cas nuclease systems that are able to specifically alter portions of a mutant form of Htt are provided. These include compositions and methods using engineered zinc finger proteins or engineered TALE proteins, *i.e.*, non-naturally occurring proteins which bind to a predetermined nucleic acid target sequence.

[0041] Thus, the methods and compositions described herein provide methods for treatment and prevention of Huntington's Disease, and these methods and compositions can comprise zinc finger transcription factors or TALE transcription

factors that are capable of modulating target genes as well as engineered zinc finger and TALE nucleases and CRISPR/Cas nuclease systems capable of modifying or editing Htt.

General

[0042] Practice of the methods, as well as preparation and use of the compositions disclosed herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See*, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolffe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

Definitions

[0043] The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.*, phosphorothioate backbones). In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analogue of A will base-pair with T.

[0044] The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally-occurring amino acids.

[0045] "Binding" refers to a sequence-specific, non-covalent interaction between macromolecules (*e.g.*, between a protein and a nucleic acid). Not all components of a binding interaction need be sequence-specific (*e.g.*, contacts with phosphate residues in a DNA backbone), as long as the interaction as a whole is sequence-specific. Such interactions are generally characterized by a dissociation constant (K_d) of 10^{-6} M^{-1} or lower. "Affinity" refers to the strength of binding: increased binding affinity being correlated with a lower K_d.

[0046] A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, *etc.*) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity.

[0047] A "zinc finger DNA binding protein" (or binding domain) is a protein, or a domain within a larger protein, that binds DNA in a sequence-specific manner through one or more zinc fingers, which are regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

[0048] A "TALE DNA binding domain" or "TALE" is a polypeptide comprising one or more TALE repeat domains/units. The repeat domains are involved in binding of the TALE to its cognate target DNA sequence. A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length and exhibits at least some sequence homology with other TALE repeat sequences within a naturally occurring TALE protein.

[0049] Zinc finger binding domains or TALE DNA binding domains can be "engineered" to bind to a predetermined nucleotide sequence, for example via engineering (altering one or more amino acids) of the recognition helix region of a naturally occurring zinc finger protein or by engineering the RVDs of a TALE protein. Therefore, engineered zinc finger proteins or TALEs are proteins that are non-naturally occurring. Non-limiting examples of methods for engineering zinc finger proteins or TALEs are design and selection. A designed zinc finger protein or TALE is a protein not occurring in nature whose design/composition results

principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data. See, for example, US Patents 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496 and U.S. Publication No. 20110301073.

[0050] A "selected" zinc finger protein or TALE is a protein not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. See *e.g.*, US 5,789,538; US 5,925,523; US 6,007,988; US 6,013,453; US 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197, WO 02/099084 and WO 2011/146121 (U.S. Patent Publication No. 20110301073).

[0051] "Recombination" refers to a process of exchange of genetic information between two polynucleotides. For the purposes of this disclosure, "homologous recombination (HR)" refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells via homology-directed repair mechanisms. This process requires nucleotide sequence homology, uses a "donor" molecule to template repair of a "target" molecule (*i.e.*, the one that experienced the double-strand break), and is variously known as "non-crossover gene conversion" or "short tract gene conversion," because it leads to the transfer of genetic information from the donor to the target. Without wishing to be bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or "synthesis-dependent strand annealing," in which the donor is used to resynthesize genetic information that will become part of the target, and/or related processes. Such specialized HR often results in an alteration of the sequence of the target molecule such that part or all of the sequence of the donor polynucleotide is incorporated into the target polynucleotide.

[0052] In the methods of the disclosure, one or more targeted nucleases as described herein create a double-stranded break in the target sequence (*e.g.*, cellular chromatin) at a predetermined site, and a "donor" polynucleotide, having homology to the nucleotide sequence in the region of the break, can be introduced into the cell. The presence of the double-stranded break has been shown to facilitate integration of

the donor sequence. The donor sequence may be physically integrated or, alternatively, the donor polynucleotide is used as a template for repair of the break via homologous recombination, resulting in the introduction of all or part of the nucleotide sequence as in the donor into the cellular chromatin. Thus, a first sequence in cellular chromatin can be altered and, in certain embodiments, can be converted into a sequence present in a donor polynucleotide. Thus, the use of the terms "replace" or "replacement" can be understood to represent replacement of one nucleotide sequence by another, (*i.e.*, replacement of a sequence in the informational sense), and does not necessarily require physical or chemical replacement of one polynucleotide by another.

[0053] In any of the methods described herein, additional pairs of zinc-finger or TALE proteins can be used for additional double-stranded cleavage of additional target sites within the cell.

[0054] In certain embodiments of methods for targeted recombination and/or replacement and/or alteration of a sequence in a region of interest in cellular chromatin, a chromosomal sequence is altered by homologous recombination with an exogenous "donor" nucleotide sequence. Such homologous recombination is stimulated by the presence of a double-stranded break in cellular chromatin, if sequences homologous to the region of the break are present.

[0055] In any of the methods described herein, the first nucleotide sequence (the "donor sequence") can contain sequences that are homologous, but not identical, to genomic sequences in the region of interest, thereby stimulating homologous recombination to insert a non-identical sequence in the region of interest. Thus, in certain embodiments, portions of the donor sequence that are homologous to sequences in the region of interest exhibit between about 80 to 99% (or any integer therebetween) sequence identity to the genomic sequence that is replaced. In other embodiments, the homology between the donor and genomic sequence is higher than 99%, for example if only 1 nucleotide differs as between donor and genomic sequences of over 100 contiguous base pairs. In certain cases, a non-homologous portion of the donor sequence can contain sequences not present in the region of interest, such that new sequences are introduced into the region of interest. In these instances, the non-homologous sequence is generally flanked by sequences of 50-1,000 base pairs (or any integral value therebetween) or any number of base pairs

greater than 1,000, that are homologous or identical to sequences in the region of interest. In other embodiments, the donor sequence is non-homologous to the first sequence, and is inserted into the genome by non-homologous recombination mechanisms.

[0056] Any of the methods described herein can be used for partial or complete inactivation of one or more target sequences in a cell by targeted integration of donor sequence that disrupts expression of the gene(s) of interest. Cell lines with partially or completely inactivated genes are also provided.

[0057] Furthermore, the methods of targeted integration as described herein can also be used to integrate one or more exogenous sequences. The exogenous nucleic acid sequence can comprise, for example, one or more genes or cDNA molecules, or any type of coding or noncoding sequence, as well as one or more control elements (*e.g.,* promoters). In addition, the exogenous nucleic acid sequence may produce one or more RNA molecules (*e.g.*, small hairpin RNAs (shRNAs), inhibitory RNAs (RNAis), microRNAs (miRNAs), *etc.*).

[0058] "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

[0059] A "cleavage half-domain" is a polypeptide sequence which, in conjunction with a second polypeptide (either identical or different) forms a complex having cleavage activity (preferably double-strand cleavage activity). The terms "first and second cleavage half-domains;" "+ and – cleavage half-domains" and "right and left cleavage half-domains" are used interchangeably to refer to pairs of cleavage half-domains that dimerize.

[0060] An "engineered cleavage half-domain" is a cleavage half-domain that has been modified so as to form obligate heterodimers with another cleavage half-domain (*e.g.,* another engineered cleavage half-domain). *See, also,* U.S. Patent Publication Nos. 2005/0064474, 20070218528 ,2008/0131962 and 2011/0201055, incorporated herein by reference in their entireties.

[0061] The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 10,000 nucleotides in length (or any integer value therebetween or thereabove), preferably between about 100 and 1,000 nucleotides in length (or any integer therebetween), more preferably between about 200 and 500 nucleotides in length.

[0062] "Chromatin" is the nucleoprotein structure comprising the cellular genome. Cellular chromatin comprises nucleic acid, primarily DNA, and protein, including histones and non-histone chromosomal proteins. The majority of eukaryotic cellular chromatin exists in the form of nucleosomes, wherein a nucleosome core comprises approximately 150 base pairs of DNA associated with an octamer comprising two each of histones H2A, H2B, H3 and H4; and linker DNA (of variable length depending on the organism) extends between nucleosome cores. A molecule of histone H1 is generally associated with the linker DNA. For the purposes of the present disclosure, the term "chromatin" is meant to encompass all types of cellular nucleoprotein, both prokaryotic and eukaryotic. Cellular chromatin includes both chromosomal and episomal chromatin.

[0063] A "chromosome," is a chromatin complex comprising all or a portion of the genome of a cell. The genome of a cell is often characterized by its karyotype, which is the collection of all the chromosomes that comprise the genome of the cell. The genome of a cell can comprise one or more chromosomes.

[0064] An "episome" is a replicating nucleic acid, nucleoprotein complex or other structure comprising a nucleic acid that is not part of the chromosomal karyotype of a cell. Examples of episomes include plasmids and certain viral genomes.

[0065] A "target site" or "target sequence" is a nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind, provided sufficient conditions for binding exist.

[0066] An "exogenous" molecule is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. "Normal presence in the cell" is determined with respect to the particular

developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a functioning version of a malfunctioning endogenous molecule or a malfunctioning version of a normally-functioning endogenous molecule.

[0067] An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and 5,422,251. Proteins include, but are not limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

[0068] An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, an exogenous protein or nucleic acid. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer. An exogeneous molecule can also be the same type of molecule as an endogenous molecule but derived from a different species than the cell is derived from. For example, a human nucleic acid sequence may be introduced into a cell line originally derived from a mouse or hamster.

[0069] By contrast, an "endogenous" molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, transcription factors and enzymes.

[0070] A "fusion" molecule is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion proteins (for example, a fusion between a ZFP or TALE DNA-binding domain and one or more activation domains) and fusion nucleic acids (for example, a nucleic acid encoding the fusion protein described *supra*). Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

[0071] Expression of a fusion protein in a cell can result from delivery of the fusion protein to the cell or by delivery of a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Trans-splicing, polypeptide cleavage and polypeptide ligation can also be involved in expression of a protein in a cell. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

[0072] A "multimerization domain", (also referred to as a "dimerization domain" or "protein interaction domain") is a domain incorporated at the amino, carboxy or amino and carboxy terminal regions of a ZFP TF or TALE TF. These domains allow for multimerization of multiple ZFP TF or TALE TF units such that larger tracts of trinucleotide repeat domains become preferentially bound by multimerized ZFP TFs or TALE TFs relative to shorter tracts with wild-type numbers of lengths. Examples of multimerization domains include leucine zippers. Multimerization domains may also be regulated by small molecules wherein the multimerization domain assumes a proper conformation to allow for interaction with another multimerization domain only in the presence of a small molecule or external

ligand. In this way, exogenous ligands can be used to regulate the activity of these domains.

[0073] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see *infra*), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0074] "Gene expression" refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[0075] "Modulation" of gene expression refers to a change in the activity of a gene. Modulation of expression can include, but is not limited to, gene activation and gene repression. Genome editing (*e.g.*, cleavage, alteration, inactivation, random mutation) can be used to modulate expression. Gene inactivation refers to any reduction in gene expression as compared to a cell that does not include a ZFP or TALE protein as described herein. Thus, gene inactivation may be partial or complete.

[0076] A "region of interest" is any region of cellular chromatin, such as, for example, a gene or a non-coding sequence within or adjacent to a gene, in which it is desirable to bind an exogenous molecule. Binding can be for the purposes of targeted DNA cleavage and/or targeted recombination. A region of interest can be present in a chromosome, an episome, an organellar genome (*e.g.*, mitochondrial, chloroplast), or an infecting viral genome, for example. A region of interest can be within the coding region of a gene, within transcribed non-coding regions such as, for example, leader sequences, trailer sequences or introns, or within non-transcribed regions, either

upstream or downstream of the coding region. A region of interest can be as small as a single nucleotide pair or up to 2,000 nucleotide pairs in length, or any integral value of nucleotide pairs.

[0077] "Eukaryotic" cells include, but are not limited to, fungal cells (such as yeast), plant cells, animal cells, mammalian cells and human cells (*e.g.*, T-cells).

[0078] The terms "operative linkage" and "operatively linked" (or "operably linked") are used interchangeably with reference to a juxtaposition of two or more components (such as sequence elements), in which the components are arranged such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. By way of illustration, a transcriptional regulatory sequence, such as a promoter, is operatively linked to a coding sequence if the transcriptional regulatory sequence controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. A transcriptional regulatory sequence is generally operatively linked in *cis* with a coding sequence, but need not be directly adjacent to it. For example, an enhancer is a transcriptional regulatory sequence that is operatively linked to a coding sequence, even though they are not contiguous.

[0079] With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a ZFP or TALE DNA-binding domain is fused to an activation domain, the ZFP or TALE DNA-binding domain and the activation domain are in operative linkage if, in the fusion polypeptide, the ZFP or TALE DNA-binding domain portion is able to bind its target site and/or its binding site, while the activation domain is able to upregulate gene expression. ZFPs fused to domains capable of regulating gene expression are collectively referred to as "ZFP-TFs" or "zinc finger transcription factors", while TALEs fused to domains capable of regulating gene expression are collectively referred to as "TALE-TFs" or "TALE transcription factors." When a fusion polypeptide in which a ZFP DNA-binding domain is fused to a cleavage domain (a "ZFN" or "zinc finger nuclease"), the ZFP DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site

and/or its binding site, while the cleavage domain is able to cleave DNA in the vicinity of the target site. When a fusion polypeptide in which a TALE DNA-binding domain is fused to a cleavage domain (a "TALEN" or "TALE nuclease"), the TALE DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the TALE DNA-binding domain portion is able to bind its target site and/or its binding site, while the cleavage domain is able to cleave DNA in the vicinity of the target site.

[0080] A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule, and/or can contain one ore more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (*e.g.*, coding function, ability to hybridize to another nucleic acid) are well-known in the art. Similarly, methods for determining protein function are well-known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. DNA cleavage can be assayed by gel electrophoresis. See Ausubel *et al., supra.* The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields *et al.* (1989) *Nature* **340:**245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

[0081] A "vector" is capable of transferring gene sequences to target cells. Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

[0082] A "reporter gene" or "reporter sequence" refers to any sequence that produces a protein product that is easily measured, preferably although not necessarily in a routine assay. Suitable reporter genes include, but are not limited to, sequences encoding proteins that mediate antibiotic resistance (e.g., ampicillin resistance, neomycin resistance, G418 resistance, puromycin resistance), sequences encoding

colored or fluorescent or luminescent proteins (e.g., green fluorescent protein, enhanced green fluorescent protein, red fluorescent protein, luciferase), and proteins which mediate enhanced cell growth and/or gene amplification (e.g., dihydrofolate reductase). Epitope tags include, for example, one or more copies of FLAG, His, myc, Tap, HA or any detectable amino acid sequence. "Expression tags" include sequences that encode reporters that may be operably linked to a desired gene sequence in order to monitor expression of the gene of interest.

DNA-binding domains

[0083] Described herein are compositions comprising a DNA-binding domain that specifically bind to a target sequence in any gene comprising a trinucleotide repeat, including, but not limited to, Htt. Any DNA-binding domain can be used in the compositions and methods disclosed herein.

[0084] In certain embodiments, the DNA binding domain comprises a zinc finger protein. Preferably, the zinc finger protein is non-naturally occurring in that it is engineered to bind to a target site of choice. *See*, for example, Beerli *et al.* (2002) *Nature Biotechnol.* **20:**135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.* **70:**313-340; Isalan *et al.* (2001) *Nature Biotechnol.* **19:**656-660; Segal *et al.* (2001) *Curr. Opin. Biotechnol.* **12:**632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* **10:**411-416; U.S. Patent Nos. 6,453,242; 6,534,261; 6,599,692; 6,503,717; 6,689,558; 7,030,215; 6,794,136; 7,067,317; 7,262,054; 7,070,934; 7,361,635; 7,253,273; and U.S. Patent Publication Nos. 2005/0064474; 2007/0218528; 2005/0267061, all incorporated herein by reference in their entireties.

[0085] An engineered zinc finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, for example, co-owned U.S. Patents 6,453,242 and 6,534,261, incorporated by reference herein in their entireties.

[0086] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0087] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0088] Selection of target sites; ZFPs and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Nos. 6,140,0815; 789,538; 6,453,242; 6,534,261; 5,925,523; 6,007,988; 6,013,453; 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197; WO 02/099084; WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496.

[0089] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein.

[0090] In certain embodiments, the DNA binding domain is an engineered zinc finger protein that binds (in a sequence-specific manner) to a target site in a Htt gene and modulates expression of Htt. The ZFPs can bind selectively to either a mutant Htt allele or a wild-type Htt sequence. Htt target sites typically include at least one zinc finger but can include a plurality of zinc fingers (*e.g.*, 2, 3, 4, 5, 6 or more

fingers). Usually, the ZFPs include at least three fingers. Certain of the ZFPs include four, five or six fingers, while some ZFPs include 8, 9, 10, 11 or 12 fingers. The ZFPs that include three fingers typically recognize a target site that includes 9 or 10 nucleotides; ZFPs that include four fingers typically recognize a target site that includes 12 to 14 nucleotides; while ZFPs having six fingers can recognize target sites that include 18 to 21 nucleotides. The ZFPs can also be fusion proteins that include one or more regulatory domains, which domains can be transcriptional activation or repression domains. In some embodiments, the fusion protein comprises two ZFP DNA binding domains linked together. These zinc finger proteins can thus comprise 8, 9, 10, 11, 12 or more fingers. In some embodiments, the two DNA binding domains are linked via an extendable flexible linker such that one DNA binding domain comprises 4, 5, or 6 zinc fingers and the second DNA binding domain comprises an additional 4, 5, or 5 zinc fingers. In some embodiments, the linker is a standard inter-finger linker such that the finger array comprises one DNA binding domain comprising 8, 9, 10, 11 or 12 or more fingers. In other embodiments, the linker is an atypical linker such as a flexible linker. The DNA binding domains are fused to at least one regulatory domain and can be thought of as a 'ZFP-ZFP-TF'architecture. Specific examples of these embodiments can be referred to as "ZFP-ZFP-KOX" which comprises two DNA binding domains linked with a flexible linker and fused to a KOX repressor and "ZFP-KOX-ZFP-KOX" where two ZFP-KOX fusion proteins are fused together via a linker.

[0091] Alternatively, the DNA-binding domain may be derived from a nuclease. For example, the recognition sequences of homing endonucleases and meganucleases such as I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce*, I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III are known. *See* also U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort *et al.* (1997) *Nucleic Acids Res.* **25:**3379–3388; Dujon *et al.* (1989) *Gene* **82:**115–118; Perler *et al.* (1994) Nucleic Acids Res. 22, 1125–1127; Jasin (1996) *Trends Genet.* **12:**224–228; Gimble *et al.* (1996) *J. Mol. Biol.* **263:**163–180; Argast *et al.* (1998) *J. Mol. Biol.* **280:**345–353 and the New England Biolabs catalogue. In addition, the DNA-binding specificity of homing endonucleases and meganucleases can be engineered to bind non-natural target sites. *See*, for example, Chevalier *et al.* (2002) *Molec. Cell* **10:**895-905; Epinat *et al.* (2003) *Nucleic Acids Res.* **31:**2952-2962; Ashworth *et al.* (2006)

Nature **441**:656-659; Paques *et al.* (2007) *Current Gene Therapy* **7**:49-66; U.S. Patent Publication No. 20070117128.

[0092] "Two handed" zinc finger proteins are those proteins in which two clusters of zinc finger DNA binding domains are separated by intervening amino acids so that the two zinc finger domains bind to two discontinuous target sites. An example of a two handed type of zinc finger binding protein is SIP1, where a cluster of four zinc fingers is located at the amino terminus of the protein and a cluster of three fingers is located at the carboxyl terminus (*see* Remacle et al, (1999) *EMBO Journal* 18 (18): 5073-5084). Each cluster of zinc fingers in these proteins is able to bind to a unique target sequence and the spacing between the two target sequences can comprise many nucleotides. Two-handed ZFPs may include a functional domain, for example fused to one or both of the ZFPs. Thus, it will be apparent that the functional domain may be attached to the exterior of one or both ZFPs (see, Figure 1C) or may be positioned between the ZFPs (attached to both ZFPs) (see, Figure 4).

[0093] Specific examples of Htt-targeted ZFPs are disclosed in Tables 1A and 1B. The first column in this table is an internal reference name (number) for a ZFP and corresponds to the same name in column 1 of Tables 2A and 2B. "F" refers to the finger and the number following "F" refers which zinc finger (*e.g.*, "F1" refers to finger 1).

Table 1A: Htt-targeted zinc finger proteins

SBS #	Design					
	F1	F2	F3	F4	F5	F6
18856	RSDDLSR (SEQ ID NO: 1)	RNDNRTK (SEQ ID NO:2)	RSDDLTR (SEQ ID NO:3)	RSDDRKT (SEQ ID NO:4)	RSADLTR (SEQ ID NO:5)	QSSDLRR (SEQ ID NO:6)
25920	RSAALSR (SEQ ID NO: 58)	RSDALAR (SEQ ID NO: 59)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSSDLRR (SEQ ID NO: 6)	NA
25921	WRSCRSA (SEQ ID NO: 62)	DRSNLSR (SEQ ID NO: 9)	QRTHLTQ (SEQ ID NO: 53)	RSAHLSR (SEQ ID NO: 46)	TSGHLSR (SEQ ID NO: 43)	NA
25923	RSDDLSR (SEQ ID NO: 1)	RNDNRTK (SEQ ID NO: 2)	WRSCRSA (SEQ ID NO: 62)	RSDNLAR (SEQ ID NO: 7)	QSGHLSR (SEQ ID NO: 41)	NA
25922	RSAALSR (SEQ ID NO: 58)	RSDALAR (SEQ ID NO: 59)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSSDLSR (SEQ ID NO: 31)	DRSHLAR (SEQ ID NO: 13)

Table 1B: Human and Mouse Htt-targeted zinc finger proteins

SBS #	Design					
	F1	F2	F3	F4	F5	F6
32468	RSDNLAR (SEQ ID NO: 7)	WRGDRVK (SEQ ID NO: 8)	DRSNLSR (SEQ ID NO: 9)	TSGSLTR (SEQ ID NO: 10)	ERGTLAR (SEQ ID NO: 11)	RSDDRKT (SEQ ID NO: 4)
32501	RSDALSR (SEQ ID NO: 12)	DRSHLAR (SEQ ID NO: 13)	RSDHLSR (SEQ ID NO: 14)	QSSDLTR (SEQ ID NO: 15)	TSGNLTR (SEQ ID NO: 16)	DRSHLAR (SEQ ID NO: 13)
31809	RSDDLSR (SEQ ID NO: 1)	RNDNRTK (SEQ ID NO: 2)	RSDDLTR (SEQ ID NO: 3)	RSDDRKT (SEQ ID NO: 4)	RSDDLTR (SEQ ID NO: 3)	QSSDLRR (SEQ ID NO: 6)
32528	QSGHLQR (SEQ ID NO: 17)	TSGNLTR (SEQ ID NO: 16)	QSGDLTR (SEQ ID NO: 18)	DRSHLAR (SEQ ID NO: 13)	RSDVLST (SEQ ID NO: 19)	VRSRLRR (SEQ ID NO: 20)
30580	RSDNLAR (SEQ ID NO: 7)	WRGDRVK (SEQ ID NO: 8)	DRSDLSR (SEQ ID NO: 22)	RSDALAR (SEQ ID NO: 59)	ERGTLAR (SEQ ID NO: 11)	RSDDRKT (SEQ ID NO: 4)
30929	DRSTLRQ (SEQ ID NO: 21)	DRSDLSR (SEQ ID NO: 22)	QSSTRAR (SEQ ID NO: 23)	RSDTLSE (SEQ ID NO: 24)	HRRSRWG (SEQ ID NO: 25)	NA
32538	DRSDLSR (SEQ ID NO: 22)	RRDTLRS (SEQ ID NO: 26)	RSDHLST (SEQ ID NO: 27)	QSAHRIT (SEQ ID NO: 28)	QSGDLTR (SEQ ID NO: 18)	DRSHLAR (SEQ ID NO: 13)
32567	RSDHLSE (SEQ ID NO: 29)	QNAHRKT (SEQ ID NO: 30)	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)
29627	DRSNLSR (SEQ ID NO: 9)	LRQDLKR (SEQ ID NO: 33)	DRSHLTR (SEQ ID NO: 34)	DRSNLTR (SEQ ID NO: 35)	RSDHLST (SEQ ID NO: 27)	QSAHRIT (SEQ ID NO: 28)
29628	TSGNLTR (SEQ ID NO: 16)	LKQMLAV (SEQ ID NO: 36)	RSDSLSA (SEQ ID NO: 37)	DRSDLSR (SEQ ID NO: 22)	RSDALST (SEQ ID NO: 38)	DRSTRTK (SEQ ID NO: 39)
29631	QSSDLSR (SEQ ID NO: 31)	DRSALAR (SEQ ID NO: 40)	QSSDLSR (SEQ ID NO: 31)	QSGHLSR (SEQ ID NO: 41)	RSDVLSE (SEQ ID NO: 42)	TSGHLSR (SEQ ID NO: 43)
29632	RSDTLSE (SEQ ID NO: 24)	KLCNRKC (SEQ ID NO: 44)	TSGNLTR (SEQ ID NO: 16)	HRTSLTD (SEQ ID NO: 45)	RSAHLSR (SEQ ID NO: 46)	QSGNLAR (SEQ ID NO: 47)
29637	DRSNLSR (SEQ ID NO: 9)	QSGNLAR (SEQ ID NO: 47)	DRSNLSR (SEQ ID NO: 9)	LKHHLTD (SEQ ID NO: 48)	QSGDLTR (SEQ ID NO: 18)	YRWLRNN (SEQ ID NO: 49)
29638	RSDHLSQ	RSAVRKN	QSSDLSR	QSGDLTR	WSTSLRA	NA

	(SEQ ID NO: 50)	(SEQ ID NO: 51)	(SEQ ID NO: 31)	(SEQ ID NO: 18)	(SEQ ID NO: 52)	
25917	DRSNLSR (SEQ ID NO: 9)	QRTHLTQ (SEQ ID NO: 53)	RSSHLSR (SEQ ID NO: 54)	TSGSLSR (SEQ ID NO: 55)	TRQNRDT (SEQ ID NO: 56)	NA
25916	DQSTLRN (SEQ ID NO: 57)	RSAALSR (SEQ ID NO: 58)	RSDALAR (SEQ ID NO: 59)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	NA
33074	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSGDLTR (SEQ ID NO: 18)	QSGDLTR (SEQ ID NO: 18)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)
33080	QSGDLTR (SEQ ID NO: 18)	QSGDLTR (SEQ ID NO: 18)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSGDLTR (SEQ ID NO: 18)	QSGDLTR (SEQ ID NO: 18)
33084	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)	RSDTLSE (SEQ ID NO: 24)	RRWTLVG (SEQ ID NO: 64)	NA	NA
33088	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)	RSAVLSE (SEQ ID NO: 148)	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)	NA
30643	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)
30648	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)
30645	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	QSGDLTR (SEQ ID NO: 18)	NA
30640	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	QSGDLTR (SEQ ID NO: 18)	NA
30657	RSDTLSE (SEQ ID NO: 24)	RRWTLVG (SEQ ID NO: 64)	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)
30642	QSGDLTR (SEQ ID NO: 18)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	NA
30646	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	QSGDLTR (SEQ ID NO: 18)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	NA
32220	RSDVLSE (SEQ ID NO: 42)	QSSDLSR (SEQ ID NO: 31)	HRSTRNR (SEQ ID NO: 32)	NA	NA	NA

32210	QSGDLTR (SEQ ID NO: 18)	QSSDLSR (SEQ ID NO: 31)	QWSTRKR (SEQ ID NO: 63)	NA	NA	NA
32215	RSDNLRE (SEQ ID NO: 65)	RSDNLSE (SEQ ID NO: 60)	KRCNLRC (SEQ ID NO: 61)	NA	NA	NA
30658	QSSDLSR (SEQ ID NO:31)	HRSTRNR (SEQ ID NO:32)	QSSDLSR (SEQ ID NO:31)	HRSTRNR (SEQ ID NO:32)	QSSDLSR (SEQ ID NO:31)	NA
32218	QSSDLSR (SEQ ID NO: 31)	QSSDLSR (SEQ ID NO: 31)	NA	NA	NA	NA
32427	ERGTLAR (SEQ ID NO:11)	TSGSLTR (SEQ ID NO:10)	RSDNLAR (SEQ ID NO:7)	DPSNRVG (SEQ ID NO:78)	RSDDLSK (SEQ ID NO:149)	DNSNRIK (SEQ ID NO:150)
32653	RSDHLSE (SEQ ID NO: 29)	QSGHLSR (SEQ ID NO: 41)	RSDDLTR (SEQ ID NO: 3)	YRWLLRS (SEQ ID NO: 66)	QSSDLSR (SEQ ID NO: 31)	RKDALVA (SEQ ID NO: 67)
32677	QSGDLTR (SEQ ID NO: 18)	RRADLSR (SEQ ID NO: 68)	DRSHLTR (SEQ ID NO: 34)	DRSHLAR (SEQ ID NO: 13)	DRSNLSR (SEQ ID NO: 9)	LAQPRNK (SEQ ID NO: 69)
33560	ERGTLAR (SEQ ID NO:11)	QSGSLTR (SEQ ID NO:84)	RSDNLAR (SEQ ID NO:7)	DDSHRKD (SEQ ID NO:151)	RSDDLSK (SEQ ID NO:149)	DNSNRIK (SEQ ID NO:150)
33583	DRSNLSR (SEQ ID NO:9)	HKQHRDA (SEQ ID NO:76)	DRSDLSR (SEQ ID NO:22)	RRTDLRR (SEQ ID NO:77)	RSANLAR (SEQ ID NO:73)	DRSHLAR (SEQ ID NO:13)
32685	RSDHLSA (SEQ ID NO: 70)	RSADRTR (SEQ ID NO: 71)	RSDVLSE (SEQ ID NO: 42)	TSGHLSR (SEQ ID NO: 43)	RSDDLTR (SEQ ID NO: 3)	TSSDRKK (SEQ ID NO: 72)
32422	RSANLAR (SEQ ID NO: 73)	RSDDLTR (SEQ ID NO: 3)	RSDTLSE (SEQ ID NO: 24)	HHSARRC (SEQ ID NO: 74)	ERGTLAR (SEQ ID NO: 11)	DRSNLTR (SEQ ID NO: 35)
32428	RSDVLST (SEQ ID NO: 19)	DNSSRTR (SEQ ID NO: 75)	DRSNLSR (SEQ ID NO: 9)	HKQHRDA (SEQ ID NO: 76)	DRSDLSR (SEQ ID NO: 22)	RRTDLRR (SEQ ID NO: 77)
32430	RSDVLST (SEQ ID NO: 19)	VRSRLRR (SEQ ID NO: 20)	ERGTLAR (SEQ ID NO: 11)	TSGSLTR (SEQ ID NO: 10)	RSDNLAR (SEQ ID NO: 7)	DPSNRVG (SEQ ID NO: 78)
32432	RSDVLST (SEQ ID NO: 19)	VRSRLRR (SEQ ID NO: 20)	ERGTLAR (SEQ ID NO: 11)	TSGSLTR (SEQ ID NO: 10)	RSDHLSA (SEQ ID NO: 70)	RSADLSR (SEQ ID NO: 79)
32714	RSDVLST (SEQ ID NO: 19)	DNSSRTR (SEQ ID NO: 75)	ERGTLAR (SEQ ID NO: 11)	QSGNLAR (SEQ ID NO: 47)	DRSHLTR (SEQ ID NO: 34)	RNDDRKK (SEQ ID NO: 80)
32733	DRSNLSR (SEQ ID NO: 9)	QKVTLAA (SEQ ID NO: 81)	RSAHLSR (SEQ ID NO: 46)	TSGNLTR (SEQ ID NO: 16)	DRSDLSR (SEQ ID NO: 22)	RRSTLRS (SEQ ID NO: 82)

30901	DRSALSR (SEQ ID NO: 83)	QSGSLTR (SEQ ID NO: 84)	QSSDLSR (SEQ ID NO: 31)	LKWNLRT (SEQ ID NO: 85)	RSDNLAR (SEQ ID NO: 7)	LKWDRQT (SEQ ID NO: 86)
31952	QSGALAR (SEQ ID NO:147)	RSDDLTR (SEQ ID NO:3)	DRSALSR (SEQ ID NO:83)	RSDHLTQ (SEQ ID NO:152)	QSGDLTR (SEQ ID NO:18)	WSTSLRA (SEQ ID NO:52)
31921	RSDSLLR (SEQ ID NO:153)	RSDDLTR (SEQ ID NO:3)	QSGDLTR (SEQ ID NO:18)	RRDWLPQ (SEQ ID NO:154)	DRSNLSR (SEQ ID NO:9)	RSDDRKT (SEQ ID NO:4)
30906	DRSHLSR (SEQ ID NO: 87)	TSGNLTR (SEQ ID NO: 16)	QSGDLTR (SEQ ID NO: 18)	DRSHLAR (SEQ ID NO: 13)	RSDVLST (SEQ ID NO: 19)	VRSRLRR (SEQ ID NO: 20)

[0094] The sequence and location for the target sites of these proteins are disclosed in Tables 2A and 2B. Tables 2A and 2B show the target sequences for the indicated zinc finger proteins. Nucleotides in the target site that are contacted by the ZFP recognition helices are indicated in uppercase letters; non-contacted nucleotides indicated in lowercase.

