



(51) International Patent Classification:

C07K 14/48 (2006.01) A61K 38/00 (2006.01)
C07K 14/475 (2006.01)

(21) International Application Number:

PCT/US2017/064934

(22) International Filing Date:

06 December 2017 (06.12.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/443,554 06 January 2017 (06.01.2017) US
62/515,746 06 June 2017 (06.06.2017) US

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,

TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))
— with sequence listing part of description (Rule 5.2(a))

(54) Title: CILIARY NEUROTROPHIC FACTOR RECEPTOR LIGANDS AND METHODS OF USING THE SAME

(57) Abstract: Provided are ciliary neurotrophic factor receptor (CNTFR) ligands. In certain aspects, a CNTFR ligand of the present disclosure exhibits increased affinity for CNTFR relative to the corresponding wild-type CNTFR ligand. In certain aspects, a CNTFR ligand of the present disclosure results in reduced binding affinity of glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), or both, for a complex including the CNTFR ligand and CNTFR, relative to the binding affinity for a complex including the corresponding wild-type CNTFR ligand and CNTFR. In certain aspects, a CNTFR ligand of the present disclosure has both of the aforementioned properties. Also provided are pharmaceutical compositions including the CNTFR ligands, as well as methods of using the CNTFR ligands.



CILIARY NEUROTROPHIC FACTOR RECEPTOR LIGANDS AND METHODS OF USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Patent Application No. 62/443,554, filed January 6, 2017, and U.S. Provisional Patent Application No. 62/515,746, filed June 6, 2017, which applications are incorporated herein by reference in their entirety.

INTRODUCTION

10 Ciliary neurotrophic factor (CNTF) was identified as a survival factor for chick ciliary neurons and belongs to the interleukin (IL)-6 family of structurally related hemato- and neuropoietic cytokines (IL-6, IL-11, cardiotrophin-like cytokine factor 1 (CLCF1), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1)). Cellular responses to CNTF and IL-6 type cytokines are elicited by different multi-unit receptor complexes that include the membrane-spanning 130-kDa glycoprotein, gp130. CNTF first binds in a 1:1
15 stoichiometry to the GPI-anchored CNTF receptor (CNTFR), which is not involved in signal transduction. Binding of CNTF to the membrane-bound or soluble CNTFR induces a heterodimer of the signal transducing β -receptors gp130 and LIF receptor (LIFR), which triggers intracellular signaling cascades.

20 Cancer is initiated and progresses within a microenvironment that is itself altered as a consequence of the tumorigenic process. Stromal cells in contact with cancer cells secrete growth factors and cytokines that may act directly by signaling to tumor cells or indirectly by recruiting other stromal components to promote tumor progression. An important aspect of this process is the expansion of cancer-associated fibroblasts (CAFs). CAFs are a diverse population of stromal cells with distinct characteristics in different
25 tumors and tissues.

 CAFs support the growth of cancer cells (e.g., lung cancer cells) *in vivo* by secretion of soluble factors that stimulate the growth of tumor cells. One such soluble factor is CLCF1. CLCF1 produced by cells in the stroma is received as a growth signal by tumor cells expressing a receptor for this protein – CNTFR. For example, functional studies have

identified a role for CLCF1-CNTFR signaling in promoting growth of non-small cell lung cancer (NSCLC).

Studies have also shown that CNTFR and its cognate ligands support the survival of neurons. For example, CNTF has been shown to have a direct neuroprotective effect on
5 degenerating motoneurons in stress-induced conditions, both in cell culture and in a rodent model of axotomy-induced apoptosis. The beneficial effect of CNTF was further supported in a mouse model of neuronopathy with motoneuron degeneration. Given these promising results, CNTF was tested in two clinical trials in 1996 with 570 and 730 patients. However, both trials reported no observable benefit of treatment, with severe adverse effects for
10 doses over 5 µg/kg. Two main reasons appeared to account for failure in these trials. First, the half-life of CNTF after intravenous injection is only ~3 min, so subcutaneously applied CNTF was unlikely to have adequately reached its target cells. Second, further research found that CNTF has another binding partner, interleukin-6 receptor (IL6R), which induces an acute-phase response on human liver cells. Attempts have been made to create CNTF
15 variants with reduced IL6R binding affinity, but these variants suffered from weak CNTFR binding, and hence limited potency.

SUMMARY

Provided are ciliary neurotrophic factor receptor (CNTFR) ligands. In certain aspects, a CNTFR ligand of the present disclosure exhibits increased affinity for CNTFR
20 relative to the corresponding wild-type CNTFR ligand. In certain aspects, a CNTFR ligand of the present disclosure results in reduced binding affinity of glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), or both, for a complex including the CNTFR ligand and CNTFR, relative to the binding affinity for a complex including the corresponding wild-type CNTFR ligand and CNTFR. In certain aspects, a CNTFR ligand of the present
25 disclosure has both of the aforementioned properties. Also provided are pharmaceutical compositions including the CNTFR ligands, as well as methods of using the CNTFR ligands.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 schematically illustrates activation of the CNTFR signaling pathway by wild-
30 type CLCF1.

FIG. 2 is a graph showing normalized expression of CNTFR in various tissues. Tissue-specific expression levels of CNTFR was acquired from Tissue-specific Gene Expression and Regulation (TiGER) database of the Bioinformatics Lab at Wilmer Eye Institute of Johns Hopkins University (<http://bioinfo.wilmer.jhu.edu/tiger/>) [10]. The gene expression pattern for each UniGene in 30 human tissues was calculated based on NCBI EST (expressed sequence tag) database. The expression level is normalized with tissue-library size. Each value for a gene in a tissue is a ratio of observed ESTs to the expected on in this tissue. The expected number of ESTs is the product of total ESTs of the gene and the fraction of total ESTs in the tissue among all ESTs in 30 tissues.

FIG. 3, panels A-D, shows data demonstrating that recombinant CLCF1 activates STAT3. The levels of p-STAT3 at Tyr705 in SH-SY5Y cell line (panels A and B) and E18 cells (panels C and D). CLCF1 activates STAT3 in both a time- (panels A and C) and concentration- (panels B and D) dependent manner.

FIG. 4, panels A and B, shows the effect of CLCF1 treatment on SH-SY5Y and E18 cells. After 24 h of pre-incubation in serum free condition, cells were treated with CLCF1 in serum free media for 72 h. CLCF1 increased SH-SY5Y (panel A) and E18 (panel B) cell survival in a concentration-dependent manner. Statistical significance was measured against the negative control *P < 0.05, **P < 0.01.

FIG. 5, panels A and B, show yeast displayed CLCF1 for protein engineering. Panel A: CLCF1 was displayed as a fusion to yeast surface mating protein Aga2p. Panel B: Flow cytometry scatter plot showing that when treated with 20 nM CNTFR-HIS the CLCF1 expressing population (as measured with chicken anti-c-myc tag antibody and anti-chicken-PE antibody) has increased binding signal for CNTFR (as measured by mouse anti-HIS Hilyte Fluor 488).

FIG. 6 depicts the amino acid sequences for colonies isolated from the highest CNTFR binding population after 3 rounds of screening of a randomly mutated yeast-displayed CLCF1 library.

FIG. 7, panels A and B, shows the characterization of affinity matured CLCF1 variants. Panel A: CNTFR binding to yeast displaying CLCF1 variants isolated from screening and wild-type (WT) CLCF1. Panel B: Apparent K_d values of yeast-displayed CLCF1 constructs.

FIG. 8, panels A-D, shows the characterization of recombinantly expressed CLCF1 variants. Panels A and B: The apparent binding affinities (K_d) of the soluble, recombinant

CLCF1 wild-type (WT) and variants were comparable to those of yeast-displayed constructs. Increased binding affinity to CNTFR leads to increased binding to gp130 (panel C) and LIFR (panel D). Significance was measured against wild-type CLCF1. *P < 0.05, **P < 0.01.

5 **FIG. 9**, panels A-D, shows data demonstrating that FRR-CLCF1 is a potent activator of STAT3 in human cell lines A549 (panel A), SH-SY5Y (panel B) and primary rat cortical neuron E18 (panel C). FRR-CLCF1 did not activate STAT3 in rat microglia (panel D) as compared to IL6.

10 **FIG. 10**, panels A and B, shows data demonstrating that FRR-CLCF1 enhances cell survival in SH-SY5Y (panel A) and E18 (panel B) cells. Significance was measured against wtCLCF1-treated sample at the same concentration. *P < 0.05, **P < 0.01.

FIG. 11, panels A and B, show a microfluidic culture platform for axonal injury. E18 cells were seeded in the somata chamber and cultured for 4 days (panel A). Axons extended past the channel without detecting dendrites (panel B).

15 **FIG. 12** shows the axonal side before, immediately after, and 48 h after axotomy of E18 cells. 2 nM FRR-CLCF1 treatment led to higher axon regrowth compared to untreated control.

20 **FIG. 13** shows the axonal side 48 h after axotomy of E18 cells. 2 nM FRR-CLCF1 treatment led to higher axon regrowth compared to untreated control and 2 nM CLCF1. Anti-tau antibody was used to fluorescently image axons.

FIG. 14 depicts the amino acid sequences for colonies isolated from the highest affinity population after 3 rounds of sorting with shuffled CLCF1 library.

25 **FIG. 15**, panels A and B, shows data relating to the characterization of affinity matured CLCF1 variants. Panel A: Soluble CNTFR binding to yeasts expressing CLCF1 constructs isolated from affinity maturation. Panel B: Apparent K_d values of yeast displayed CLCF1 constructs.

FIG. 16 shows data relating to soluble CLCF1 binding to albumin (50 mg/ml) coated surfaces. CLCF1 binds the least to milk coated surface.

30 **FIG. 17**, panels A and B, shows data relating to the characterization of soluble CLCF1 variants. Panel A: Binding interaction of soluble CLCF1 constructs with soluble CNTFR. Panel B: K_d values of soluble CLCF1 constructs.

FIG. 18, panels A and B, show data demonstrating that CLCF1 constructs with Y22C, W169L, and K180R exhibit decreased binding to gp130 (panel A) while LIFR binding increased (panel B). Significance was measured against wtCLCF1. *P < 0.05, **P < 0.01.

FIG. 19, panels A and B, shows data demonstrating that F151A and K154A in CLCF1 does not influence gp130 binding (panel A) while they decrease binding to LIFR (panel B). Significance was measured against wtCLCF1. *P < 0.05, **P < 0.01.

FIG. 20 shows engineered CLCF1 variants with different binding specificities for the beta receptors.

FIG. 21, panels A and B, provides data demonstrating that CLCF1 constructs that bind to CNTFR with high affinity and different beta receptor binding affinities activate STAT3 differently. Panel A: Phosphorylation of STAT3 (705) induced by different CLCF1 variants. Panel B: Phosphorylation of STAT3 when wtCLCF1 is combined with different CLCF1 variants.

FIG. 22, panels A and B, provides data demonstrating that enCLCF1 inhibits cell survival enhancing effect of wtCLCF1 in A549 (panel A) and H23 (panel B). Significance was measured against non-treated control. *P < 0.05, **P < 0.01.

FIG. 23, panels A and B, provides data demonstrating that enCLCF1 treatment inhibits *in vivo* growth of A549 xenograft model. Tumor burden in *in vivo* model of NSCLC using A549 in tumor volume (panel A), and fold change of the individual tumors from day 1 (panel B). Significance was measured against PBS treated control. *P<0.05, **P < 0.01, ***P<0.001, ****P<0.0001.

FIG. 24, panels A-D, provide data demonstrating that CLCF1 and CNTFR are expressed in non-small cell lung cancer (NSCLC).

DETAILED DESCRIPTION

Provided are ciliary neurotrophic factor receptor (CNTFR) ligands. In certain aspects, a CNTFR ligand of the present disclosure exhibits increased affinity for CNTFR relative to the corresponding wild-type CNTFR ligand. In certain aspects, a CNTFR ligand of the present disclosure results in reduced binding affinity of glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), or both, for a complex including the CNTFR ligand and CNTFR, relative to the binding affinity for a complex including the corresponding wild-type CNTFR ligand and CNTFR. In certain aspects, a CNTFR ligand of the present

disclosure has both of the aforementioned properties. Also provided are pharmaceutical compositions including the CNTFR ligands, as well as methods of using the CNTFR ligands.

Before the CNTFR ligands, compositions and methods of the present disclosure are described in greater detail, it is to be understood that the ligands, compositions and methods are not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the ligands, compositions and methods will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the ligands, compositions and methods. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the ligands, compositions and methods, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the ligands, compositions and methods.

Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the ligands, compositions and methods belong. Although any ligands, compositions and methods similar or equivalent to those described herein can also be used in the practice or testing of the ligands, compositions and methods, representative illustrative ligands, compositions and methods are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the materials and/or methods in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present ligands, compositions and methods are not entitled to antedate such publication, as the date of publication provided may be different from the actual publication date which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

It is appreciated that certain features of the ligands, compositions and methods, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the ligands, compositions and methods, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed, to the extent that such combinations embrace operable processes and/or compositions. In addition, all sub-combinations listed in the embodiments describing such variables are also specifically embraced by the present ligands, compositions and methods and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present methods. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

CNTFR LIGANDS

As summarized above, aspects of the present disclosure include ciliary neurotrophic factor receptor (CNTFR) ligands. CNTFR (also referred to as CNTF receptor subunit α) is a member of the type 1 cytokine receptor family. CNTFR is the ligand-specific component of a tripartite receptor for ciliary neurotrophic factor (CNTF), as well as other ligands such as cardiotrophin-like cytokine factor 1 (CLCF1) and neuropoetin (NP). Binding of wild-type ligand to CNTFR recruits the transmembrane components of the receptor, gp130 and leukemia inhibitory factor receptor (LIFR), facilitating signal transduction. Wild-type amino acid sequences for human CNTFR, CNTF, CLCF1 and NP are provided in Table 1.

10 Table 1: Wild-Type Human CNTFR and CNTFR Ligand Amino Acid Sequences

	Amino Acid Sequence
Wild-Type Human CNTFR (SEQ ID NO:1)	MAAPVPWACCAVLAAAAVVYAQRHSPQEAPHVQYERLGSDVTLPCGTA NWDAAVTWRVNGTDLAPDLLNGSQLVLHGLELGHSGLYACFHRDSWHLR HQVLLHVGLPPREPVLSCRSNTYPKGFYCSWHLPTPTYIPNTFNVTVLH GSKIMVCEKDPALKNRCHIRYMHLFSTIKYKVSISVSNALGHNATAITF DEFTIVKPDPPENVVARPVPSNPRRLEVTWQTPSTWPDPEFPLKFFLR YRPLILDQWQHVELSDGTAHTITDAYAGKEYIIQVAAKDNEIGTWSWDS VAAHATPWTEEPRLTTEAQAETTTSTTSSLAPPPTTKICDPGELGSG GGPSAPFLVSPITLALAAAAATASSLLI
Wild-Type Human CNTF (SEQ ID NO:2)	MAFTEHSPLTPHRRDLCSRSIWLARKIRSDLTALTESYVKHQGLNKNIN LDSADGMPVASTDQWSELTEAERLQENLQAYRTFHVLLARLLEDQQVHF TPTEGDFHQAIHTLLLQVAAFAYQIEELMILLEYKIPRNEADGMPINVG DGGLEFEEKLWGLKVLQELSQWTVRSIHDLRFISSHQTGIPARGSHYIAN NKKM
Wild-Type Human CLCF1 (SEQ ID NO:3)	MDLRAGDSWGMLACLCTVLWHLPAVPALNRTGDPGPGPSIQKTYDLTRY LEHQLRSLAGTYLNYLGPPFNPDNPPRLGAETLPRATVDLEVWRSLN DKLRLTQNYEAYSHLLCYLRGLNRQAATAELRRSLAHFCTSLQGLLGS AGVMAALGYPLPQPLPGTEPTWTPGPAHSDFLQKMDDFWLLKELQTWLW RSKDFNRLKKKMQPPAAVTLHLGAHGF
Wild-Type Human NP (SEQ ID NO:4)	MYCLLATPLCLLSLLLPPLSPAAPISPSEPIGQAYSLALYMQKNTSALL QTYLQHQGSPFSDPGFSAPELQLSTLPSAAVSFKTWHAMEDAERLSRAQ GAFLALTQHLQLVGDDQSYLNPGSPILLAQLGAARLRAQGLLGNMAAIM TALGLPIPPEEDTLGFVPFGASAFERKCRGYIVTREYGHWTDRVRLA LLKAKYSA

As used herein, a "CNTFR ligand" is a polypeptide that binds to CNTFR and is a variant of a wild-type CNTFR ligand, such as a variant CNTF ligand, a variant CLCF1

ligand, or a variant NP ligand. By “variant” is meant the CNTFR ligand includes one or more mutations relative to the corresponding wild-type CNTFR ligand. For example, a CNTF ligand of the present disclosure (which may be referred to as a “variant CNTF” or an “engineered CNTF”) includes one or more mutations relative to wild-type CNTF, a CLCF1
5 ligand of the present disclosure (which may be referred to as a “variant CLCF1” or an “engineered CLCF1”) includes one or more mutations relative to wild-type CLCF1, etc. As used throughout the present disclosure, a “mutation” or “mutations” may include one or more amino acid substitutions, one or more amino acid deletions (e.g., truncations), one or more amino acid insertions, or any combination thereof, in the CNTFR ligand relative to the
10 corresponding wild-type CNTFR ligand.