Table 2A: Target sites on human and mouse Htt

SBS #	Target Site
18856	AcGCTGCGCCGGCGGAGGCGgggccgcg (SEQ ID NO:88)
25920	gcGCTCAGCAGGTGGTGaccttgtggac_(SEQ ID NO:103)
25921	atGGTGGGAGAGACTGTgaggcggcagc_(SEQ ID NO:104)
25923	tgGGAGAGacTGTGAGGCGgcagctggg(SEQ ID NO:105)
25922	atGGCGCTCAGCAGGTGGTGaccttgtg_(SEQ ID NO:106)

Table 2B: Target sites on human and mouse Htt

SBS #	Target Site
32468	agCCGGCCGTGGACTCTGAGccgaggtg_(SEQ ID NO:89)
32427	cgCACTCGcCGCGAGgGTTGCCgggacg_(SEQ ID NO:155)
32501	gtGGCGATGCGGGGGGCGTGgtgaggta_(SEQ ID NO:90)
31809	acGCTGCGCCGGCGGAGGCGgggccgcg_(SEQ ID NO:88)
32528	ccGGGACGGGTCCAaGATGGAcggccgc_(SEQ ID NO:91)
30580	agCCGGCCGTGGACTCTGAGccgaggtg_(SEQ ID NO:89)
30929	ccGTCCCGGCAGCCCCCacggcgccttg_(SEQ ID NO:92)
30658	ctGCTGCTGCTGCTGCTgctggaaggac_(SEQ ID NO:108)
32538	cgGGTCCAAGATGGACGGCCgctcaggt_(SEQ ID NO:93)
32567	ctGCTGCTGCTGCTGGAAGGacttgagg_(SEQ ID NO:94)
29627	tcAGATGGGACGGCGCTGACctggctgg_(SEQ ID NO:95)
29628	ctGCCATGGACCTGAATGATgggaccca_(SEQ ID NO:96)
29631	gtGGTCTGGGAGCTGTCGCTgatgggcg_(SEQ ID NO:97)
29632	ccGAAGGGCCTGATtCAGCTGttacccc_(SEQ ID NO:98)
29637	aaCTTGCAAGTAACaGAAGACtcatcct_(SEQ ID NO:99)

29638	ctTGTACAGCTGTGAGGgtgagcataat_(SEQ ID NO:100)
25917	gcCATGGTGGGAGAGACtgtgaggcggc_(SEQ ID NO:101)
25916	ctCAGCAGGTGGTGACCttgtggacatt_(SEQ ID NO:102)
33074	agCAGCAGcaGCAGCAgCAGCAGcagca_(SEQ ID NO:157)
33080	caGCAGCAgCAGCAGcaGCAGCAgcagc_(SEQ ID NO:107)
33084	tgCTGCTGctGCTGCTgctgctggaagg_(SEQ ID NO:109)
33088	ctGCTGCTgCTGctGCTGCTgctggaag_(SEQ ID NO:158)
30643	caGCAGCAGCAGCAgCAGCAGcagcagc_(SEQ ID NO:107)
30648	agCAGCAGCAGCAGCAGCAGcagcagca_(SEQ ID NO:157)
30645	caGCAGCAGCAgCAGCAGcagcagcagc_(SEQ ID NO:107)
30640	caGCAGCAGCAGCAGCAgcagcagcagc_(SEQ ID NO:107)
30657	ctGCTGCTGCTGCTgCTGCTGgaaggac_(SEQ ID NO:108)
30642	caGCAGCAGCAGCAGCAgcagcagcagc_(SEQ ID NO:107)
30646	caGCAGCAGCAgCAGCAGcagcagcagc_(SEQ ID NO:107)
32220	ctGCTGCTgCTGctgctgctgctggaagg_(SEQ ID NO:109)
32210	caGCAGCAGCAgcagcagcagcagcagc_(SEQ ID NO:107)
32215	agCAGCAGCAGcagcagcagcagcagca(SEQ ID NO:110)
32218	tGCTGCTgctgctgctgctgctggaagg(SEQ ID NO:111)
32653	ggCTGGCTTTTGCGGGAAGGggcggggc (SEQ ID NO:112)
32677	gaATTGACaGGCGGAtGCGTCGtcctct_(SEQ ID NO:113)
33560	cgCACTCGcCGCGAGgGTTGCCgggacg_(SEQ ID NO:155)
33583	gcGGCGAGtGCGTCCCGTGACgtcatgc_(SEQ ID NO:158)
32685	atTCTGCGGGTCTGGCGTGGcctcgtct_(SEQ ID NO:114)
32422	gtGACGTCATGCCGGCGGAGacgaggcc_(SEQ ID NO:115)
32428	gtGCGTCCCGTGACGTCATGccggcgga_(SEQ ID NO:116)
32430	gcCGCGAGgGTTGCCGGGACGggcccaa_(SEQ ID NO:117)
32432	ccGCGAGGGTTGCCGGGACGggcccaag_(SEQ ID NO:118)
32714	caTCGGGCagGAAGCCGTCATGgcaacc_(SEQ ID NO:119)
32733	tcCTGCCCGATGGGACAGACcctgaaga_(SEQ ID NO:120)
30901	gtACTGAGcAATGCTGTAGTCagcaatc_(SEQ ID NO:121)
31952	ccTGTCCAgAGGGTCGCGGTAcctccct_(SEQ ID NO:159)
31921	tgCCGGACCTGGCAGCGGCGgtggtggc_(SEQ ID NO:160)
30906	ccGGGACGGGTCCAaGATGGAcggccgc_(SEQ ID NO:91)

[0095] In certain embodiments, the DNA-binding domain comprises a naturally occurring or engineered (non-naturally occurring) TAL effector (TALE) DNA binding domain. *See, e.g.,* U.S. Patent Publication No. 20110301073, incorporated by reference in its entirety herein. The plant pathogenic bacteria of the genus *Xanthomonas* are known to cause many diseases in important crop plants. Pathogenicity of *Xanthomonas* depends on a conserved type III secretion (T3S) system which injects more than 25 different effector proteins into the plant cell. Among these injected proteins are transcription activator-like effectors (TALE) which mimic plant transcriptional activators and manipulate the plant transcriptome (see Kay *et al* (2007) *Science* 318:648-651). These proteins contain a DNA binding domain

and a transcriptional activation domain. One of the most well characterized TALEs is AvrBs3 from *Xanthomonas campestgris* pv. *Vesicatoria* (see Bonas *et al* (1989) *Mol Gen Genet* 218: 127-136 and WO2010079430). TALEs contain a centralized domain of tandem repeats, each repeat containing approximately 34 amino acids, which are key to the DNA binding specificity of these proteins. In addition, they contain a nuclear localization sequence and an acidic transcriptional activation domain (for a review see Schornack S, *et al* (2006) *J Plant Physiol* 163(3): 256-272). In addition, in the phytopathogenic bacteria *Ralstonia solanacearum* two genes, designated brg11 and hpx17 have been found that are homologous to the AvrBs3 family of *Xanthomonas* in the *R. solanacearum* biovar 1 strain GMI1000 and in the biovar 4 strain RS1000 (See Heuer *et al* (2007) *Appl and Envir Micro* 73(13): 4379-4384). These genes are 98.9% identical in nucleotide sequence to each other but differ by a deletion of 1,575 bp in the repeat domain of hpx17. However, both gene products have less than 40% sequence identity with AvrBs3 family proteins of *Xanthomonas*.

[0096] Specificity of these TALEs depends on the sequences found in the tandem repeats. The repeated sequence comprises approximately 102 bp and the repeats are typically 91-100% homologous with each other (Bonas *et al, ibid*). Polymorphism of the repeats is usually located at positions 12 and 13 and there appears to be a one-to-one correspondence between the identity of the hypervariable diresidues at positions 12 and 13 with the identity of the contiguous nucleotides in the TALE's target sequence (see Moscou and Bogdanove, (2009) *Science* 326:1501 and Boch *et al* (2009) *Science* 326:1509-1512). Experimentally, the code for DNA recognition of these TALEs has been determined such that an HD sequence at positions 12 and 13 leads to a binding to cytosine (C), NG binds to T, NI to A, C, G or T, NN binds to A or G, and IG binds to T. These DNA binding repeats have been assembled into proteins with new combinations and numbers of repeats, to make artificial transcription factors that are able to interact with new sequences and activate the expression of a non-endogenous reporter gene in plant cells (Boch *et al, ibid*). Engineered TAL proteins have been linked to a *Fok*I cleavage half domain to yield a TAL effector domain nuclease fusion (TALEN) exhibiting activity in a yeast reporter assay (plasmid based target). Christian *et al* ((2010)< *Genetics* epub 10.1534/genetics.110.120717). *See, also,* U.S. Patent Publication No. 20110301073, incorporated by reference in its entirety.

[0097] Specific examples of designed dimerization domains to be used with ZFPs or TALE proteins are listed in Table 3. The amino acid sequences of two types of domain, coiled-coil (CC) and dimerizing zinc finger (DZ) are listed.

Table 3: Designed dimerization domains

Design name	Amino acid sequence
DZ1	TKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEH (SEQ ID NO:122)
	TKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEH(SEQ ID NO:122)
DZ2	FKCEHCRILFLDHVMFTIHMGCHGFRDPFKCNMCGEKCDGPVGLFVHMARNAH(SEQ ID NO:123)
	TKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEH(SEQ ID NO:122)
DZ3	FKCEHCRILFLDHVMFTIHMGCHGFRDPFKCNMCGEKCDGPVGLFVHMARNAH(SEQ ID NO:123)
	HHCQHCDMYFADNILYTIHMGCHGYENPFECNICGYHSQDRYEFSSHIVRGEH(SEQ ID NO:124)
DZ4	HHCQHCDMYFADNILYTIHMGCHSCDDVFKCNMCGEKCDGPVGLFVHMARNAHGEKPTKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEH(SEQ ID NO:125)
	FKCEHCRILFLDHVMFTIHMGCHGFRDPFKCNMCGEKCDGPVGLFVHMARNAHGEKPFYCEHCEITFRDVVMYSLHKGYHGFRDPFECNICGYHSQDRYEFSSHIVRGEH(SEQ ID NO:126)
CC1	AQLEKELQALEKKLAQLEWENQALEKELAQ(SEQ ID NO:127)
	AQLKKKLQANKKELAQLKWKLQALKKKLAQ(SEQ ID NO:128)
CC2	EQLEKKLQALEKKLAQLEWKNQALEKKLAQ(SEQ ID NO:129)
	ALKKELQANKKELAQLKWELQALKKELAQ(SEQ ID NO:130)
CC3	EQLEKKLQALEKKLAQLEWKNQALEK(SEQ ID NO:131)
	ELQANKKELAQLKWELQALKKELAQ(SEQ ID NO:132)
CC4	EQLEKKLQALEKKLAQLEWKNQA(SEQ ID NO:133)
	QANKKELAQLKWELQALKKELAQ(SEQ ID NO:134)
CC5	EQLEKKLQALEKKLAQLEWKNQALEKKLAQ(SEQ ID NO:129)

	ALKKELQANKKELAQLKWELQALKKELAQ(SEQ ID NO:130)
CC6	EQLEKKLQALEKKLAQLEWKNQALEKKLAQ(SEQ ID NO:129)
	ALKKELQANKKELAQLKWELQALKKELAQ(SEQ ID NO:130)
CC7	EQLEKKLQALEKKLAQLEWKNQALEKKLAQ(SEQ ID NO:129)
	ALKKELQANKKELAQLKWELQALKKELAQ(SEQ ID NO:130)

Fusion proteins

[0098] Fusion proteins comprising DNA-binding proteins (*e.g.*, ZFPs or TALEs) as described herein and a heterologous regulatory (functional) domain (or functional fragment thereof) are also provided. Common domains include, *e.g.*, transcription factor domains (activators, repressors, co-activators, co-repressors), silencers, oncogenes (*e.g.*, myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members etc.); DNA repair enzymes and their associated factors and modifiers; DNA rearrangement enzymes and their associated factors and modifiers; chromatin associated proteins and their modifiers (*e.g.* kinases, acetylases and deacetylases); and DNA modifying enzymes (*e.g.*, methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases, endonucleases) and their associated factors and modifiers. U.S. Patent Application Publication Nos. 20050064474; 20060188987 and 2007/0218528 for details regarding fusions of DNA-binding domains and nuclease cleavage domains, incorporated by reference in their entireties herein

[0099] Suitable domains for achieving activation include the HSV VP16 activation domain (*see*, *e.g.*, Hagmann *et al.*, *J. Virol.* 71, 5952-5962 (1997)) nuclear hormone receptors (*see, e.g.*, Torchia *et al.*, *Curr. Opin. Cell. Biol.* 10:373-383 (1998)); the p65 subunit of nuclear factor kappa B (Bitko & Barik, *J. Virol.* 72:5610-5618 (1998) and Doyle & Hunt, *Neuroreport* 8:2937-2942 (1997)); Liu et al., *Cancer Gene Ther.* 5:3-28 (1998)), or artificial chimeric functional domains such as VP64 (Beerli *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* 95:14623-33), and degron (Molinari *et al.*, (1999) *EMBO J.* 18, 6439-6447). Additional exemplary activation domains include, Oct 1, Oct-2A, Sp1, AP-2, and CTF1 (Seipel *et al.*, *EMBO J.* 11, 4961-4968 (1992) as well as p300, CBP, PCAF, SRC1 PvALF, AtHD2A and ERF-2. *See*, for example, Robyr *et al.* (2000) *Mol. Endocrinol.* 14:329-347; Collingwood *et al.* (1999) *J. Mol. Endocrinol.* 23:255-275; Leo *et al.* (2000) *Gene* 245:1-11; Manteuffel-

Cymborowska (1999) *Acta Biochim. Pol.* 46:77-89; McKenna *et al.* (1999) *J. Steroid Biochem. Mol. Biol.* 69:3-12; Malik *et al.* (2000) *Trends Biochem. Sci.* 25:277-283; and Lemon *et al.* (1999) *Curr. Opin. Genet. Dev.* 9:499-504. Additional exemplary activation domains include, but are not limited to, OsGAI, HALF-1, C1, AP1, ARF-5,-6,-7, and -8, CPRF1, CPRF4, MYC-RP/GP, and TRAB1. *See*, for example, Ogawa *et al.* (2000) *Gene* 245:21-29; Okanami *et al.* (1996) *Genes Cells* 1:87-99; Goff *et al.* (1991) *Genes Dev.* 5:298-309; Cho *et al.* (1999) *Plant Mol. Biol.* 40:419-429; Ulmason *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:5844-5849; Sprenger-Haussels *et al.* (2000) *Plant J.* 22:1-8; Gong *et al.* (1999) *Plant Mol. Biol.* 41:33-44; and Hobo *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:15,348-15,353.

[0100] It will be clear to those of skill in the art that, in the formation of a fusion protein (or a nucleic acid encoding same) between a DNA-binding domain and a functional domain, either an activation domain or a molecule that interacts with an activation domain is suitable as a functional domain. Essentially any molecule capable of recruiting an activating complex and/or activating activity (such as, for example, histone acetylation) to the target gene is useful as an activating domain of a fusion protein. Insulator domains, localization domains, and chromatin remodeling proteins such as ISWI-containing domains and/or methyl binding domain proteins suitable for use as functional domains in fusion molecules are described, for example, in co-owned U.S. Patent Applications 2002/0115215 and 2003/0082552 and in co-owned WO 02/44376.

[0101] Exemplary repression domains include, but are not limited to, KRAB A/B, KOX, TGF-beta-inducible early gene (TIEG), v-erbA, SID, MBD2, MBD3, members of the DNMT family (*e.g.*, DNMT1, DNMT3A, DNMT3B), Rb, and MeCP2. *See*, for example, Bird *et al.* (1999) *Cell* 99:451-454; Tyler *et al.* (1999) *Cell* 99:443-446; Knoepfler *et al.* (1999) *Cell* 99:447-450; and Robertson *et al.* (2000) *Nature Genet.* 25:338-342. Additional exemplary repression domains include, but are not limited to, ROM2 and AtHD2A. *See*, for example, Chem *et al.* (1996) *Plant Cell* 8:305-321; and Wu *et al.* (2000) *Plant J.* 22:19-27.

[0102] Fusion molecules are constructed by methods of cloning and biochemical conjugation that are well known to those of skill in the art. Fusion molecules comprise a DNA-binding domain and a functional domain (*e.g.*, a transcriptional activation or repression domain). Fusion molecules also optionally

comprise nuclear localization signals (such as, for example, that from the SV40 medium T-antigen) and epitope tags (such as, for example, FLAG and hemagglutinin). Fusion proteins (and nucleic acids encoding them) are designed such that the translational reading frame is preserved among the components of the fusion.

[0103] Fusions between a polypeptide component of a functional domain (or a functional fragment thereof) on the one hand, and a non-protein DNA-binding domain (*e.g.*, antibiotic, intercalator, minor groove binder, nucleic acid) on the other, are constructed by methods of biochemical conjugation known to those of skill in the art. *See*, for example, the Pierce Chemical Company (Rockford, IL) Catalogue. Methods and compositions for making fusions between a minor groove binder and a polypeptide have been described. Mapp *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:3930-3935.

[0104] In certain embodiments, the target site bound by the DNA binding domain is present in an accessible region of cellular chromatin. Accessible regions can be determined as described, for example, in co-owned International Publication WO 01/83732. If the target site is not present in an accessible region of cellular chromatin, one or more accessible regions can be generated as described in co-owned WO 01/83793. In additional embodiments, the DNA-binding domain of a fusion molecule is capable of binding to cellular chromatin regardless of whether its target site is in an accessible region or not. For example, such DNA-binding domains are capable of binding to linker DNA and/or nucleosomal DNA. Examples of this type of "pioneer" DNA binding domain are found in certain steroid receptor and in hepatocyte nuclear factor 3 (HNF3). Cordingley *et al.* (1987) *Cell* 48:261-270; Pina *et al.* (1990) *Cell* 60:719-731; and Cirillo *et al.* (1998) *EMBO J.* 17:244-254.

[0105] The fusion molecule may be formulated with a pharmaceutically acceptable carrier, as is known to those of skill in the art. *See*, for example, Remington's Pharmaceutical Sciences, 17th ed., 1985; and co-owned WO 00/42219.

[0106] The functional component/domain of a fusion molecule can be selected from any of a variety of different components capable of influencing transcription of a gene once the fusion molecule binds to a target sequence via its DNA binding domain. Hence, the functional component can include, but is not limited to, various transcription factor domains, such as activators, repressors, co-activators, co-repressors, and silencers.

[0107] Additional exemplary functional domains are disclosed, for example, in co-owned US Patent No. 6,534,261 and US Patent Application Publication No. 2002/0160940.

[0108] Functional domains that are regulated by exogenous small molecules or ligands may also be selected. For example, RheoSwitch® technology may be employed wherein a functional domain only assumes its active conformation in the presence of the external RheoChem™ ligand (*see* for example US 20090136465). Thus, the ZFP or TALE may be operably linked to the regulatable functional domain wherein the resultant activity of the ZFP-TF or TALE-TF is controlled by the external ligand.

Nucleases

[0109] In certain embodiments, the fusion protein comprises a DNA-binding binding domain and cleavage (nuclease) domain. As such, gene modification can be achieved using a nuclease, for example an engineered nuclease. Engineered nuclease technology is based on the engineering of naturally occurring DNA-binding proteins. For example, engineering of homing endonucleases with tailored DNA-binding specificities has been described. (*see,* Chames *et al.* (2005) *Nucleic Acids Res* 33(20):e178; Arnould *et al.* (2006) *J. Mol. Biol.* 355:443-458). In addition, engineering of ZFPs has also been described. See, *e.g.*, U.S. Patent Nos. 6,534,261; 6,607,882; 6,824,978; 6,979,539; 6,933,113; 7,163,824; and 7,013,219.

[0110] In addition, ZFPs and TALEs have been fused to nuclease domains to create ZFNs and TALENs –functional entities that are able to recognize their intended nucleic acid target through their engineered (ZFP or TALE) DNA binding domains and cause the DNA to be cut near the ZFP or TALE DNA binding site via the nuclease activity. See, *e.g.*, Kim *et al.* (1996) *Proc Natl Acad Sci USA* 93(3):1156-1160. More recently, ZFNs have been used for genome modification in a variety of organisms. *See*, for example, United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20060063231; and International Publication WO 07/014275.

[0111] Thus, the methods and compositions described herein are broadly applicable and may involve any nuclease of interest. Non-limiting examples of nucleases include meganucleases, TALENs and zinc finger nucleases. The nuclease

may comprise heterologous DNA-binding and cleavage domains (*e.g.*, zinc finger nucleases; TALENs; meganuclease DNA-binding domains with heterologous cleavage domains) or, alternatively, the DNA-binding domain of a naturally-occurring nuclease may be altered to bind to a selected target site (*e.g.*, a meganuclease that has been engineered to bind to site different than the cognate binding site).

[0112] In certain embodiments, the nuclease is a meganuclease (homing endonuclease). Naturally-occurring meganucleases recognize 15-40 base-pair cleavage sites and are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cyst box family and the HNH family. Exemplary homing endonucleases include I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce*, I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III. Their recognition sequences are known. *See* also U.*S. Pa*tent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort *et al.* (1997) *Nucleic Acids Res.* **25:**3379–3388; Dujon *et al.* (1989) *Gene* **82:**115–118; Perler *et al.* (1994) Nucleic Acids Res. 22, 1125–1127; Jasin (1996) *Trends Genet.* **12:**224–228; Gimble *et al.* (1996) *J. Mol. Biol.* **263:**163–180; Argast *et al.* (1998) *J. Mol. Biol.* **280:**345–353 and the New England Biolabs catalogue.

[0113] DNA-binding domains from naturally-occurring meganucleases, primarily from the LAGLIDADG family, have been used to promote site-specific genome modification in plants, yeast, Drosophila, mammalian cells and mice, but this approach has been limited to the modification of either homologous genes that conserve the meganuclease recognition sequence (Monet *et al.* (1999), *Biochem. Biophysics. Res. Common.* 255: 88-93) or to pre-engineered genomes into which a recognition sequence has been introduced (Route *et al.* (1994), *Mol. Cell. Biol.* 14:8096-106; Chilton *et al.* (2003) *Plant Physiology* 133:956-65; Puchta *et al.* (1996), *Proc. Natl. Acad. Sci. USA* 93: 5055-60; Rong *et al.* (2002), *Genes Dev.* 16: 1568-81; Gouble *et al.* (2006), *J. Gene Med.* 8(5):616-622). Accordingly, attempts have been made to engineer meganucleases to exhibit novel binding specificity at medically or biotechnologically relevant sites (Porteus *et al.* (2005), *Nat. Biotechnol.* 23: 967-73; Sussman *et al.* (2004), *J. Mol. Biol.* 342: 31-41; Epinat *et al.* (2003), *Nucleic Acids Res.* 31: 2952-62; Chevalier *et al.* (2002) *Molec. Cell* **10:**895-905; Epinat *et al.* (2003) *Nucleic Acids Res.* **31:**2952-2962; Ashworth *et al.* (2006) *Nature* **441:**656-659; Paques *et al.* (2007) *Current Gene Therapy* **7:**49-66; U.S. Patent Publication Nos.

20070117128; 20060206949; 20060153826; 20060078552; and 20040002092). In addition, naturally-occurring or engineered DNA-binding domains from meganucleases have also been operably linked with a cleavage domain from a heterologous nuclease (*e.g., Fok*I).

[0114] In other embodiments, the nuclease is a zinc finger nuclease (ZFN). ZFNs comprise a zinc finger protein that has been engineered to bind to a target site in a gene of choice and cleavage domain or a cleavage half-domain.

[0115] As described in detail above, zinc finger binding domains can be engineered to bind to a sequence of choice. *See*, for example, Beerli *et al.* (2002) *Nature Biotechnol.* **20:**135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.* **70:**313-340; Isalan *et al.* (2001) *Nature Biotechnol.* **19:**656-660; Segal *et al.* (2001) *Curr. Opin. Biotechnol.* **12:**632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* **10:**411-416. An engineered zinc finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. *See*, for example, co-owned U.S. Patents 6,453,242 and 6,534,261, incorporated by reference herein in their entireties.

[0116] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0117] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length (*e.g.*, TGEKP (SEQ ID NO:135), TGGQRP (SEQ ID NO:136), TGQKP (SEQ ID NO:137), and/or TGSQKP (SEQ ID NO:138)). *See*, *e.g.*, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino

acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein. *See, also*, U.S. Provisional Patent Publication No. 20110287512.

[0118] The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR Associated) nuclease system is a recently engineered nuclease system based on a bacterial system that can be used for genome engineering. It is based on part of the adaptive immune response of many bacteria and archea. When a virus or plasmid invades a bacterium, segments of the invader's DNA are converted into CRISPR RNAs (crRNA) by the 'immune' response. This crRNA then associates, through a region of partial complementarity, with another type of RNA called tracrRNA to guide the Cas9 nuclease to a region homologous to the crRNA in the target DNA called a "protospacer." Cas9 cleaves the DNA to generate blunt ends at the DSB at sites specified by a 20-nucleotide guide sequence contained within the crRNA transcript. Cas9 requires both the crRNA and the tracrRNA for site specific DNA recognition and cleavage. This system has now been engineered such that the crRNA and tracrRNA can be combined into one molecule (the "single guide RNA"), and the crRNA equivalent portion of the single guide RNA can be engineered to guide the Cas9 nuclease to target any desired sequence (see Jinek*et al* (2012) *Science* 337, p. 816-821, Jinek*et al*, (2013), *eLife* 2:e00471, and David Segal, (2013) *eLife* 2:e00563). Thus, the CRISPR/Cas system can be engineered to create a DSB at a desired target in a genome, and repair of the DSB can be influenced by the use of repair inhibitors to cause an increase in error prone repair.

[0119] Nucleases such as ZFNs, TALENs and/or meganucleases also comprise a nuclease (cleavage domain, cleavage half-domain). As noted above, the cleavage domain may be heterologous to the DNA-binding domain, for example a zinc finger DNA-binding domain and a cleavage domain from a nuclease or a meganuclease DNA-binding domain and cleavage domain from a different nuclease. Heterologous cleavage domains can be obtained from any endonuclease or exonuclease. Exemplary endonucleases from which a cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. *See*, for example, 2002-2003 Catalogue, New England Biolabs, Beverly, MA; and Belfort *et al.* (1997) *Nucleic Acids Res.* **25:**3379-3388. Additional enzymes which cleave DNA are known (*e.g.*, S1 Nuclease; mung bean nuclease;

pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease; *see* also Linn *et al.* (eds.) *Nucleases*, Cold Spring Harbor Laboratory Press,1993). One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains.

[0120] Similarly, a cleavage half-domain can be derived from any nuclease or portion thereof, as set forth above, that requires dimerization for cleavage activity. In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. Alternatively, a single protein comprising two cleavage half-domains can be used. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof). In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins to their respective target sites places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any integral number of nucleotides or nucleotide pairs can intervene between two target sites (*e.g.*, from 2 to 50 nucleotide pairs or more). In general, the site of cleavage lies between the target sites.

[0121] Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.*, Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme *Fok* I catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. *See*, for example, US Patents 5,356,802; 5,436,150 and 5,487,994; as well as Li *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89:**4275-4279; Li *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90:**2764-2768; Kim *et al.* (1994a) *Proc. Natl. Acad. Sci. USA* **91:**883-887; Kim *et al.* (1994b) *J. Biol. Chem.* **269:**31,978-31,982. Thus, in one embodiment, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered.

[0122] An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is *Fok* I. This particular enzyme is active as a dimer. Bitinaite *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95:** 10,570-10,575. Accordingly, for the purposes of the present disclosure, the portion of the *Fok* I enzyme used in the disclosed fusion proteins is considered a cleavage half-domain. Thus, for targeted double-stranded cleavage and/or targeted replacement of cellular sequences using zinc finger- or TALE-*Fok* I fusions, two fusion proteins, each comprising a *Fok*I cleavage half-domain, can be used to reconstitute a catalytically active cleavage domain. Alternatively, a single polypeptide molecule containing a zinc finger binding domain and two *Fok* I cleavage half-domains can also be used. Parameters for targeted cleavage and targeted sequence alteration using zinc finger- or TALE-*Fok* I fusions are provided elsewhere in this disclosure.

[0123] A cleavage domain or cleavage half-domain can be any portion of a protein that retains cleavage activity, or that retains the ability to multimerize (*e.g.*, dimerize) to form a functional cleavage domain.

[0124] Exemplary Type IIS restriction enzymes are described in International Publication WO 07/014275, incorporated herein in its entirety. Additional restriction enzymes also contain separable binding and cleavage domains, and these are contemplated by the present disclosure. *See*, for example, Roberts *et al.* (2003) *Nucleic Acids Res.* **31:**418-420.

[0125] In certain embodiments, the cleavage domain comprises one or more engineered cleavage half-domain (also referred to as dimerization domain mutants) that minimize or prevent homodimerization, as described, for example, in U.S. Patent Publication Nos. 20050064474 and 20060188987 and in U.S. Application No. 11/805,850 (filed May 23, 2007), the disclosures of all of which are incorporated by reference in their entireties herein. Amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of *Fok* I are all targets for influencing dimerization of the *Fok* I cleavage half-domains. Exemplary engineered cleavage half-domains of *Fok* I that form obligate heterodimers include a pair in which a first cleavage half-domain includes mutations at amino acid residues at positions 490 and 538 of *Fok* I and a second cleavage half-domain includes mutations at amino acid residues 486 and 499.