In certain aspects, a CNTFR ligand of the present disclosure includes 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, or 20 or more mutations (e.g., amino acid substitution(s)),
15 relative to the corresponding wild-type CNTFR ligand. In some embodiments, a CNTFR ligand of the present disclosure includes 20 or less, 19 or less, 18 or less, 17 or less, 16 or less, 15 or less, 14 or less, 13 or less, 12 or less, 11 or less, 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2, or 1 mutation (e.g., amino acid substitution(s)), relative to the corresponding wild-type CNTFR ligand.

According to certain embodiments, a CNTFR ligand of the present disclosure includes an amino acid sequence that has 70% or greater, 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater identity to the corresponding wild-type CNTFR ligand, or a fragment thereof, such as a CNTFR ligand fragment having a length of from 100-120 amino acids, 120-140 amino acids, 140-160
25 amino acids, 160-180 amino acids, 180-200 amino acids, or 200-220 amino acids.

In certain aspects, a CNTFR ligand of the present disclosure binds to CNTFR with a K_D of less than or equal to about 10^{-5} M, less than or equal to about 10^{-6} M, less than or equal to about 10^{-7} M, less than or equal to about 10^{-8} M, or less than or equal to about 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M or less. Such affinities may be readily determined using
30 conventional techniques, such as by equilibrium dialysis; by using surface plasmon resonance (SPR) technology (e.g., the BIAcore 2000 instrument, using general procedures outlined by the manufacturer); by radioimmunoassay; or by another method set forth in the examples below or known to the skilled artisan.

In certain embodiments, a CNTFR ligand of the present disclosure exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand. By “increased binding affinity” or “greater binding affinity” is meant that the CNTFR ligand exhibits tighter binding (as indicated by a lower K_D value) to CNTFR as compared to the
5 corresponding wild-type CNTFR ligand. By way of example, in certain aspects, when the CNTFR ligand is a variant CLCF1 ligand, the binding affinity of the CLCF1 ligand for CNTFR has a K_D value that is 20 nM or less.

In certain aspects, the CNTFR ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand is a CLCF1 ligand (which may
10 be referred to as a “variant CLCF1” or an “engineered CLCF1”). In some embodiments, such a CLCF1 ligand may include one or more mutations at amino acid positions 86, 96, 148, 169, 180, or any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3. By way of example, such a CLCF1 ligand may include one or more mutations selected from L86F, Q96R, H148R, W169L, K180R, and any
15 combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3. Non-limiting examples of CLCF1 variants exhibiting increased binding affinity for CNTFR, as well as strategies for identifying additional such variants, are described in the Experimental section below.

In some embodiments, a CNTFR ligand of the present disclosure results in reduced
20 binding affinity of glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), or both, for a complex including the CNTFR ligand and CNTFR, relative to the binding affinity for a complex including the corresponding wild-type CNTFR ligand and CNTFR. As used herein, “reduced binding affinity”, “decreased binding affinity”, or “lower binding affinity” means that gp130, LIFR, or gp130 and LIFR, exhibits less tight binding (as indicated by a
25 higher K_D value) to a complex including the CNTFR ligand and CNTFR, as compared to a complex including the corresponding wild-type CNTFR ligand and CNTFR. The complex may consist of the CNTFR ligand bound to CNTFR, or may include additional components. For example, the CNTFR ligand may result in reduced binding affinity of gp130 for a complex consisting of the CNTFR ligand bound to CNTFR, or such a complex that further
30 includes LIFR. Similarly, the CNTFR ligand may result in reduced binding affinity of LIFR for a complex consisting of the CNTFR ligand bound to CNTFR, or such a complex that further includes gp130.

In certain aspects, a CNTFR ligand of the present disclosure results in reduced binding affinity of gp130 for a complex comprising the CNTFR ligand and CNTFR. In some embodiments, such a ligand is a CLCF1 ligand that includes one or more mutations at amino acid positions 22, 169, 180, or any combination thereof, relative to a CLCF1 ligand
5 having the amino acid sequence set forth in SEQ ID NO:3. By way of example, such a CLCF1 ligand may include one or more mutations selected from Y22C, W169L, K180R, and any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3. Non-limiting examples of CLCF1 variants resulting in reduced binding affinity of gp130 for a complex including the CLCF1 variant and CNTFR, as well as
10 strategies for identifying additional such variants, are described in the Experimental section below.

According to certain embodiments, a CNTFR ligand of the present disclosure results in reduced binding affinity of LIFR for a complex comprising the CNTFR ligand and CNTFR. In certain aspects, such a ligand is a CLCF1 ligand that includes one or more mutations at
15 amino acid positions 151, 154, or both, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3. By way of example, such a CLCF1 ligand may include one or more mutations selected from the group consisting of: F151A, K154A, or both, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3. Non-limiting examples of CLCF1 variants resulting in reduced binding affinity of LIFR for a
20 complex including the CLCF1 variant and CNTFR, as well as strategies for identifying additional such variants, are described in the Experimental section below.

In certain aspects, a CNTFR ligand of the present disclosure exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand and results in reduced binding affinity of gp130, LIFR, or both, for a complex comprising the CNTFR
25 ligand and CNTFR.

Methods are available for measuring the binding affinity of a CNTFR ligand to CNTFR, or for measuring the binding affinity of gp130 or LIFR for a complex that includes the CNTFR ligand and CNTFR. For example, surface plasmon resonance (SPR) technology (e.g., using a BIAcoreTM 2000 instrument), KinExA® kinetic exclusion assay
30 (Sapidyne Instruments), Bio-Layer Interferometry (BLI) technology (e.g., ForteBio Octet®), or other similar assay/technology may be employed to determine whether a CNTFR ligand exhibits a desired binding affinity. Suitable approaches for measuring binding affinity in the

context of the present disclosure include, e.g., those described in Hunter, S.A. and Cochran, J.R. (2016) *Methods Enzymol.* 580:21-44.

In some embodiments, in a direct binding assay, an equilibrium binding constant (K_D) may be measured using a CNTFR ligand, gp130, or LIFR conjugated to a fluorophore or radioisotope, or a CNTFR ligand, gp130, or LIFR that contains an N- or C-terminal epitope tag for detection by a labeled antibody. If labels or tags are not feasible or desired, a competition binding assay can be used to determine the half-maximal inhibitory concentration (IC_{50}), the amount of unlabeled CNTFR ligand, gp130, or LIFR at which 50% of the maximal signal of the labeled competitor is detectable. A K_D value can then be calculated from the measured IC_{50} value.

The amino acid sequences of two non-limiting examples of CNTFR ligands of the present disclosure are provided in Table 2 below.

Table 2: Amino Acid Sequences of Two Example CNTFR Ligands

	Amino Acid Sequence
Example CNTFR Ligand (CLCF1 variant – “FRR-CLCF1”) (SEQ ID NO:5) (L86F, Q96R, H148R)	LNRTGDPGPGPSIQKTYDLTRYLEHQLRSLAGTYLNYL GPPFNEPDFNPPRLGAETLPRATVDLEVWRSNDKLRL TQNYEAYSHFLCYLRGLNRRRAATAELRRSLAHFCTSLQ GLLGSIAGVMAALGYPLPQPLPGTEPTWTPGPARSDFL QKMDDFWLLKELQTWLWRSKDFNRLKKKMQPPAAAVT LHLGAHGF
Example CNTFR Ligand (CLCF1 variant – “enCLCF1”) (SEQ ID NO:6) (Y22C, L86F, Q96R, H148R, F151A, K154A, W169L, K180R)	LNRTGDPGPGPSIQKTYDLTRCLEHQLRSLAGTYLNYL GPPFNEPDFNPPRLGAETLPRATVDLEVWRSNDKLRL TQNYEAYSHFLCYLRGLNRRRAATAELRRSLAHFCTSLQ GLLGSIAGVMAALGYPLPQPLPGTEPTWTPGPARS D AL Q A MDDFWLLKELQTWL L RSKDFNRLK R KMQPPAAAVT LHLGAHGF

The example CNTFR ligands in Table 2 are CLCF1 variants. Both variants exhibit increased binding affinity for CNTFR relative to wild-type CLCF1. See Example 1 below. The second variant additionally results in reduced binding affinity of gp130 and LIFR to a complex that includes this variant and CNTFR. See Example 2 below.

In some embodiments, a CNTFR ligand of the present disclosure is any of the CNTFR ligands presented in Table 2, the Experimental section below, and any of FIGs. 6-8, FIG. 14, FIG. 15, and FIG. 20. In some embodiments, such a CNTFR ligand is present in a fusion protein (e.g., fused to an Fc domain), conjugate (e.g., conjugated to PEG, a drug, and/or the like), or combination thereof, as described elsewhere herein.

In certain aspects, a CNTFR ligand of the present disclosure binds to CNTFR and has 70% or greater, 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater amino acid sequence identity to any of the CNTFR ligands presented in Table 2, the Experimental section below, and any of FIGs. 6-8, FIG. 14, FIG. 15, and FIG. 20. In some embodiments, such a CNTFR ligand is present in a fusion protein (e.g., fused to an Fc domain), conjugate (e.g., conjugated to PEG, a drug, and/or the like), or combination thereof, as described elsewhere herein.

In certain aspects, a CNTFR ligand of the present disclosure is a CLCF1 variant that binds to CNTFR and includes an amino acid substitution selected from L86F, Q96R, H148R, and any combination thereof, where the CLCF1 variant includes 70% or greater, 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:5 ("FRR-CLCF1"). In some embodiments, such a CNTFR ligand is present in a fusion protein (e.g., fused to an Fc domain), conjugate (e.g., conjugated to PEG, a drug, and/or the like), or combination thereof, as described elsewhere herein.

In certain aspects, a CNTFR ligand of the present disclosure is a CLCF1 variant that binds to CNTFR and includes an amino acid substitution selected from Y22C, L86F, Q96R, H148R, F151A, K154A, W169L, K180R, and any combination thereof, where the CLCF1 variant includes 70% or greater, 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, or 99% or greater amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:6 ("enCLCF1"). In some embodiments, such a CNTFR ligand is present in a fusion protein (e.g., fused to an Fc domain), conjugate (e.g., conjugated to PEG, a drug, and/or the like), or combination thereof, as described elsewhere herein.

Engineering/Development and Production of CNTFR Ligands

Also provided by the present disclosure are methods of engineering/developing additional CNTFR ligands having one or more desired functionalities. The manner in which

the CNTFR ligands are developed may vary. Rational and combinatorial approaches may be used to engineer CNTFR ligands with novel properties, e.g., increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand, reduced binding affinity of gp130, LIFR, or both, for a complex including the CNTFR ligand and CNTFR, any combination thereof, etc. For example, to develop a CNTFR ligand, a library of CNTFR ligands (e.g., variant CNTFs, variant CLCF1s, variant NPs, etc.) may be created and screened, e.g., by bacterial display, phage display, yeast surface display, fluorescence-activated cell sorting (FACS), and/or any other suitable screening method.

Yeast surface display is a powerful combinatorial technology that has been used to engineer proteins with novel molecular recognition properties, increased target binding affinity, proper folding, and improved stability. In this platform, libraries of protein variants are generated and screened in a high-throughput manner to isolate mutants with desired biochemical and biophysical properties. As demonstrated in the Examples section below, the present inventors have successfully employed yeast surface display for engineering CNTFR ligands with increased binding affinities for CNTFR, as well as CNTFR ligands that result in reduced binding affinities of gp130 and LIFR for a complex including the CNTFR ligand and CNTFR. Yeast surface display benefits from quality control mechanisms of the eukaryotic secretory pathway, chaperone-assisted folding, and efficient disulfide bond formation.

One example approach for developing a CNTFR ligand having a desirable property of interest involves genetically fusing a CNTFR ligand to the yeast mating agglutinin protein Aga2p, which is attached by two disulfide binds to the yeast cell wall protein Aga1p. This Aga2p-fusion construct, and a chromosomally integrated Aga1p expression cassette, may be expressed under the control of a suitable promoter, such as a galactose-inducible promoter. N- or C-terminal epitope tags may be included to measure cell surface expression levels by flow cytometry using fluorescently labeled primary or secondary antibodies. This construct represents the most widely used display format, where a terminus of the CNTFR ligand (or other protein to be engineered) is fused to Aga2p, but several alternative variations of the yeast surface display plasmid have been described and may be employed to develop a CNTFR ligand of the present disclosure. One of the benefits of this screening platform over panning-based methods used with phage or mRNA display is that two-color FACS can be used to quantitatively discriminate clones that differ by as little as two-fold in binding affinity to a particular target.

To selectively mutate CNTFR ligands at the DNA level, an example approach is error prone PCR, which can be used to introduce mutations by any number of altered reaction conditions including using a polymerase that does not possess proofreading (i.e. exonuclease) activity, using mixtures of triphosphate derivatives of nucleoside analogues, using altered ratios of dNTPs, varying concentrations of magnesium or manganese, or the like. Alternatively, degenerate codons can be introduced by oligonucleotide assembly using, e.g., overlap extension PCR. Next, the genetic material may be amplified using flanking primers with sufficient overlap with the yeast display vector for homologous recombination in yeast. These methods allow CNTFR ligand libraries to be created at relatively low cost and effort. Synthetic libraries and recent methods have been developed that allow defined control over library composition.

In certain aspects, a display library (e.g., a yeast display library) is screened for binding to the target of interest (e.g., CNTFR) by FACS. Two-color FACS may be used for library screening, where one fluorescent label can be used to detect the c-myc epitope tag and the other to measure interaction of the CNTFR ligand against the binding target of interest. Different instrument lasers and/or filter sets can be used to measure excitation and emission properties of the two fluorophores at single-cell resolution. This enables yeast expression levels to be normalized with binding. That is, a CNTFR ligand that exhibits poor yeast expression but binds a high amount of a target can be distinguished from a CNTFR ligand that is expressed at high levels but binds weakly to a target. Accordingly, a two-dimensional flow cytometry plot of expression versus binding will result in a diagonal population of yeast cells that bind to target. High-affinity binders can be isolated using library sort gates. Alternatively, in an initial sort round it could be useful to clear the library of undesired clones that do not express full-length protein.

Following enrichment of CNTFR ligand libraries for clones encoding CNTFR ligands of interest, the yeast plasmids are recovered and sequenced. Additional rounds of FACS can be performed under increased sorting stringency. The binding affinities or kinetic off-rates of individual yeast-displayed CNTFR ligand clones may then be measured.

Once CNTFR ligands of interest have been identified by surface display (e.g., yeast surface display), the engineered CNTFR ligands may be produced using a suitable method. According to certain embodiments, the CNTFR ligand is produced by solid phase peptide synthesis. CNTFR ligand polypeptide sequences may be synthesized using solid phase peptide chemistry on an automated synthesizer. For example, standard 9-

fluorenylmethyloxycarbonyl (Fmoc)-based solid phase peptide chemistry may be employed. Solid phase synthesis may be followed by purification, e.g., by reversed-phase high-performance liquid chromatography (RP-HPLC).

5 In certain aspects, the CNTFR ligands are produced using a recombinant DNA approach. Strategies have been developed for producing proteins such as CNTFR ligands using recombinant methods in a variety of host cell types. For example, functional CNTFR ligands may be produced with barnase as a genetic fusion partner, which promotes folding in the *E. coli* periplasmic space and serves as a useful purification handle. According to certain embodiments, the CNTFR ligand is expressed in yeast (e.g., the yeast strain *Pichia*
10 *pastoris* or *Saccharomyces cerevisiae*) or mammalian cells (e.g. human embryonic kidney cells or Chinese hamster ovary cells). The expression construct may encode one or more tags (e.g., a C-terminal hexahistidine tag for purification by, e.g., metal chelating chromatography (Ni-NTA)). Size exclusion chromatography or high performance liquid chromatography may then be used to remove aggregates, misfolded multimers, and the
15 like.

Aspects of the present disclosure include nucleic acids that encode the CNTFR ligands of the present disclosure. That is, provided are nucleic acids that encode any of the CNTFR ligands described herein. In certain aspects, such a nucleic acid is present in an expression vector. The expression vector includes a promoter operably linked to the
20 nucleic acid encoding the CNTFR ligand, the promoter being selected based on the type of host cell selected to express the CNTFR ligand. Suitable expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors contain selection markers (e.g., ampicillin-resistance, hygromycin-resistance, tetracycline resistance, kanamycin resistance,
25 neomycin resistance, and/or the like) to permit detection of those cells transformed with the desired DNA sequences.

Also provided are host cells that include a nucleic acid that encodes any of the CNTFR ligands described herein, as well as any expression vectors including the same. *Escherichia coli* is an example of a prokaryotic host cell that can be used for cloning a
30 nucleic acid encoding a CNTFR ligand of the present disclosure. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences

compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, are also useful for expression. *Saccharomyces* (e.g., *S. cerevisiae*) and *Pichia* are examples of suitable yeast host cells, with suitable vectors having expression control sequences (e.g., promoters), an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

In addition to microorganisms, mammalian cells (e.g., mammalian cells grown in *in vitro* cell culture) can also be used to express and produce the CNTFR ligands of the present disclosure. Suitable mammalian host cells include human cell lines, non-human primate cell lines, rodent (e.g., mouse, rat) cell lines, and the like. Suitable mammalian cell lines include, but are not limited to, HeLa cells (e.g., American Type Culture Collection (ATCC) No. CCL-2), CHO cells (e.g., ATCC Nos. CRL9618, CCL61, CRL9096), 293 cells (e.g., ATCC No. CRL-1573), Vero cells, NIH 3T3 cells (e.g., ATCC No. CRL-1658), Huh-7 cells, BHK cells (e.g., ATCC No. CCL10), PC12 cells (ATCC No. CRL1721), COS cells, COS-7 cells (ATCC No. CRL1651), RAT1 cells, mouse L cells (ATCC No. CCL1.3), human embryonic kidney (HEK) cells (ATCC No. CRL1573), HLHepG2 cells, and the like. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Examples of suitable expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papilloma virus, cytomegalovirus and the like.

Once synthesized (either chemically or recombinantly), the CNTFR ligands can be purified according to standard procedures known in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, high performance liquid chromatography (HPLC) purification, gel electrophoresis, and the like. A subject CNTFR

ligand can be substantially pure, e.g., at least about 80% to 85% pure, at least about 85% to 90% pure, at least about 90% to 95% pure, or 98% to 99%, or more, pure, e.g., free from contaminants such as cell debris, macromolecules other than the CNTFR ligand, etc.

Fusion Proteins and Conjugates

5 In certain aspects, provided are CNTFR ligands (e.g., any of the CNTFR ligands described herein) stably associated with (e.g., fused, conjugated, or otherwise attached to) a heterologous moiety.

In some embodiments, provided are fusion proteins in which a CNTFR ligand is fused to a heterologous polypeptide. Heterologous polypeptides of interest include, but are not limited to, an Fc domain (e.g., a human or mouse Fc domain), an albumin, a transferrin, XTEN, a homo-amino acid polymer, a proline-alanine-serine polymer, an elastin-like peptide, or any combination thereof. In certain aspects, the heterologous polypeptide increases the stability and/or serum half-life of the CNTFR ligand upon its administration to an individual in need thereof, as compared to the same CNTFR ligand which is not fused to the heterologous polypeptide. In certain aspects, provided are fusion proteins that include any of the CNTFR ligands of the present disclosure fused to a human Fc domain (e.g., a full-length human Fc domain or fragment thereof). According to certain embodiments, such a fusion protein finds use, e.g., in administering to an individual in need thereof in accordance with the methods of the present disclosure, e.g., an individual having a cell proliferative disorder associated with CNTFR signaling, an individual having a neurodegenerative disorder, and/or the like. A non-limiting example of a human Fc domain that may be fused to any of the CNTFR ligands of the present disclosure is a human IgG1 Fc domain having the sequence set forth in Table 3 below (SEQ ID NO:7), or a fragment thereof.

25 Table 3: Amino Acid Sequence of an Example Human Fc Domain

	Amino Acid Sequence
Example Human Fc Domain (SEQ ID NO:7)	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

According to certain embodiments, provided are conjugates in which a CNTFR ligand of the present disclosure is conjugated to a moiety. Moieties of interest include, but are not limited to, polyethylene glycol (PEG), an anti-cancer drug, a detectable label, and combinations thereof.

5 Anti-cancer drugs of interest include agents that inhibit cell proliferation and/or kill cancer cells. Such agents may vary and include cytostatic agents and cytotoxic agents (e.g., an agent capable of killing a target cell tissue with or without being internalized into a target cell). In certain aspects, the therapeutic agent is a cytotoxic agent selected from an enediyne, a lexitropsin, a duocarmycin, a taxane, a puromycin, a dolastatin, a maytansinoid, 10 and a vinca alkaloid. In some embodiments, the cytotoxic agent is paclitaxel, docetaxel, CC-1065, CPT-11 (SN-38), topotecan, doxorubicin, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretastatin, calicheamicin, maytansine, maytansine DM1, maytansine DM4, DM-1, an auristatin or other dolastatin derivatives, such as auristatin E or auristatin F, AEB (AEB-071), AEVB (5-benzoylvaleric acid-AE ester), AEFP (antibody-endostatin fusion protein), MMAE (monomethylauristatin E), 15 MMAF (monomethylauristatin F), pyrrolobenzodiazepines (PBDs), eleutherobin, netropsin, or any combination thereof. According to certain embodiments, the agent is a protein toxin selected from hemiasterlin and hemiasterlin analogs such as HTI-286 (e.g., see USPN 7,579,323; WO 2004/026293; and USPN 8,129,407, the full disclosures of which are 20 incorporated herein by reference), abrin, brucine, cicutoxin, diphtheria toxin, batrachotoxin, botulism toxin, shiga toxin, endotoxin, *Pseudomonas* exotoxin, *Pseudomonas* endotoxin, tetanus toxin, pertussis toxin, anthrax toxin, cholera toxin, falcarinol, fumonisin B1, fumonisin B2, aflatoxin, maurotoxin, agitoxin, charybdotoxin, margatoxin, slo toxin, scyllatoxin, hefutoxin, calciseptine, taicatoxin, calcicludeine, geldanamycin, gelonin, lotaustralin, 25 ocratoxin A, patulin, ricin, strychnine, trichothecene, zearlenone, and tetradoxin. Enzymatically active toxins and fragments thereof which may be employed include diphtheria A chain, non-binding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and 30 PAP-S), *Momordica charantia* inhibitor, curcumin, croton, *Saponaire officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes.

Detectable labels include labels that may be detected in an application of interest (e.g., *in vitro* and/or *in vivo* research and/or clinical applications). Detectable labels of

interest include radioisotopes, enzymes that generate a detectable product (e.g., horseradish peroxidase, alkaline phosphatase, etc.), fluorescent proteins, paramagnetic atoms, and the like. In certain aspects, the CNTFR ligand is conjugated to a specific binding partner of detectable label (e.g., conjugated to biotin such that detection may occur via a detectable label that includes avidin/streptavidin).

According to certain embodiments, the agent is a labeling agent that finds use in *in vivo* imaging, such as near-infrared (NIR) optical imaging, single-photon emission computed tomography (SPECT)/CT imaging, positron emission tomography (PET), nuclear magnetic resonance (NMR) spectroscopy, or the like. Labeling agents that find use in such applications include, but are not limited to, fluorescent labels, radioisotopes, and the like. In certain aspects, the labeling agent is a multi-modal *in vivo* imaging agent that permits *in vivo* imaging using two or more imaging approaches (e.g., see Thorp-Greenwood and Coogan (2011) *Dalton Trans.* 40:6129-6143).

In certain aspects, the labeling agent is an *in vivo* imaging agent that finds use in near-infrared (NIR) imaging applications, which agent is selected from a Kodak X-SIGHT dye, Pz 247, DyLight 750 and 800 Fluors, Cy 5.5 and 7 Fluors, Alexa Fluor 680 and 750 Dyes, IRDye 680 and 800CW Fluors. According to certain embodiments, the labeling agent is an *in vivo* imaging agent that finds use in SPECT imaging applications, which agent is selected from ^{99m}Tc , ^{111}In , ^{123}In , ^{201}Tl , and ^{133}Xe . In certain aspects, the labeling agent is an *in vivo* imaging agent that finds use in positron emission tomography (PET) imaging applications, which agent is selected from ^{11}C , ^{13}N , ^{15}O , ^{18}F , ^{64}Cu , ^{62}Cu , ^{124}I , ^{76}Br , ^{82}Rb and ^{68}Ga .

Linkers that find use in the conjugates of the present disclosure include ester linkers, amide linkers, maleimide or maleimide-based linkers; valine-citrulline linkers; hydrazone linkers; N-succinimidyl-4-(2-pyridyldithio)butyrate (SPDB) linkers; Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linkers; vinylsulfone-based linkers; linkers that include polyethylene glycol (PEG), such as, but not limited to tetraethylene glycol; linkers that include propanoic acid; linkers that include caproic acid, and linkers including any combination thereof.

Numerous strategies are available for linking a CNTFR ligand to a moiety of interest through a linker. For example, the moiety of interest may be derivatized by covalently attaching the linker to the drug, where the linker has a functional group capable of reacting with a "chemical handle" on the CNTFR ligand. The functional group on the linker may vary

and may be selected based on compatibility with the chemical handle on the CNTFR ligand. According to one embodiment, the chemical handle on the CNTFR ligand is provided by incorporation of an unnatural amino acid having the chemical handle into the CNTFR ligand. Such an unnatural amino acid may be incorporated into a CNTFR ligand, e.g., via chemical
5 synthesis or recombinant approaches, e.g., using a suitable orthogonal amino acyl tRNA synthetase-tRNA pair for incorporation of the unnatural amino acid during translation in a host cell.

The functional group of an unnatural amino acid present in the CNTFR ligand may be an azide, alkyne, alkene, amino-oxy, hydrazine, aldehyde, nitron, nitrile oxide,
10 cyclopropene, norbornene, iso-cyanide, aryl halide, boronic acid, or other suitable functional group, and the functional group on the linker is selected to react with the functional group of the unnatural amino acid (or vice versa).

COMPOSITIONS

Also provided are compositions that include a CNTFR ligand of the present
15 disclosure. The compositions may include, e.g., any of the CNTFR ligands described herein.

In certain aspects, the compositions include a CNTFR ligand of the present disclosure present in a liquid medium. The liquid medium may be an aqueous liquid medium, such as water, a buffered solution, and the like. One or more additives such as a
20 salt (e.g., NaCl, MgCl₂, KCl, MgSO₄), a buffering agent (a Tris buffer, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 2-(N-Morpholino)ethanesulfonic acid (MES), 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES), 3-(N-Morpholino)propanesulfonic acid (MOPS), N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), etc.), a protease inhibitor, glycerol, and the like may be
25 present in such compositions.

Pharmaceutical compositions are also provided. The pharmaceutical compositions include any of the CNTFR ligands of the present disclosure, and a pharmaceutically-acceptable carrier. The pharmaceutical compositions generally include a therapeutically effective amount of the CNTFR ligand. By "therapeutically effective amount" is meant a
30 dosage sufficient to produce a desired result, e.g., an amount sufficient to effect beneficial or desired therapeutic (including preventative) results, such as a reduction in cellular proliferation in an individual having a cell proliferative disorder associated with CNTFR

signaling, reduction in neurodegeneration in an individual having a neurodegenerative disorder, and/or the like.

A CNTFR ligand of the present disclosure can be incorporated into a variety of formulations for therapeutic administration. More particularly, the CNTFR ligand can be formulated into pharmaceutical compositions by combination with appropriate pharmaceutically acceptable excipients or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, injections, inhalants and aerosols.

Formulations of the CNTFR ligands of the present disclosure suitable for administration to an individual (e.g., suitable for human administration) are generally sterile and may further be free of detectable pyrogens or other contaminants contraindicated for administration to an individual according to a selected route of administration.

In pharmaceutical dosage forms, the CNTFR ligand can be administered alone or in appropriate association, as well as in combination, with other pharmaceutically-active compounds. The following methods and excipients are merely examples and are in no way limiting.

For oral preparations, the CNTFR ligand can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The CNTFR ligands can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or non-aqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The pharmaceutical composition may be in a liquid form, a lyophilized form or a liquid form reconstituted from a lyophilized form, where the lyophilized preparation is to be reconstituted with a sterile solution prior to administration. The standard procedure for reconstituting a lyophilized composition is to add back a volume of pure water (typically equivalent to the volume removed during lyophilization); however solutions comprising

antibacterial agents may be used for the production of pharmaceutical compositions for parenteral administration.

An aqueous formulation of the CNTFR ligand may be prepared in a pH-buffered solution, e.g., at pH ranging from about 4.0 to about 7.0, or from about 5.0 to about 6.0, or alternatively about 5.5. Examples of buffers that are suitable for a pH within this range include phosphate-, histidine-, citrate-, succinate-, acetate-buffers and other organic acid buffers. The buffer concentration can be from about 1 mM to about 100 mM, or from about 5 mM to about 50 mM, depending, e.g., on the buffer and the desired tonicity of the formulation.

10 METHODS OF USE

Also provided are methods of using the CNTFR ligands and compositions of the present disclosure. In certain aspects, provided are methods that include administering a CNTFR ligand or composition of the present disclosure to an individual (e.g., a mammal, such as a human). According to certain embodiments, provided are methods that include administering to an individual in need thereof a therapeutically effective amount of a CNTFR ligand or pharmaceutical composition of the present disclosure. In certain aspects, the individual in need thereof has a cell proliferative disorder associated with CNTFR signaling, and the administering is effective in treating the cell proliferative disorder. For example, a CNTFR ligand that results in reduced affinity of gp130 and/or LIFR for a complex that includes the CNTFR ligand and CNTFR (which ligand may optionally exhibit increased binding affinity for CNTFR relative to the corresponding wild-type ligand) may be employed to inhibit CNTFR signaling in an individual having a cell proliferative disorder associated with CNTFR signaling. In certain aspects, the cell proliferative disorder is cancer.

For example, in some embodiments, a CNTFR ligand or pharmaceutical composition of the present disclosure inhibits growth, metastasis and/or invasiveness of a cancer cell(s) in a host when the CNTFR ligand or pharmaceutical composition is administered in an effective amount. By "cancer cell" is meant a cell exhibiting a neoplastic cellular phenotype, which may be characterized by one or more of, for example, abnormal cell growth, abnormal cellular proliferation, loss of density dependent growth inhibition, anchorage-independent growth potential, ability to promote tumor growth and/or development in an immunocompromised non-human animal model, and/or any appropriate indicator of cellular transformation. "Cancer cell" may be used interchangeably herein with "tumor cell",

“malignant cell” or “cancerous cell”, and encompasses cancer cells of a solid tumor, a semi-solid tumor, a primary tumor, a metastatic tumor, and the like.

Cancers which may be treated using the methods of the present disclosure include, but are not limited to, solid tumors, lung cancer (e.g., non-small cell lung cancer (NSCLC), breast cancer, prostate cancer, pancreatic cancer, colorectal carcinoma, renal cell carcinoma, Hodgkin’s lymphoma, Non-Hodgkin’s lymphoma, anaplastic large cell lymphoma, acute myelogenous leukemia, multiple myeloma, and any other type of cancer which may be treated using a CNTFR ligand or pharmaceutical composition of the present disclosure.

In certain aspects, the individual in need thereof has a neurodegenerative disorder, and the administering is effective in treating the neurodegenerative disorder. For example, a CNTFR ligand (e.g., a CNTFR ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type ligand) may be used to stimulate CNTFR signaling in a manner that is neuroprotective, e.g., inhibits neurodegeneration. Neurodegenerative disorders that may be treated according to the methods of the present disclosure include, but are not limited to, Alzheimer’s Disease (AD), Parkinson’s Disease (PD), Lewy body dementia, frontotemporal dementia, amyotrophic lateral sclerosis (ALS), Huntington disease, and prion diseases. As such, the individual to which the CNTFR ligand is administered may have any of the aforementioned neurodegenerative diseases.

The CNTFR ligand may be administered alone (e.g., in monotherapy) or in combination (e.g., in combination therapy) with one or more additional therapeutic agents.

In some embodiments, an effective amount of the CNTFR ligand (or pharmaceutical composition including same) is an amount that, when administered alone (e.g., in monotherapy) or in combination (e.g., in combination therapy) with one or more additional therapeutic agents, in one or more doses, is effective to reduce the symptoms of a cell proliferative disorder (e.g., cancer) or neurodegenerative disorder in the individual by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or more, compared to the symptoms in the individual in the absence of treatment with the CNTFR ligand or pharmaceutical composition.

In certain aspects, the methods of the present disclosure inhibit growth, metastasis and/or invasiveness of cancer cells in the individual when the CNTFR ligand or pharmaceutical composition is administered in an effective amount.