[0126] Thus, in one embodiment, a mutation at 490 replaces Glu (E) with Lys (K); the mutation at 538 replaces Iso (I) with Lys (K); the mutation at 486 replaced Gln (Q) with Glu (E); and the mutation at position 499 replaces Iso (I) with Lys (K). Specifically, the engineered cleavage half-domains described herein were prepared by mutating positions 490 (E→K) and 538 (I→K) in one cleavage half-domain to produce an engineered cleavage half-domain designated "E490K:I538K" and by mutating positions 486 (Q→E) and 499 (I→L) in another cleavage half-domain to produce an engineered cleavage half-domain designated "Q486E:I499L". The engineered cleavage half-domains described herein are obligate heterodimer mutants in which aberrant cleavage is minimized or abolished. *See, e.g.,* U.S. Patent Publication No. 2008/0131962 and 2011/0201055, the disclosure of which is incorporated by reference in its entirety for all purposes. In certain embodiments, the engineered cleavage half-domain comprises mutations at positions 486, 499 and 496 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Gln (Q) residue at position 486 with a Glu (E) residue, the wild type Iso (I) residue at position 499 with a Leu (L) residue and the wild-type Asn (N) residue at position 496 with an Asp (D) or Glu (E) residue (also referred to as a "ELD" and "ELE" domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490, 538 and 537 (numbered relative to wild-type FokI), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue, the wild type Iso (I) residue at position 538 with a Lys (K) residue, and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as "KKK" and "KKR" domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490 and 537 (numbered relative to wild-type FokI), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as "KIK" and "KIR" domains, respectively). (See US Patent Publication No. 20110201055).

[0127] Engineered cleavage half-domains described herein can be prepared using any suitable method, for example, by site-directed mutagenesis of wild-type cleavage half-domains (*Fok* I) as described in U.S. Patent Publication Nos. 20050064474 and 20080131962.

[0128] Alternatively, nucleases may be assembled *in vivo* at the nucleic acid target site using so-called "split-enzyme" technology (*see e.g.* U.S. Patent Publication No. 20090068164). Components of such split enzymes may be expressed either on separate expression constructs, or can be linked in one open reading frame where the individual components are separated, for example, by a self-cleaving 2A peptide or IRES sequence. Components may be individual zinc finger binding domains or domains of a meganuclease nucleic acid binding domain.

[0129] In some embodiments, the DNA binding domain is an engineered domain from a TAL effector similar to those derived from the plant pathogens Xanthomonas (see Boch *et al*, (2009) *Science* 326: 1509-1512 and Moscou and Bogdanove, (2009) *Science*326: 1501) and Ralstonia (see Heuer *et al* (2007) *Applied and Environmental Microbiology* 73(13): 4379-4384). Also, see PCT publication WO2010/079430.

[0130] Nucleases (*e.g.*, ZFNs or TALENs) can be screened for activity prior to use, for example in a yeast-based chromosomal system as described in WO 2009/042163 and 20090068164. Nuclease expression constructs can be readily designed using methods known in the art. See, *e.g.*, United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20060063231; and International Publication WO 07/014275. Expression of the nuclease may be under the control of a constitutive promoter or an inducible promoter, for example the galactokinase promoter which is activated (de-repressed) in the presence of raffinose and/or galactose and repressed in presence of glucose.

Delivery

[0131] The proteins (*e.g.*, ZFPs, TALEs, CRISPR/Cas), polynucleotides encoding same and compositions comprising the proteins and/or polynucleotides described herein may be delivered to a target cell by any suitable means including, for example, by injection of ZFP-TF, TALE-TF proteins or by use of ZFN or TALEN encoding mRNA. Suitable cells include but not limited to eukaryotic and prokaryotic cells and/or cell lines. Non-limiting examples of such cells or cell lines generated from such cells include COS, CHO (*e.g.*, CHO-S, CHO-K1, CHO-DG44, CHO-DUXB11, CHO-DUKX, CHOK1SV), VERO, MDCK, WI38, V79, B14AF28-G3, BHK, HaK, NS0, SP2/0-Ag14, HeLa, HEK293 (*e.g.*, HEK293-F, HEK293-H,

HEK293-T), and perC6 cells as well as insect cells such as *Spodoptera fugiperda* (Sf), or fungal cells such as *Saccharomyces*, *Pichia* and *Schizosaccharomyces*. In certain embodiments, the cell line is a CHO-K1, MDCK or HEK293 cell line. Suitable cells also include stem cells such as, by way of example, embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells, neuronal stem cells and mesenchymal stem cells.

[0132] Methods of delivering proteins comprising zinc finger proteins as described herein are described, for example, in U.S. Patent Nos. 6,453,242; 6,503,717; 6,534,261; 6,599,692; 6,607,882; 6,689,558; 6,824,978; 6,933,113; 6,979,539; 7,013,219; and 7,163,824, the disclosures of all of which are incorporated by reference herein in their entireties.

[0133] Zinc finger, TALE or CRISPR/Cas proteins as described herein may also be delivered using vectors containing sequences encoding one or more of the zinc finger, TALE or CRISPR/Cas protein(s). Any vector systems may be used including, but not limited to, plasmid vectors, retroviral vectors, lentiviral vectors, adenovirus vectors, poxvirus vectors; herpesvirus vectors and adeno-associated virus vectors, etc. *See, also,* U.S. Patent Nos. 6,534,261; 6,607,882; 6,824,978; 6,933,113; 6,979,539; 7,013,219; and 7,163,824, incorporated by reference herein in their entireties. Furthermore, it will be apparent that any of these vectors may comprise one or more zinc finger or TALE protein-encoding sequences. Thus, when one or more ZFPs,TALEs or CRISPR/Cas proteins are introduced into the cell, the sequences encoding the ZFPs, TALEs or CRISPR/Cas proteins may be carried on the same vector or on different vectors. When multiple vectors are used, each vector may comprise a sequence encoding one or multiple ZFPs, TALEs or CRISPR/Cas systems.

[0134] Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding engineered ZFPs, TALEs or CRISPR/Cas systems in cells (*e.g.*, mammalian cells) and target tissues. Such methods can also be used to administer nucleic acids encoding ZFPs, TALEs or a CRISPR/Cas system to cells *in vitro*. In certain embodiments, nucleic acids encoding the ZFPs, TALEs or CRISPR/Cas system are administered for *in vivo* or *ex vivo* gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Viral vector delivery systems include DNA and RNA viruses, which have either episomal

or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds.) (1995); and Yu *et al., Gene Therapy* 1:13-26 (1994).

[0135] Methods of non-viral delivery of nucleic acids include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, naked RNA, artificial virions, and agent-enhanced uptake of DNA. Sonoporation using, *e.g.*, the Sonitron 2000 system (Rich-Mar) can also be used for delivery of nucleic acids. In a preferred embodiment, one or more nucleic acids are delivered as mRNA. Also preferred is the use of capped mRNAs to increase translational efficiency and/or mRNA stability. Especially preferred are ARCA (anti-reverse cap analog) caps or variants thereof. See US patents US7074596 and US8153773, incorporated by reference herein.

[0136] Additional exemplary nucleic acid delivery systems include those provided by Amaxa Biosystems (Cologne, Germany), Maxcyte, Inc. (Rockville, Maryland), BTX Molecular Delivery Systems (Holliston, MA) and Copernicus Therapeutics Inc, (*see* for example US6008336). Lipofection is described in *e.g.*, U.S. Patent Nos. 5,049,386; 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (*e.g.*, Transfectam™ and Lipofectin™ and Lipofectamine™ RNAiMAX). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

[0137] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al., Cancer Gene Ther.* 2:291-297 (1995); Behr *et al., Bioconjugate Chem.* 5:382-389 (1994); Remy *et al., Bioconjugate Chem.* 5:647-654 (1994); Gao *et al., Gene Therapy* 2:710-722 (1995);

Ahmad *et al., Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[0138] Additional methods of delivery include the use of packaging the nucleic acids to be delivered into EnGeneIC delivery vehicles (EDVs). These EDVs are specifically delivered to target tissues using bispecific antibodies where one arm of the antibody has specificity for the target tissue and the other has specificity for the EDV. The antibody brings the EDVs to the target cell surface and then the EDV is brought into the cell by endocytosis. Once in the cell, the contents are released (*see* MacDiarmid *et al* (2009) *Nature Biotechnology* 27(7):643).

[0139] The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding engineered ZFPs, TALEs or CRISPR/Cas systems take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of ZFPs, TALEs or CRISPR/Cas systems include, but are not limited to, retroviral, lentivirus, adenoviral, adeno-associated, vaccinia and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[0140] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system depends on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon mouse leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and

combinations thereof (*see, e.g.,* Buchscher *et al.*, *J. Virol.* 66:2731-2739 (1992); Johann *et al., J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al.*, *Virol.* 176:58-59 (1990); Wilson *et al.*, *J. Virol.* 63:2374-2378 (1989); Miller *et al.*, *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

[0141] In applications in which transient expression is preferred, adenoviral based systems can be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and high levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, *e.g.*, in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (*see, e.g.*, West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

[0142] At least six viral vector approaches are currently available for gene transfer in clinical trials, which utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

[0143] pLASN and MFG-S are examples of retroviral vectors that have been used in clinical trials (Dunbar *et al., Blood* 85:3048-305 (1995); Kohn *et al., Nat. Med.* 1:1017-102 (1995); Malech *et al., PNAS* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al., Science* 270:475-480 (1995)). Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors. (Ellem *et al., Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al., Hum. Gene Ther.* 1:111-2 (1997).

[0144] Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene

expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system. (Wagner *et al., Lancet* 351:9117 1702-3 (1998), Kearns *et al., Gene Ther.* 9:748-55 (1996)). Other AAV serotypes, including AAV1, AAV3, AAV4, AAV5, AAV6, AAV8AAV 8.2, AAV9, and AAV rh10 and pseudotyped AAV such as AAV2/8, AAV2/5 and AAV2/6 can also be used in accordance with the present invention.

[0145] Replication-deficient recombinant adenoviral vectors (Ad) can be produced at high titer and readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and/or E3 genes; subsequently the replication defective vector is propagated in human 293 cells that supply deleted gene function in *trans*. Ad vectors can transduce multiple types of tissues *in vivo*, including nondividing, differentiated cells such as those found in liver, kidney and muscle. Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Sterman *et al., Hum. Gene Ther.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker *et al., Infection* 24:1 5-10 (1996); Sterman *et al., Hum. Gene Ther.* 9:7 1083-1089 (1998); Welsh *et al., Hum. Gene Ther.* 2:205-18 (1995); Alvarez *et al., Hum. Gene Ther.* 5:597-613 (1997); Topf *et al., Gene Ther.* 5:507-513 (1998); Sterman *et al., Hum. Gene Ther.* 7:1083-1089 (1998).

[0146] Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by a producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host (if applicable), other viral sequences being replaced by an expression cassette encoding the protein to be expressed. The missing viral functions are supplied in *trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess inverted terminal repeat (ITR) sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which

contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, *e.g.*, heat treatment to which adenovirus is more sensitive than AAV.

[0147] In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. Accordingly, a viral vector can be modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the outer surface of the virus. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc. Natl. Acad. Sci. USA* 92:9747-9751 (1995), reported that Moloney mouse leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other virus-target cell pairs, in which the target cell expresses a receptor and the virus expresses a fusion protein comprising a ligand for the cell-surface receptor. For example, filamentous phage can be engineered to display antibody fragments (*e.g.*, FAB or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences which favor uptake by specific target cells.

[0148] Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.*, lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

[0149] *Ex vivo* cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to

those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a ZFP, TALE or CRISPR/Cas system nucleic acid (gene. cDNA or mRNA), and re-infused back into the subject organism (*e.g.*, patient). In a preferred embodiment, one or more nucleic acids are delivered as mRNA. Also preferred is the use of capped mRNAs to increase translational efficiency and/or mRNA stability. Especially preferred are ARCA (anti-reverse cap analog) caps or variants thereof. See U.S. patents 7,074,596 and 8,153,773, incorporated by reference herein in their entireties. Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (*see, e.g.*, Freshney *et al., Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

[0150] In one embodiment, stem cells are used in *ex vivo* procedures for cell transfection and gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34+ cells *in vitro* into clinically important immune cell types using cytokines such a GM-CSF, IFN-γ and TNF-α are known (*see* Inaba *et al., J. Exp. Med.* 176:1693-1702 (1992)).

[0151] Stem cells are isolated for transduction and differentiation using known methods. For example, stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4+ and CD8+ (T cells), CD45+ (panB cells), GR-1 (granulocytes), and Iad (differentiated antigen presenting cells) (*see* Inaba *et al., J. Exp. Med.* 176:1693-1702 (1992)).

[0152] Stem cells that have been modified may also be used in some embodiments. For example, neuronal stem cells that have been made resistant to apoptosis may be used as therapeutic compositions where the stem cells also contain the ZFP TFs of the invention. Resistance to apoptosis may come about, for example, by knocking out BAX and/or BAK using BAX- or BAK-specific TALENs or ZFNs (see, U.S. Patent Publication No. 20100003756) in the stem cells, or those that are disrupted in a caspase, again using caspase-6 specific ZFNs for example. These cells can be transfected with the ZFP TFs or TALE TFs that are known to regulate mutant or wild-type Htt.

[0153] Vectors (*e.g.*, retroviruses, adenoviruses, liposomes, etc.) containing therapeutic ZFP nucleic acids can also be administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[0154] Methods for introduction of DNA into hematopoietic stem cells are disclosed, for example, in U.S. Patent No. 5,928,638. Vectors useful for introduction of transgenes into hematopoietic stem cells, *e.g.*, $CD34^+$ cells, include adenovirus Type 35.

[0155] Vectors suitable for introduction of transgenes into immune cells (*e.g.*, T-cells) include non-integrating lentivirus vectors. *See*, for example, Ory *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93:**11382-11388; Dull *et al.* (1998) *J. Virol.* **72:**8463-8471; Zuffery *et al.* (1998) *J. Virol.* **72:**9873-9880; Follenzi *et al.* (2000) *Nature Genetics* **25:**217-222.

[0156] 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 available, as described below (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed., 1989).

[0157] As noted above, the disclosed methods and compositions can be used in any type of cell including, but not limited to, prokaryotic cells, fungal cells, Archaeal cells, plant cells, insect cells, animal cells, vertebrate cells, mammalian cells and human cells. Suitable cell lines for protein expression are known to those of skill in the art and include, but are not limited to COS, CHO (*e.g.*, CHO-S, CHO-K1, CHO-DG44, CHO-DUXB11), VERO, MDCK, WI38, V79, B14AF28-G3, BHK, HaK, NS0, SP2/0-Ag14, HeLa, HEK293 (*e.g.*, HEK293-F, HEK293-H, HEK293-T), perC6, insect cells such as *Spodoptera fugiperda* (Sf), and fungal cells such as

Saccharomyces, *Pischia* and *Schizosaccharomyces*. Progeny, variants and derivatives of these cell lines can also be used.

Applications

[0158] The disclosed compositions and methods can be used for any application in which it is desired to modulate the Htt allele, including but not limited to, therapeutic and research applications.

[0159] Diseases and conditions which Htt repressing ZFP TFs or TALE TFs can be used as therapeutic agents include, but are not limited to, Huntington's disease. Additionally, methods and compositions comprising ZFNs or TALENs specific for mutant alleles of Htt can be used as a therapeutic for the treatment of Huntington's disease.

[0160] ZFP-TFs or TALE TFs that repress a HD Htt allele may also be used in conjunction with ZFP-TFs or TALE-TFs that activate neutrotrophic factors including, but not limited to, GDNF and BDNF. These ZFPs or TALEs (or polynucleotides encoding these ZFPs or TALEs) may be administered concurrently (*e.g.*, in the same pharmaceutical compositions) or may be administered sequentially in any order.

[0161] Methods and compositions for the treatment of Huntington's disease also include stem cell compositions wherein a mutant copy of the Htt allele within the stem cells has been modified to a wild-type Htt allele using a Htt-specific ZFN or TALEN.

[0162] The methods and compositions of the invention are also useful for the design and implementation of *in vitro* and *in vivo* models, for example, animal models of trinucleotide repeate disorders, which allows for the study of these disorders. Non-limiting examples of suitable in vitro models include cells or cell lines from any organism, including fibroblasts. Non-limiting examples of suitable animals for use as animal models include, invertebrates (*C. elegans, drosophila)*, rodents (*e.g.*, rat or mouse), primates (*e.g.*, non-human primates).

EXAMPLES

Example 1: Design and Construction of Htt-targeted zinc finger protein transcription factors (ZFP-TFs) and ZFNs

[0163] Zinc finger proteins targeted to Htt were engineered essentially as described in U.S. Patent No. 6,534,261. Tables 1A and 1B show the recognition helices of the DNA binding domain of exemplary Htt-targeted ZFPs, while Tables 2A and 2B show the target sequences of these ZFPs.

[0164] ZFPs with one contiguous array of zinc fingers were designed to target sites completely within the CAG repeat region (Figure 1B). Such ZFPs may bind longer, mutant tracts with higher affinity and/or a higher net occupancy, achieving selective repression of the mutant allele. ZFNs were also designed that targeted sites which lay partially or wholly outside of the CAG region (Figure 1A), and which will therefore bind to the wild type and mutant allele equally, and regulate expression from both alleles with similar efficiency. When designing zinc finger proteins to recognize the CAG region, a set of one- and two-finger modules can be employed in a 'mix and match' combination. Those modules are shown below in Table 2C.

Table 2C: Zinc finger recognition helices used in ZFP-TFs targeting CAG repeats

Target site	F2	F3	SEQ ID NO: (F2+F3)
CAGCAG	RSDNLSE	KRCNLRC	161
CAGCAG	RSDNLSE	KPYNLRT	162
CAGCAG	RSDNLSE	RLWNRKQ	163
CAGCAG	RSDNLSV	RRWNLRA	164
CAGCAG	RSDNLSV	RKWNRDS	165
CAGCAG	RSDNLSE	NTSPLML	166
CAGCAG	RSDNLSE	RRYNLVK	167
CTGCTG	RSDTLSE	RRWTLVG	168
GCAGCA	QSSDLSR	QWSTRKR	169
GCAGCA	RSAHLSR	QSGDLTR	170
GCAGCA	QSGDLTR	QSGDLTR	171
GCAGCA	QSGDLTR	QSSDLRR	172
GCTGCT	QSSDLSR	QSSDLRR	173
GCTGCT	QSSDLSR	HRSTRNR	174
AGC	MACCRYA	none	175
CAG	RSANLRE	none	176

CAG	RNADRKK	none	177
CTG	RSDVLSE	none	42
CTG	RSAVLSE	none	148
GCA	QSGDLTR	none	18
GCA	QSSDLRR	none	6
GCA	QNATRIK	none	178
GCT	QSSDLSR	none	31
AAG	RSDNLRE	none	65

[0165] Multimerizing ZFP TFs are also constructed as described above except that the vector also contains sequences encoding 1 or more protein interaction domains (also called dimerization or protein interaction domains) that enable multimerization of the expressed protein along a tract of trinucleotide repeats that is operably linked to the sequences encoding the ZFP TF. *See,* Figure 1D and Figure 10. Table 3 shows dimerization domain designs that are used with ZFPs targeted to the CAG repeat region. Figure 10C shows protein sequences of the four ZFP monomer scaffolds that are designed to multimerize via interactions between dimerizing zinc fingers (DZ), DZ1 – DZ4. Designs are based on work described in *Mol. Syst. Biol.* (2006) 2:2006.2011. Figure 10D shows protein sequences of the seven ZFP monomer scaffolds that are designed to multimerize via interactions between coiled-coils (CC), CC1 – CC7. The design of CC#1 is based on the work described in (*J. Am. Chem. Soc.* (2001), 123:3151-3152), while CC#2, CC#3 and CC#4 are based on (*J. Am. Chem. Soc.* (2000), 122:5658-5659). CC and DZ domains allow the ZFP to polymerize within the major groove of a CAG tract (depicted in figure 10B). By choosing a finger array and dimerization domains with appropriate binding properties, efficient binding will occur only to the expanded CAG tract of a disease allele.

[0166] ZFP-TFs were constructed as fusion proteins comprising a nuclear localization sequence, the engineered zinc finger DNA-binding domain (Tables 1A and 1B), targeted to the Htt allele, and a KRAB repression domain from the human KOX1 protein. See, Fig 1A, 1B and 1D. The designed DNA-binding domains contain 3-6 finger modules, recognizing 9-18 bp sequences (Tables 2A and 2B). Nucleotides in the target site that are contacted by the ZFP recognition helices are indicated in uppercase letters; non-contacted nucleotides indicated in lowercase. ZFP-ZFP-TF molecules were also constructed where two ZFP DNA binding domains

were fused with a flexible linker and fused to a KRAB repression domain (Figure 1E). DNA binding domains were chosen from Tables 2A and 2B.

Example 2: Repression of both alleles of Htt in human and mouse cells.

[0167] To repress both alleles of the Htt (non-allele-specific), ZFPs were designed to bind to the Htt promoter and exon 1 region, wherein the target site was not entirely within the CAG repeat. *See,* Figure 1A. To test the activity of the Htt repressing ZFP TFs, the ZFP TFs were transfected into human cells and expression of Htt was monitored using real-time RT-PCR.

[0168] Human HEK293 cells (Graham *et al* (1977) *J Gen Virol* 36 :59-74) were cultured in DMEM supplemented with 10% FBS and $1e^5$ cells were transfected with 1 μg of plasmid DNA encoding indicated ZFP-KOX fusions by Amaxa Nucleofector® following the manufacturer's instructions.

[0169] Transfected cells were incubated for 2 days, and the levels of endogenous human Huntingtin (Htt) and normalization control beta-actin (ACTB) were analyzed by real-time PCR using Hs00918176_m1 and 4352935E primers and probes (Applied Biosystems), respectively, according to standard protocols. Htt levels were expressed as Htt/ACTB ratios normalized to that of the mock-transfected samples (set as 1).

[0170] As shown in Figure 2A, Htt-targeted ZFPs repressed Htt expression. Western blot analyses were done using standard protocols to confirm the reduction in Htt protein level (Figure 2B); p65 protein was used as loading control.

[0171] Mouse Htt-specific ZFP TFs repressors were transiently transfected into Neuro2A cells (Klebe & Ruddle (1969) *J. Cell Biol.* 43: 69A) using the Lipofectamine® 2000 kit (Invitrogen) according to manufacturer's protocols. mHtt and ACTB mRNA levels were measured at 48 hours after transfection using ABI Taqman® primer/probe set Mm01213820 m1 and 4352933E, respectively. mHtt/ACTB ratios for ZFP transfected samples were normalized to that of the GFP control (set as 1).

[0172] As shown in Figure 2C, the ZFPs repressed mouse Htt expression. In addition, mouse Htt-specific ZFP-TF repressors can repress mouse Htt in immortalized striatal cells, ST*Hdh*(Q111/Q7), derived from Htt knock-in mice (Trettel *et al.* (2000) *Hum. Mol. Genet 9: 2799-2809*). *See,* Figures 2D and 2E.

mRNA for indicated ZFPs were generated using the mMessage mMachine kit (Ambion), and 0.1, 0.5 or 2 ug of these mRNAs were transfected using Amaxa nucleofector as described above. Cells were harvested 48 hours after transfection for mHtt and ACTB expression analysis as described above. More significant repression in the striatal cells compared to that in Neuro2A cells was observed and may be a result of enhanced transfection efficiency achieved via mRNA transfection in straital cells.

Example 3: Selective Repression of mutant Htt in human and mouse cells

[0173] To achieve selective repression of the mutant Htt allele, ZFPs were designed to bind within the CAG repeat. Figure 1B shows one type of such ZFPs, with a contiguous array of zinc fingers linked to a repression domain (*e.g.* the KRAB domain from KOX1); these ZFPs can be designed with appropriate affinity such that threshold occupancy required from transcriptional repression can only be established on expanded CAG repeats. Figures 1C, 1D and 1E show threeother examples of ZFP design that can allow specific binding to the expanded CAG repeats.

[0174] ZFPs designed as illustrated in Figure 1B were introduced into HEK293 cells and Htt expression evaluated. ZFP-encoding constructs were transfected into HEK293 cells using FugeneHD using standard protocols. Seventy-two hours after transfection total RNA was isolated and the levels of endogenous human Huntingtin (Htt) relative to internal control beta-actin (ACTB) were analyzed by real-time PCR using Hs00918176_m1 and 4352935E primers and probes (Applied Biosystems), respectively. Htt/ACTB ratios for ZFP transfected samples were normalized to that of the GFP control (set as 1).

[0175] As shown in Figure 3A, ZFP repressors (fused with KRAB repression domain) designed to bind to CAG repeats (Figure 1B), either to top or bottom strand, in HEK293 cells, effectively repressed Htt expression. Figure 3A depicts repressors of transcription where expression was measure in duplicate transfections (separate bars in the Figure) and multiple real-time PCR assays completed (error bars). Different levels of repression by individual ZFPs suggest that they have different affinity to the CAG repeat region. Because Htt alleles in HEK293 cells have 16 and 17 CAG, this result also suggests that "weaker" ZFPs, such as 30640, do not repress Htt alleles with wild-type (unexpanded) CAG repeat length effectively.

[0176] To test whether ZFPs such as 30640 can repress transcription of Htt alleles with expanded CAG repeats, luciferase reporters controlled by Htt promoter/exon 1 fragment that contains different CAG repeat lengths were constructed. First, the human Htt promoter/exon1 fragment was amplified from HEK293 genomic DNA using forward primer:
5' GAAGATCTCACTTGGGGTCCTCAGGTCGTGCCGAC (SEQ ID NO:139)
and reverse primer:
5' GTATCCAAGCTTCAGCTTTTCCAGGGTCGCCTAGGCGGTCT. (SEQ ID NO:140).

[0177] The forward primer introduces a BglII site, the reverse primer changes the first ATG of Htt into TAG and creates an AvrII site, and also includes a HindIII site. The PCR product was digested with BglII and HindIII and ligated to pRL-TK vector (Promega) that was digested with the same enzymes to generate the construct pRL-Htt. Then the human Htt exon1 fragment (coding sequence minus first ATG) was amplified from HEK293 genomic DNA or genomic DNA from HD patients with expanded CAG repeats using forward primer:
5' GCCTAGGCGACCCTGGAAAAGCTGATGAAGGCC (SEQ ID NO:141)
and reverse primer: 5'
5' GTATCCAAGCTTGAGCTGCAGCGGGCCCAAACTCACG (SEQ ID NO:142).

[0178] The forward primer introduces an AvrII site, the reverse primer introduces a HindIII site. The PCR product was digested with AvrII and HindIII and ligated to pRL-Htt vector that was digested with the same enzymes. Clones with 10, 17, 23 or 47 CAG repeats (pRL-Htt-CAG(x)) were identified by sequencing.

[0179] pRL-Htt-CAG(x) reporters (300 ng) and pGL3-promoter reporter (100ng, used as normalization control, Progema) were transfected into HEK293 cells with or without 100 ng of ZFP 30640 expression vector. Firefly (pGL reporter) and renilla (pRL reporter) luciferase activities were measured 24 hours after transfection. Renilla luciferase levels were normalized to those of firefly luciferase from the same transfected sample, and further normalized to the renilla/firefly ratio of the "reporter only" sample.

[0180] As shown in Figure 3B, repression of the luciferase reporters by ZFP-TF 30640 increases with the length of the CAG repeat, suggesting that ZFPs with DNA binding affinities similar to that of 30640 can repress Htt promoter activity via

an expanded CAG repeat, and the level of repression is dependent on the CAG repeat lengths.

[0181] Figure 3C shows a similar experiment as in Figure 3B, except the "strong" ZFP-TF 30657 was also tested, and both 30640 and 30657 were tested at multiple doses as indicated. At every dose level, 30640 gave more repression of the pRL-Htt-CAG47 reporter than the pRL-Htt-CAG23 reporter (CAG repeat length-dependent repression), while 30657 repressed both reporters to similar levels. On the pRL-Htt-CAG23 reporter, 30640 gave less repression than 30657 at every dose level, recapitulating the difference in their activities on the endogenous Htt allele with normal CAG repeat length (HEK293 cells, Figure 3A); but on the pRL-Htt-CAG47 reporter, 30640 and 30657 gave similar repression at every dose level, suggesting that "weaker" ZFPs such as 30640 can efficiently repress Htt promoter through an expanded CAG repeat, most likely because only an expanded CAG target can allow threshold occupancy required for repression to be established by such ZFPs.

[0182] Figure 3D shows ZFP-TFs 30640 and 30657 (fused to the KRAB repression domain of KOX1) can repress the knock-in Htt allele (CAG111) in immortalized striatal cells derived from the Hdh(Q111/Q7) knock-in mice, demonstrating the ZFPs such as 30640, which drives CAG repeat length-dependent repression of luciferase reporters, can also repress expression from an endogenous Htt allele that has expanded CAG repeat. mRNA for indicated ZFPs were generated using the mMessage mMachine kit (Ambion), and transfected into Hdh(Q111/Q7) cells at indicated doses using Amaxa nucleofector. To detect expression from the wt mouse Htt allele, forward primer CAGGTCCGGCAGAGGAACC (SEQ ID NO:193) and reverse primer TTCACACGGTCTTTCTTGGTGG (SEQ ID NO:194) were used in the real-time RT-PCR; to detect expression from the knockin Htt allele, forward primer GCCCGGCTGTGGCTGA (SEQ ID NO:195) and reverse primer TTCACACGGTCTTTCTTGGTGG (SEQ ID NO:196) were used.

[0183] Figure 3E show the result of testing the ZFP-TFs 30640 and 30657 in an HD patient-derived fibroblast line (GM21756, Coriell) that has 15 and 70 CAGs on the normal and mutant Htt allele, respectively. A SNP-based allele-specific real-time PCR assay was first established to allow specific detection from the wild type or the mutant Htt allele. The phasing of the SNP (rs363099 T/C) was determined by Carroll *et al.* (*Mol Ther.* (2011) 19:2178-85); "T" is on the normal allele and "C" is on the

mutant allele. To detect Htt expression from the mutant allele (099C), cDNA from the fibroblast was amplified by real-time PCR (SsoFast EvaGreen Supermix, Bio-Rad) using forward primer 099C.F (5'AGTTTGGAGGGTTTCTC, SEQ ID NO:143) and reverse primer 099.R5 (5' TCGACTAAAGCAGGATTTCAGG, SEQ ID NO:144); the annealing/extension temperature was 55.6 °C. To detect Htt expression from the wild type allele (099T), real-time PCR of the fibroblast cDNA were done using forward primer 099T.F (5'AGTTTGGAGGGTTTCTT, SEQ ID NO:145), reverse primer 099.R5 and the 3'phosphorylated blocker oligo 099T.BL (5'AGGGTTTCTCCGCTCAGC-3'phos, SEQ ID NO:146); the annealing/extension temperature was 58.3°C. Total human Huntingtin (hHtt, both wt and mutant allele) levels and normalization control beta-actin (ACTB) levels were analyzed by real-time PCR using primer/probe Hs00918176_m1 and 4352935E (Applied Biosystems), respectively. For the experiment shown in Figure 3E, mRNA for indicated ZFPs were generated using the mMessage mMachine kit (Ambion), 1 ug of mRNA was transfected using Amaxa nucleofector as above. Cells were harvested 48 hours after transfection; mRNA levels from the normal (CAG15, 099T),the mutant (CAG70, 099C) Htt allele and total Htt (hHtt) were quantified as described above and normalized to the levels of ACTB; the Htt/ACTB ratios for each sample was further normalized to that of the mock-transfected sample. The "strong" CAG-targeted ZFP 30657 repressed both alleles, as expected (based on its activity in HEK293 cells Fig.3A). ZFP 30640, which showed CAG repeat length-dependent repression of the reporters, gave <10% repression of the wild-type allele while repressed the mutant allele >90%. The levels of total Htt in each sample were consistent with those of wt and mutant Htt levels in the same sample.

[0184] ZFP-30640 was also tested in a normal fibroblast line as well as other HD fibroblast lines that contain different CAG repeat length in the Htt gene (see Figure 3F). Htt expression from each allele was detected as described above. No Htt repression was observed in the normal fibroblast line (CAG18/18). In contrast, excellent allelic discrimination was observed in the CAG 15/67 and CAG15/70 lines at both a high and low dose of transfected 30640 mRNA ; similar results were obtain for the two HD fibroblast lines with intermediate CAG repeat length on the mutant allele (CAG 18/44 and CAG 18/45) - wherein the expanded allele is repressed by ~80% at both the high and low doses of 30640, yet the CAG18 allele remained

unaffected. Taken together, these data indicate that allele-specific repressors such as 30640 can maintain strong CAG allele length selectivity in the context of more prevalent disease genotypes such as CAG18/44 and CAG18/45.

[0185] Western blot analysis was used to show that that ZFPs such as 30640 selectively down-regulated mutant Htt protein levels in two patient-derived fibroblast lines, confirming allele-specific regulation that was shown by qPCR assays (see Figure 3G). ZFPs were delivered by mRNA transfection (Amaxa nucleofection) at a 300 ng dose into 4 replicates of 1.5e5 cells and pooled prior to plating in 12-well plates. At 48 hours, cells were washed and harvested for protein extract preparation. Approximately 2.5 ug of extract was loaded onto 5% Tris-acetate gels and detected by MAB2166 (Millipore). Additionally, the same samples were loaded on a 4-15% Tris-HCl gels (Bio-Rad) and transferred using standard methods for detection by an anti B-Actin (1:20,000, Sigma) as loading controls. Based on qPCR studies that measure Htt mRNA, 30640 is an allele-specific repressor targeting the CAG repeat; 32528 is biallelic repressors targeting the transcription start site (TSS), and 30657 is CAG-targeted repressor that repress both Htt alleles at the dose that was used. Western blot showed that 30640 specifically reduced the levels of mutant Htt (upper band) in both HD patient-derived cell lines, while 32528 and 30657 repressed both alleles similarly.

Example 4: Additional CAG-targeted ZFP designs that drive allele specific repression of Htt.

[0186] Figure 4A shows the results of testing ZFP-TFs 30640, 30643, 30645 and 33074 (all targeted to the CAG repeat and uses the KRAB repression domain) in a CAG18/45 HD fibroblast line. Different amounts of ZFP mRNA were transfected using Amaxa nucleofector as indicated, expression of the mutant Htt (right bar), wild type Htt (middle bar) and total Htt (both alleles, left bar) were measured as described above at 24 hours after transfection. ZFPs 30640, 30645 and 33074 drive allele-specific repression over the entire 3 ug - 10 ng ZFP mRNA dose range; while 30643 appears to repress both alleles significantly at doses that are 30 ng or higher, and begins to exhibit allele selectivity at the 10 ng dose.

[0187] Figure 4B shows the results of testing ZFP 30643, 30648, 30657 and 30658 (all targeted to CAG repeat and uses the KRAB repression domain) in a CAG15/70 HD fibroblast line. Different amounts of ZFP mRNA were transfected

using Amaxa nucleofector as indicated, expression of the mutant Htt (right bar), wild type Htt (middle bar) and total Htt (both alleles, left bar) were measured as described above at 24 hours after transfection. Compared to the ZFPs that were tested in the previous figure (30640, 30645 and 33074), these ZFPs drive mutant Htt-specific repression at lower doses. These results suggest that depend on ZFP expression levels that can be achieved in vivo (e.g. in the brains of HD patients), allele-specific repression of mutant Htt can be achieved using appropriate ZFP designs.

Example 5: Repression of alternate CAG-containing genes

[0188] Using the RNAs isolated in Example 3 (Fig. 3E), repression of other CAG repeat containing genes was analyzed, and the results are depicted in Figure 5. The expression levels of the following genes was examined using real-time PCR and normalized to that of Actin: Ataxin 2 ("ATXN2"); Dynamin ("DNM1"); F-box only protein 11 ("FBXO11"), nitrate reductase ("NAP"); Origin recognition complex subunit 4 ("ORC4"); phosphokinase ("PHK"); OCT3/4 protein ("POU3"); THAP domain containing, apoptosis associated protein 2 ("THAPII"); TATA binding protein ("TBP"); and stanniocalcin 1("STC1"). In addition, the location of the CAG repeat sequence relative to the transcriptional start site (TSS) was noted, and is indicated in Figure 3F as "TSS@", where "+" indicates base position of CAG repeats that are downstream of TSS, and "-" indicates base position of CAG repeats that are upstream of TSS. Also, the number of CAG repeats ("#CAG") is indicated for each gene.

[0189] The data demonstrate that repression of the mutant expanded Htt allele by 30640 is highly specific, and only a subset of CAG repeat-containing genes whose CAG repeats are relatively close to their respective transcroption start sites maybe repression targets of ZFPs such as 30640.

Example 6: Genome-wide specificity of allele-specific ZFP repressors of Htt

[0190] HD fibroblasts (CAG18/45) were transfected to study genome-wide specificity of CAG-targeted ZFPs by microarray analysis (Figure 6). ZFPs were delivered by mRNA transfection (Amaxa nucleofection) at the indicated doses - ZFPs 30640, 30645 and 33074 were transfected in sextuplicate at the 75 ng, 15 ng and 15 ng dose, respectively; GFP-Kox mRNA (150 ng) was transfected as a control, and was used as carrier to bring the total amount of transfected mRNA to 150 ng in all

samples. Expression from CAG18 (099T, middle bars) and CAG45 (099C, right bars) alleles was measured by allele-specific qPCR reagents at 24 hours after transfection as described above where each of the samples (1-6) are biological replicates (separate transfections). Htt levels were normalized to those of GAPDH. Mutant allele-specific repression of Htt was observed for all three ZFPs. The four most similar replicates were then chosen for microarray analysis (Affymetrix HGU133plus2.0) as follows: GFP replicate 1, 3, 4 and 6; 30640 replicate 2, 3, 5 and 6; 30645 replicate 2, 3, 5 and 6; and 33074 replicate 1, 3, 4 and 5 were used for microarray analysis. Robust Multi-array Average (RMA) was used to normalize raw signals from each probe set; ZFP-transfected samples were compared to GFP-transfected samples using T-test; "change" calls were made on genes (probe sets) with >2 fold difference relative to control samples and T-test P-value<0.05. Based on that criterion, 30640 repressed only two genes, stanniocalcin 1 (STC1) and extended synaptotagmin-like protein 1 (ESYT1); 30645 and 33074 only repressed one gene each, STC1 and interleukin 17 receptor A (ILR17RA), respectively. Htt was not detected as a repressed (>2-fold repression) gene because the Htt probe set on the array detects both wt and mutant Htt mRNA. This experiment demonstrates that mutant Htt-specific ZFPs, when expressed at levels that drive efficient allele-specific repression of Htt, can operate with very high specificity genome-wide.

Example 7: Allele specific repression in HD neural stem cells (NSCs)

[0191] HD iPSC/ESCs were passaged with accutase and cultured on matrigel coated plates in E8 media (Life Technologies). Neural stem cells were derived using StemPro Neural Induction Medium (Life Technologies). Briefly, iPSC/ESCs were seeded into geltrex coated 6 well dish with 200,000 cells/well and when 10-20% confluent the medium was changed to StemPro Neural Induction Medium. Medium was changed every 2 days and NSC harvested and expanded on day 7. StemPro NSC SFM medium (Life Technologies) was used to culture NSCs. HD NSCs(CAG17/69, derived from Coriell GM23225 iPSC) were transfected with 1.5 or 0.5 μg ZFP mRNA using nucleofection. Forty-eight hours post transfection cells were harvested and expression quantified by RT-PCR. Allele-specific detection of Htt expression was performed using a SNP (rs1143646)-based genotyping assay #4351376 (Applied Biosystems). At the ZFP doses that were tested, 30640 gave allele-specific

repression of mutant Htt, 30643 gave ~50% repression of wt Htt and ~90% repression of mutant Htt, and 30648 repressed both alleles (Figure 7); the behavior of these ZFPs is consistent with that in HD fibroblasts (Figure 4). The total Htt levels (middle bars) for each sample is consistent with levels of mutant and wt Htt levels.

Example 8: Htt repression in differentiated HD neurons

[0192] HD NSCs were passaged with accutase on geltrex coated plates. Neuron differentiation was induced by changing medium to neural differentiation medium containing StemPRO NSC SFM medium without (bFGF and EGF). Medium was changed every 3-4 days for up to 21 days. Neurons were derived from NSC (CAG17/48, derived from HD ESCs) by culture in neural differentiation medium. On day 15 post neural induction cells were transfected with 1.0 or 0.5 μg ZFP mRNA using nucleofection. Forty-eight hours post transfection cells were harvested and gene expression quantified by RT-PCR. Because this patient line does not contain a SNP that allows qPCR-based allele-specific detection of wt and mutant Htt, only total Htt levels can be measured. Because we showed that 30640 and 33074 does not repress the CAG18 or CAG17 allele in HD fibroblasts and NSCs, the levelsof total Htt observed in 30640- and 33074-treated samples are consistent with allele-specific repression of the mutant allele (CAG48). More potent repression by 30643 and 30648 at the ZFP doses tested is also consistent with the behavior of these ZFPs in HD fibroblasts (Figure 8).

Example 9: A CAG targeted repressor represses mutant Htt transgene in R6/2 mice

[0193] R6/2 mice (which carries a transgene of mutant human Htt exon 1 with ~120 CAG repeat, *see* Mangiarini *et al*, (1996) *Cell* 15:197) received stereotactic, bilateral striatal injections of 3e10 vector genome of recombinant AAV2/6 encoding either ZFP 30640-KOX or GFP driven by a CMV promoter. Mice were injected at 5 weeks of age and sacrificed for molecular analysis at 8 weeks of age. Left and rightstriata were dissected from each hemisphere and snap frozen. To assess repression of the mutant Htt transgene, total RNA was extracted from each striatum with TRIzol Plus (Life Technologies) followed by cDNA synthesis using High Capacity RT (Life Technologies). Subsequently, R6/2 transgene expression was

measured by qPCR and normalized to the geometric mean of three reference genes (Atp5b, Eif4a2, UbC) as previously described by Benn *et al.* ((2008) *Molecular Neurodegeneration*: 3, 17). We observed statistically significant repression ($P < 0.001$) of the mutant Htt transgene in four ZFP-treated striata relative to the four GFP-treated control striata (Figure 9). The average R6/2 repression was 64.9% of the GFP-treated controls. Because complete coverage of the striatum was not achieved using a single stereotactic injection and AAV2/6 preferentially transduces neuronal cells, the fold of repression observed (~35%) is likely an underestimate of actual repression in cells that were transduced with the AAV vector.

Example 10: Selective Repression of mutant Htt using ZFPs with dimerization/multimerization domains

[0194] In order to engineer zinc finger transcription factors to better discriminate between short CAG and long GAG repeats, we sought to both decrease the DNA-binding affinity of individual zinc finger transcription factors and increase the interaction strength between different copies of fusion protein bound to adjacent subsites within the CAG repeat. In order to decrease the DNA-binding affinity of individual zinc finger transcription factors, we generated zinc finger domains with fewer zinc fingers and/or with amino acid sequences expected to bind DNA with less than optimal affinity. In order increase the interaction strength between different copies of fusion protein bound to adjacent subsites within the CAG repeat, we fused various dimerization domains to our zinc finger transcription factors. The dimerization domains can interact in a "parallel" fashion and yield "head-to-head" or "tail-to-tail" dimers of fusion proteins that contain them. One potential dimerization strategy requires an array of identical ZFP-transcription factor fusions that bind in a "head-to-tail" orientation and thus this strategy requires dimerization domains that interact in an "anti-parallel" fashion. *See, e.g.,* McClain *et al.* (2001) *J. Am. Chem. Soc.* 123:3151-3152) and dimerizing zinc finger peptides (Giesecke *et al.* (2006), *Molecular Systems Biology* 2:2006.2011).

[0195] Dimerization contructs CC1 and CC2 were based on pairs of antiparallel coiled coils (McClain *et al* , *ibid*, Ghosh *et al.* (2000) *J Am Chem Soc* 122:5658-5659). Dimerization constructs CC3 and CC4 were truncated versions of CC2 that lack either 4 residues or 7 residues respectively. Dimerization constructs

DZ1, DZ2, DZ3, and DZ4 were based on pairs of dimerizing zinc finger domains (Giesecke *et al*, *ibid*). In each case, one member of the pair was fused to the N-terminus of the zinc finger DNA binding domain and the other member of the pair was fused to the C-terminus of the zinc finger DNA binding domain. Short linkers rich in glycine and serine residues were used to fuse the dimerization domains to the zinc finger binding domain. Additional embodiments of the invention utilize linkers with alternate lengths and/or amino acid composition. Linkers with one or more residues removed or with one or more glycine or serine residues changes to other amino acid residues will reduce the flexibility of these linkers and may result in improved discrimination between long and short CAG repeats.

[0196] To achieve selective repression of the mutant Htt allele, ZFPs were designed as illustrated in Figure 1D and Figure 10A and 4B. Figure 10C and 10D show the sequences of the multimerization domains. Figure 11A and 11B depict the results that experiments designed to measure the ability of the ZFP-TFs comprising the CC and DZ domains, respectively to repress their targets. For these experiments, indicated ZFP constructs (50 ng) were co-transfected with pRL-Htt-CAG17 (200ng), pGL3-Htt-CAG47 (200ng) and pVax-SEAP (secreted alkaline phosphatase, 10 ng, used as normalization control) into HEK293 cells. Luciferase activity and secreted alkaline phosphatase activities were measured 24 hours after transfection. The Renilla luciferase (CAG17)/SEAP and firefly luciferase (CAG47)/SEAP ratios for each sample were normalized to those of the reporter-only samples. The pGL3-Htt-CAG47 reporter was constructed in the same way as the pRL-Htt-CAG47 reporter (see Example 3), except the pGL-promoter construct (Promega) was used instead of the pRL-TK construct.

[0197] As shown in Figure 11A, 3 ZFPs, when tested asone or more CC domain-containing constructs, enhanced repression of one or both reporters when compared to constructs with the same ZFP but no CC domains. Figure 11B shows that DZ1 and DZ3 domains enhanced repression of 32220 on both reporters.

[0198] These results suggest that the CC and DZ domains can in general increase affinity of multimerized ZFPs and that design of the DNA binding domain and the dimerization domain may yield optimal CAG-repeat length discrimination.

Example 11: Selective repression of mutant Htt with ZFP-ZFP-Kox designs

[0199] ZFP TFs were tested in HD fibroblasts that were of the ZFP-ZFP-KOX design. In these experiments, the two ZFP DNA binding domains were linked together with a flexible linker (LRQKDAARGSAAMAERPFQ, SEQ ID NO:179) and fused to a KOX repression domain. The linker was placed between the conserved histidines and cysteines. The proteins were tested as described above using ZFP mRNA at indicated doses. These results in the CAG18/45 (Figure 12A) and CAG20/41 (Figure 12B) HD fibroblast lines demonstrated that linking less active ZFP DNA binding domains in this fashion can result in composite ZFPs that drive allele-specific repression.

Example 12: Activation of Htt in mouse cells

[0200] ZFPs as described herein were also evaluated for their ability to activate Htt expression. ZFPs targeted to the +200 to +467 bp region (relative to the transcription start site) of mouse Htt were fused to the transcriptional activation domain of NFκB p65 subunit. This targeting region was chosen because this fragment was replaced by the corresponding sequence (majority of exon 1 and some intron 1 sequence) from human Htt in various knock-in mouse models of HD (Menalled *et al.* (2003) *J. Comp. Neurol* 4651:11-26; Wheeler *et al.* (2000) *Hum Mol Genet* 8:115-122), therefore ZFPs targeted to this region can selectively activate the wild type allele in those animals but not the knock-in allele.

[0201] ZFPs were transfected into Neuro2A cells (both Htt alleles are wild type in these cells), mouse Htt and ACTB mRNA levels were measured as described in Example 2 (duplicate transfections and multiple assays).

[0202] As shown in Figure 13, an increase in Htt mRNA levels as compared to a mock transfection was detected using both ZFP-TFs. *See,* Figure 13A. Increased Htt protein levels were confirmed by Western blot. *See,* Figure 13B.

[0203] The generation of the knock-in Htt allele is illustrated in Figure 13C; sequence alignment (Figure 13D) shows divergence between the mouse sequence that was replaced and the corresponding human sequence. Figure 13E shows that when such ZFP activators were transfected into immortalized striatal cells derived from HdhQ111/Q7 knock-in mice, only the wild type Htt was selectively activated.

Example 13: Regulation of Htt expression *in vivo*

[0204] To test efficacy of the Htt-specific ZFP TFs *in vivo*, AAV2 vectors encoding the ZFPs are produced. These AAV2 based constructs are then delivered to the brains of mice. For human Htt-specific ZFP TFs, AAV vectors are delivered to R6.2 mice or BAC HD mice (C57Bl/6 or FVB/N strains) to assess the repression of the human transgene, as well as change in HD-like phenotypes. For mouse Htt-specific ZFPs (activators or repressors), AAV vectors are delivered to wild-type mice (C57Bl/6 or FVB/N) or human Htt knock-in mice (HdhQ111/Q7, HdhQ140/Q7 or HdhQ175/Q7) to assess the activation or repression of the endogenous mouse Htt expression. For ZFPs that preferentially targeting the CAG-expanded allele, AAV vectors are delivered to R6.2 mice or human Htt knock-in mice (HdhQ111/Q7, HdhQ140/Q7 or HdhQ175/Q7) to examine the selective repression of wt vs. expanded Htt allele. Following sacrifice, brain tissues are analyzed for Htt expression by Taqman real-time RT-PCR, and demonstrate that the Htt genes are modulated by ZFP-TFs.

Example 14: Co-transfection of a neurotrophic factor and a HD Htt allele-specific ZFP TF

[0205] The Htt-specific ZFP TFs identified above are co-transfected with ZFP TFs-specific for a brain neurotrophic factor. The ZFP TF specific for brain neurotrophic factors used are specific for either GDNF or BDNF.

Example 15: Design and Construction of Htt-targeted zinc finger nucleases (ZFNs)

[0206] ZFNs targeting human Htt and mouse Htt were designed to target the sequences flanking the CAG repeats, sequences near the first coding ATG, the stop codon, as well as in early exons. ZFNs were designed and incorporated into plasmids or adenoviral vectors essentially as described in Urnov *et al.* (2005) *Nature* 435(7042):646-651, Perez *et al* (2008) *Nature Biotechnology* 26(7): 808-816, and U.S. Patent Publication 2008/0131962.

Example 16: Cleavage activity of Htt-specific ZFNs

[0207] To test cleavage activity, plasmids encoding the pairs of human Htt-specific ZFNs described above were transfected into K562 cells. K562 cells were obtained from the American Type Culture Collection and grown as recommended in F-12 medium (Invitrogen) supplemented with 10% qualified fetal calf serum (FCS, Cyclone). Cells were disassociated from plastic ware using TrypLE Select™ protease (Invitrogen). For transfection, one million K562 cells were mixed with 2μg of the zinc-finger nuclease plasmid and 100μL Amaxa Solution T. Cells were transfected in an Amaxa Nucleofector II™ using program U-23 and recovered into 1.4mL warm F-12 medium + 10% FCS.

[0208] Genomic DNA was harvested and a portion of the Htt locus encompassing the intended cleavage site was PCR amplified. PCR using the Accuprime HiFi polymerase from InVitrogen was performed as follows: after an initial 3 minute denaturation at 94°C, 30 cycles of PCR are performed with a 30 second denaturation step at 94°C followed by a 30 second annealing step at 58°C followed by a 30 second extension step at 68°C. After the completion of 30 cycles, the reaction was incubated at 68°C for 7 minutes, then at 10°C indefinitely.

[0209] The genomic DNA from the K562 Htt-specific ZFN treated cells was examined by the Surveyor™ nuclease (Transgenomic) as described, for example, in U.S. Patent Publication Nos. 20080015164; 20080131962 and 20080159996.

[0210] Plasmids encoding the pairs of mouse Htt-specific ZFNs were tested in similar fashion in Neuro-2a cells.

[0211] Figure 14A and B show that the ZFNs were capable of targeting the Htt genes with a gene modification efficiency of between 8-40%, assayed as described previously by the amount of indels observed.

Example 17: Targeted integration of varying lengths of trinucleotide repeats

[0212] The Htt-specific ZFNs with the greatest cleaving activity for sequences flanking the CAG repeat as described above are used in a targeted integration strategy to introduce varying lengths of CAG repeat into a wild-type copy of Htt. Donors are constructed that contain 50, 80, 109 and 180 repeat CAG units. These donors are then transfected into K562 cells with plasmids encoding the Htt-specific ZFNs as described above. Verification of donor integration is achieved by genomic DNA

isolation, PCR amplification (as described above) followed by sequencing of the region of interest.

[0213] ZFNs identified in the K562 cells which result in targeted integration of the donor alleles into the Htt allele are used to insert the variable length donor nucleic acids into human embryonic stem cells (hESC). Successful donor integration is verified by genomic DNA isolation, PCR and sequencing as described above.

Example 18: Disruption/knock-out of wild-type and/or mutant Htt

[0214] ZFNs that cleave in early exons can result in small insertion or deletions (in-dels) as a result non-homologous end joining (NHEJ), this can generate cell models with one or both alleles of Htt disrupted,

[0215] Indicated ZFN pairs were prepared as described above and tested for cleavage activity using the Cel I mismatch as described for Example 8. These ZFN pairs target early exons of human Htt, and thus may be used to knock-out either the wild-type or a mutant Htt allele.

[0216] As shown in Figure 14A, ZFP pairs 29627/29628, 29631/29632 (exon 12) and 29637/29638 (exon 18) cleaved the Htt gene and can thus be utilized for generating knock-out cell lines.

Example 19: Expression tagging of wild-type and HD Htt alleles.

[0217] ZFNs with the greatest cleaving activity for the first or last coding exon are used to tag the wild-type and mutant Htt allele with different reporter proteins. Donor DNAs for each reporter (A and B) are designed based on the cleavage site of the lead ZFN pair(s) to allow targeted integration of the reporter gene to produce an in-frame fusion to Htt. Donor DNAs are co-transfected with the lead ZFN pair(s) into K562 cells for selecting the donor DNA construct that gives the highest frequency of integration.

[0218] ZFN pairs were prepared as described above and tested for cleavage activity using the Cel I mismatch as described for Example 8. The ZFN pairs used target the 3' end of the Htt coding sequence, and thus may be used to target either a wild-type or a mutant Htt allele. As shown in Figure 14B, ZFP pairs 25917/25916, 25920/25921 and 25923/25922 were capable of cleaving the Htt gene and can thus be utilized for the introduction of a reporter tag.

[0219] The selected donor DNA construct for reporter A along with corresponding ZFNs are delivered to cells derived from subjects carrying mutant Htt gene (e.g. fibroblasts, induced pluripotent cells) Clones are derived and screened for the target integration of the reporter A. Heterozygous events are desired and the targeted alleles are identified by PCR. Clones containing a single reporter-tagged Htt allele and unmodified ZFN target sequence on the other allele are selected; the donor construct for reporter B and corresponding ZFNs are transfected to tag the second allele with the reporter B.

[0220] The resulting mouse embryonic stem cell clone contains the wild-type Htt allele and mutant allele tagged with two different markers that allow tracking of expression from each allele; these cells are used to generate mouse models of trinucleotide repeat disorders using standard protocols.

Example 20: Construction of active TALE-TF proteins against Htt.

[0221] TALE DNA binding domains were linked to the KRAB repression domain from the Kox1 protein (TALE TF) and used to test repression of the Htt gene in HD patient (CAG 20/41)-derived fibroblasts. The construction of the TALE proteins was done as described previously (see co-owned US Patent publication 20110301073 and co-owned US Patent application 13/679,684, both of which are incorporated herein by reference), and were constructed with three different C-terminal architectures: +63, +231 and +278 as described in US20110301073. To construct the TALE TF expression plasmids, the TALEN expression plasmids described previously (see US20110301073) were used except that the *Fok*I domain used for in the TALENs was replaced with the KRAB repression domain. The linkages of the C-terminus of the TALE protein and the KRAB domain are shown below, where the KRAB domain sequence is indicated by underline. The ***bold and italicized*** text indicates the triple flag tag, the **bold** text indicates the nuclear localization sequence, "[repeats]" indicates the location of the TALE repeat unit array (full repeats plus the C-terminal half repeat), and the wavy underlined portion shows the sequence of the KRAB domain:

TALE-C63-Kox1:
M***DYKDHDGDYKDHDIDYKDDDDK***MA**PKKKRKV**GIHGVPMVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGF
THAHIVALSQHPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKI
AKRGGVTAVEAVHAWRNALTGAPLN[repeats]GGRPALESIVAQLSRPDPALAALTNDHLVALACLGGRPALD

AVKKGLPHAPALIKRTNRRIPERTSHRVAGSGMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVM
LENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSV (SEQ ID NO:197)

TALE-C231-Kox1:
M*DYKDHDGDYKDHDIDYKDDDDK*MA**PKKKRKV**GIHGVPMVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGF
THAHIVALSQHPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKI
AKRGGVTAVEAVHAWRNALTGAPLN[repeats]GGRPALESIVAQLSRPDPALAALTNDHLVALACLGGRPALD
AVKKGLPHAPALIKRTNRRIPERTSHRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSLERDLDAPSPTHEGDQRRASSRKRSR
SDRAVTGPSAQQSFEVRAPEQRDALHLPLSWRVKRPRTSIGGGLPDPGSGMDAKSLTAWSRTLVTFKDVFVDFTR
EEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSV
(SEQ ID NO:198)

TALE-C278-Kox1
M*DYKDHDGDYKDHDIDYKDDDDK*MA**PKKKRKV**GIHGVPMVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGF
THAHIVALSQHPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKI
AKRGGVTAVEAVHAWRNALTGAPLN[repeats]GGRPALESIVAQLSRPDPALAALTNDHLVALACLGGRPALD
AVKKGLPHAPALIKRTNRRIPERTSHRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSLERDLDAPSPTHEGDQRRASSRKRSR
SDRAVTGPSAQQSFEVRAPEQRDALHLPLSWRVKRPRTSIGGGLPDPTPTAADLAASSTVMREQDEDPFAGAADD
FPAFNEEELAWLMELLPQGSGMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLG
YQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSV (SEQ ID NO:199)

[0222] Base recognition was achieved using the canonical RVD-base correspondences (the "TALE code": NI for A, HD for C, NN for G (NK in half repeat), NG for T). In some of the TALE TFs, the protein is designed to bind the sense (5'-3') strand of the DNA, while in others, the TALE TF is designed to bind to the anti-sense (3'-5') strand. This set of TALE TFs was designed to target the CAG repeats of the Htt gene. TALE DNA binding proteins often preferentially interact with a 'T' nucleotide base at the 5' end of the target, and so since the targets are CAG repeat regions, it can be predicted that the proteins that bind to the anti-sense DNA strand, and thus CTG repeat sequences with the base 'T' at the 5' 3nd of the target, might have better binding affinity and specificity and thus repressor activity.

[0223] The targets and numeric identifiers for the TALE TFs tested are shown below in Table 4. Numeric identifiers are labeled "SBS#", specificity for the Sense or Antisense strand is indicated ("S/A"), as well as the target, the number of repeat units or RVDs and the type of C-terminus.

Table 4: Htt specific TALE-TFs

SBS#	S/A	Target (5′-3′)	SEQ ID NO	RVDs	C term
102449	S	gcAGCAGCAGCAGCAGCAGca	200	17	+63
102450	S	gcAGCAGCAGCAGCAGca	201	14	+63
102451	S	gcAGCAGCAGCAGca	202	11	+63
102452	S	gcAGCAGCAGca	203	8	+63
102453	A	ctGCTGCTGCTGCTGCTGCtg	204	17	+63
102454	A	ctGCTGCTGCTGCTGCtg	205	14	+63
102455	A	ctGCTGCTGCTGCtg	206	11	+63
102456	A	ctGCTGCTGCtg	207	8	+63
102457	S	gcAGCAGCAGCAGCAGCAGca	200	17	+231
102458	S	gcAGCAGCAGCAGCAGca	201	14	+231
102459	S	gcAGCAGCAGCAGca	202	11	+231
102460	S	gcAGCAGCAGca	203	8	+231
102462	A	ctGCTGCTGCTGCTGCtg	205	14	+231
102463	A	ctGCTGCTGCTGCtg	206	11	+231
102464	A	ctGCTGCTGCtg	207	8	+231
102466	S	gcAGCAGCAGCAGCAGca	201	14	+278
102467	S	gcAGCAGCAGCAGca	202	11	+278
102468	S	gcAGCAGCAGca	203	8	+278
102469	A	ctGCTGCTGCTGCTGCTGCtg	204	17	+278
102470	A	ctGCTGCTGCTGCTGCtg	205	14	+278
102471	A	ctGCTGCTGCTGCtg	206	11	+278
102472	A	ctGCTGCTGCtg	207	8	+278

[0224] The TALE TFs in the table were then tested for Htt repression in HD patient (CAG 20/41) derived fibroblasts, and the results are shown in Figure 15. In this experiment, the cells were transfected with either1000, 100 or 10 ng of TALE-TF encoding mRNA. The results for each TALE TF assayed are shown in groups of three, representing the three transfected mRNA amounts. In each grouping, there are also three samples: the left bar indicates the total Htt expression, the middle bar indicates the expression from the CAG20 Htt allele, and the right bar indicates the expression from the CAG41 Htt allele. The data demonstrates that there are some TALE TFs that were able to repress both Htt alleles (see for example 102454), while other TALE TFs were able to selectively inhibit the mutant Htt with the extended CAG repeat (see for example 102451 and 102472).

[0225] All patents, patent applications and publications mentioned herein are hereby incorporated by reference in their entirety.

[0226] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications can be practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

CLAIMS

What is claimed is:

1. A non-naturally occurring zinc finger protein that binds to an Htt gene, the zinc finger protein comprising 4, 5 or 6 zinc finger domains ordered F1 to F4, F1 to F5 and F1 to F6. wherein the zinc finger domains comprise the recognition helix regions sequences shown in a single row of Table 1B.

2. The zinc finger protein of claim 1, wherein the zinc finger protein binds entirely or partially outside the CAG repeat region of the Htt gene.

3. The zinc finger protein of claim 1, wherein the zinc finger protein binds to sequences within the CAG repeat region of the Htt gene.

4. The zinc finger protein of any of claims 1 to 3, further comprising a dimerization domain that allows multimerization of zinc finger proteins when bound to DNA.

5. A fusion protein comprising a zinc finger protein of any of claims 1 to 4 and a functional domain.

6. The fusion protein of claim 5, wherein the functional domain is selected from the group consisting of a transcriptional activation domain, a transcriptional repression domain, and a nuclease domain.

7. A polynucleotide encoding one or more zinc finger proteins of claims 1 to 4 or one or more fusion proteins of claims 5 or 6.

8. A host cell comprising one or more zinc finger proteins of claims 1 to 4, one or more fusion proteins of claims 5 or 6, or one or more polynucleotides according to claim 7.

9. A pharmaceutical composition comprising one or more zinc finger proteins of claims 1 to 4, one or more fusion proteins of claims 5 or 6, or one or more polynucleotides according to claim 7.

10. A method of modifying expression of an Htt gene in a cell, the method comprising administering to the cell one or more polynucleotides encoding one or more fusion proteins according to claim 5.