5 The CNTFR ligand or pharmaceutical composition may be administered to an individual using any available method and route suitable for drug delivery, including *in vivo* and *ex vivo* methods, as well as systemic and localized routes of administration. Conventional and pharmaceutically acceptable routes of administration include intranasal, intramuscular, intra-tracheal, subcutaneous, intradermal, topical application, ocular, intravenous, intra-arterial, nasal, oral, and other enteral and parenteral routes of
10 administration. Routes of administration may be combined, if desired, or adjusted depending upon the CNTFR ligand and/or the desired effect. The CNTFR ligands or pharmaceutical compositions may be administered in a single dose or in multiple doses. In some embodiments, the CNTFR ligand or pharmaceutical composition is administered intravenously. In some embodiments, the CNTFR ligand or pharmaceutical composition is
15 administered by injection, e.g., for systemic delivery (e.g., intravenous infusion) or to a local site.

A variety of individuals are treatable according to the subject methods. Generally such subjects are “mammals” or “mammalian,” where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore
20 (e.g., dogs and cats), rodentia (e.g., mice, guinea pigs, and rats), and primates (e.g., humans, chimpanzees, and monkeys). In some embodiments, the individual is a human.

By “treating” or “treatment” is meant at least an amelioration of the symptoms associated with the cell proliferative disorder (e.g., cancer) or neurodegenerative disorder of the individual, where amelioration is used in a broad sense to refer to at least a reduction in
25 the magnitude of a parameter, e.g. symptom, associated with the cell proliferative disorder or neurodegenerative disorder being treated. As such, treatment also includes situations where the cell proliferative disorder or neurodegenerative disorder, or at least symptoms associated therewith, are completely inhibited, e.g., prevented from happening, or stopped, e.g., terminated, such that the individual no longer suffers from the cell proliferative disorder
30 or neurodegenerative disorder, or at least the symptoms that characterize the cell proliferative disorder or neurodegenerative disorder.

Dosing is dependent on severity and responsiveness of the disease state to be treated. Optimal dosing schedules can be calculated from measurements of drug

accumulation in the body of the patient. The administering physician can determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual CNTFR ligands, and can generally be estimated based on EC₅₀s found to be effective in *in vitro* and *in vivo* animal models, etc. In
5 general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the subject undergo maintenance therapy to prevent the recurrence of the disease state, where the
10 CNTFR ligand or pharmaceutical composition is administered in maintenance doses, ranging from 0.01 µg to 100 g per kg of body weight, once or more daily, to once every several months, once every six months, once every year, or at any other suitable frequency.

The therapeutic methods of the present disclosure may include administering a single type of CNTFR ligand to a subject, or may include administering two or more types of
15 CNTFR ligands to a subject by administration of a cocktail of different CNTFR ligands.

In some embodiments, the methods include, prior to the administering the CNTFR ligand or pharmaceutical composition, identifying the individual as having a cell proliferative disorder associated with CNTFR signaling, or a neurodegenerative disorder. Identifying the individual as having a cell proliferative or neurodegenerative disorder associated with
20 CNTFR signaling may be carried out using a variety of approaches and combinations thereof. In certain aspects, the identifying is based on CNTFR ligand abundance in a sample obtained from the individual. The CNTFR ligand may be one or more of CNTF, CLCF1, NP, etc., and any combinations thereof. In certain aspects, the sample includes cancer-associated fibroblasts (CAFs, such as normal lung fibroblasts (NLFs)), and
25 identifying the individual as having a cell proliferative or neurodegenerative disorder associated with CNTFR signaling is based on the level of CLCF1 expression in the CAFs. In some embodiments, the CNTFR ligand abundance is quantified using a soluble CNTFR polypeptide as a CNTFR ligand capture agent.

According to certain embodiments, the identifying is based on CNTFR abundance
30 and/or the abundance of CNTFR-gp130-LIFR tripartite receptor complexes in a sample obtained from the individual. In certain aspects, the identifying is based on the level of CNTFR signaling in a sample obtained from the individual. The level of CNTFR signaling may be based on the phosphorylation status of one or more CNTFR signaling pathway

molecules. For example, the present inventors have determined that binding of CNTFR to CLCF1 results in activation of the Jak-STAT and Ras-Raf-MEK-ERK signaling pathways, as schematically illustrated in FIG. 1. As such, the abundance, activity, phosphorylation status, and/or the like of any of Jak, STAT, Ras, Raf, MEK, ERK, or any combination thereof, may be assessed to determine aberrant CNTFR signaling in the individual.

The identifying may be based on ligands, CNTFR molecules, CNTFR-gp130-LIFR tripartite receptor complexes, etc. quantified using any suitable approaches. According to certain embodiments, the identifying is based on an immunoassay. A variety of suitable immunoassay formats are available, including ELISA, flow cytometry assays, immunohistochemistry on tissue section samples, immunofluorescence on tissue section samples, Western analysis, and/or the like.

In some embodiments, the identifying is based on nucleic acid sequencing. For example, the number of sequencing reads corresponding to an mRNA encoding a protein of interest may be used to determine the expression level of the protein. In certain aspects, the sequencing is performed using a next-generation sequencing system, such as on a sequencing platform provided by Illumina® (e.g., the HiSeq™, MiSeq™ and/or Genome Analyzer™ sequencing systems); Ion Torrent™ (e.g., the Ion PGM™ and/or Ion Proton™ sequencing systems); Pacific Biosciences (e.g., the PACBIO RS II sequencing system); Life Technologies™ (e.g., a SOLiD sequencing system); Roche (e.g., the 454 GS FLX+ and/or GS Junior sequencing systems); or any other sequencing platform of interest. Protocols for isolating nucleic acids from tissue or fluid samples, as well as protocols for preparing sequencing libraries having sequencing adapters appropriate for the desired sequencing platform are readily available.

In some embodiments, methods that include identifying the individual as having a cell proliferative or neurodegenerative disorder associated with CNTFR signaling further include obtaining the sample from the individual.

The sample obtained from the individual may be any sample suitable for determining whether the individual has a cell proliferative or neurodegenerative disorder associated with CNTFR signaling. In certain aspects, the sample is a fluid sample, such as whole blood, serum, plasma, or the like. In some embodiments, the sample is a tissue sample. Tissue samples of interest include, but are not limited to, tumor biopsy samples, and the like.

A variety of suitable approaches are available to identify an individual as having a neurodegenerative disorder. In certain aspects, the identifying comprises neuroimaging of

the individual, electroencephalography (EEG), biomarker analysis (e.g., measuring a biomarker (such as a misfolded protein) in blood or urine of the individual), or any combination thereof.

KITS

5 Also provided by the present disclosure are kits. According to certain embodiments, the kits include a therapeutically effective amount of any of the CNTFR ligands described herein, or any of the pharmaceutical compositions described herein, and instructions for administering the CNTFR ligand or pharmaceutical composition to an individual in need thereof (e.g., an individual identified as having a cell proliferative disorder associated with
10 CNTFR signaling, or a neurodegenerative disorder). In certain aspects, the kits include a CNTFR ligand or a pharmaceutical composition of the present disclosure, present in a container. The container may be a tube, vial, or the like. According to certain embodiments, the kit includes the CNTFR ligand or the pharmaceutical composition present in one or more unit dosages, such as 1, 2 or more, 3 or more, 4 or more, 5 or more, etc. unit
15 dosages.

Components of the kits may be present in separate containers, or multiple components may be present in a single container.

The instructions for administering the CNTFR ligand or pharmaceutical composition to an individual may be recorded on a suitable recording medium. For example, the
20 instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g., portable flash drive, DVD, CD-ROM,
25 diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, the means for obtaining the instructions is recorded on a suitable substrate.

30 In some embodiments, provided are kits that include a CNTFR ligand capture agent and instructions for using the capture agent to quantify a CNTFR ligand abundance present in a biological sample. The CNTFR ligand may be one or more of CNTF, CLCF1, NP, etc.,

and any combinations thereof. In some embodiments, the CNTFR ligand abundance is quantified using a soluble CNTFR polypeptide as a CNTFR ligand capture agent.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

5 Example 1 – Engineering Variant CNTFR Ligands Exhibiting Increased Binding Affinity for CNTFR

It was hypothesized that variant CNTFR ligands could be employed as a therapeutic to increase CNTFR signaling (e.g., to treat a neurodegenerative disorder), and that a CNTFR ligand having a greater binding affinity for CNTFR as compared the corresponding
10 wild-type CNTFR ligand would be desirable in this context. In this particular example, CLCF1 variants were generated and assessed for binding affinity to CNTFR, although the materials and methods employed in this example are readily applicable to other CNTFR ligands, such as CNTF and neuropoetin (NP1).

Introduction

15 While the detection of amyloid plaques or neurofibrillary tangles (NFTs) have been known as the hallmarks of Alzheimer's Disease (AD) and has diagnostic value, their exact role in the pathogenesis of AD remains controversial. For example, people with substantial plaque burdens can have normal condition, suggesting that plaque loads may not necessarily correlate with functional impairments. Major anti-amyloid treatments that have
20 been tested can be divided into A β fragments for active immunization, A β targeting antibodies for passive immunization, small molecules that target amyloid precursor protein (APP) cleavage enzyme, presenilin, and small molecules that target another APP cleavage enzyme, BACE1. All phase III clinical trials for treatments targeting β amyloid have failed to show cognitive improvement although some led to reductions of β amyloid. These studies
25 do not necessarily disprove the amyloid hypothesis, however, orthogonal strategies that directly induce neuronal survival, neurogenesis, or synaptogenesis to slow or compensate for neuronal damages with well-established mechanisms may provide a critical stand-alone or adjuvant treatment.

Neurotrophic factors, or neurotrophins, are a group of growth factors that regulate pathways involved in pro-survival and pro-functional responses in neurons. They are a critical component during development and maintenance of the vertebrate nervous system. Much evidence shows the importance of neurotrophins in AD. For example, inhibition of nerve growth factor (NGF) by antibodies led to pathological phenotypes similar to those observed in AD in a mouse model. Alterations in NGF and brain-derived neurotrophic factor (BDNF) levels are found in AD patients as well as in other disorders such as Down's syndrome (DS), PD, and HD. Pre-clinical and clinical studies are currently underway to use NGF, BDNF, GDNF, neurturin, and neurotrophin-3 (NT-3) as therapeutic agents for neurological disorders, but discovery of optimal drug targets and effective therapeutics that act on these targets without toxicity still remains a major challenge.

Neurodegenerative diseases are complex disorders and the failures of monotherapy suggest more potent approaches are needed. In part, this is because the blood brain barrier (BBB) makes it difficult for proteins to cross from the blood into the brain. Moreover, infusion through direct intracerebroventricular injection has been associated with significant side effects. Equally important is target specificity, since heavy crosstalk between multiple circuits of the brain makes it especially vulnerable to off-target toxicities. To address this need, described herein is a novel engineered growth factor ligand with optimized neuro-receptor targeting properties, with the goal of maximizing efficacy with reduced side effects.

Ciliary neurotrophic factor receptor (CNTFR) is highly expressed in the brain and the peripheral nervous system (FIG. 2), and supports the survival of neurons. Ciliary neurotrophic factor (CNTF), the first ligand discovered for CNTFR, was shown to have a direct neuroprotective effect on degenerating neurons in stress-induced conditions, both in cell culture and in a rodent model of axotomy-induced apoptosis. The beneficial effect of CNTF was further supported in preclinical studies involving animal models of AD, HD, and ALS. Administration of soluble CNTF was tested in phase I clinical trials as a therapy for HD and ALS, but reported no observable benefit. In these studies, the treatment dose was limited by adverse effects that are linked to the activation of interleukin-6 receptor (IL6R) by CNTF, which induces an acute-phase response on human liver cells. However, these results suggest that mono-specificity for CNTFR may improve the safety profile of CNTF.

Described herein is an alternative strategy to develop an effective CNTFR targeting ligand with reduced toxicity. Cardiotrophin-like cytokine 1 (CLCF1) is a related agonist ligand for CNTFR. CLCF1 and CNTF activate CNTFR through the same mechanism of

dimerization of the beta receptors glycoprotein 130 (gp130) and leukemia inhibitory factor receptor (LIFR). Unlike CNTF, CLCF1 does not naturally bind to IL6R, and thus is known to act only through CNTFR. This specificity for CNTFR will mitigate side effects caused by off-target activation of IL6R, making CLCF1 a more suitable therapeutic molecule than CNTF.

5 However, CLCF1 has lower binding affinity for CNTFR, and thus is a weaker agonist than CNTF.

Described herein are engineered CLCF1 variants that surpass natural CLCF1 and CNTF in potency, and directly overcome the IL6R specificity limitations of CNTF. These variants were mined from a yeast displayed library of millions of CLCF1 mutants that were

10 screened in a high-throughput manner to identify those with tighter binding against CNTFR. Some of these variants bind even more strongly to CNTFR than CNTF, potentially allowing lower doses to be administered to further decrease side effects.

Recombinant human CLCF1 activates STAT3 in human neuroblastoma cells and rat cortical neurons

Human neuroblastoma (SH-SY5Y) cells have morphological and biochemical similarity to primary neurons when differentiated and thus are used as a model cell line for *in vitro* studies. A recent study reported that SH-SY5Y cells express CNTFR and respond to CNTF treatment by phosphorylating STAT3 at Tyr705. Recombinant human CLCF1 was prepared. Briefly, human CLCF1 sequence was cloned into bacterial expression plasmid

20 pET28b and transformed and expressed in Rosetta-gami 2(DE)3 cells. The expressed protein was collected and purified from inclusion body using reverse-phase high performance liquid chromatography (HPLC). Indeed, when treated with CLCF1, SH-SY5 Y phosphorylated STAT3 (Tyr705), which peaked 20 min after treatment and gradually decreased (FIG. 3, panel A). STAT3 activation was also concentration dependent (FIG. 3, panel B). To test CLCF1 treatment on primary neurons, rat embryonic cortical neurons (E18

25 cells) were harvested at day 18. Rat CNTFR has 94% sequence homology to human CNTFR, suggesting high likelihood of cross-reactivity. When treated with CLCF1, E18 cells showed STAT3 activation that was both time- and concentration-dependent, confirming their suitability for our *in vitro* studies (FIG. 3, panels C and D).

Recombinant human CLCF1 increases cell survival in serum starved conditions

To examine the effect of CLCF1 on cell survival, serum starved E18 cells were treated with different concentrations of CLCF1. After 72 hours of treatment, CLCF1-treated cells displayed higher survival compared to non-treated control in concentration dependent manner (FIG. 4). Together with the phosphorylation assay results, these experiments indicated the neuro-protective effect of human CLCF1 on both human and rat cells with neuronal origin.

Engineering high affinity CLCF1 variants using yeast surface display

Yeast display is a robust and versatile technology that allows engineering and quantitative, real-time assessment of proteins. It has been implemented to engineer a variety of proteins including antibodies, receptors, ligands, and enzymes. The open reading frame of the CLCF1 gene was cloned into the yeast surface display plasmid pCTcon2 and transformed into EBY100 (FIG. 5, panel A). The transformed yeast demonstrated low expression of CLCF1 compared to CNTFR, which may suggest lower stability or solubility of CLCF1 (FIG. 5, panel B). The CLCF1 expressing population bound 20 nM wild-type CNTFR-HIS (wtCNTFR-HIS), confirming its functionality.

Error prone PCR was used to create a randomly mutated yeast-displayed CLCF1 library with an estimated diversity of about 1×10^8 . The resulting library was transformed and displayed on yeast. Using equilibrium binding conditions and screened by flow cytometric sorting (FACS), high throughput screening of the CLCF1 library using wtCNTFR-HIS was performed to enrich variants showing increased CLCF1 binding. Expression signal was used to normalize CNTFR binding to distinguish high expressers from high affinity binders. Screening stringency was increased throughout sorting by decreasing the concentration of wtCNTFR-HIS incubated with the library. After 4 rounds of FACS a consensus mutation, Q96R ($K_d = 7.9$ nM), emerged as shown in FIG. 6. Quantitative yeast-displayed binding studies suggested that two additional mutations, L86F ($K_d = 3.1$ nM) and H148R ($K_d = 4.2$ nM), additively increased the binding for CNTFR with the variant with all three mutations leading to 10-fold higher apparent binding affinity ($K_d = 0.83$ nM). Thus a variant containing the three mutations (named FRR-CLCF1) was chosen for further characterization (FIG. 7).