11. The method of claim 10, wherein the Htt gene comprises at least one mutant allele.

12. The method of claim 10, wherein the Htt gene is wild-type.

13. The method of any of claims 10 to 12, wherein the fusion protein comprises a nuclease domain and expression of the Htt gene is inactivated.

14. A method of modifying an Htt gene in a cell, the method comprising, administering to the cell one or more polynucleotides encoding one or more fusion proteins according to claim 5, wherein the one or more fusion proteins comprises a nuclease domain and wherein the sequence of the Htt gene is modified.

15. A method of treating Huntington's Disease, the method comprising administering to the cell one or more polynucleotides encoding one or more fusion proteins according to claim 5 to a subject in need thereof.

16. A method of generating a model system for the study of Huntington's Disease, the method comprising modifying an Htt gene according to the method of claim 14.

17. The method of claim 16, wherein the Htt gene is modified to comprise one or more mutant alleles.

18. The method of claim 17, wherein the mutant alleles comprise expanded trinucleotide repeats.

19. The method of any of claims 16 to 18, wherein the cell comprises an embryonic stem cell.

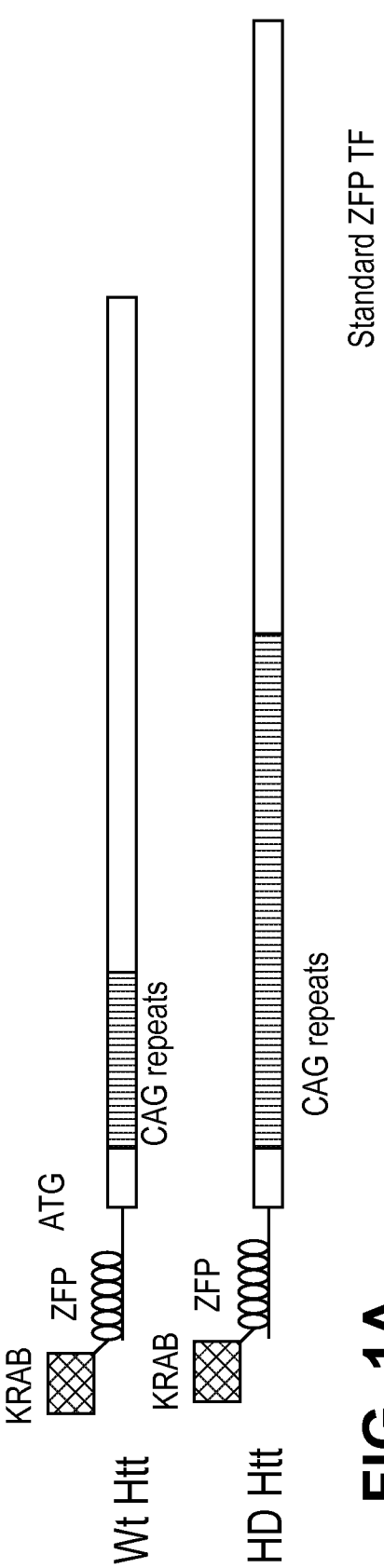

KRAB
ZFP
ATG
CAG repeats
Wt Htt
KRAB
ZFP
CAG repeats
HD Htt
Standard ZFP TF

FIG. 1A

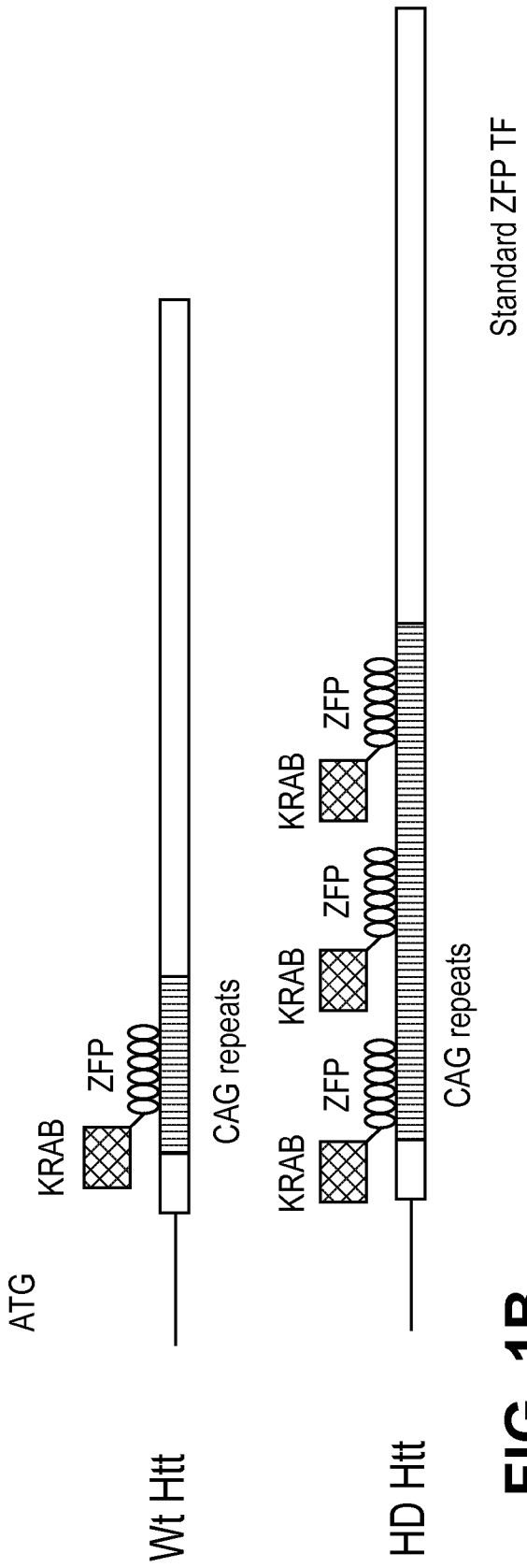

ATG
KRAB
ZFP
CAG repeats
Wt Htt
KRAB
ZFP
KRAB
ZFP
KRAB
ZFP
CAG repeats
HD Htt
Standard ZFP TF

FIG. 1B

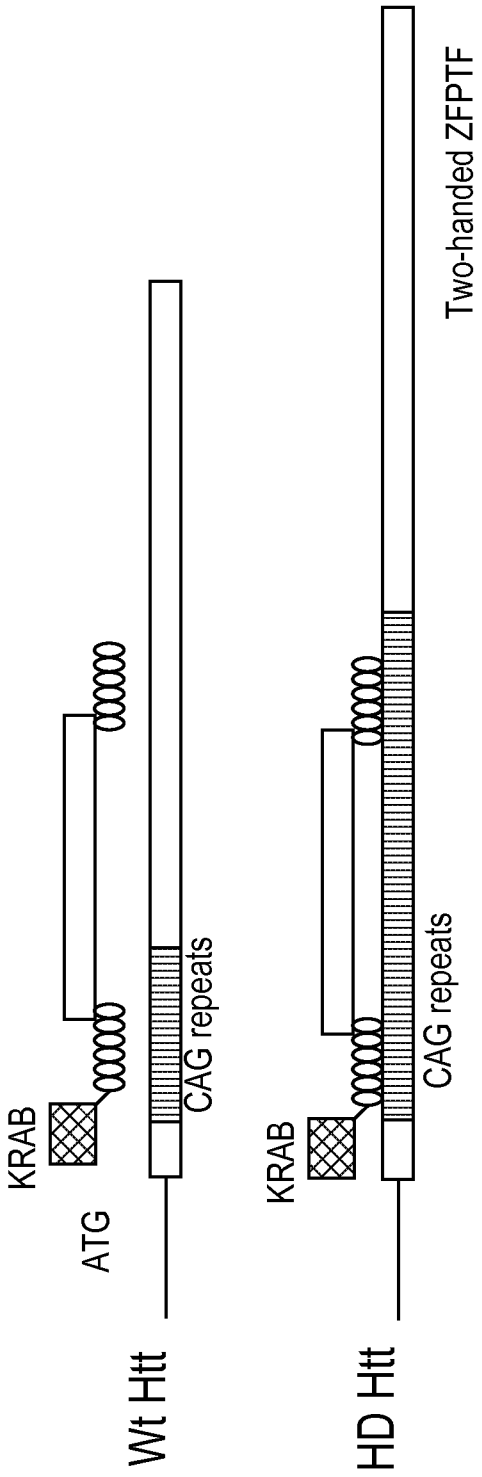

KRAB
ATG
CAG repeats
Wt Htt
KRAB
CAG repeats
HD Htt
Two-handed ZFPTF

FIG. 1C

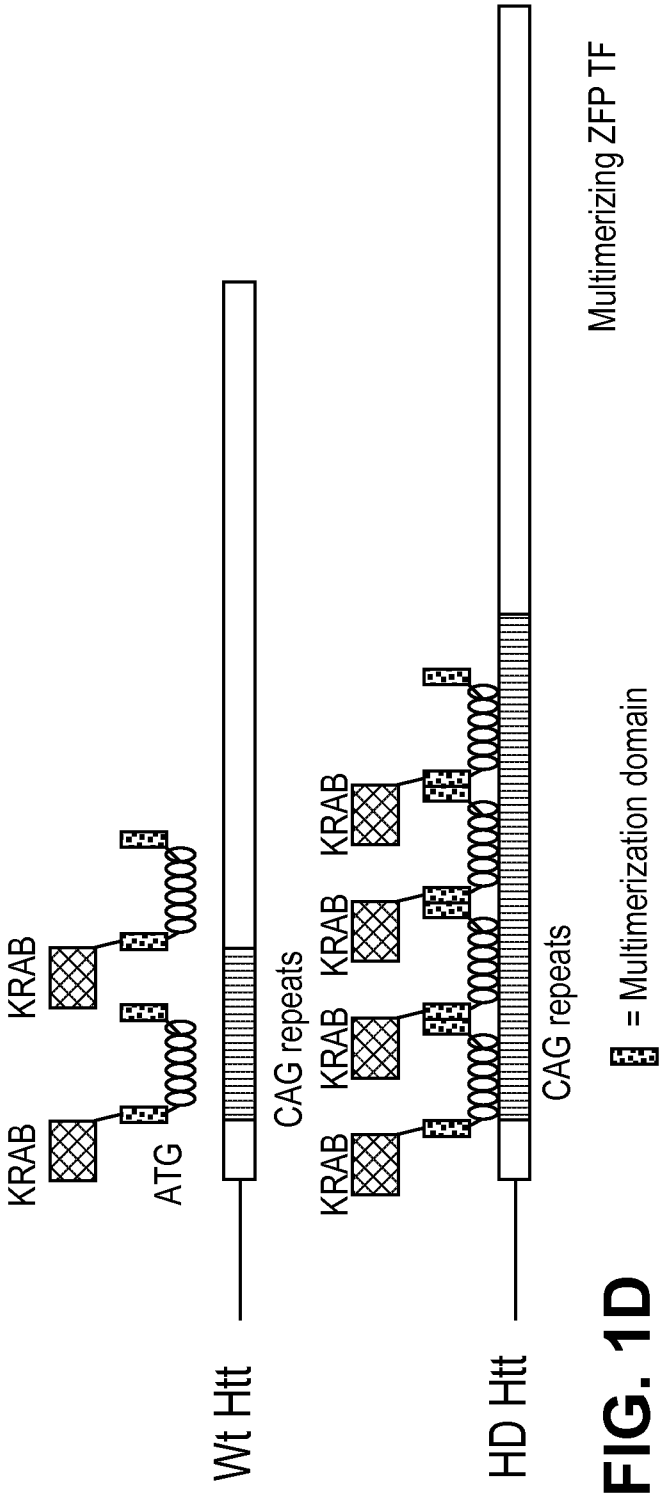

KRAB
KRAB
ATG
CAG repeats
Wt Htt
KRAB
KRAB
KRAB
KRAB
CAG repeats
HD Htt
Multimerizing ZFP TF
= Multimerization domain

FIG. 1D

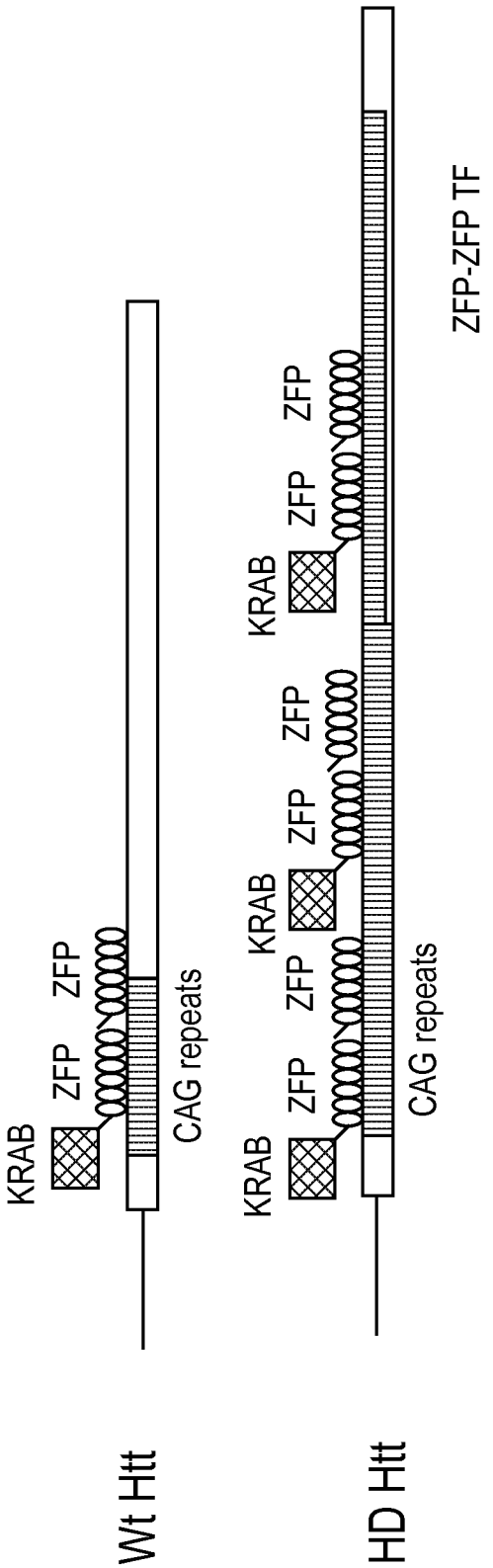

KRAB
ZFP
ZFP
CAG repeats
Wt Htt
KRAB
ZFP
ZFP
KRAB
ZFP
ZFP
KRAB
ZFP
ZFP
CAG repeats
HD Htt
ZFP-ZFP TF

FIG. 1E

FIG. 2A

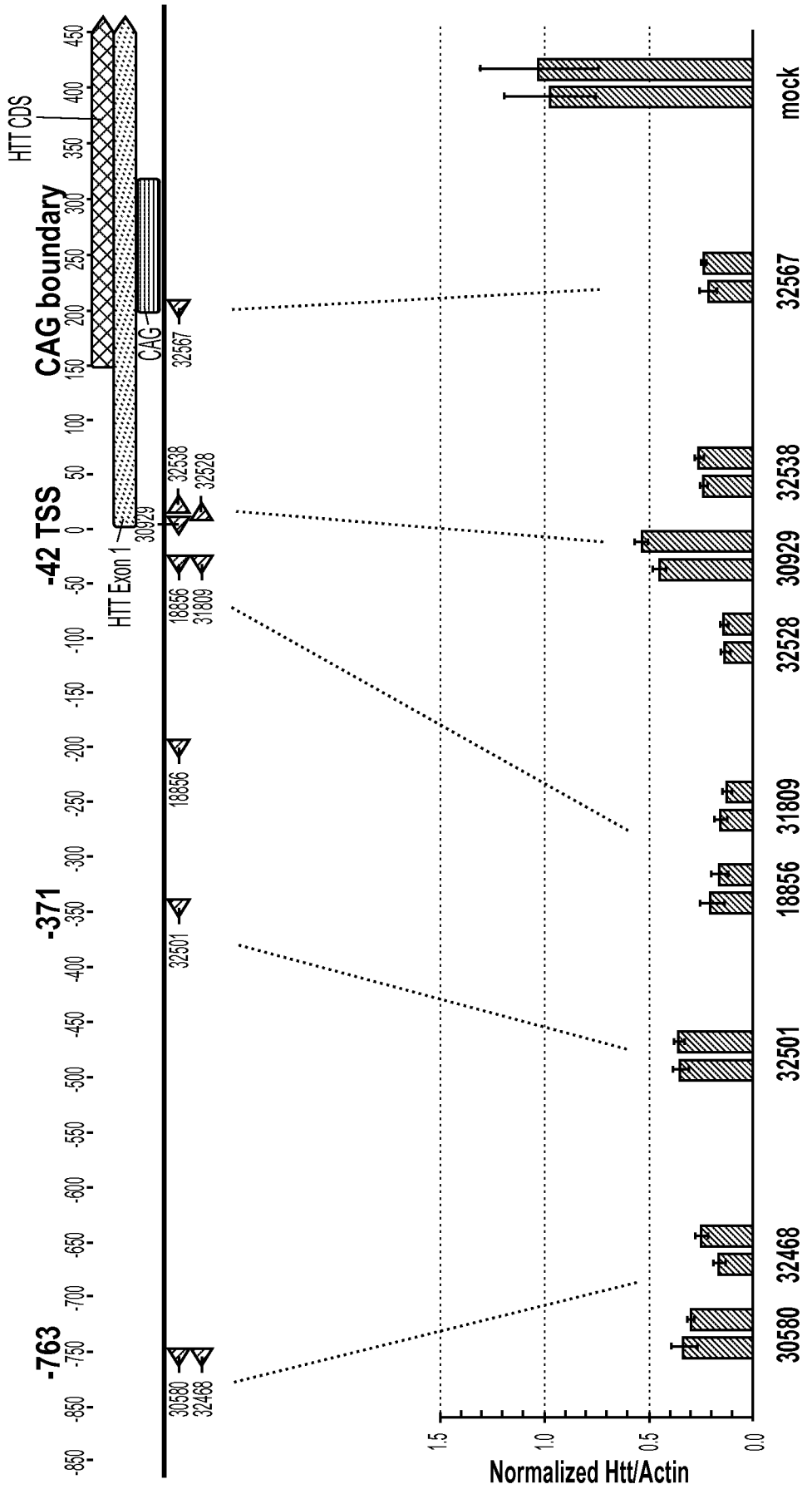

HTT CDS
CAG boundary
-42 TSS
-371
-763
HTT Exon 1
CAG
30929
32567
32538
32528
18856
31809
32501
30580
32468
mock
Normalized Htt/Actin
1.5
1.0
0.5
0.0

FIG. 2B

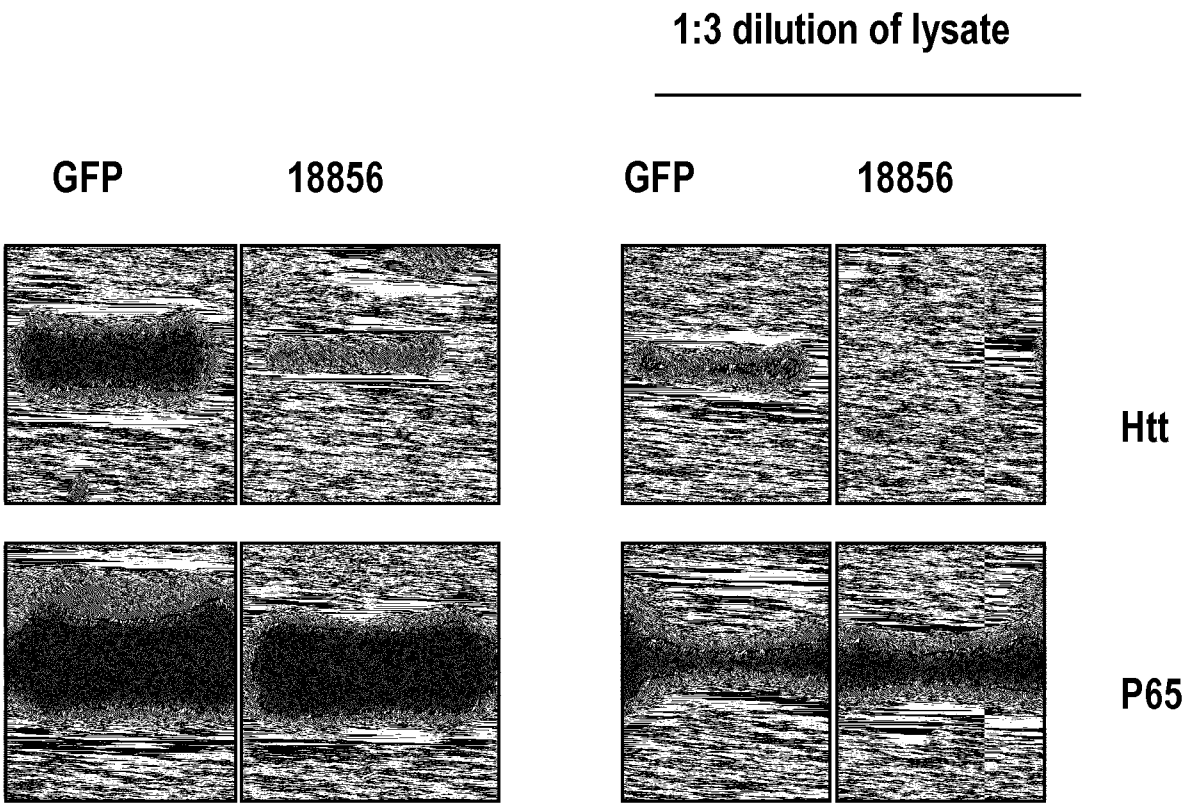


FIG. 2C

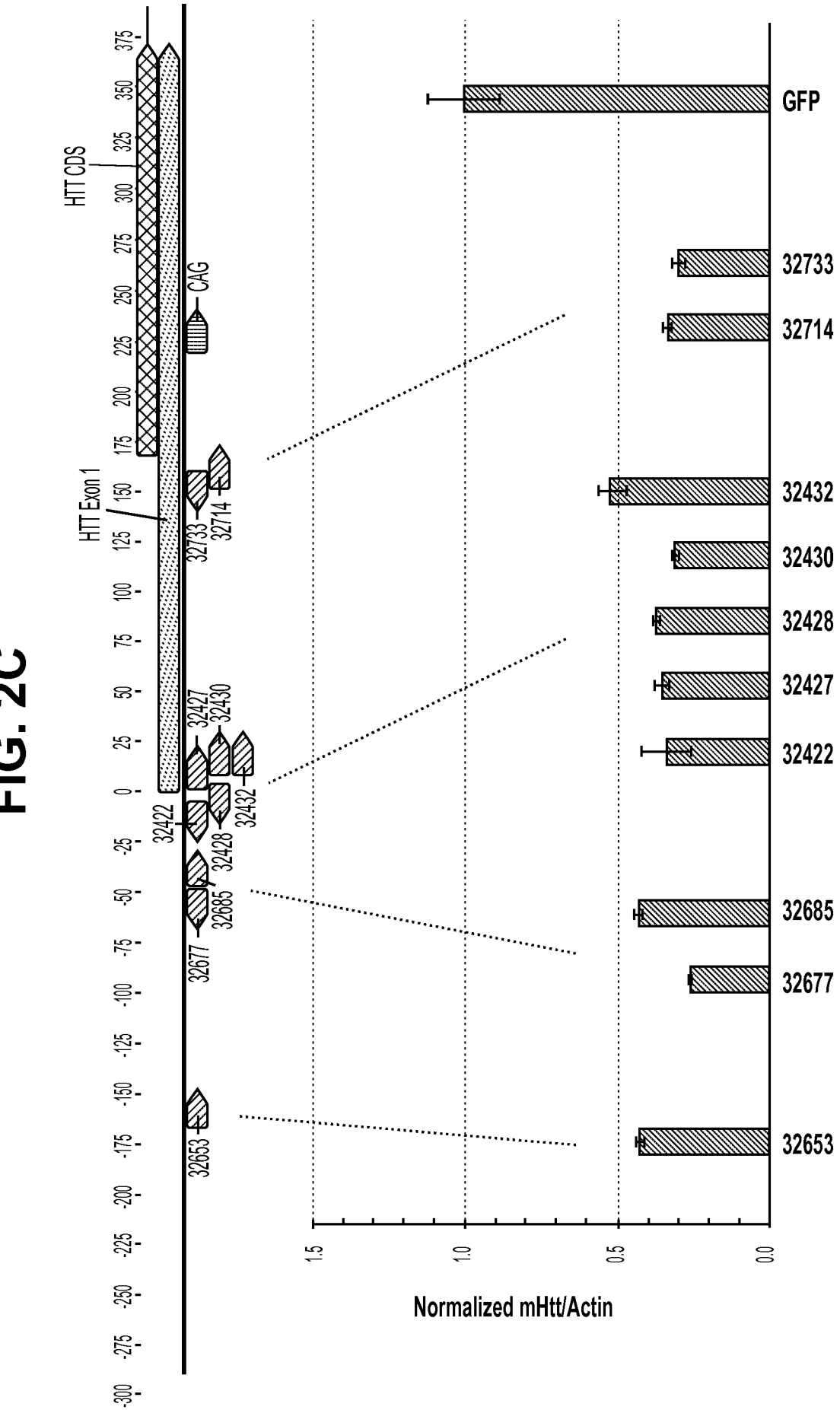

HTT CDS
HTT Exon 1
CAG
375
350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
-250
-275
-300
32733
32714
32427
32430
32422
32432
32428
32685
32677
32653
GFP
32733
32714
32432
32430
32428
32427
32422
32685
32677
32653
1.5
1.0
0.5
0.0
Normalized mHtt/Actin

FIG. 2D

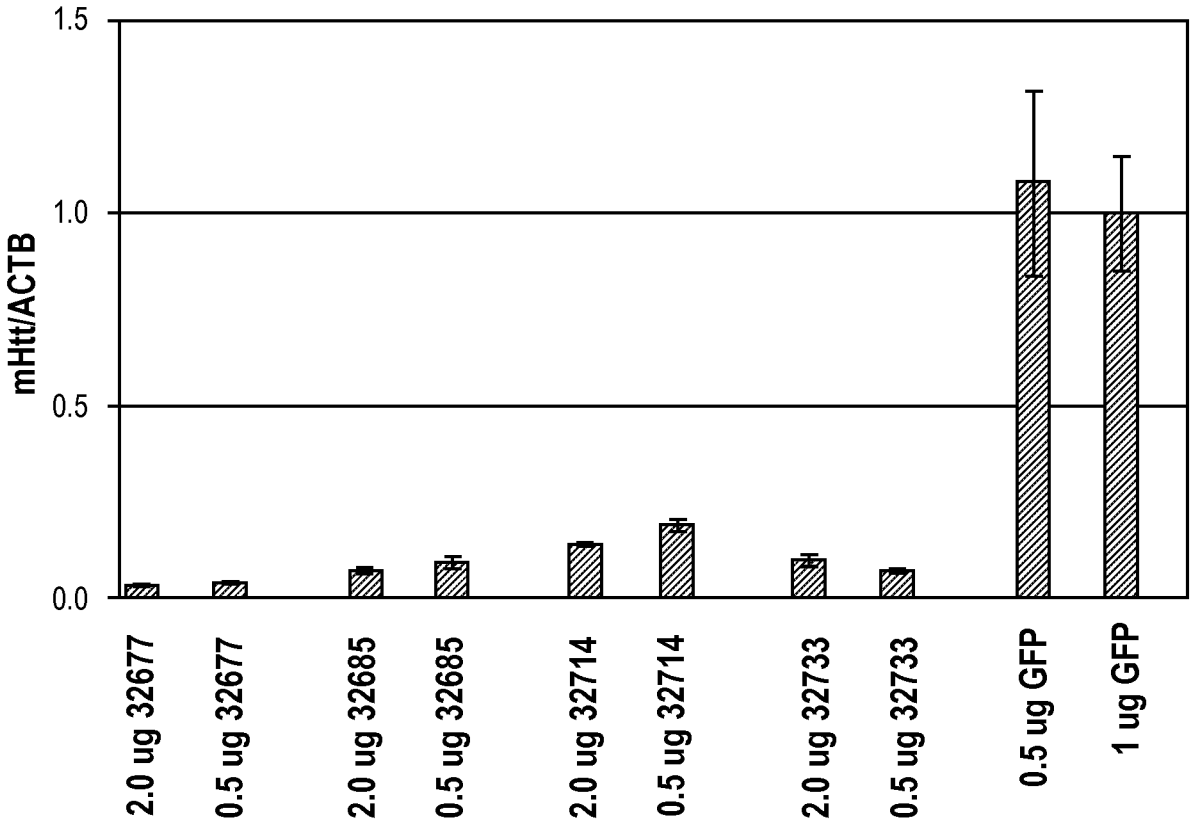
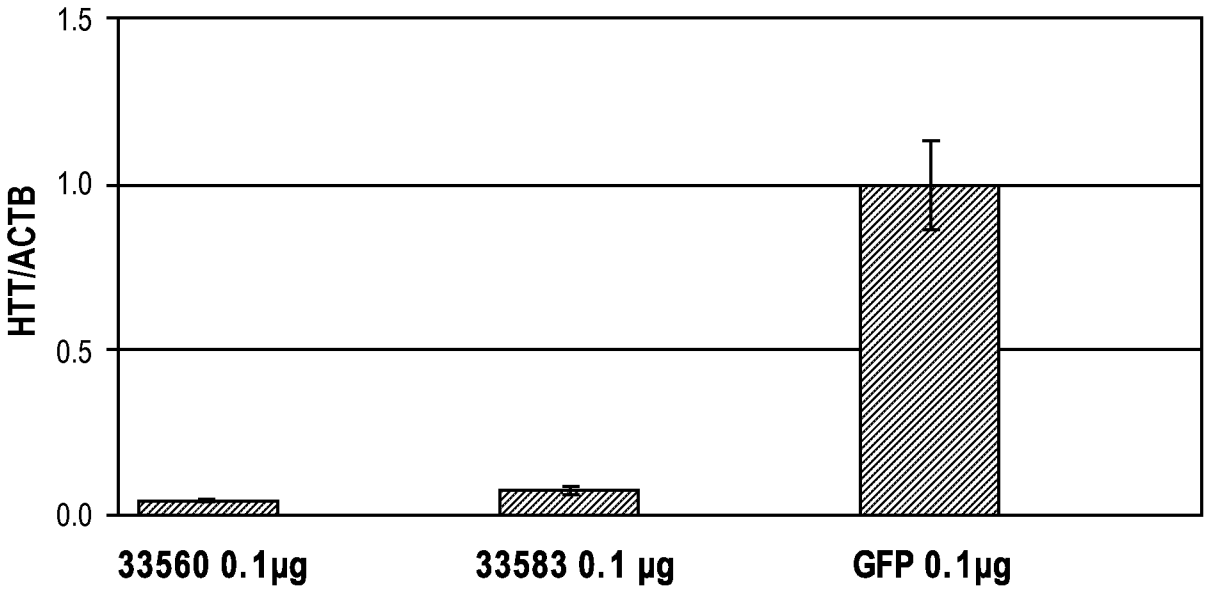

1.5
1.0
0.5
0.0
mHtt/ACTB
2.0 ug 32677
0.5 ug 32677
2.0 ug 32685
0.5 ug 32685
2.0 ug 32714
0.5 ug 32714
2.0 ug 32733
0.5 ug 32733
0.5 ug GFP
1 ug GFP

FIG. 2E



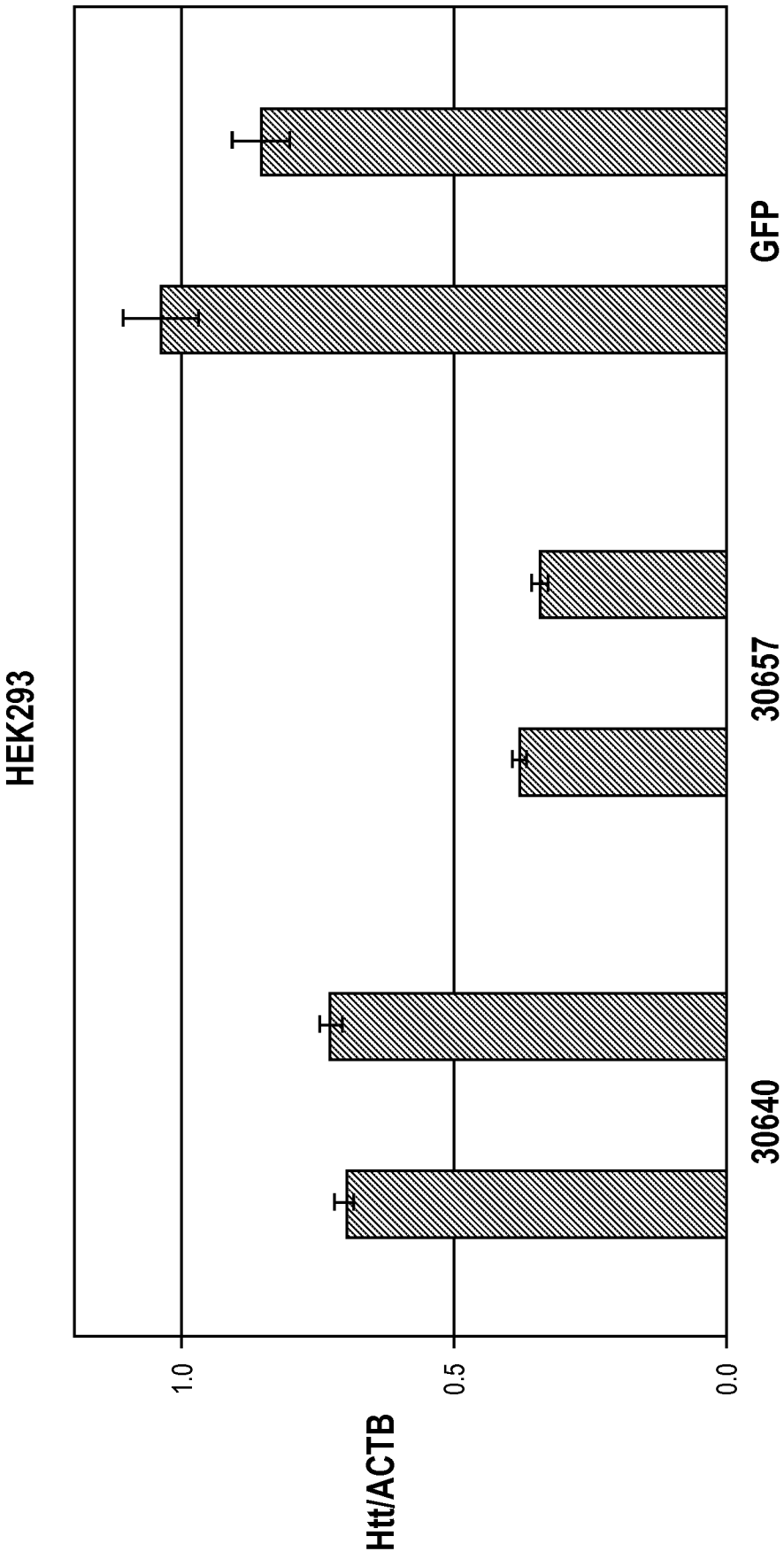


FIG. 3A

FIG. 3B

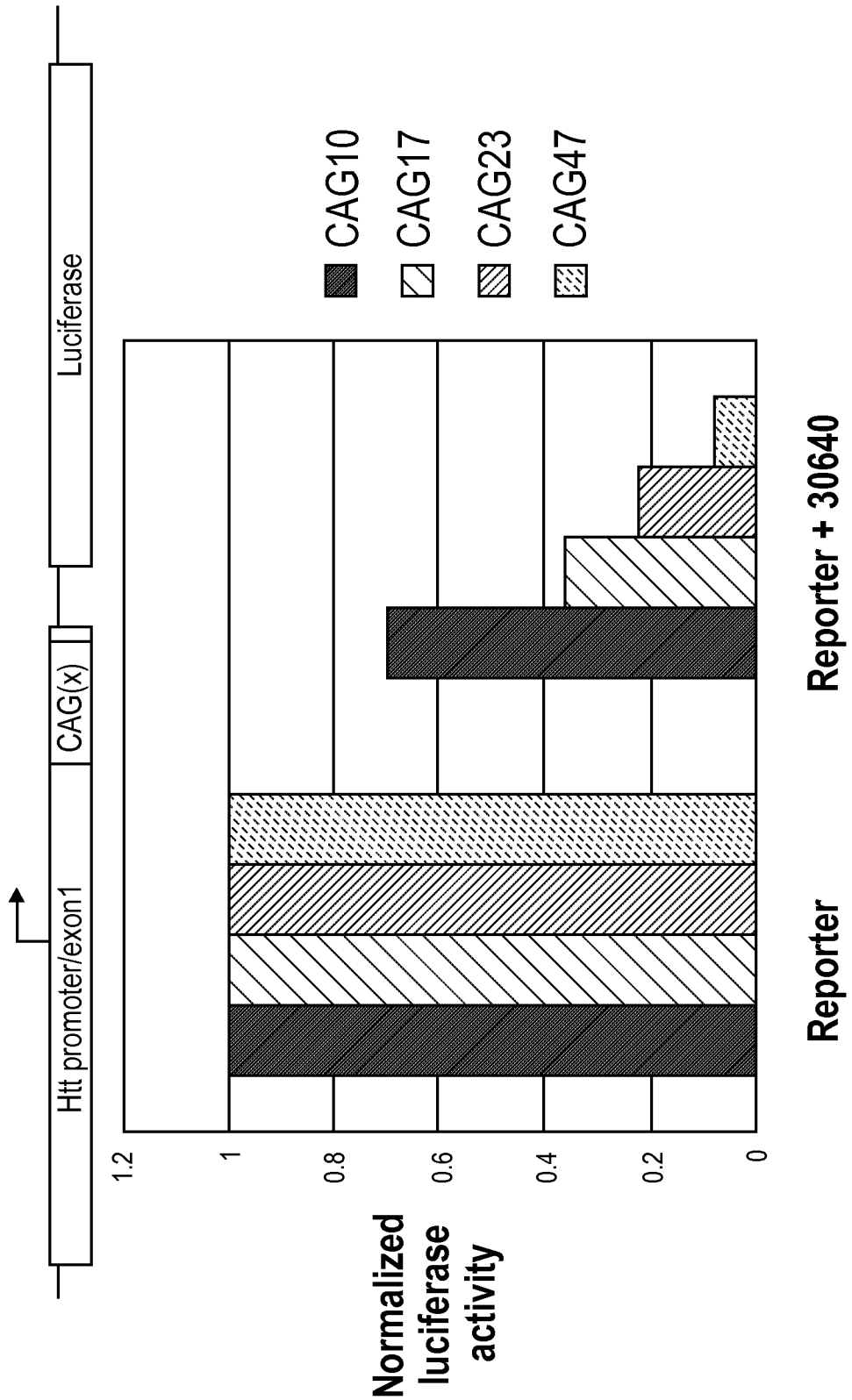

Htt promoter/exon1
CAG(x)
Luciferase
CAG10
CAG17
CAG23
CAG47
Reporter
Reporter + 30640
Normalized luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0

FIG. 3C

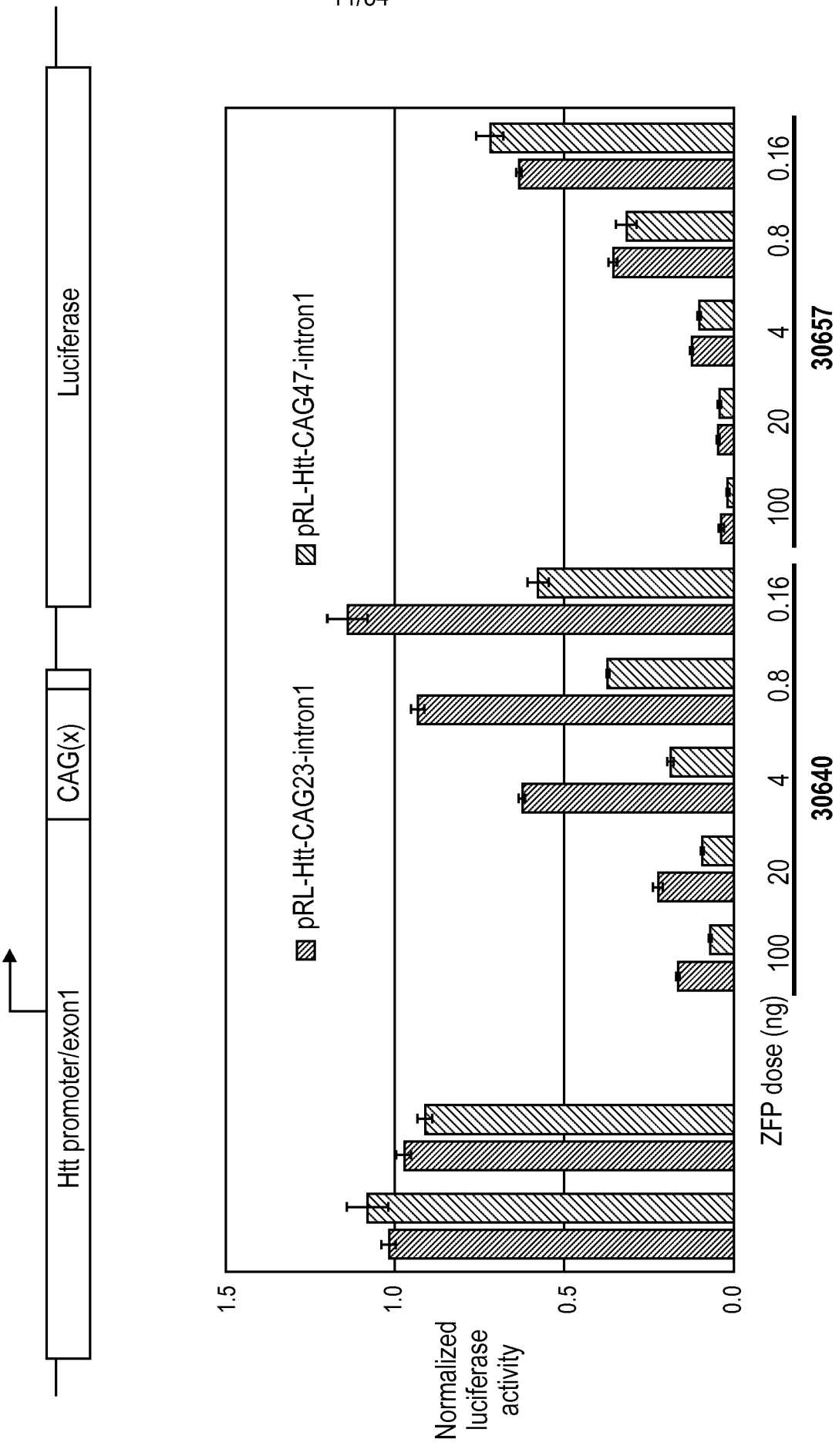

Htt promoter/exon1
CAG(x)
Luciferase
pRL-Htt-CAG23-intron1
pRL-Htt-CAG47-intron1
Normalized luciferase activity
1.5
1.0
0.5
0.0
ZFP dose (ng)
100
20
4
0.8
0.16
100
20
4
0.8
0.16
30640
30657

FIG. 3D

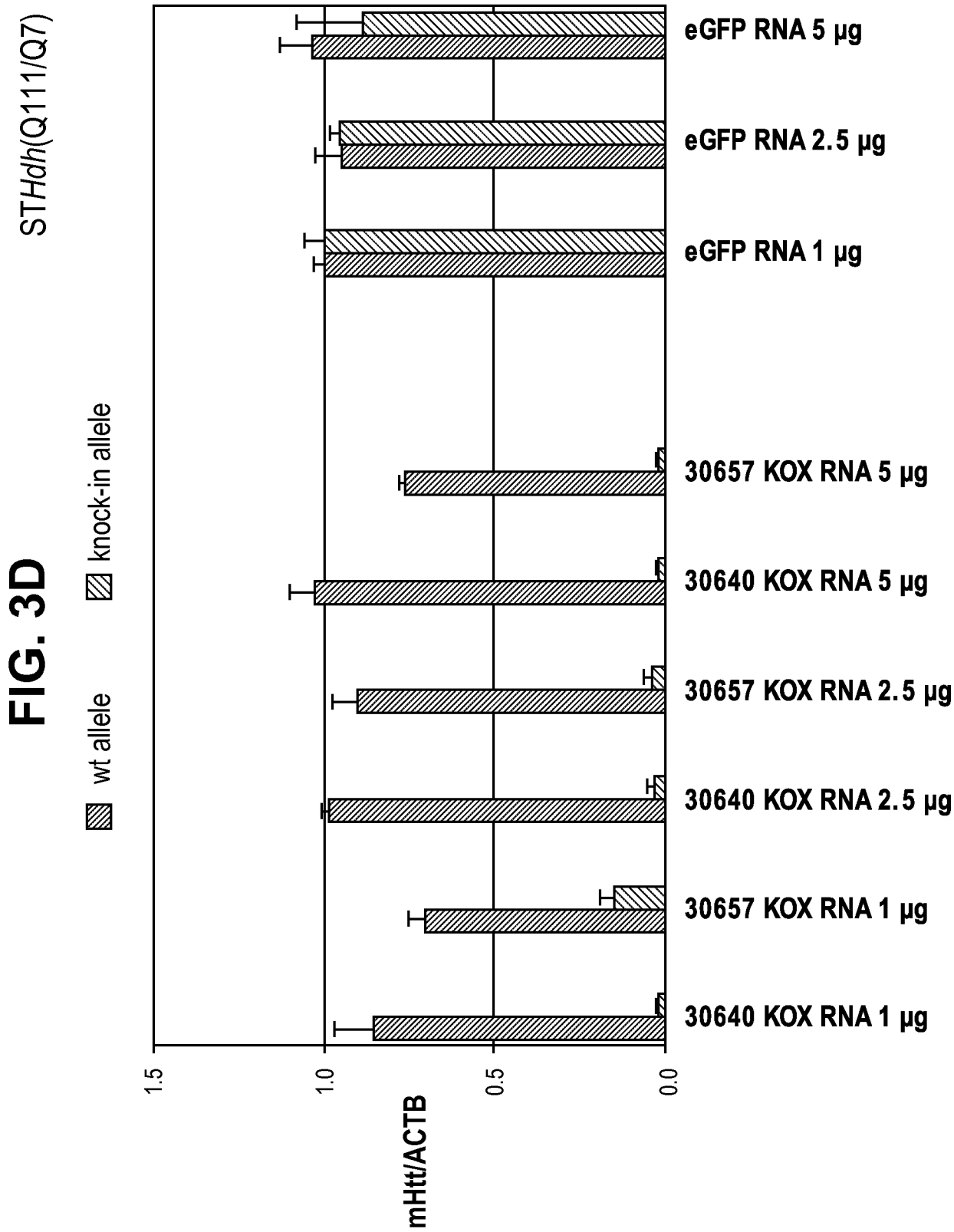

STHdh(Q111/Q7)
knock-in allele
wt allele
eGFP RNA 5 µg
eGFP RNA 2.5 µg
eGFP RNA 1 µg
30657 KOX RNA 5 µg
30640 KOX RNA 5 µg
30657 KOX RNA 2.5 µg
30640 KOX RNA 2.5 µg
30657 KOX RNA 1 µg
30640 KOX RNA 1 µg
1.5
1.0
0.5
0.0
mHtt/ACTB

FIG. 3E

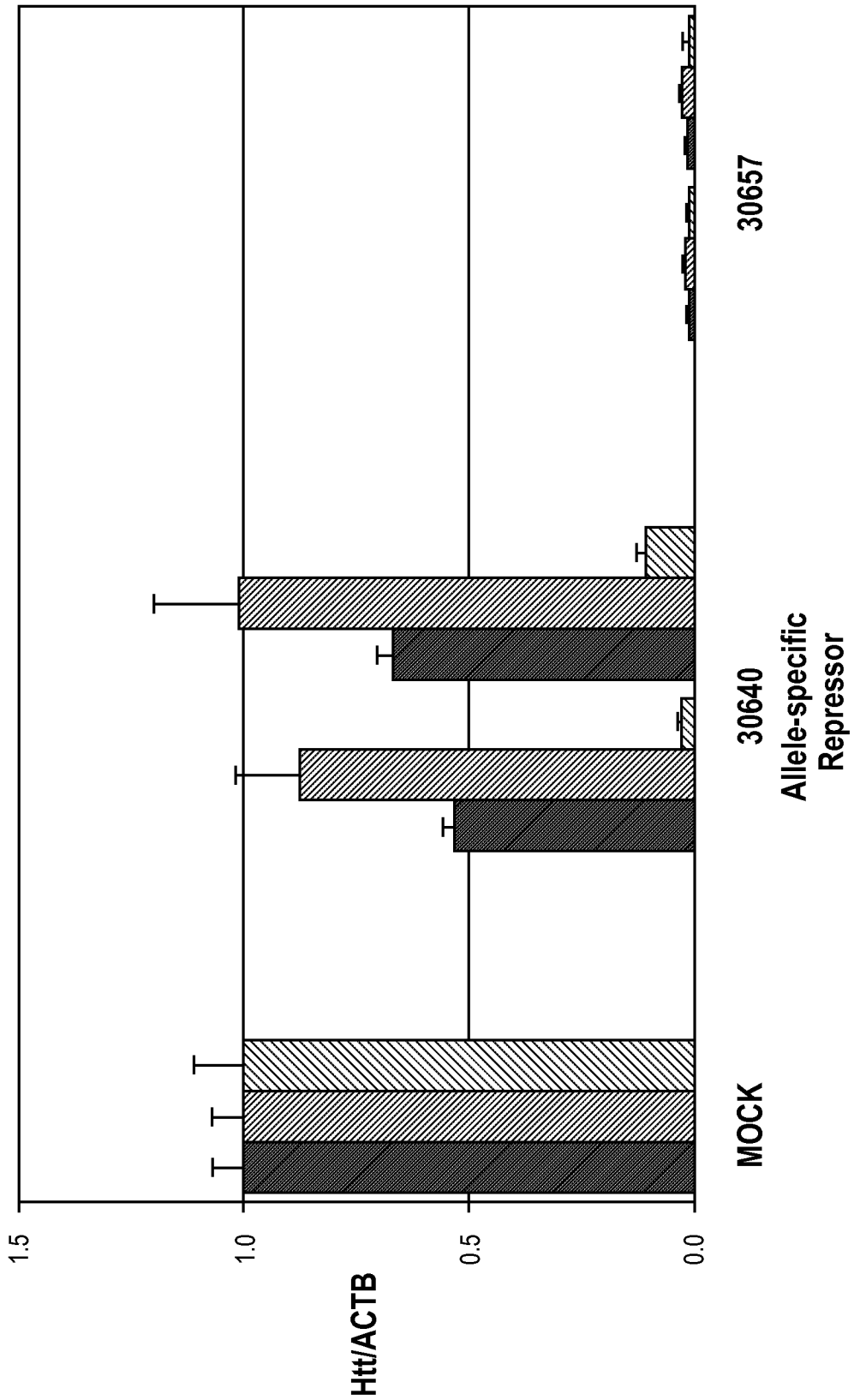

hHtt
099T (CAG15)
099C (CAG70)
Htt/ACTB
1.5
1.0
0.5
0.0
MOCK
30640
Allele-specific
Repressor
30657

FIG. 3F

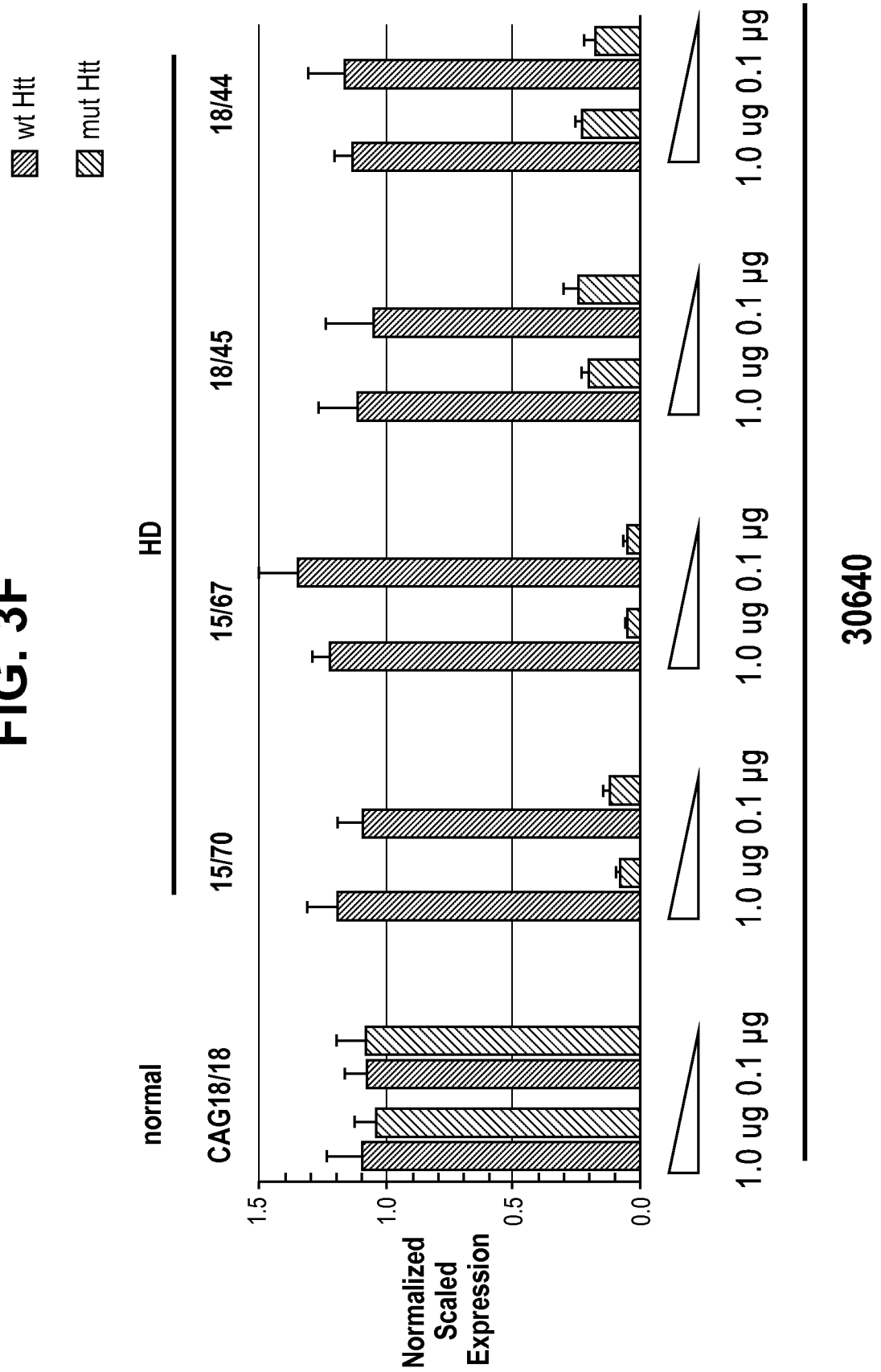
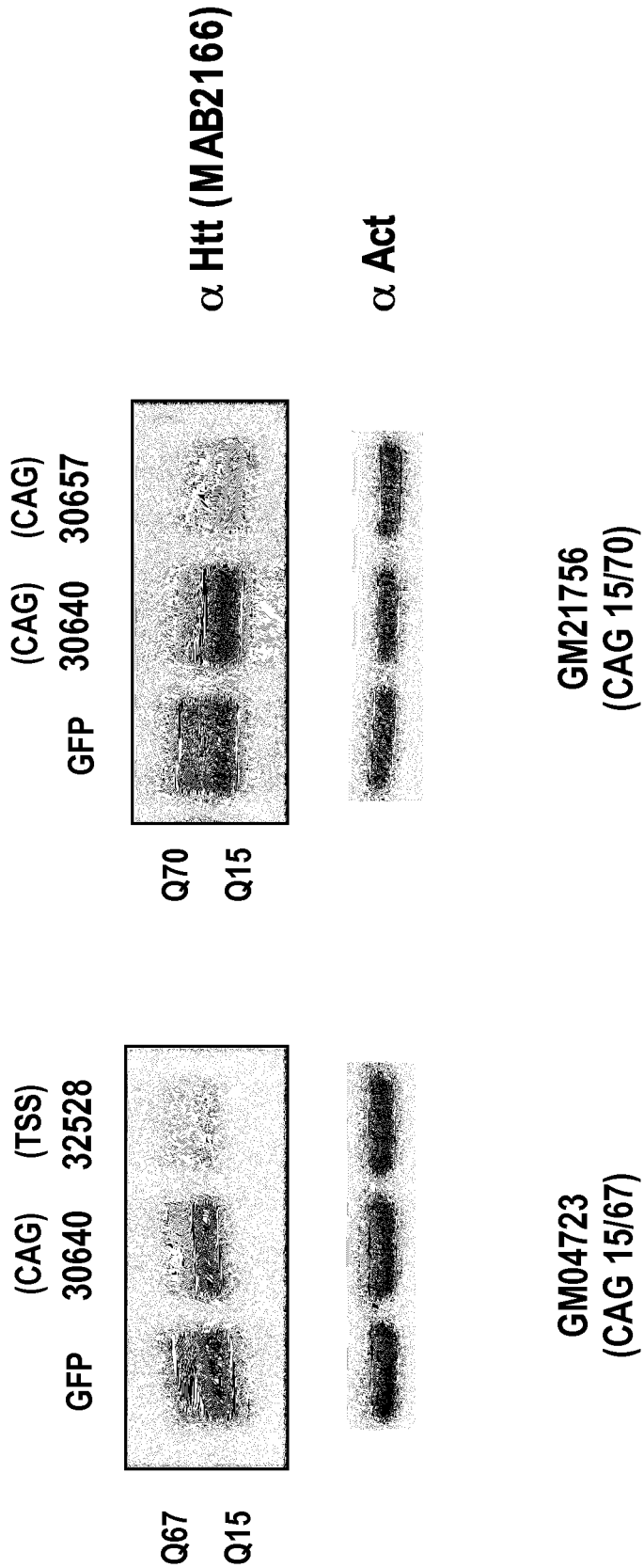

wt Htt
mut Htt
normal
HD
CAG18/18
15/70
15/67
18/45
18/44
1.0 ug 0.1 µg
1.0 ug 0.1 µg
1.0 ug 0.1 µg
1.0 ug 0.1 µg
1.0 ug 0.1 µg
30640
Normalized Scaled Expression
0.0
0.5
1.0
1.5

FIG. 3G


α Htt (MAB2166)
α Act
GFP (CAG) 30640 (CAG) 30657
Q70
Q15
GM21756
(CAG 15/70)
GFP (CAG) 30640 (TSS) 32528
Q67
Q15
GM04723
(CAG 15/67)

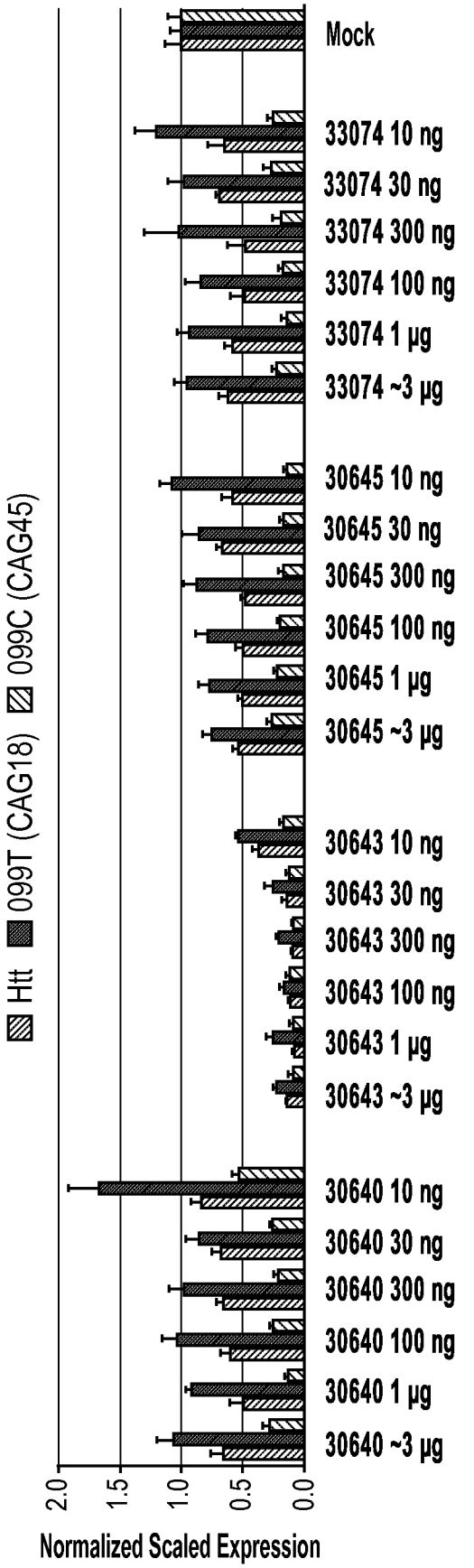

Mock
33074 10 ng
33074 30 ng
33074 300 ng
33074 100 ng
33074 1 µg
33074 ~3 µg
30645 10 ng
30645 30 ng
30645 300 ng
30645 100 ng
30645 1 µg
30645 ~3 µg
30643 10 ng
30643 30 ng
30643 300 ng
30643 100 ng
30643 1 µg
30643 ~3 µg
30640 10 ng
30640 30 ng
30640 300 ng
30640 100 ng
30640 1 µg
30640 ~3 µg
Htt
099T (CAG18)
099C (CAG45)
2.0
1.5
1.0
0.5
0.0
Normalized Scaled Expression