Binding affinities of recombinant engineered CLCF1 variants

The CLCF1 variants isolated from screening were cloned into the pET28b bacterial expression plasmid and expressed and purified. To measure binding affinity, recombinant CLCF1 variants or wild-type CLCF1 were incubated with wtCNTFR-Fc at room temperature for 4 hours. A sandwich ELISA was performed using an anti-mouse Fc antibody as a capturing agent for wtCNTFR-Fc, followed by an anti-CLCF1 antibody as a detection agent for the bound complex. Compared to wild-type CLCF1 (wtCLCF1), FRR-CLCF1 exhibited higher binding affinity, with an apparent K_d of approximately 0.83 nM (FIG. 8, panels A and B). ELISA was also used to measure binding interactions with the gp130 and LIFR beta receptors. FRR-CLCF1 complexed with CNTFR displayed substantially increased binding for gp130 and LIFR compared to wtCLCF1 (FIG. 8, panels C and D). These results confirmed that binding of CLCF1 and CNTFR drives their interaction with the beta receptors and demonstrates that higher binding affinity for CNTFR can lead to higher tripartite receptor complex formation.

Engineered CLCF1 is a potent activator of STAT3 and induces enhanced cell survival

To determine whether the enhanced tripartite receptor complex formation driven by FRR-CLCF1 effectively leads to increased downstream signaling, we tested its effect on SH-SY5Y cells and human non-small cell lung cancer (NSCLC) cells. When treated with FRR-CLCF1, the cells showed higher phosphorylation of STAT3 at Tyr705 (FIG. 9, panels A and B). FRR-CLCF1 demonstrated higher STAT3 activation in E18 cells as well, indicating that affinity maturation to human CNTFR also increased the potency for rat neurons (FIG. 9, panel C). Microglia are the major inflammatory cells in the brain. Activated microglia produce inflammatory mediators such as nitric oxide, tumor necrosis factor- α , and prostaglandins and this can damage the surrounding tissues. Since STAT3 has been shown to be involved in microglia activation and inflammation in the brain can lead to adverse effect, the effect of FRR-CLCF1 on STAT3 activation in microglia was tested. In the range of concentrations tested, no activation of STAT3 was detected using FRR-CLCF1 while IL6 led to a robust STAT3 activation (FIG. 9, panel D). Importantly, FRR-CLCF1 treatment led to higher cell survival in SH-SY5Y and E18 cells (FIG. 10) suggesting that the engineered CLCF1 is a promising candidate for neuronal survival.

Engineered CLCF1 enhances rat cortical neuron axon regeneration

Axon formation is the basis of neuronal connectivity. In a damaged brain, damaged axons can sprout nerve endings and rewire the network to compensate for damage, which is part of a process called neuroplasticity. Different growth factors and cytokines are known to be involved in this process. To test whether FRR-CLCF1 can also facilitate axonal regeneration, a microfluidic device was used for compartmentalizing, isolating, and directing the growth of axons. Rat embryonic neurons (E18) were seeded and cultured for 4 days in the somata chamber, leading to outgrowth of axons into the axon chamber, while dendrites stayed within the somata chamber (FIG. 11, panels A and B). Using this device as an *in vitro* model of axonal injury, selective lesion of axons were performed by aspirating only the contents of the axon chamber. It was observed that treatment with FRR-CLCF1 led to higher regrowth of axons within 48 hours compared to untreated control (FIG. 12). Furthermore, the level of axonal regeneration induced by FRR-CLCF1 was higher than that of wtCLCF1 and comparable to CNTF (FIG. 13).

Materials and Methods

Cells and Reagents

SH-SY5Y growth media was Dulbecco's Modified Eagle Media (Invitrogen) with 10% fetal bovine serum (FBS) (American Type Culture Collection), 1% penicillin-streptomycin. Embryonic rat cortical neurons (E18) were plated in plating media (Neurobasal (Gibco), 2% B27 (Gibco), 1% Glutamax, and 1% FBS) for overnight and cultured in culture media (Neurobasal (Gibco), 2% B27 (Gibco), 1% Glutamax, and 10% FBS).

Preparation of the yeast-displayed CLCF1 library

Yeast-displayed protein library was prepared as reported in Van Deventer and Wittrup (2014) *Methods Mol Biol* 1131:151-181. Briefly, DNA encoding human CLCF1 was cloned into the pCTcon2 yeast display plasmid using NheI and BamHI restriction sites. A DNA library was created by error-prone PCR using the CLCF1 sequence as a template, and mutations were introduced by low-fidelity Taq polymerase (Invitrogen) and 55 mM MgCl₂. Separate PCR reactions were performed using different concentrations of MnCl₂ (0, 0.01, 0.05, 0.1, and 0.15 mM). Products from these reactions were purified using gel electrophoresis. Purified mutant cDNA and linearized plasmid were electroporated into EBY100 yeast, where they were assembled *in vivo* through homologous recombination.

Library size was estimated to 8.1×10^7 by dilution plating of yeast colonies. The transformed cells were selected in SDCAA media and induced for expression in SGCAA media.

Library Screening

5 Yeast displaying high-affinity CNTFR variants were isolated from the library using fluorescence-activated cell sorting (FACS). FACS was carried out after equilibrium binding where yeasts were incubated at room temperature in phosphate-buffered saline with 1mg/mL BSA (PBSA) with the following concentrations of HIS tagged CNTFR: for sort 1, 20 nM CNTFR for 3h; for sort 2, 2 nM CN TFR for 6h; for sort 3, 0.5 nM CNTFR for 12 h.
10 After incubation with CNTFR, yeast were pelleted, washed and resuspended in PBSA with a 1:500 dilution of chicken anti-c-Myc (Invitrogen) for 30 min at 4C. Yeast were then washed and pelleted, and secondary labeling was performed on ice for 30 min using PBSA with a 1:100 dilution of goat anti-chicken PE and mouse anti-HIS Hilyte Fluor 488. Sorted clones were propagated and subjected to further rounds of FACS. After the last round of screening
15 plasmid DNA was recovered using a Zymoprep kit (Zymo Research Corp), transformed into DH10B electrocompetent cells, and isolated using plasmid miniprep kit. Sequencing was performed by MC Lab. Samples were analyzed on a FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (Treestar Inc).

Yeast cell surface CLCF1-CNTFR binding assays

20 Yeast displaying CLCF1 constructs were incubated with varying concentrations of CNTFR for 12 h at room temperature to reach equilibrium binding. This was followed by washing with BPBS and resuspension in PBSA with 1:500 ratio of chicken anti-c-Myc (Invitrogen) for 30 min at 4C. Yeast were then washed and pelleted, and secondary labeling was performed on ice for 30 min using PBSA with 1:100 dilution of goat anti-chicken PE
25 (Santa Cruz) and mouse anti-HIS Hilyte Fluor 488 (Anaspec). Then samples were washed and analyzed by flow cytometry using Accuri (BD Biosciences). Samples were analyzed on BD Bioscience software, and data were analyzed using FlowJo software. K_d values were determined by fitting to a four-parameter sigmoidal curve. Error bars represent the standard deviation of triplicate experiments.

ELISA-based CLCF1-CNTFR binding assays

To measure the solution-phase binding interaction between the CLCF1 constructs and CNTFR, different concentrations of soluble, recombinant CLCF1 variants and wtCLCF1 were incubated with 2 nM CNTFR-Fc in BPBS for 12 h at room temperature. The mixture
5 was then added to 96-well plates coated with anti-mouse-Fc antibody for 1 h followed by washing with BPBS twice. Subsequently, the wells were incubated with 1:1000 diluted anti-CLCF1 rabbit antibody for 2 h at room temperature then washed four times with BPBS. The wells were incubated with a 1:1000 diluted HRP conjugated anti-rabbit antibody for 2 h at room temperature, and washed four times with BPBS. 1-Step Ultra TMB ELISA was used
10 for the readout, which was measured using a plate reader (Tecan).

To detect binding interactions between CLCF1-CNTFR complex and gp130 or LIFR, 2 nM soluble CLCF1 variants or wtCLCF1 were incubated with 2 nM CNTFR-HIS and gp130-Fc or LIFR-Fc for 3 h at room temperature. The mixture was then added to 96-well plates coated with anti-HIS rabbit antibody for 1 h followed by washing with BPBS twice.
15 Subsequently, the wells were incubated with 1:1000 diluted HRP conjugated anti-mouse antibodies for 2 hr at room temperature, washed four times with BPBS. 1-Step Ultra TMB ELISA was used for the readout, which was measured using a plate reader (Tecan).

Phosphorylation assays

A549, SH-SY5Y, and E18 cells were grown to 50% confluence in 6-well plates. The
20 cells were serum starved overnight (12 hr) before incubation with varying concentrations of CLCF1 variants or wtCLCF1 for the indicated times at 37°C in 5% CO₂. The treated cells were lysed with NP-40 buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, and 1% IGEPAL/NP40) containing protease and phosphatase inhibitors (Thermo Fisher Scientific) for 1 h at 4°C. Then equal amounts of lysate were loaded on Bis-Tris gels (GenScript) and
25 transferred onto nitrocellulose membrane (Thermo Fisher Scientific). The blotted membrane was blocked with 5% BSA. Anti-STAT3 antibodies were from Cell Signaling. Anti-β-tubulin antibody was from Covance. All secondary antibodies were purchased from Jackson ImmunoResearch. SuperSignal West Femto Maximum Sensitivity Substrate was used as a HRP substrate (Thermo Fisher Scientific). Chemiluminescence was detected using the
30 ChemiDoc XRS System.

Cell survival assays

5 5×10^3 SH-SY5Y and 2×10^4 E18 cells were seeded and grown for 24 h, and serum starvation was induced by incubating for 24 h in DMEM with 0.1% BSA for SH-SY5Y, and neurobasal with 0.1% BSA for E18. Varying concentrations of wtCLCF1, FRR-CLCF1, and CNTF were then added and incubated for 72 h at 37°C and 5% CO₂. Next, AlamarBlue reagent (Thermo Fisher Scientific) was added to each well and incubated for 1 h at 37°C and 5% CO₂. 560EX nm/590EM nm. Error bars represent the standard deviation of triplicate wells. Data was measured against negative control with only media.

Microfluidic culture platform for *in vitro* axonal injury assay

10 Microfluidic culture devices that compartmentalize neurons were used as reported. Taylor et al. (2005) *Nat Methods* 2:599-605. Briefly, the PDMS and glass coverslips were sterilized with 70% ethanol. The cleaned glass coverslips were immersed in sterile solution of 1.0 mg/mL poly(L-lysine) (PLL) in water for 24 h before use. Sealing the PDMS piece to the PLL coated glass coverslip by conformal contact formed the enclosed channels. The device was filled with culture media for 3 h. Immediately before cell seeding culture media was aspirated and 3×10^6 cells/mL in 20 μ L of culture media were added to each of the somal side of the chamber. After incubating in a humidified incubator for 10 min to allow cells to attach rest of the chamber were filled with 150 μ L of culture media. To create axonal injury vacuum aspiration was applied in the axonal compartment for 5 s. Quickly 150 μ L of culture media mixed with wtCLCF1, FRR-CLCF1, and CNTF were added to the empty reservoirs.

25 For immunohistochemistry, the devices were separated from the slides, and the cultures were fixed using 4% paraformaldehyde for 30 min at room temperature. The cultures were then washed twice with phosphate-buffered saline (PBS) for 5 min and permeabilized using PBS with 0.2% Triton X-100 for 30 min. To block nonspecific binding, the slides were incubated in PBS with 0.2% Triton X-100 with 5% BSA for 1 h. The primary antibodies were incubated in PBS with 0.2% Triton X-100 and 5% BSA at room temperature for 1 h. Monoclonal MAP2 antibody was purchased from Sigma-Aldrich, and polyclonal Tau antibody was purchased from Abcam.

Statistical Analysis

30 Difference between groups in all experiments were examined for statistical significance using a two-tailed Student's t-test. $P < 0.05$ was considered significant.

Example 2 – Engineering Variant CNTFR Ligands for Reduced Binding of gp130 and/or LIFR

It was hypothesized that variant CNTFR ligands could be employed as a therapeutic to reduce CNTFR signaling, e.g., to treat a cell proliferative disorder, such as cancer. In this particular example, CLCF1 variants were generated and assessed for binding affinity to gp130 and/or LIFR, although the materials and methods employed in this example are readily applicable to other CNTFR ligands, such as CNTF and neuropoetin (NP1).

Screening shuffled CLCF1 library

Experimental and computational studies have shown that it is critical for a small protein to bind to its target with affinity in the low nM to pM range to have therapeutic efficacy on short time scale. To further enhance the binding affinity of CLCF1 to effectively compete against the wild type CLCF1 (wtCLCF1), another library was created and screened starting from the final population sorted in Example 1. Instead of introducing additional mutations, a PCR based recombination approach was used to shuffle segments from randomly selected variants to determine whether combining lowly enriched mutations, in addition to the mutations in FRR-CLCF1, can further enhance the binding affinity for CNTFR. From the previously sorted randomly mutagenized CLCF1 library (FIG. 6), 20 clones were randomly selected to be shuffled using the Staggered Extension Process (StEP) method. In StEP, primers anneal and extend in a step whose brief duration and suboptimal extension temperature limit primer extension. The partially extended primers randomly reanneal to different parent sequences throughout the multiple cycles, creating various recombinants. To impose increased stringency with the shuffled library, a combination of equilibrium binding and kinetic off-rate screens was used. After 3 rounds of screening different combinations of five consensus mutations (L86F, Q96R, H148R, W169L, K180R) emerged (FIG. 14).

Characterization of shuffled CLCF1

Quantitative yeast-displayed binding studies suggested that each of these mutations contributed to the binding affinity for CLCF1, and when combined with one additional mutation, Y22C, the resulting variant demonstrated the highest binding affinity (FIG. 15). CNTFR activates gp130 and LIFR by first, binding to CLCF1, then binding to gp130 and LIFR to form a tripartite receptor complex. Unexpectedly, yeast-displayed CLCF1 did not

interact with the beta receptors even in the presence of soluble CNTFR (data not shown). Given relatively smaller size of CLCF1 (20kDa) compared to other receptor subunits, it was hypothesized that steric hindrance imposed by fusing CLCF1 to yeast Aga2 prevented CLCF1-CNTFR to bind to the beta receptors.

5 To test for interactions with the beta receptors each of the variants were produced as soluble constructs by recombinant expression in *E. coli* and purified using reverse-phase high-performance liquid chromatography (RP-HPLC). Interestingly, soluble CLCF1 variants adhered to BSA coated plates but not milk coated plates, suggesting that they may interact with albumin (FIG. 16). A wide variety of molecules are known to interact with albumin to
10 increase their serum half-life and although it has not been reported, binding of CLCF1 to albumin may play an important role *in vivo*.

Shuffled CLCF1 has reduced binding for gp130

Consistent with the yeast displayed constructs, among the soluble constructs the clone with all 6 mutations demonstrated the highest binding affinity for CNTFR (FIG. 17).
15 Indeed, when the variants were produced as soluble constructs, they bound to yeast displayed CNTFR, soluble gp130 and LIFR. This was subsequently confirmed using a cell-free ELISA method. Y22C, W169L, and K180R each contributed to binding to CNTFR, decreasing K_d of FRR-CLCF1 from 869 pM to 464 pM, 640 pM, and 559 pM respectively. A variant with all of the mutations combined showed the highest binding affinity with the K_d of
20 115 pM (FIG. 18). Unexpectedly, Y22C, W169L, and K180R decreased binding for gp130 with the combined mutations leading to the lowest binding while the mutations led to increased LIFR binding through increased CNTFR binding (FIG. 18). Because reduced binding interaction with beta receptors is desirable for antagonizing activation of CNTFR-CLCF1 mediated pathways, the clone with all six mutations, which was named sfCLCF1
25 (shuffled CLCF1) was selected for further studies.

Alanine substitution to residues F151 and K154 leads to reduced binding for LIFR

Despite the decreased binding for gp130, even minimal interaction with the beta receptors, combined with the increased affinity for CNTFR, can activate the downstream
30 signaling pathways. To further reduce interactions with the beta receptors, modifications that would decrease the binding of LIFR was sought. Evolutionarily IL-6 cytokines have the

conserved sequence FXXK, which has been shown to contribute to LIFR binding. It has been suggested that F151 and K154 may also be important residues for CLCF1 binding to LIFR, although this had not been experimentally confirmed. Peret et al. (2004) *J Biol Chem* 279:43961-43970. Substitution of these residues with alanine effectively decreased the LIFR signal both on wtCLCF1 and engineered CLCF1 constructs (FIG. 19).

Engineered CLCF1 does not activate STAT3 and competitively inhibits wtCLCF1 induced STAT3 activation and cell survival

Based on the results from screening and alanine substitutions, constructs with different binding properties were prepared (FIG. 20). To examine how these constructs activate the downstream signaling pathways, they were treated on A549 cells. As shown in Example 1, a variant with mutations that increased affinity for CNTFR but did not decrease binding for gp130 (FRR-CLCF1) increased STAT3 phosphorylation (FIG. 6, panel A). The construct that contained gp130 reducing mutation but increased affinity for CNTFR (sfCLCF1) showed partial agonist activity while those containing LIFR reducing mutations (FRRAA-CLCF1 and sfAA-CLCF1) showed no detectable STAT3 activation. When these constructs were co-incubated with wtCLCF1, the construct with high affinity for CNTFR and reduced binding for gp130 and LIFR (sfAA-CLCF1) decreased STAT3 activation (FIG. 6, panel B). Therefore, sfAA-CLCF1 was chosen for further characterization and named “enCLCF1” (engineered CLCF1) for convenience. The amino acid sequence of enCLCF1 is provided in Table 2 above (SEQ ID NO:6).

enCLCF1 decreases in vitro cell survival

To test the effect of enCLCF1 treatment on cell survival, NSCLC cells A549 and H23 were serum starved overnight and treated with 50 nM wtCLCF1 and different concentrations of enCLCF1 for 72 h. In a concentration-dependent manner, enCLCF1 inhibited CLCF1 induced survival in both cell lines (FIG. 22).

In vivo effects of enCLCF1

To test the *in vivo* effect of enCLCF1 treatment, a study was conducted using 7 pairs of mice bearing subcutaneous A549 xenograft tumors. A549 cells were subcutaneously injected and grown on two opposing flanks of each mouse. After the tumor sizes grew to approximately 100 mm³, the mice were injected with 1 mg per body weight dose of an enCLCF1 or saline control, three times weekly for 4 weeks. Treatment with the enCLCF1

significantly decreased tumor growth compared saline control and the treatment displayed no adverse events or signs of toxicity with this dosing regimen (FIG. 23).

Materials and Methods

Cells and Reagents

5 A549 was purchased from ATCC. A549 growth media was Dulbecco's Modified Eagle Media (Invitrogen) with 10% fetal bovine serum (FBS) (American Type Culture Collection), 1% penicillin-streptomycin. H23 cells were grown in RPMI-1640 Medium with 10% FBS, 1% penicillin-streptomycin. Anti-CLCF1 and anti-CNTFR antibodies were purchased from Abcam. Anti-HIS Hilyte Fluor 488 antibody was purchased from Anaspec
10 and anti-mouse Alexa 488 antibody was from Invitrogen. Anti-STAT3 antibodies were from Cell Signaling. Anti- β -tubulin antibody was from Covance. All secondary antibodies were purchased from Jackson ImmunoResearch, and 1-Step Ultra TMB ELISA was from Pierce.

Recombinant CLCF1 production

cDNA of CLCF1 constructs were clones into pET28b plasmid with inducible lac
15 promoter using BsaI and XhoI restriction sites and amplified in DH10B cells. For expression, purified plasmids were transformed into Rosetta gami cells. Inclusion body was solubilized in 60% ddH₂O, 40% acetonitrile, 0.1% TFA) containing 5 mM DTT. Then reversed-phase high-performance liquid chromatography (RP-HPLC) was used to purify CLCF1. Protein purity was further analyzed using SDS-PAGE and quantified using a Nanodrop 2000
20 (Thermo Scientific). 39,549 was used for the extinction coefficient.

Soluble CNTFR, LIFR and gp130 production

cDNA of CNTFR (1-342), LIFR (1-534), and gp130 (1-619) genes were cloned into Add2 plasmid and amplified in DH10B cells. For expression, purified plasmids were transformed into human embryonic kidney 293 (HEK 293) cells using polyethylenimine
25 (PEI). Briefly, PEI was dissolved in dH₂O to 1g/L. For 500 mL transfection volume, 0.5 mg of purified DNA and 1 mL of PEI was dissolved in 10 mL of OptiPro Serum Free Media each, then mixed immediately. After 15 minutes the solution was added dropwise to 500 mL of cells. The cells were incubated on a rotary shaker at 120 RPM in a humidified incubator at 37°C and 5% CO₂. Fc fusion proteins were purified using protein A column and HIS tag
30 fusion proteins were purified using nickel column. The proteins were then further purified using size exclusion FPLC. Following extinction coefficients were used: 70,275 for soluble

CNTRF variants, 206,410 for CNTRF-Fc variants, 130,470 for soluble gp130, 326,800 for gp130-Fc, 98,610 for soluble LIFR, and 263,080 for LIFR-Fc.

Preparation of yeast-displayed CLCF1 library

From the population isolated in the final sort round with randomly mutagenized CLCF1 library (Example 1), 20 non-repeating sequences were selected randomly. 1 ng of each of the templates was combined with 1 ng of wtCLCF1 sequence and 21 ng total template was mixed with the final concentrations of 0.15 μ M each primer, 1X PCR buffer, 200 μ M dNTP mix, 1.5 mM MgCl₂, 2.5 U Taq polymerase in sterile dH₂O to 50 μ L. The extension protocol was run for 100 cycles using the following parameters: 94°C for 30 s (denaturation) and 55°C for 10 s. Products from these reactions were purified using gel electrophoresis. Purified mutant cDNA and linearized plasmid were electroporated in EBY100 yeast, where they were assembled in vivo through homologous recombination. Library size was estimated to 7.4×10^7 by dilution plating.

Library screening

FACS rounds for shuffled library was done using a single round of equilibrium binding sort using 0.5 nM of CNTRF followed by two rounds of kinetic off-rate sorts. For kinetic off-rate sorts yeasts were incubated with 2 nM CNTRF for 2 h at room temperature, after which cells were washed twice to remove excess unbound CNTRF and resuspended in PBSA containing 20 nM wtCLCF1 to render unbinding events irreversible. For the length of the unbinding steps, 10 h was used for sort 2 and 24 h was used for sort 3. Labeled yeasts were sorted by FACS using a BD Aria II flow cytometer and BD FACSCalibur. Sorts were conducted such that the 0.5-1% of clones with the highest CLCF1 binding/c-Myc expression ratio (to normalize by expression) were selected, enriching the library for clones with the highest binding affinity to CLCF1. Sorted clones were propagated and subjected to further rounds of FACS. After the last screening, plasmid DNA was recovered using a Zymoprep kit (Zymo Research Corp), transformed into DH10B electrocompetent cells, and isolated using plasmid miniprep kit. Sequencing was performed. Samples were analyzed on a FACSCalibur and data were analyzed using FlowJo software.

Cell-free binding assays

96-well plates were coated with 10 μ g/mL of anti-HIS antibody overnight and blocked with 5% milk for 1 h. The plates were then washed twice with BPBS. Different

concentrations of soluble constructs of HIS-tagged CLCF1 variants were incubated with 2 nM CNTFR-Fc in BPBS for 12 h at room temperature. The mixture was then added to 96-well plates coated with anti-HIS antibody for 1 h followed by washing with BPBS twice. Subsequently, the wells were incubated with 1:1000 diluted HRP conjugated anti-mouse antibody for 1 h at room temperature then washed four times with PBS. 1-Step Ultra TMB ELISA (Thermo Fisher Scientific) was used for the readout detected using a plate reader (Tecan).

Phosphorylation assays

A549 cells were grown until 50% confluence in 6-well plates. The cells were incubated in 2 nM or 10 nM of CLCF1 constructs for 20 min at 37°C in 5% CO₂, then lysed with NP-40 buffer containing protease and phosphatase inhibitor (Thermo Scientific). Equal amounts of lysate were loaded on Bis-Tris gels and transferred onto nitrocellulose membrane. Western Blot analysis was performed with the reagents above. Chemiluminescence was detected using the ChemiDoc XRS System (Bio-Rad). NP-40 buffer was composed of 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, and 1% IGEPAL/NP40. For analyzing competitive inhibition of wtCLCF1 mediated STAT3 activation, 2 nM or 10 nM of CLCF1 constructs were mixed with 40 nM of wtCLCF1 to treat A549 cells. The rest of the steps are same as above.

Cell survival assays

5×10^3 A549 and H23 cells were seeded and grown for 24 h, and serum starvation was induced by incubating for 24 h in DMEM with 0.1% BSA. CLCF1 and CNTFR constructs were then added and incubated for 72 h at 37°C and 5% CO₂. Next, AlamarBlue reagent was added to each well and incubated for 1 h at 37°C and 5% CO₂. The cell metabolic activity was detected by measuring fluorescence using 560EX nm/590EM nm. Error bars represent the standard deviation of triplicate wells. Data was measured against negative control with only media.

In vivo tumor models

To generate NSCLC xenograft model, 1×10^6 A549 cells were injected subcutaneously in the two lower flanks of NSG mice (NOD, SCID, gamma). The tumors were allowed to grow to on average 100 mm³ before dosing. enCLCF1 was administered at

1 mg per kg body weight three times weekly via intraperitoneal injection for 31 days and the tumor volume was calculated using $\text{volume} = \pi/6 \times (\text{length}) \times (\text{width})^2$.

Example 3 – Expression of CLCF1 and CNTFR in lung cancer

While cancer-associated fibroblasts (CAFs) express CLCF1 and may be the source for this cytokine *in vivo*, the present study determined that NSCLC cell lines also secrete CLCF1, suggesting the existence of both paracrine and autocrine signaling mechanisms for this cytokine (FIG. 24, panel A). The receptor for CLCF1, CNTFR, was also determined to be expressed on all NSCLC cell lines and patient-derived xenograft (PDX) models tested (FIG. 24, panels B and C). Expression of CNTFR was also observed by immunohistochemistry in PDX models and in tumors generated in the KrasG12D;P53f/f genetically-engineered mouse model (FIG. 24, panel D). Taken together these results suggest that the CLCF1-CNTFR signaling axis is active in lung adenocarcinoma and that it may have a role in oncogenesis, particularly in tumors driven by oncogenic Kras.

Notwithstanding the appended claims, the disclosure is also defined by the following clauses:

1. A ciliary neurotrophic factor receptor (CNTFR) ligand selected from the group consisting of:

a CNTFR ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand,

a CNTFR ligand that results in reduced binding affinity of glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), or both, for a complex comprising the CNTFR ligand and CNTFR, relative to the binding affinity for a complex comprising the corresponding wild-type CNTFR ligand and CNTFR, and

a CNTFR ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand and results in reduced binding affinity of gp130, LIFR, or both, for a complex comprising the CNTFR ligand and CNTFR, relative to the binding affinity for a complex comprising the corresponding wild-type CNTFR ligand and CNTFR.

2. The CNTFR ligand of Clause 1, wherein the CNTFR ligand is a ciliary neurotrophic factor (CNTF) ligand, a cardiotrophin-like cytokine factor 1 (CLCF1) ligand, or a neuropoetin (NP) ligand.

5

3. The CNTFR ligand of Clause 1 or Clause 2, wherein the CNTFR ligand exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand.

4. The CNTFR ligand of Clause 3, wherein the CNTFR ligand is a CLCF1 ligand comprising one or more mutations at amino acid positions 86, 96, 148, 169, 180, or any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

5. The CNTFR ligand of Clause 4, wherein the CLCF1 ligand comprises one or more amino acid substitutions selected from the group consisting of: L86F, Q96R, H148R, W169L, K180R, and any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

6. The CNTFR ligand of any one of Clauses 1 to 5, wherein the CNTFR ligand results in reduced binding affinity of gp130, LIFR, or both, for a complex comprising the CNTFR ligand and CNTFR.

7. The CNTFR ligand of Clause 6, wherein the CNTFR ligand results in reduced binding affinity of gp130 for a complex comprising the CNTFR ligand and CNTFR.

25

8. The CNTFR ligand of Clause 7, wherein the CNTFR ligand is a CLCF1 ligand comprising one or more mutations at amino acid positions 22, 169, 180, or any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

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9. The CNTFR ligand of Clause 8, wherein the CLCF1 ligand comprises one or more amino acid substitutions selected from the group consisting of: Y22C, W169L, K180R, and

any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

10. The CNTFR ligand of any one of Clauses 6 to 9, wherein the CNTFR ligand results in reduced binding affinity of LIFR for a complex comprising the CNTFR ligand and CNTFR.

11. The CNTFR ligand of Clause 10, wherein the CNTFR ligand is a CLCF1 ligand comprising one or more mutations at amino acid positions 151, 154, or both, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

12. The CNTFR ligand of Clause 11, wherein the CLCF1 ligand comprises one or more amino acid substitutions selected from the group consisting of: F151A, K154A, and F151A and K154A, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

13. The CNTFR ligand of any one of Clauses 1 to 12, wherein the CNTFR ligand is fused to a heterologous polypeptide.

14. The CNTFR ligand of Clause 13, wherein the heterologous polypeptide is an Fc domain, an albumin, a transferrin, XTEN, a homo-amino acid polymer, a proline-alanine-serine polymer, an elastin-like peptide, or any combination thereof.

15. The CNTFR ligand of Clause 14, wherein the heterologous polypeptide is an Fc domain.

16. The CNTFR ligand of Clause 15, wherein the Fc domain is a human Fc domain.

17. The CNTFR ligand of any one of Clauses 1 to 16, wherein the CNTFR ligand is conjugated to a moiety.

18. The CNTFR ligand of Clause 17, wherein the moiety is polyethylene glycol (PEG), an anti-cancer drug, a detectable label, or any combination thereof.

19. A pharmaceutical composition, comprising:
the ciliary neurotrophic factor receptor (CNTFR) ligand of any one of Clauses 1 to 18; and
a pharmaceutically acceptable carrier.

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20. A method, comprising:
administering to an individual in need thereof a therapeutically effective amount of the ciliary neurotrophic factor receptor (CNTFR) ligand of any one of Clauses 1 to 18, or the pharmaceutical composition of Clause 19.

10

21. The method according to Clause 20, wherein the individual in need thereof has a cell proliferative disorder associated with CNTFR signaling, and the administering is effective in treating the cell proliferative disorder.

- 15 22. The method according to Clause 21, wherein the cell proliferative disorder is cancer.

23. The method according to Clause 22, wherein the cancer is lung cancer.

- 20 24. The method according to Clause 23, wherein the lung cancer is non-small cell lung cancer (NSCLC).

- 25 25. The method according to Clause 20, wherein the individual in need thereof has a neurodegenerative disorder, and the administering is effective in treating the neurodegenerative disorder.

26. The method according to any one of Clauses 20 to 25, further comprising, prior to the administering, identifying the individual as having a disorder associated with CNTFR signaling.

- 30 27. The method according to Clause 26, wherein the identifying is based on CNTFR ligand abundance in a sample obtained from the individual.

28. The method according to Clause 27, wherein the abundance is of a CNTFR ligand selected from the group consisting of: CNTF, CLCF1, NP, and any combination thereof.

29. The method according to Clause 27 or Clause 28, wherein the CNTFR ligand
5 abundance is quantified using a soluble CNTFR polypeptide as a CNTFR ligand capture agent.

30. The method according to any one of Clauses 26 to 29, wherein the identifying is based on CNTFR abundance in a sample obtained from the individual.

10 31. The method according to any one of Clauses 26 to 30, wherein the identifying is based on the level of CNTFR signaling in a sample obtained from the individual.

32. The method according to Clause 31, wherein the level of CNTFR signaling in the
15 sample is determined based on the phosphorylation status of one or more CNTFR signaling pathway molecules.

33. The method according to any one of Clauses 27 to 32, wherein the identifying is based on an immunoassay.

20 34. The method according to any one of Clauses 27 to 32, wherein the identifying is based on nucleic acid sequencing.

35. The method according to any one of Clauses 27 to 34, wherein the sample is a
25 tissue sample.

36. The method according to any one of Clauses 27 to 34, wherein the sample is a fluid sample.

30 37. The method according to any one of Clauses 27 to 36, further comprising obtaining the sample from the individual.

38. A nucleic acid that encodes the CNTFR ligand of any one of Clauses 1 to 18.

39. An expression vector comprising the nucleic acid of Clause 38.

40. A host cell comprising the CNTFR ligand of any one of Clauses 1 to 18, the nucleic
5 acid of Clause 38, the expression vector of Clause 39, or any combination thereof.

41. The host cell of Clause 40, wherein the host cell is a prokaryotic cell.

42. The host cell of Clause 40, wherein the host cell is a eukaryotic cell.

10 43. The host cell of Clause 42, wherein the eukaryotic cell is a mammalian cell.

44. The host cell of Clause 43, wherein the mammalian cell is a human cell.

15 45. A nucleic acid that encodes a ciliary neurotrophic factor receptor (CNTFR) ligand
fused to a cell surface display protein.

46. The nucleic acid of Clause 45, wherein the CNTFR ligand is a ciliary neurotrophic
factor (CNTF) ligand, a cardiotrophin-like cytokine factor 1 (CLCF1) ligand, or a neuropoetin
20 (NP) ligand.