FIG. 4A

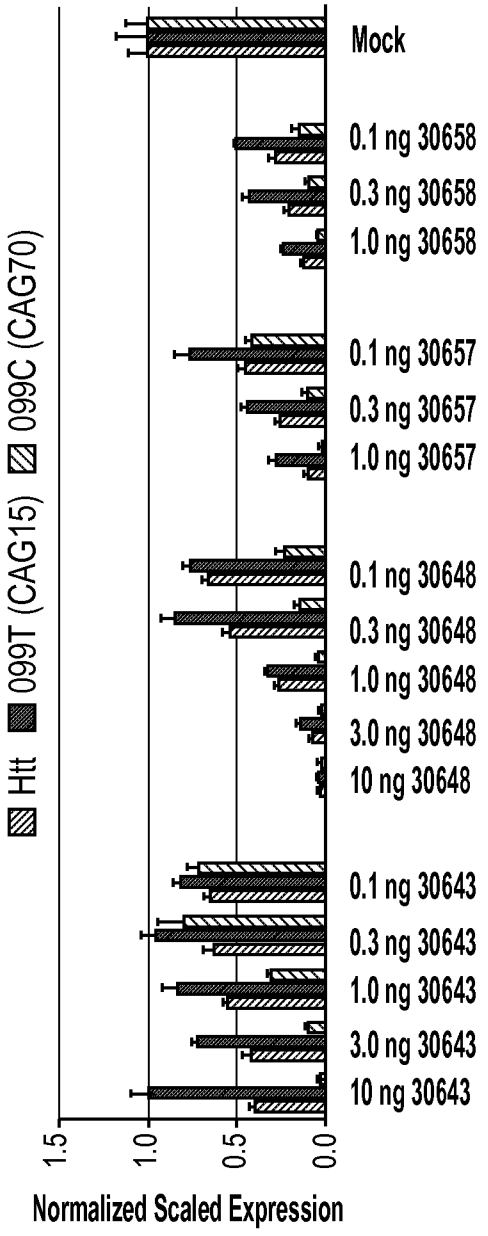
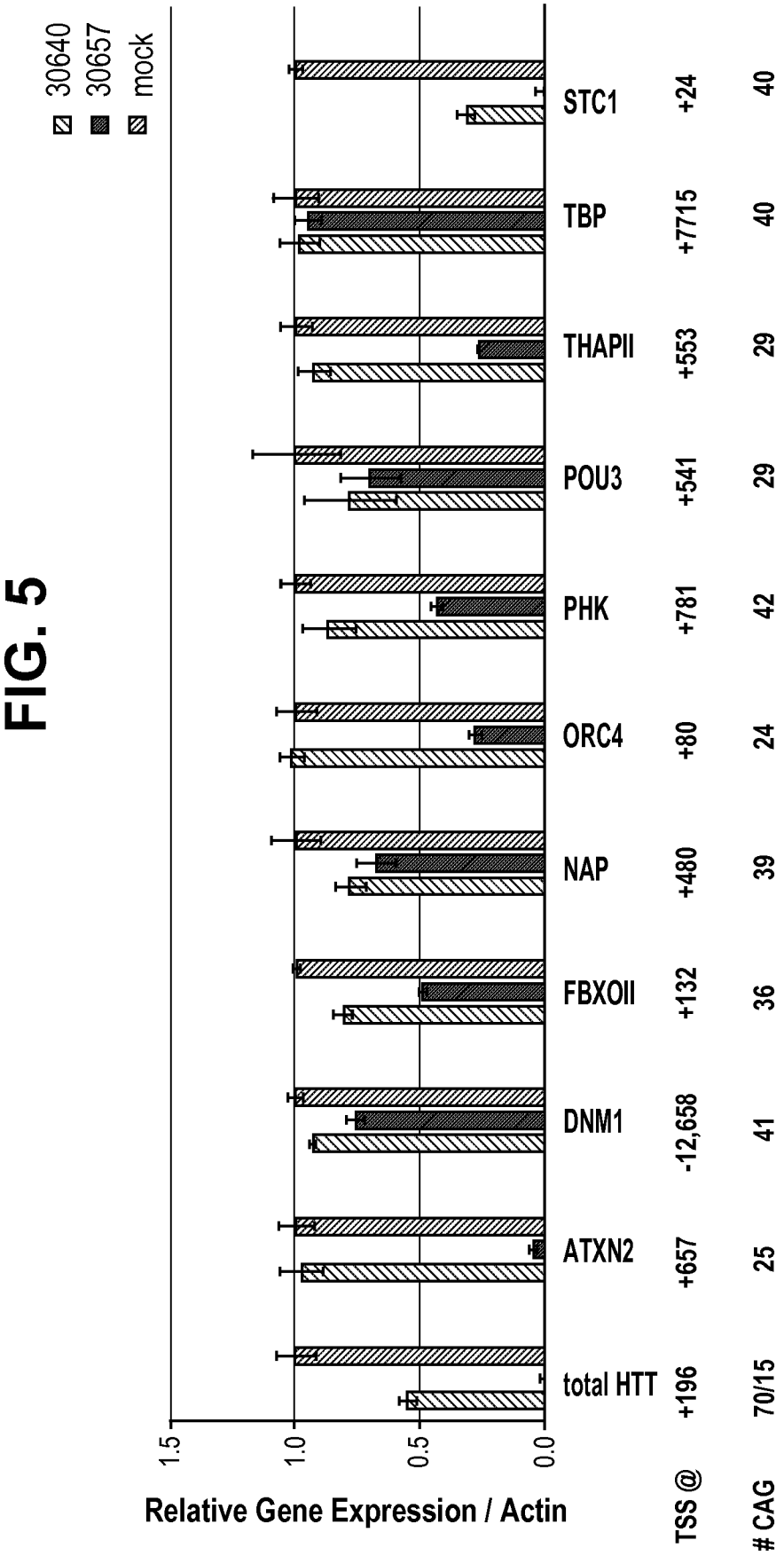

Mock
0.1 ng 30658
0.3 ng 30658
1.0 ng 30658
0.1 ng 30657
0.3 ng 30657
1.0 ng 30657
0.1 ng 30648
0.3 ng 30648
1.0 ng 30648
3.0 ng 30648
10 ng 30648
0.1 ng 30643
0.3 ng 30643
1.0 ng 30643
3.0 ng 30643
10 ng 30643
Htt
099T (CAG15)
099C (CAG70)
1.5
1.0
0.5
0.0
Normalized Scaled Expression

FIG. 4B

FIG. 5


30640
30657
mock
STC1
TBP
THAPII
POU3
PHK
ORC4
NAP
FBXOII
DNM1
ATXN2
total HTT
+24
+7715
+553
+541
+781
+80
+480
+132
-12,658
+657
+196
40
40
29
29
42
24
39
36
41
25
70/15
TSS @
CAG
1.5
1.0
0.5
0.0
Relative Gene Expression / Actin

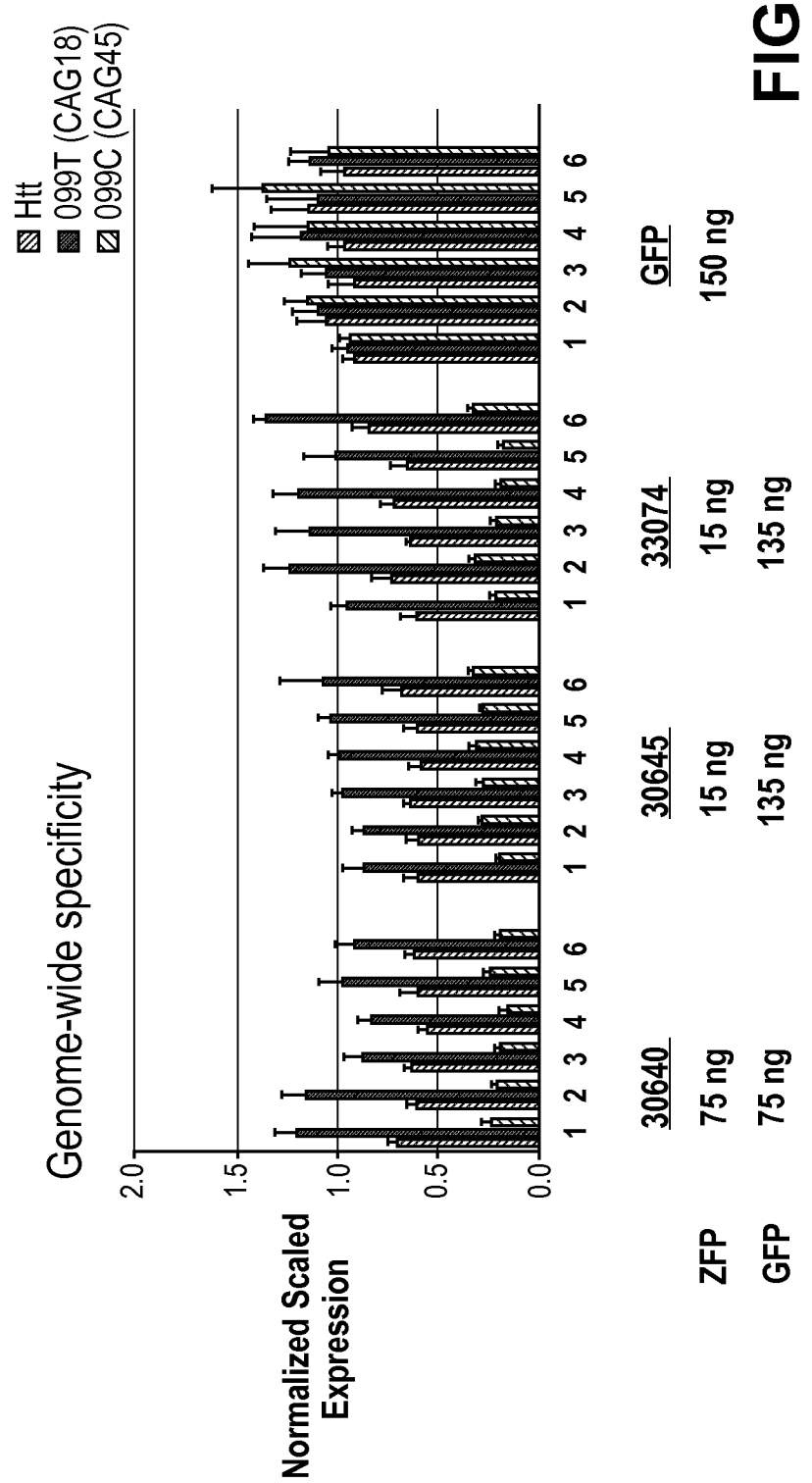


FIG. 6A

ZFP	# of down-regulated genes	# of up-regulated genes	cell line tested
30640	2	0	HD fibroblast
30645	1	0	HD fibroblast
33074	1	0	HD fibroblast

FIG. 6B

FIG. 7

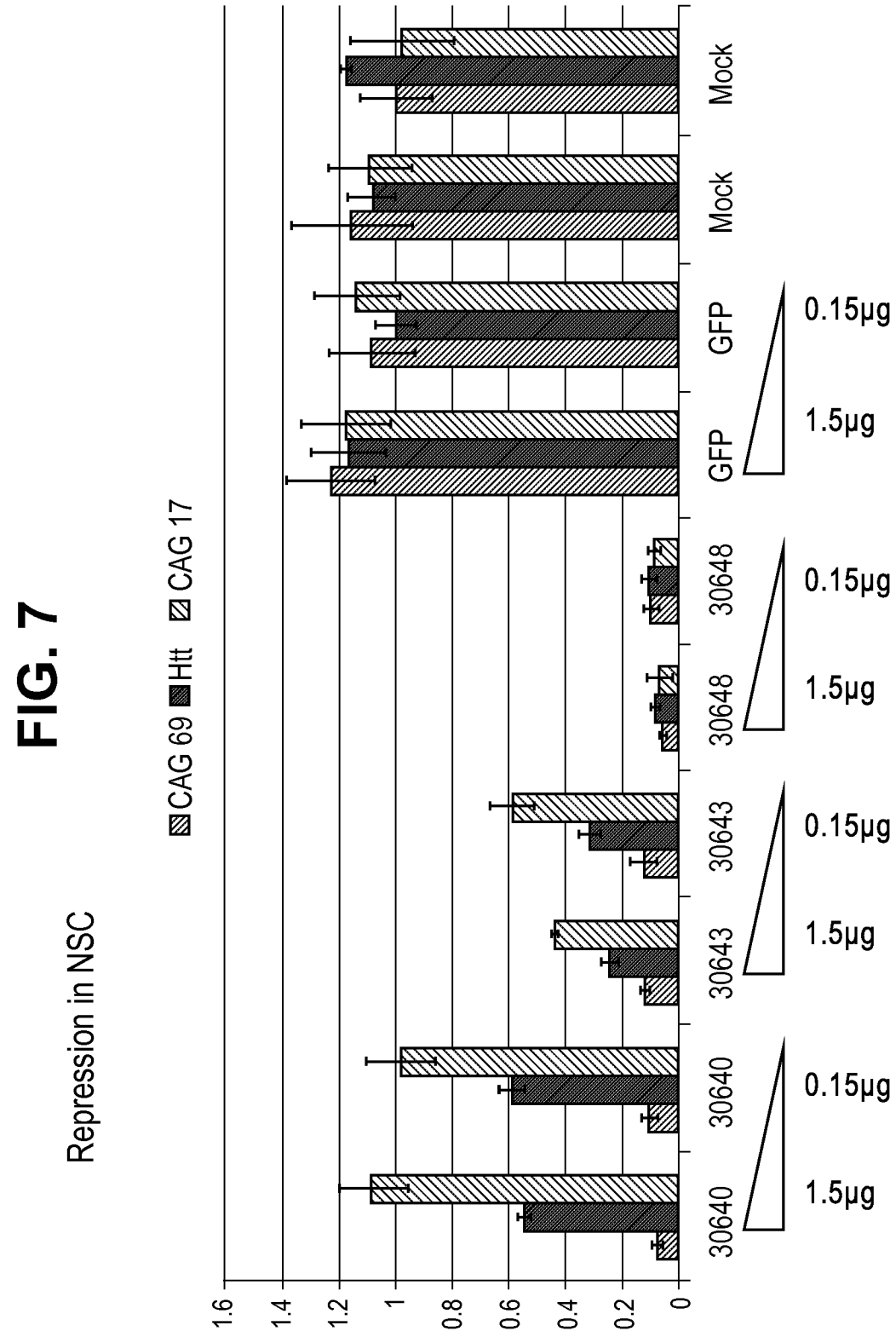
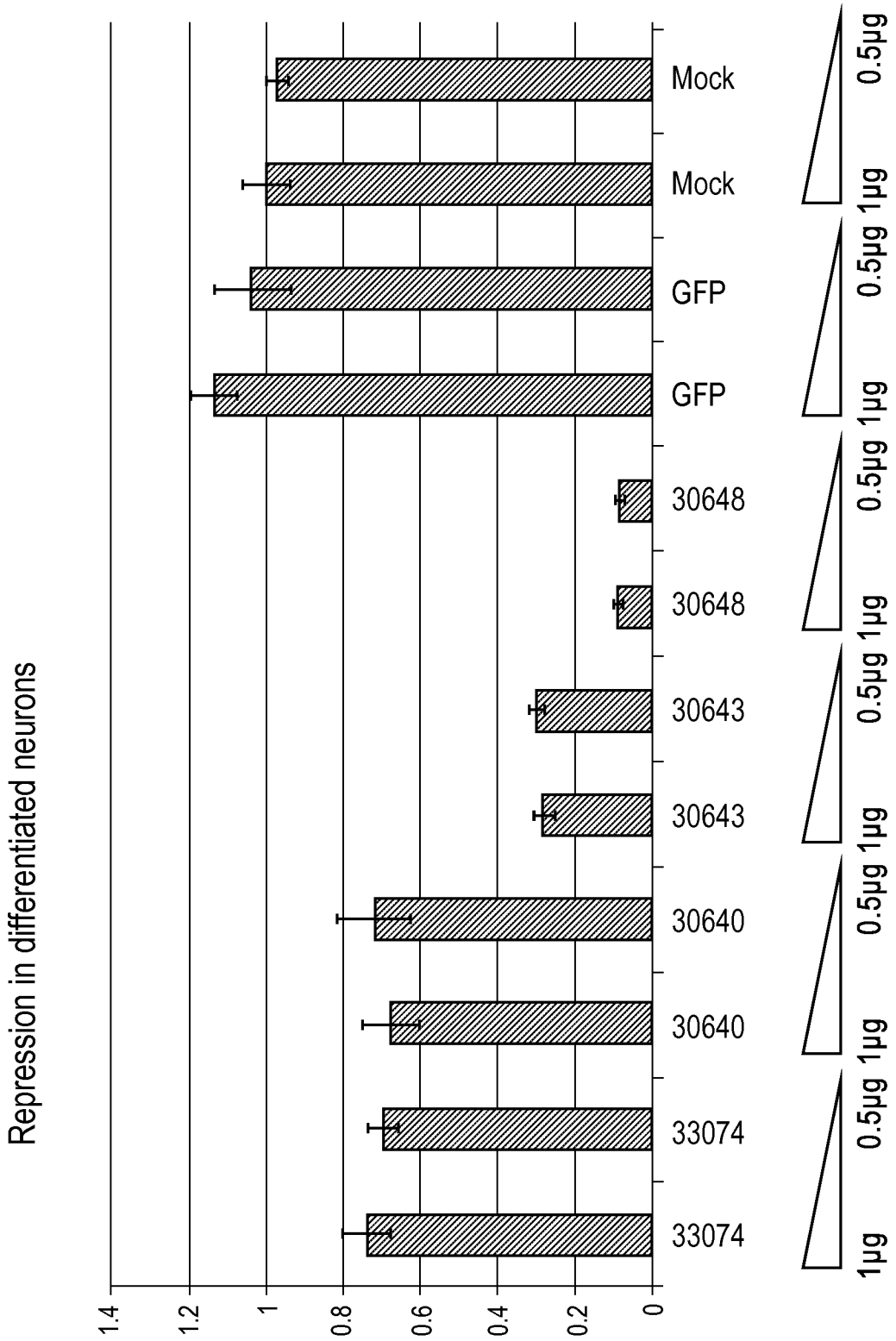

Repression in NSC
CAG 69
Htt
CAG 17
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Mock
GFP
GFP
30648
30648
30643
30643
30640
30640
0.15µg
1.5µg
0.15µg
1.5µg
0.15µg
1.5µg
0.15µg
1.5µg

FIG. 8


Repression in differentiated neurons
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Mock
GFP
GFP
30648
30648
30643
30643
30640
30640
33074
33074
0.5µg
0.5µg 1µg
0.5µg 1µg
0.5µg 1µg
0.5µg 1µg
0.5µg 1µg
1µg

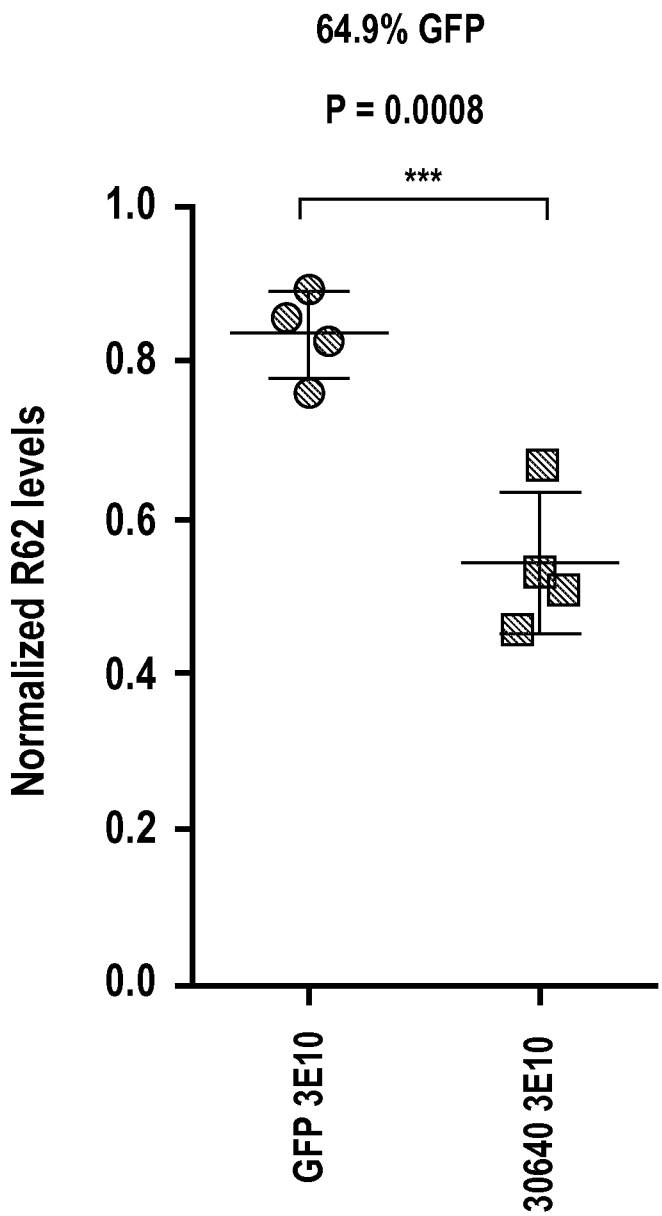

64.9% GFP
P = 0.0008
***
Normalized R62 levels
1.0
0.8
0.6
0.4
0.2
0.0
GFP 3E10
30640 3E10

FIG. 9

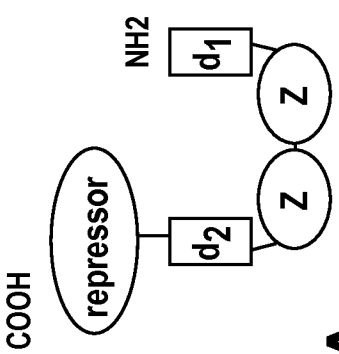


FIG. 10A

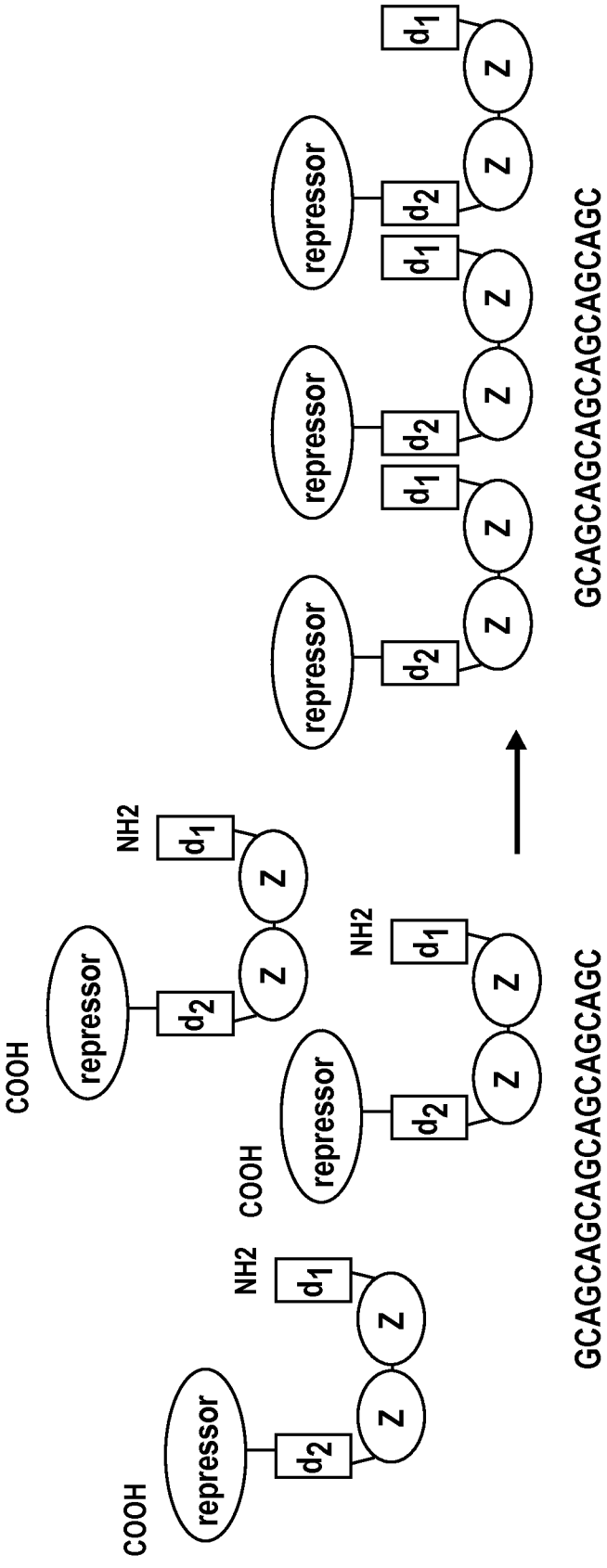


FIG. 10B

FIG. 10C

DZ#1:

*MAPKKKRKVGIH*GVLRGAATKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHSGVP**[ZFP]**
GSGGTKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHLRQKDAARSRSGMDAKSLTAWSRT
LVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSVDYKDDDDK

DZ#2

*MAPKKKRKVGIH*GVLRGAAFKCEHCRILFLDHVMFTIHMGCHGFRDPFKCNMCGEKCDGPVGLFVHMARNAHSGVP**[ZFP]**
GSGGTKCVHCGIVFLDEVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHLRQKDAARSRSGMDAKSLTAWSRT
LVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSVDYKDDDDK

DZ#3

*MAPKKKRKVGIH*GVLRGAAFKCEHCRILFLDHVMFTIHMGCHGFRDPFKCNMCGEKCDGPVGLFVHMARNAHSGVP**[ZFP]**
GSGGHHCQHCDMYFADNILYTIHMGCHGYENPFECNICGYHSQDRYEFSSHIVRGEHLRQKDAARSRSGMDAKSLTAWSRTLVTFKDVFVDF
TREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSVDYKDDDDK

DZ#4

*MAPKKKRKVGIH*GVLRGAAHHCQHCDMYFADNILYTIHMGCHSCDDVFKCNMCGEKCDGPVGLFVHMARNAHGEKPTKCVHCGIVFLD
EVMYALHMSCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHSGVP**[ZFP]**GSGGFKCEHCRILFLDHVMFTIHMGCHGFRDPFKCNMC
GEKCDGPVGLFVHMARNAHGEKPFYCEHCEITFRDVVMYSLHKGYHGFRDPFECNICGYHSQDRYEFSSHIVRGEHLRQKDAARSRSGM
DAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAF
EIKSSVDYKDDDDK

FIG. 10D

CC#1:

*MAPKKKRKVGIH*GVLRGAAGGAQLEKELQALEKKLAQLEWENQALEKELAQGGSGVP[**ZFP**]GSGGAQLKKKLQANKKELAQLKWKLQALK KKLAQGGLRQKDAARSRSGMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEP WLVEREIHQETHPDSETAFEIKSSVDYKDDDDK

CC#2:

*MAPKKKRKVGIH*GVLRGAAGGEQLEKKLQALEKKLAQLEWKNQALEKKLAQGGSGVP[**ZFP**]GSGGALKKELQANKKELAQLKWELQALKK ELAQGGLRQKDAARSRSGMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPW LVEREIHQETHPDSETAFEIKSSVDYKDDDDK

CC#3:

*MAPKKKRKVGIH*GVLRGAAGGEQLEKKLQALEKKLAQLEWKNQALEKGGSGVP[**ZFP**]GSGGELQANKKELAQLKWELQALKKELAQGGL RQKDAARSRSGMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREI HQETHPDSETAFEIKSSVDYKDDDDK

CC#4:

*MAPKKKRKVGIH*GVLRGAAGGEQLEKKLQALEKKLAQLEWKNQAGGSGVP[**ZFP**]GSGGQANKKELAQLKWELQALKKELAQGGLRQKD AARSRSGMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQE THPDSETAFEIKSSVDYKDDDDK.

FIG. 10D (CONT.)

CC2-ZFP-KOX:

*MAPKKKRKVGI*HGVLAAGGEQLEKKLQALEKKLAQLEWKNQALEKKLAQGGSGVP[**ZFP**]GSGGALKKELQANKKELAQLKWELQALKKELAQGS
GMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEI
KSSVDYKDDDDK

CC5-ZFP-KOX:

*MAPKKKRKVGI*HGVLAAGGEQLEKKLQALEKKLAQLEWKNQALEKKLAQGVP[**ZFP**]GSGGALKKELQANKKELAQLKWELQALKKELAQGS
GMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAF
EIKSSVDYKDDDDK

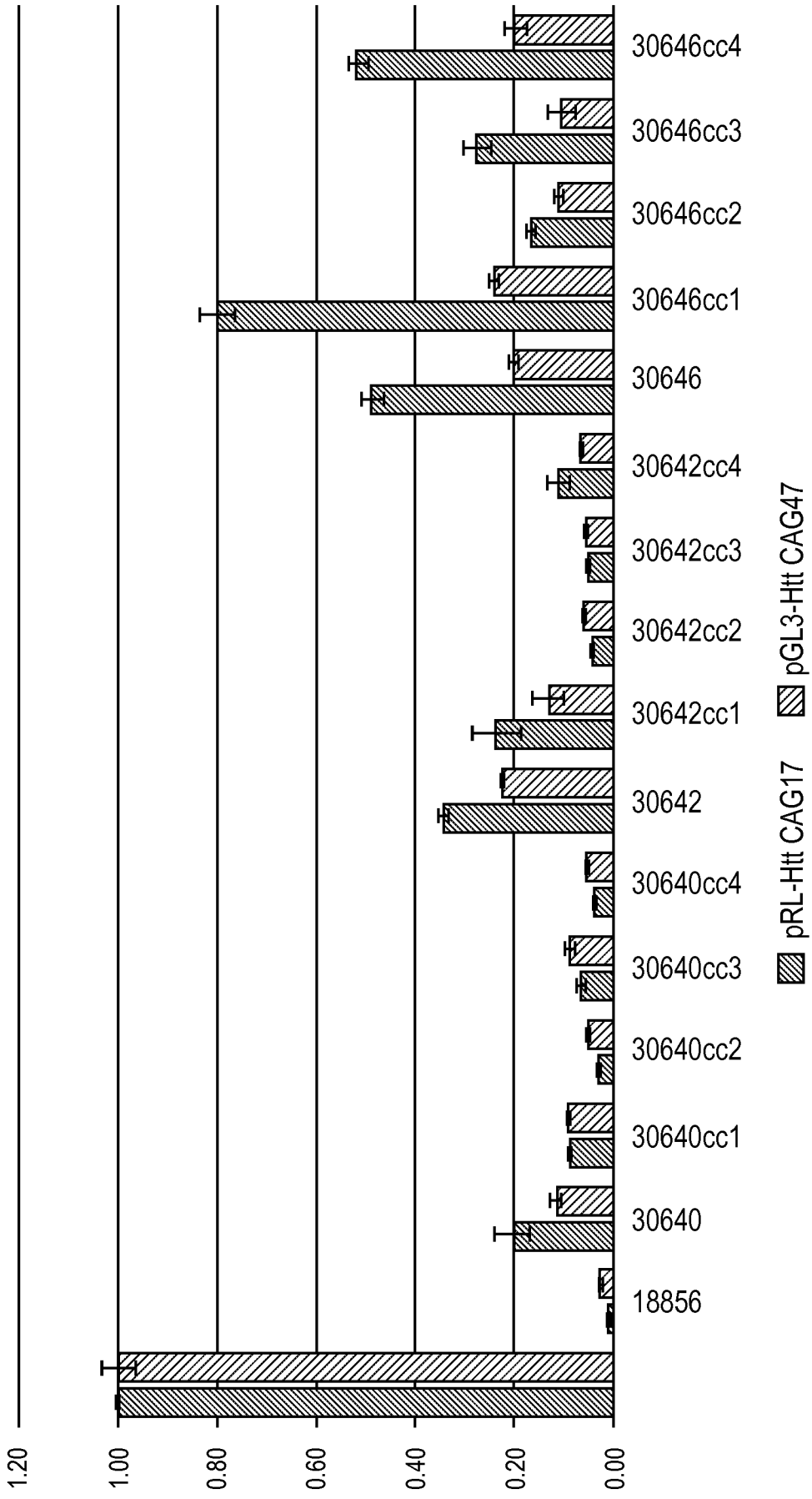
CC6-ZFP-KOX:

*MAPKKKRKVGI*HGVLAAGGEQLEKKLQALEKKLAQLEWKNQALEKKLAQGGSGVP[**ZFP**]GSALKKELQANKKELAQLKWELQALKKELAQGS
GMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAF
EIKSSVDYKDDDDK

CC7-ZFP-KOX:

*MAPKKKRKVGI*HGVLAAGGEQLEKKLQALEKKLAQLEWKNQALEKKLAQGVP[**ZFP**]GSALKKELQANKKELAQLKWELQALKKELAQGS
GMDAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQIVYRNVMLENYKNLVSLGYQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETA
FEIKSSVDYKDDDDK

FIG. 11A


30646cc4
30646cc3
30646cc2
30646cc1
30646
30642cc4
30642cc3
30642cc2
30642cc1
30642
30640cc4
30640cc3
30640cc2
30640cc1
30640
18856
1.20
1.00
0.80
0.60
0.40
0.20
0.00
pRL-Htt CAG17
pGL3-Htt CAG47

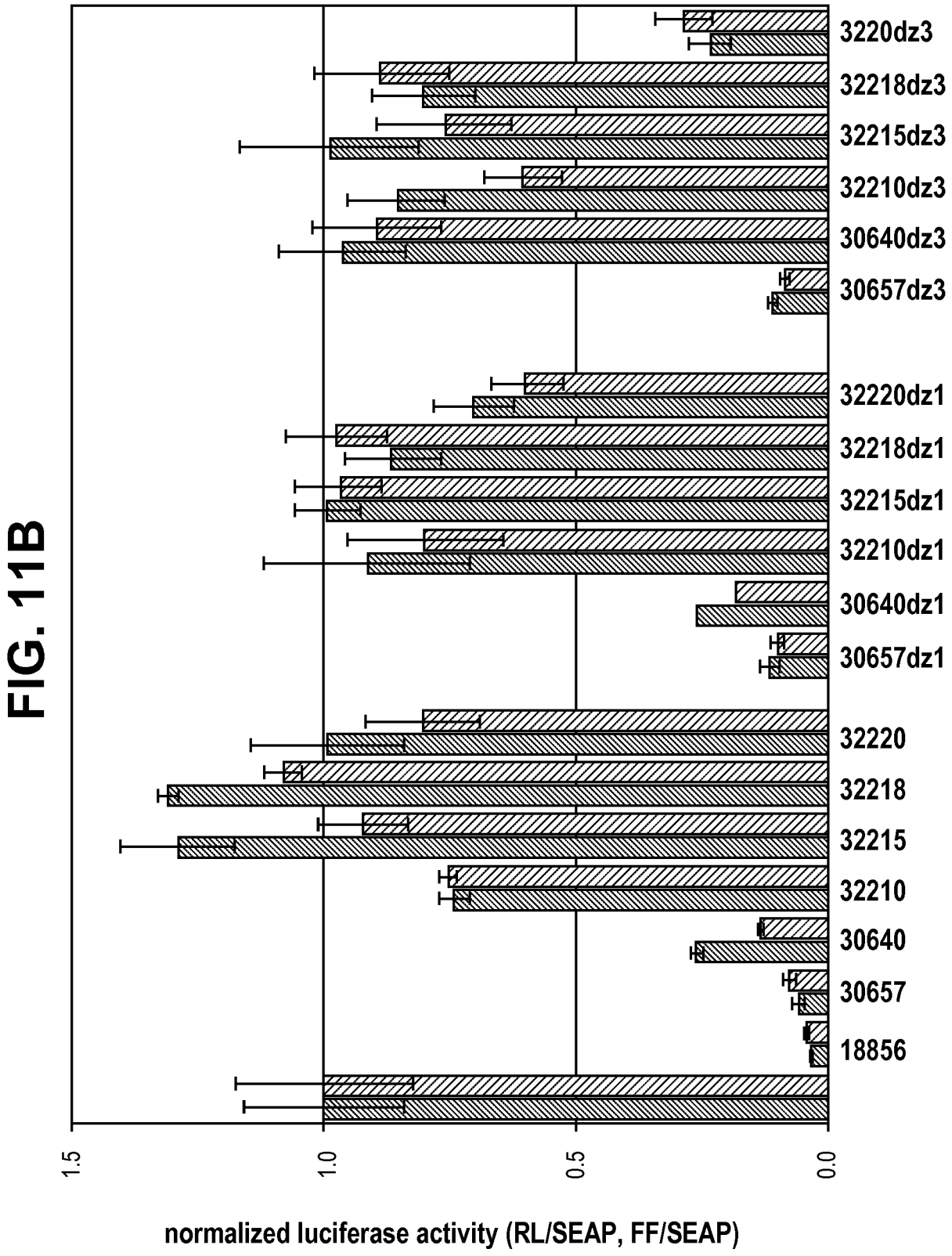

3220dz3
32218dz3
32215dz3
32210dz3
30640dz3
30657dz3
32220dz1
32218dz1
32215dz1
32210dz1
30640dz1
30657dz1
32220
32218
32215
32210
30640
30657
18856
1.5
1.0
0.5
0.0
normalized luciferase activity (RL/SEAP, FF/SEAP)

FIG. 11B

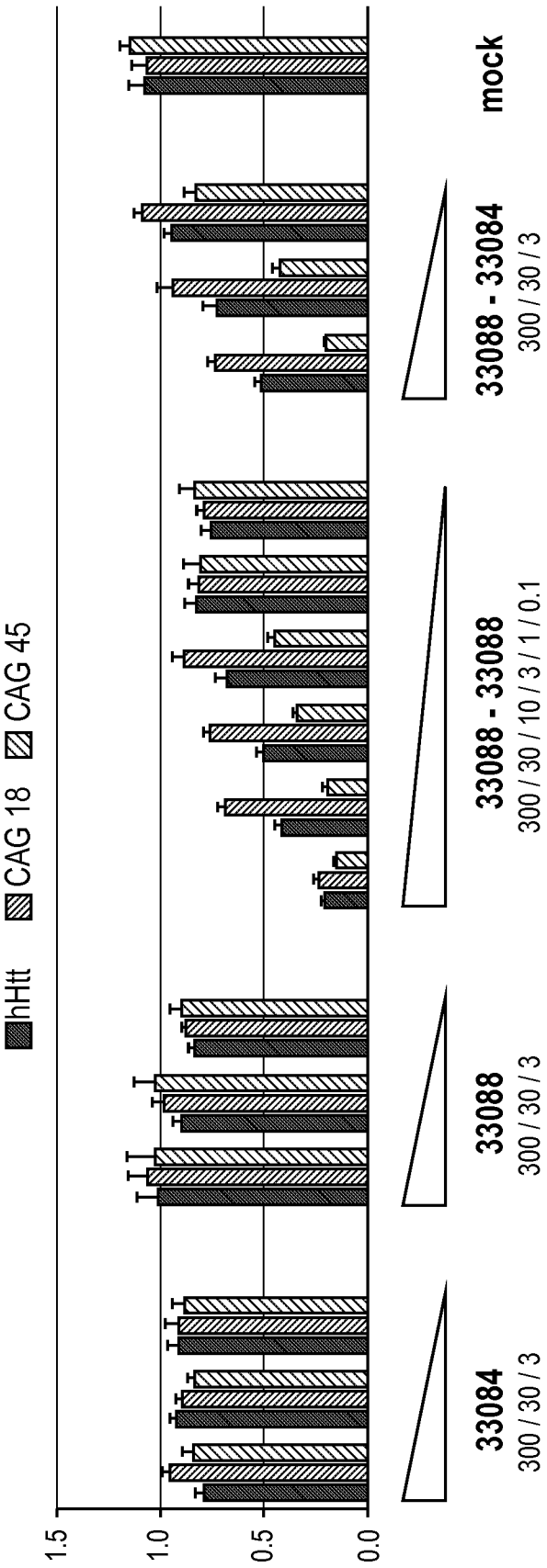


FIG. 12A

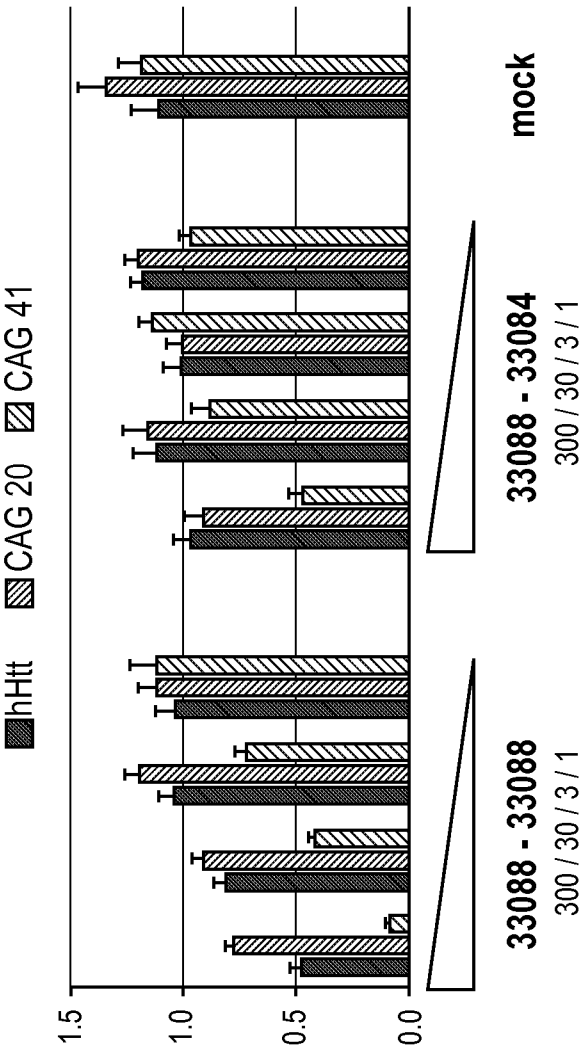


FIG. 12B

FIG. 13A

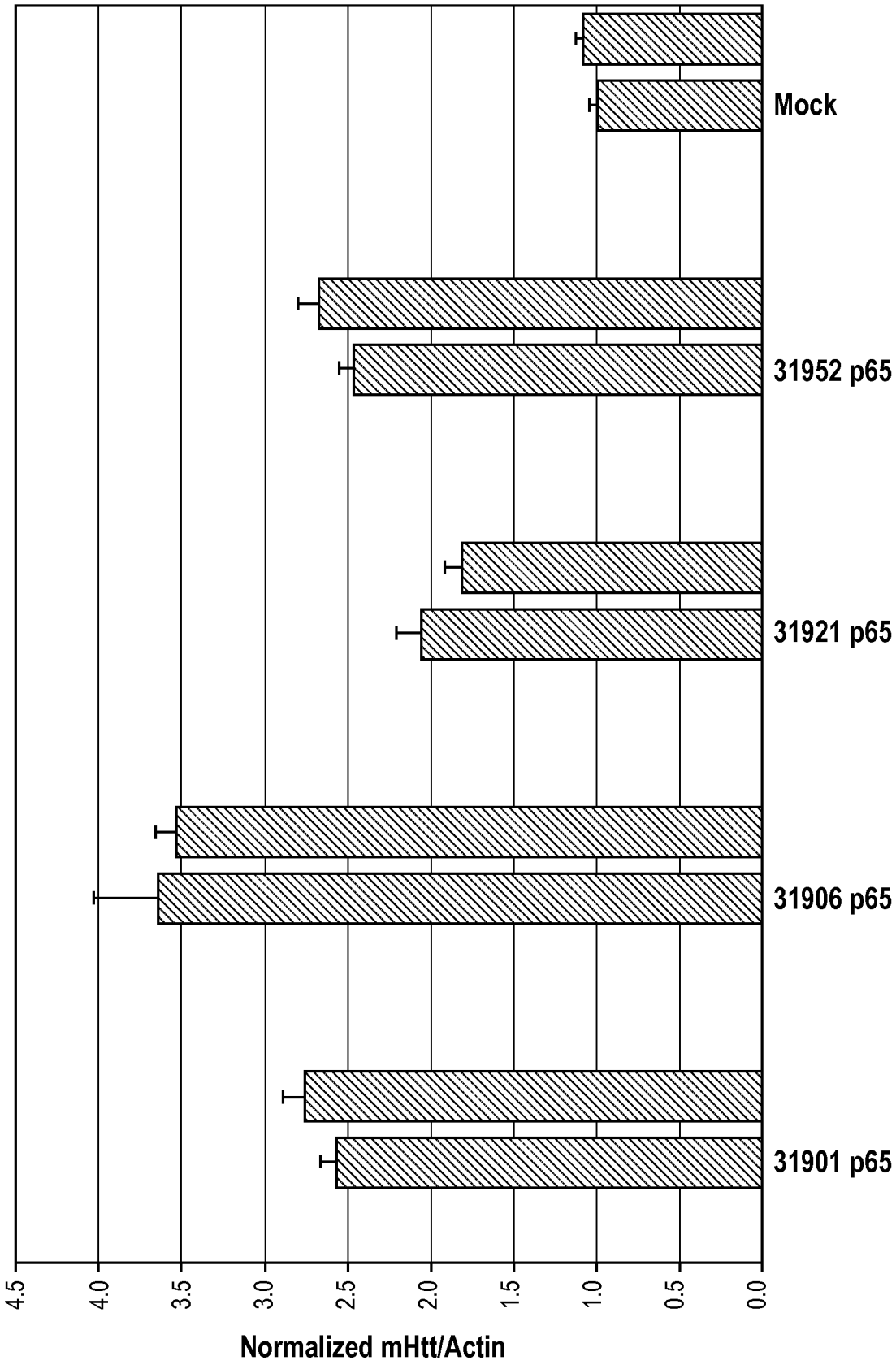
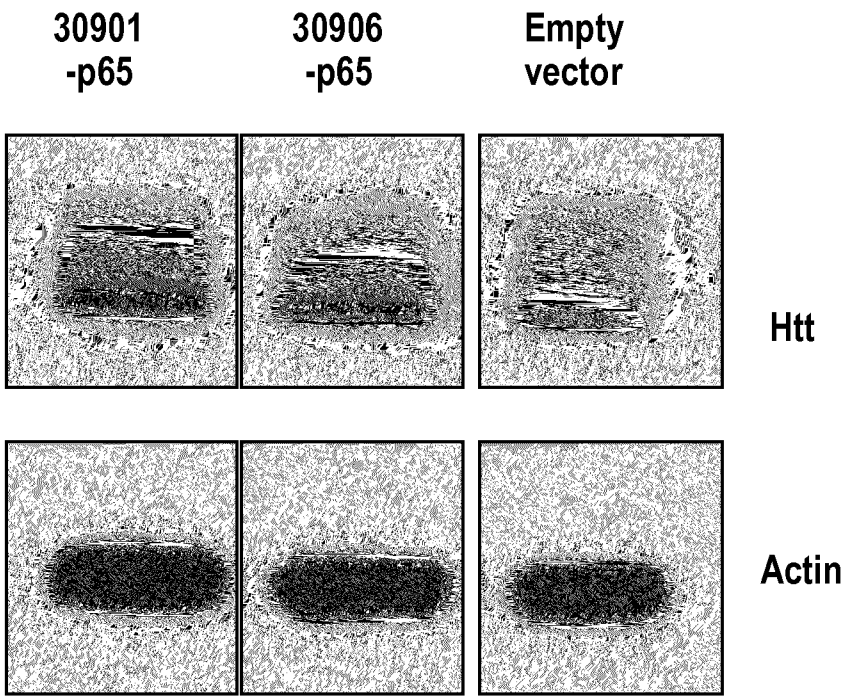

Mock
31952 p65
31921 p65
31906 p65
31901 p65
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Normalized mHtt/Actin

FIG. 13B



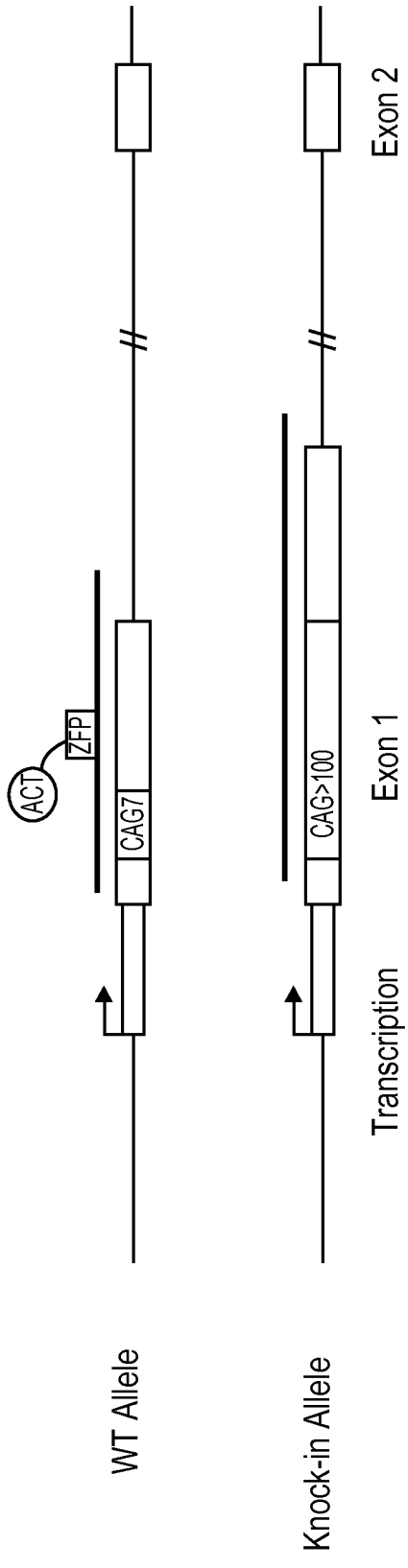


FIG. 13C

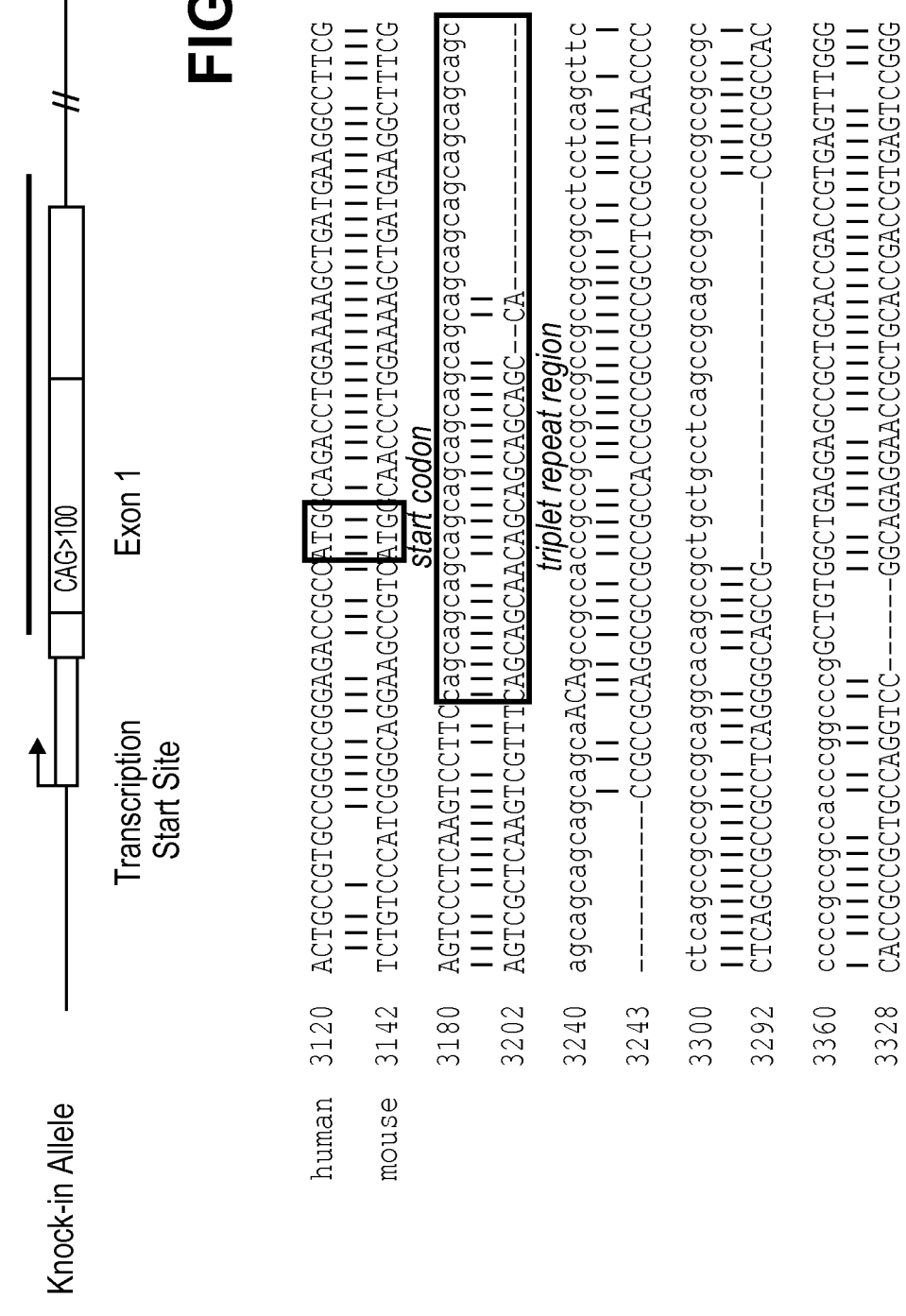
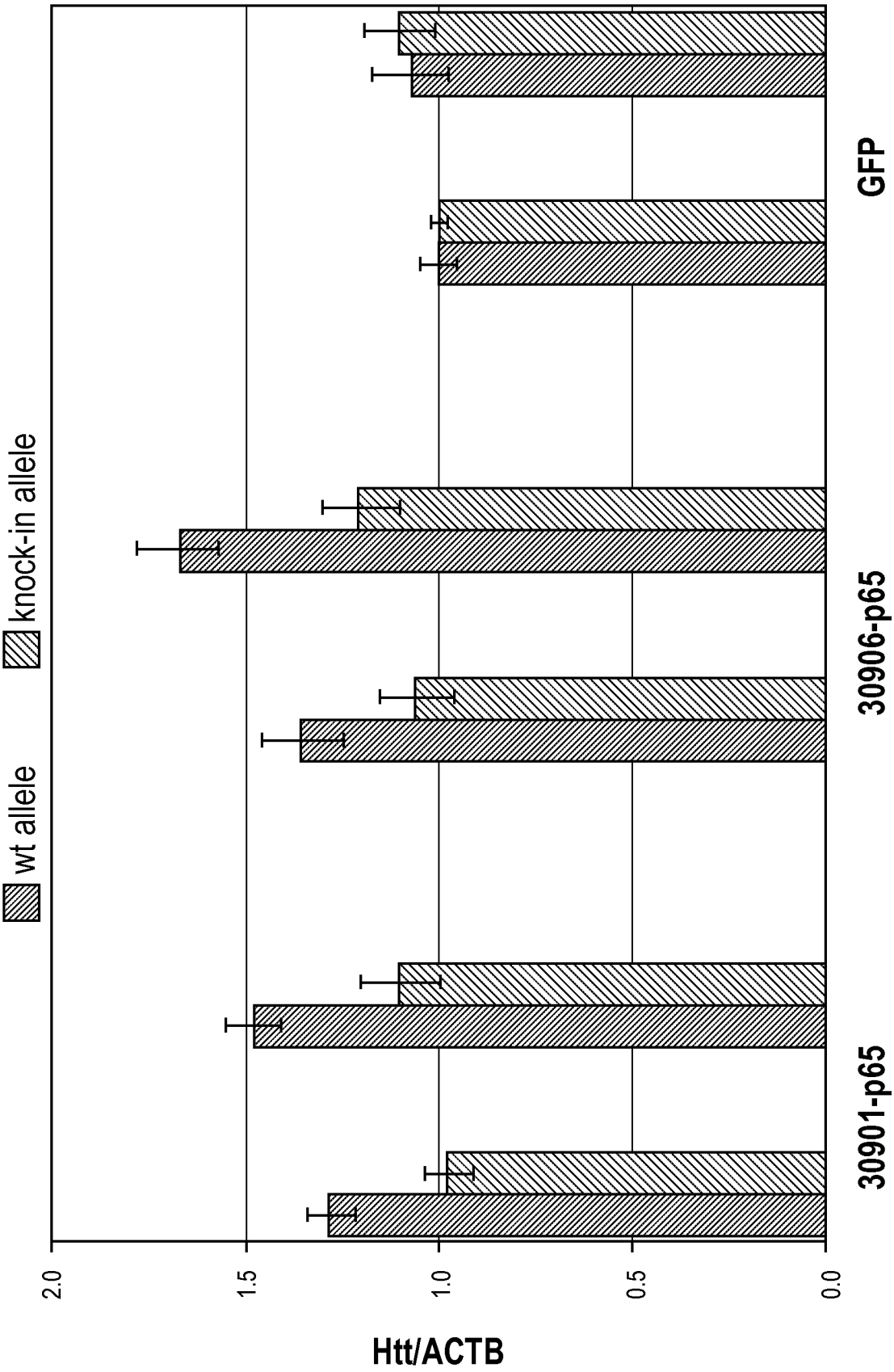
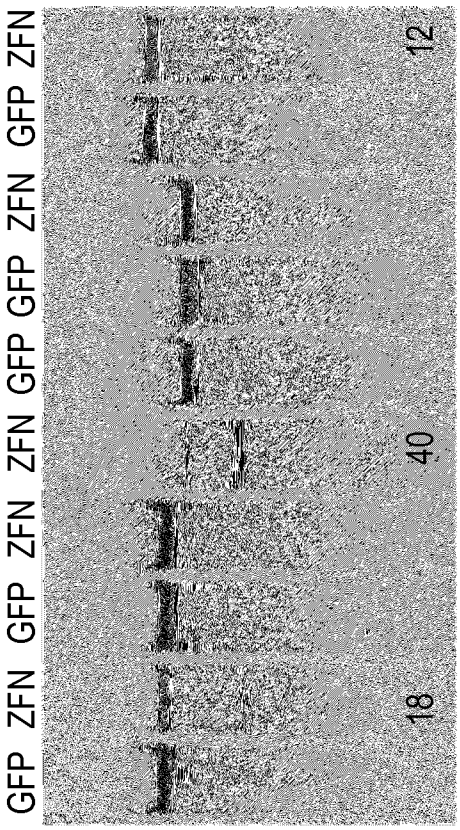


FIG. 13D

FIG. 13E


wt allele
knock-in allele
2.0
1.5
1.0
0.5
0.0
Htt/ACTB
30901-p65
30906-p65
GFP



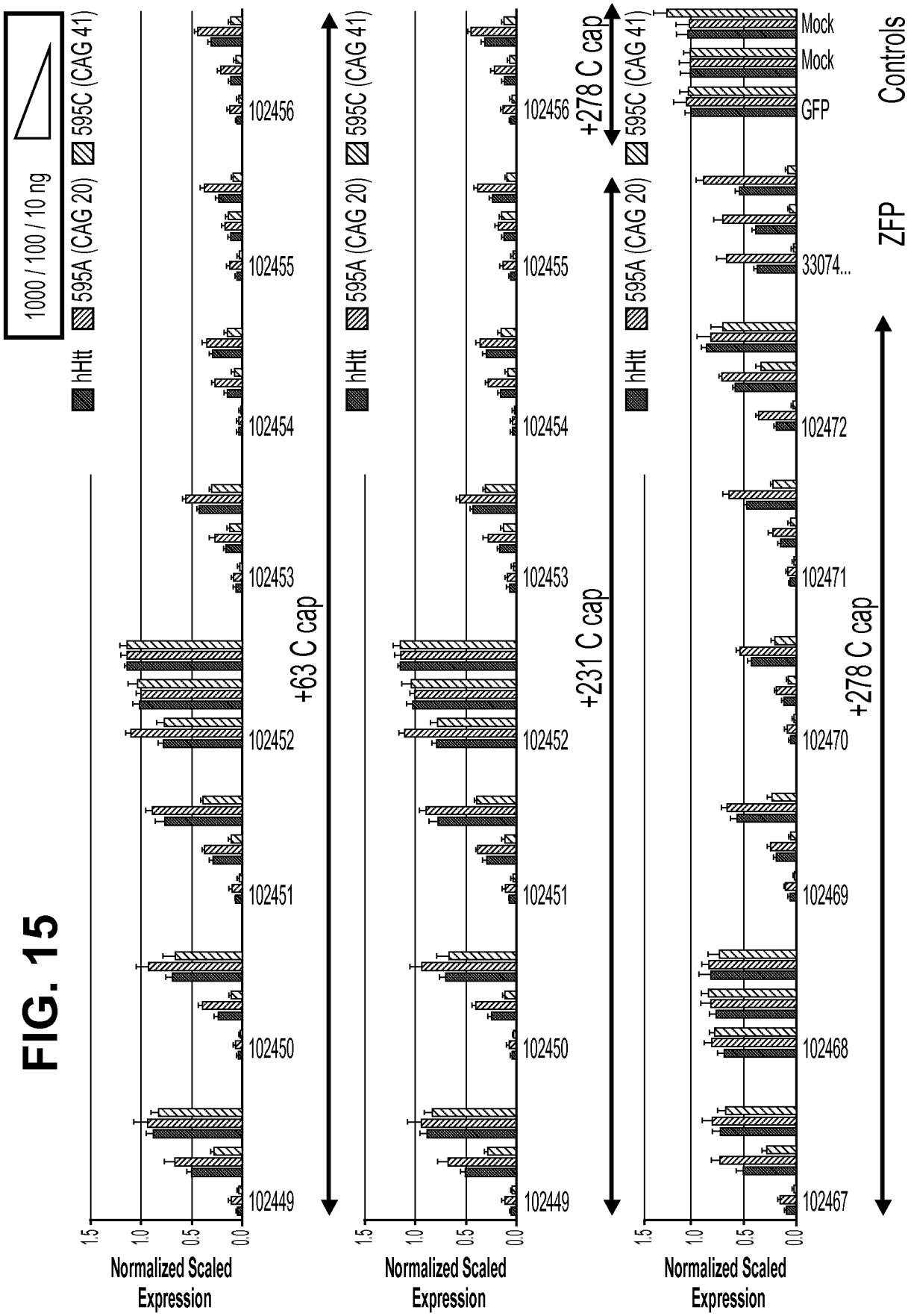
a: 29627/29628
b: 29631/29632
c: 29637/29638

FIG. 14A

d: 25917/25916
e: 25921/25920
f: 25923/25922

FIG. 14B

FIG. 15


1000 / 100 / 10 ng
hHtt
595A (CAG 20)
595C (CAG 41)
102456
102455
102454
102453
102452
102451
102450
102449
+63 C cap
+231 C cap
+278 C cap
Mock
Mock
GFP
33074...
102472
102471
102470
102469
102468
102467
Controls
ZFP
1.5
1.0
0.5
0.0
Normalized Scaled Expression

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/28348

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; C12N 9/22 (2013.01)
USPC - 435/6.1, 69.1, 199
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)
IPC(8) - C12Q 1/68; C12N 9/22 (2013.01)
USPC: 435/6.1, 69.1, 199

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase; PubWEST (PGPB, USPT, USOC, EPAB, JPAB); Google and PubMed
Search Terms: htt, huntingtin, Hungtington's disease, trinucleotide repeat, ZFN, zinc finger nuclease.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/0082093 A1 (GREGORY et al.) 07 April 2011 (07.04.2011) para [0029], [0084], [0086], [0112], [0170], [0173]; Table 1, SBS#18856; SEQ ID NOs: 3, 24-27 and 33	1-4
A	MITTELMAN, D. et al. Zinc-finger directed double-strand breaks within CAG repeat tracts promote repeat instability in human cells. Proc. Natl. Acad. Sci. USA. 16 June 2009, Vol. 106, No. 24, pages 9607-9612, entire document.	1-4

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
14 May 2013 (14.05.2013)	31 MAY 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/28348

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore 6.4.1: SEQ ID NOs: 1-4 and 6-16.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/28348

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.: 5-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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.