47. A nucleic acid that encodes ciliary neurotrophic factor receptor (CNTFR) fused to a
cell surface display protein.

25 48. The nucleic acid of any one of Clauses 45 to 47, wherein the cell surface display
protein is selected from the group consisting of: a bacterial surface display protein, a phage
display protein, and a yeast display protein.

49. The nucleic acid of Clause 48, wherein the cell surface display protein is a yeast
30 display protein.

50. The nucleic acid of Clause 49, wherein the yeast display protein is Aga2p.

51. An expression vector comprising the nucleic acid of any one of Clauses 45 to 50.

52. A host cell comprising the nucleic acid of any one of Clauses 45 to 50, or the expression vector of Clause 51.

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53. A CNTFR ligand fused to a cell surface display protein, or CNTFR fused to a cell surface display protein, encoded by the nucleic acid of any one of Clauses 45 to 50 or the expression vector of Clause 51.

Accordingly, the preceding merely illustrates the principles of the present disclosure. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

WHAT IS CLAIMED IS:

1. A ciliary neurotrophic factor receptor (CNTFR) ligand selected from the group consisting of:
 - a CNTFR ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand,
 - a CNTFR ligand that results in reduced binding affinity of glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), or both, for a complex comprising the CNTFR ligand and CNTFR, relative to the binding affinity for a complex comprising the corresponding wild-type CNTFR ligand and CNTFR, and
 - a CNTFR ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand and results in reduced binding affinity of gp130, LIFR, or both, for a complex comprising the CNTFR ligand and CNTFR, relative to the binding affinity for a complex comprising the corresponding wild-type CNTFR ligand and CNTFR.
2. The CNTFR ligand of Claim 1, wherein the CNTFR ligand is a ciliary neurotrophic factor (CNTF) ligand, a cardiotrophin-like cytokine factor 1 (CLCF1) ligand, or a neuropoetin (NP) ligand.
3. The CNTFR ligand of Claim 1 or Claim 2, wherein the CNTFR ligand exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand.
4. The CNTFR ligand of Claim 3, wherein the CNTFR ligand is a CLCF1 ligand comprising one or more mutations at amino acid positions 86, 96, 148, 169, 180, or any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.
5. The CNTFR ligand of Claim 4, wherein the CLCF1 ligand comprises one or more amino acid substitutions selected from the group consisting of: L86F, Q96R, H148R,

W169L, K180R, and any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

6. The CNTFR ligand of any one of Claims 1 to 5, wherein the CNTFR ligand results in reduced binding affinity of gp130, LIFR, or both, for a complex comprising the CNTFR ligand and CNTFR.

7. The CNTFR ligand of Claim 6, wherein the CNTFR ligand results in reduced binding affinity of gp130 for a complex comprising the CNTFR ligand and CNTFR.

8. The CNTFR ligand of Claim 7, wherein the CNTFR ligand is a CLCF1 ligand comprising one or more mutations at amino acid positions 22, 169, 180, or any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

9. The CNTFR ligand of Claim 8, wherein the CLCF1 ligand comprises one or more amino acid substitutions selected from the group consisting of: Y22C, W169L, K180R, and any combination thereof, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

10. The CNTFR ligand of any one of Claims 6 to 9, wherein the CNTFR ligand results in reduced binding affinity of LIFR for a complex comprising the CNTFR ligand and CNTFR.

11. The CNTFR ligand of Claim 10, wherein the CNTFR ligand is a CLCF1 ligand comprising one or more mutations at amino acid positions 151, 154, or both, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

12. The CNTFR ligand of Claim 11, wherein the CLCF1 ligand comprises one or more amino acid substitutions selected from the group consisting of: F151A, K154A, and F151A and K154A, relative to a CLCF1 ligand having the amino acid sequence set forth in SEQ ID NO:3.

13. The CNTFR ligand of any one of Claims 1 to 12, wherein the CNTFR ligand is fused to a heterologous polypeptide.
14. The CNTFR ligand of Claim 13, wherein the heterologous polypeptide is an Fc domain, an albumin, a transferrin, XTEN, a homo-amino acid polymer, a proline-alanine-serine polymer, an elastin-like peptide, or any combination thereof.
15. The CNTFR ligand of Claim 14, wherein the heterologous polypeptide is an Fc domain.
16. The CNTFR ligand of Claim 15, wherein the Fc domain is a human Fc domain.
17. The CNTFR ligand of any one of Claims 1 to 16, wherein the CNTFR ligand is conjugated to a moiety.
18. The CNTFR ligand of Claim 17, wherein the moiety is polyethylene glycol (PEG), an anti-cancer drug, a detectable label, or any combination thereof.
19. A pharmaceutical composition, comprising:
the ciliary neurotrophic factor receptor (CNTFR) ligand of any one of Claims 1 to 18; and
a pharmaceutically acceptable carrier.
20. A method, comprising:
administering to an individual in need thereof a therapeutically effective amount of the ciliary neurotrophic factor receptor (CNTFR) ligand of any one of Claims 1 to 18, or the pharmaceutical composition of Claim 19.
21. The method according to Claim 20, wherein the individual in need thereof has a cell proliferative disorder associated with CNTFR signaling, and the administering is effective in treating the cell proliferative disorder.

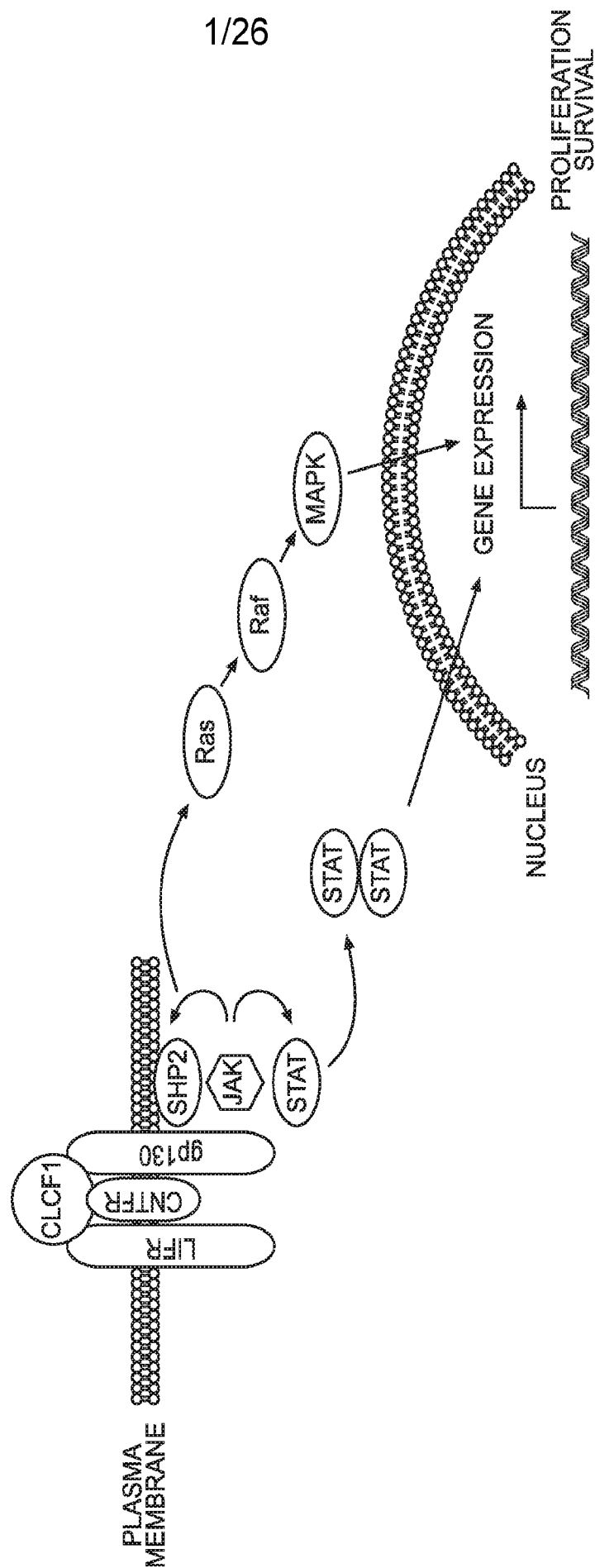
22. The method according to Claim 21, wherein the cell proliferative disorder is cancer.
23. The method according to Claim 22, wherein the cancer is lung cancer.
24. The method according to Claim 23, wherein the lung cancer is non-small cell lung cancer (NSCLC).
25. The method according to Claim 20, wherein the individual in need thereof has a neurodegenerative disorder, and the administering is effective in treating the neurodegenerative disorder.
26. The method according to any one of Claims 20 to 25, further comprising, prior to the administering, identifying the individual as having a disorder associated with CNTFR signaling.
27. The method according to Claim 26, wherein the identifying is based on CNTFR ligand abundance in a sample obtained from the individual.
28. The method according to Claim 27, wherein the abundance is of a CNTFR ligand selected from the group consisting of: CNTF, CLCF1, NP, and any combination thereof.
29. The method according to Claim 27 or Claim 28, wherein the CNTFR ligand abundance is quantified using a soluble CNTFR polypeptide as a CNTFR ligand capture agent.
30. The method according to any one of Claims 26 to 29, wherein the identifying is based on CNTFR abundance in a sample obtained from the individual.
31. The method according to any one of Claims 26 to 30, wherein the identifying is based on the level of CNTFR signaling in a sample obtained from the individual.

32. The method according to Claim 31, wherein the level of CNTFR signaling in the sample is determined based on the phosphorylation status of one or more CNTFR signaling pathway molecules.
33. The method according to any one of Claims 27 to 32, wherein the identifying is based on an immunoassay.
34. The method according to any one of Claims 27 to 32, wherein the identifying is based on nucleic acid sequencing.
35. The method according to any one of Claims 27 to 34, wherein the sample is a tissue sample.
36. The method according to any one of Claims 27 to 34, wherein the sample is a fluid sample.
37. The method according to any one of Claims 27 to 36, further comprising obtaining the sample from the individual.
38. A nucleic acid that encodes the CNTFR ligand of any one of Claims 1 to 18.
39. An expression vector comprising the nucleic acid of Claim 38.
40. A host cell comprising the CNTFR ligand of any one of Claims 1 to 18, the nucleic acid of Claim 38, the expression vector of Claim 39, or any combination thereof.
41. The host cell of Claim 40, wherein the host cell is a prokaryotic cell.
42. The host cell of Claim 40, wherein the host cell is a eukaryotic cell.
43. The host cell of Claim 42, wherein the eukaryotic cell is a mammalian cell.
44. The host cell of Claim 43, wherein the mammalian cell is a human cell.

45. A nucleic acid that encodes a ciliary neurotrophic factor receptor (CNTFR) ligand fused to a cell surface display protein.
46. The nucleic acid of Claim 45, wherein the CNTFR ligand is a ciliary neurotrophic factor (CNTF) ligand, a cardiotrophin-like cytokine factor 1 (CLCF1) ligand, or a neuropoetin (NP) ligand.
47. A nucleic acid that encodes ciliary neurotrophic factor receptor (CNTFR) fused to a cell surface display protein.
48. The nucleic acid of any one of Claims 45 to 47, wherein the cell surface display protein is selected from the group consisting of: a bacterial surface display protein, a phage display protein, and a yeast display protein.
49. The nucleic acid of Claim 48, wherein the cell surface display protein is a yeast display protein.
50. The nucleic acid of Claim 49, wherein the yeast display protein is Aga2p.
51. An expression vector comprising the nucleic acid of any one of Claims 45 to 50.
52. A host cell comprising the nucleic acid of any one of Claims 45 to 50, or the expression vector of Claim 51.
53. A CNTFR ligand fused to a cell surface display protein, or CNTFR fused to a cell surface display protein, encoded by the nucleic acid of any one of Claims 45 to 50 or the expression vector of Claim 51.

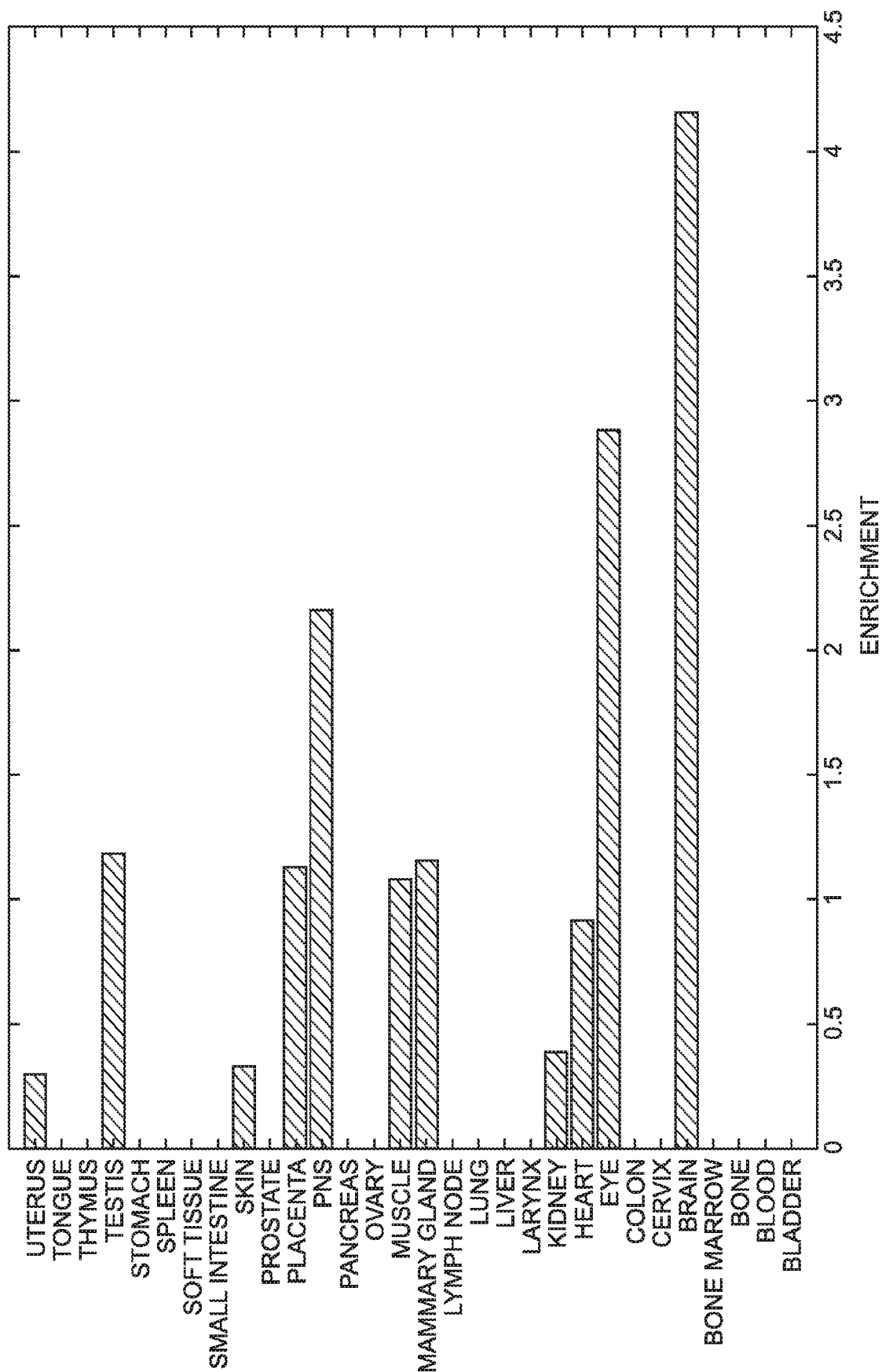
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FIG. 1



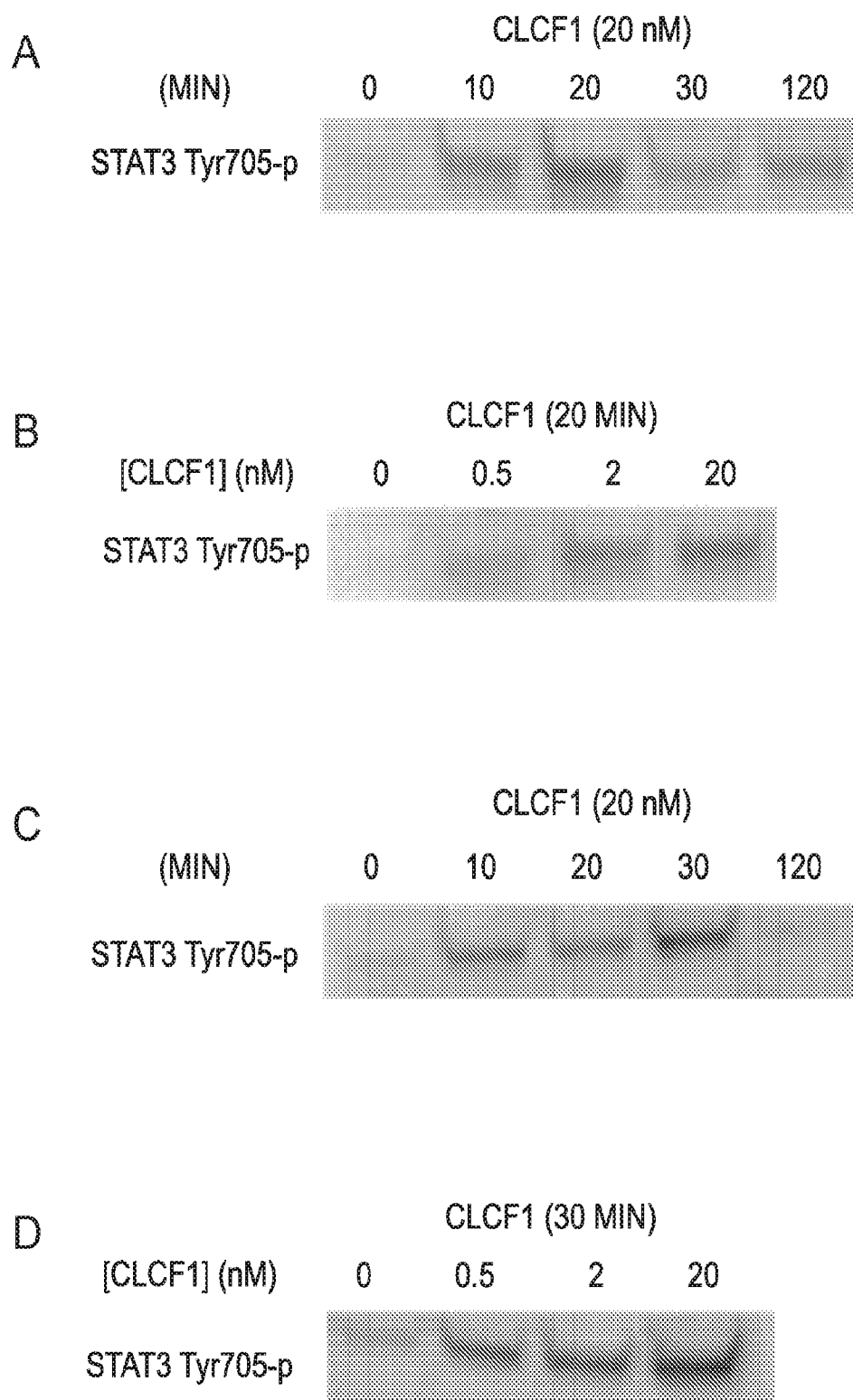
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FIG. 2



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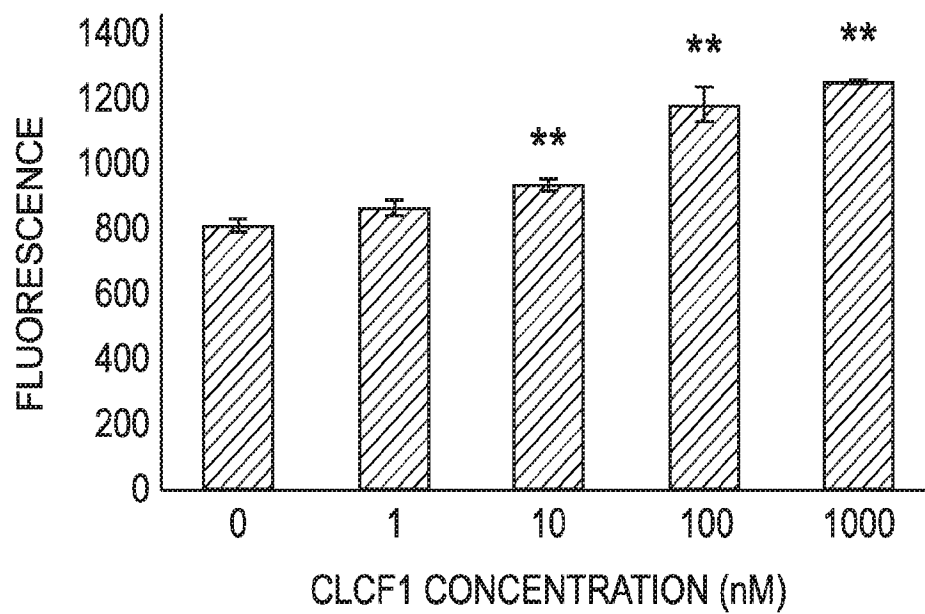
FIG. 3



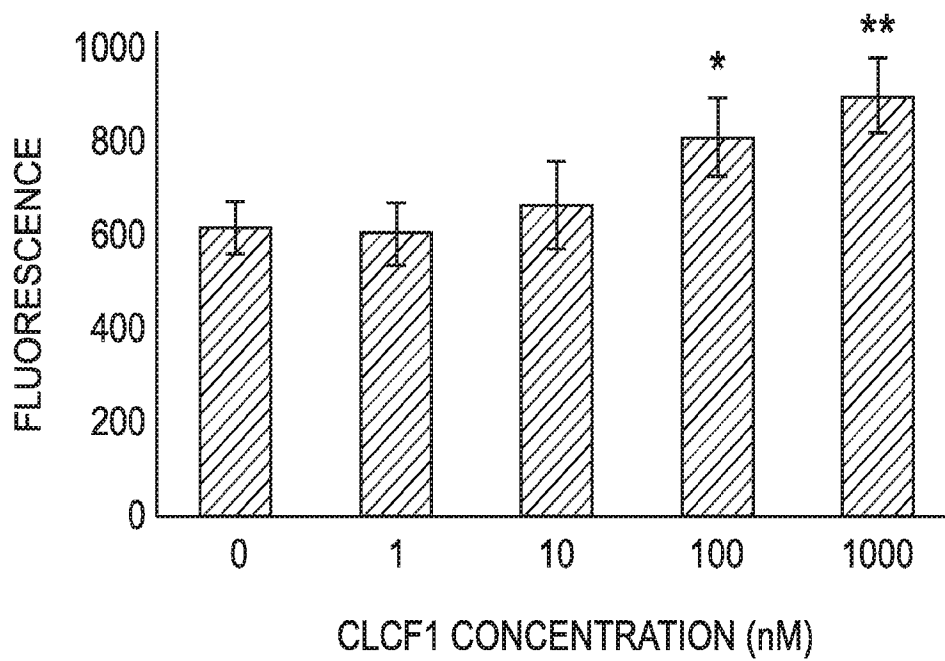
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FIG. 4

A

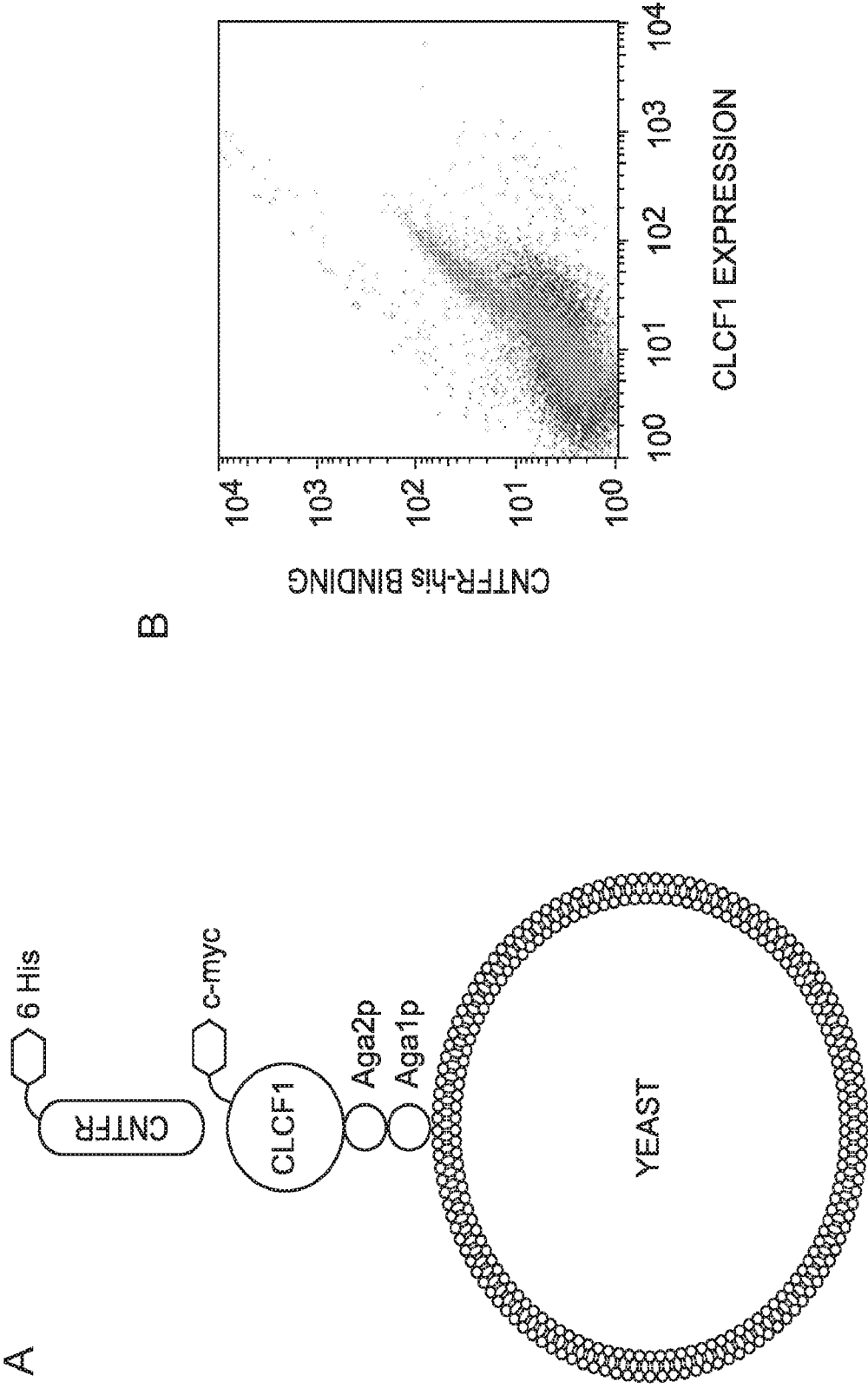


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FIG. 5



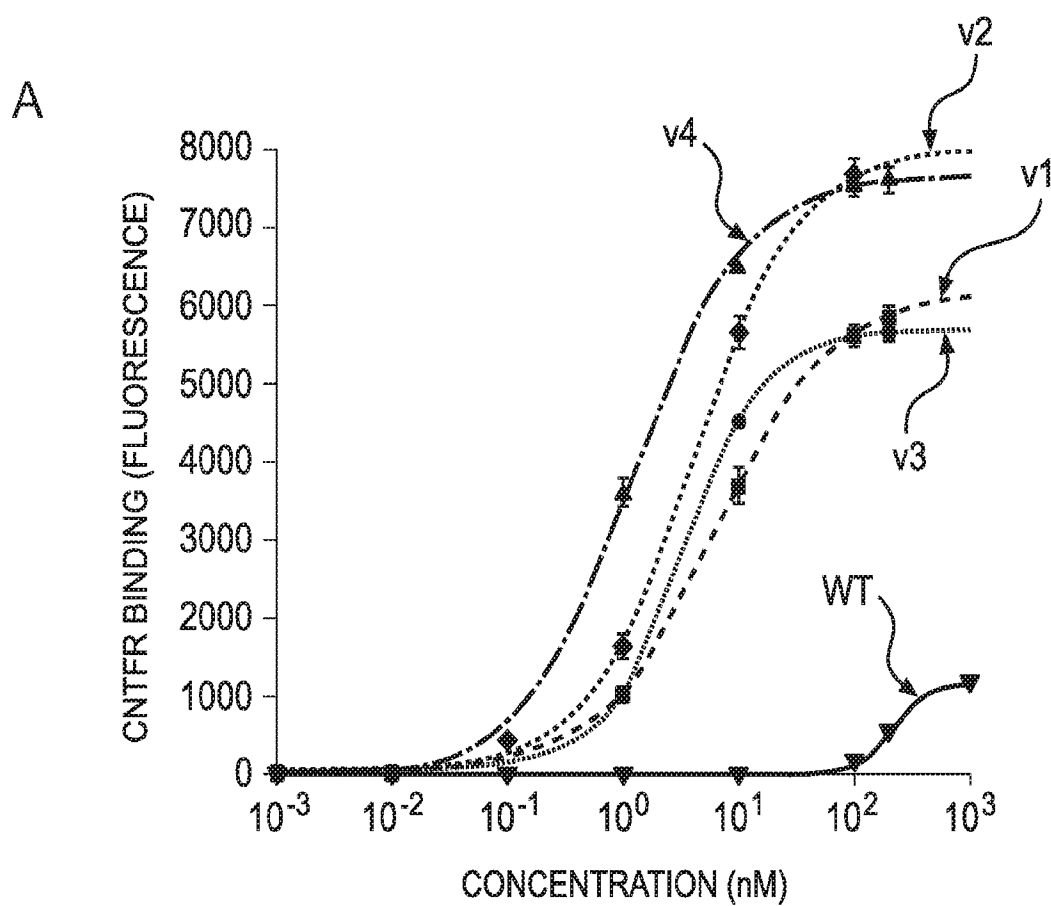
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FIG. 6

SAMPLE	8	25	32	55	62	69	71	74	86	88	96	99	107	108	112	119	133	142	148	156	158	162	166	169	173	179	180	183	184	189
WT	P	H	G	E	V	S	S	L	L	C	Q	T	A	H	S	S	Q	W	H	D	F	K	T	W	K	K	K	Q	P	V
#1											R																			
#2								Q											R					L						
#3							S													E										
#4																														
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FIG. 7

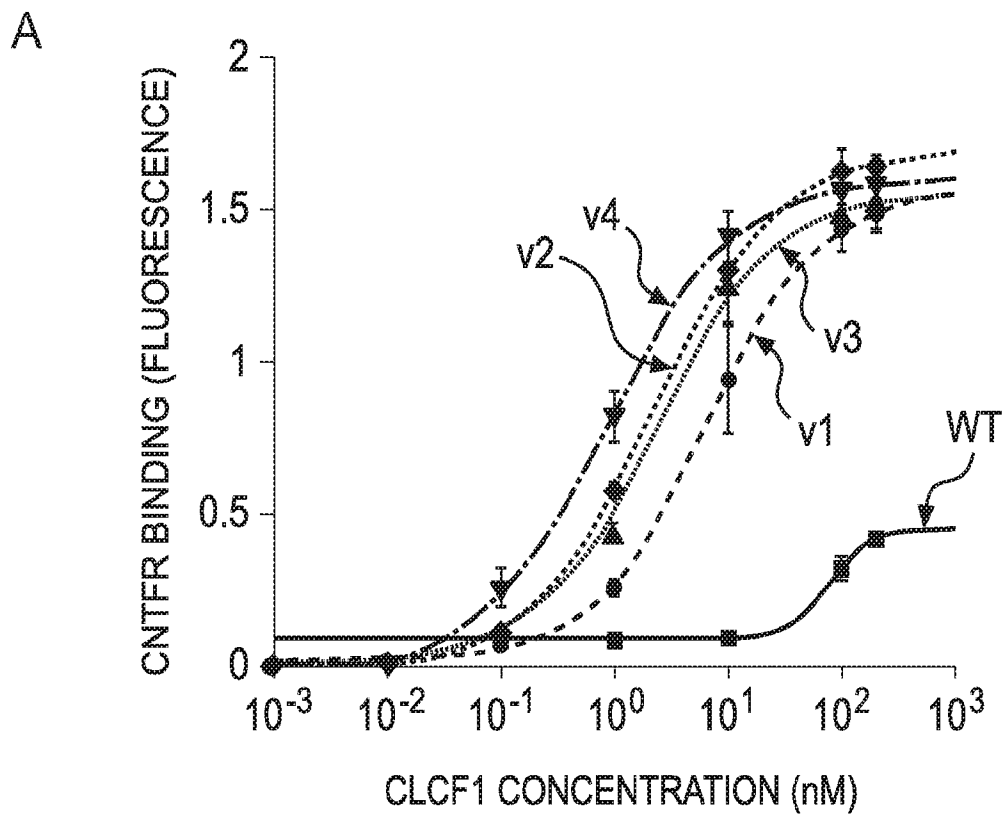


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	RESIDUE NO.			CNTFR AFFINITY
	86	96	148	
WT	L	Q	H	K _d (nM)
v1		R		7.9 ± 1.6
v2	F	R		3.1 ± 0.1
v3		R	R	4.2 ± 0.3
v4	F	R	R	0.83 ± 0.06

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FIG. 8

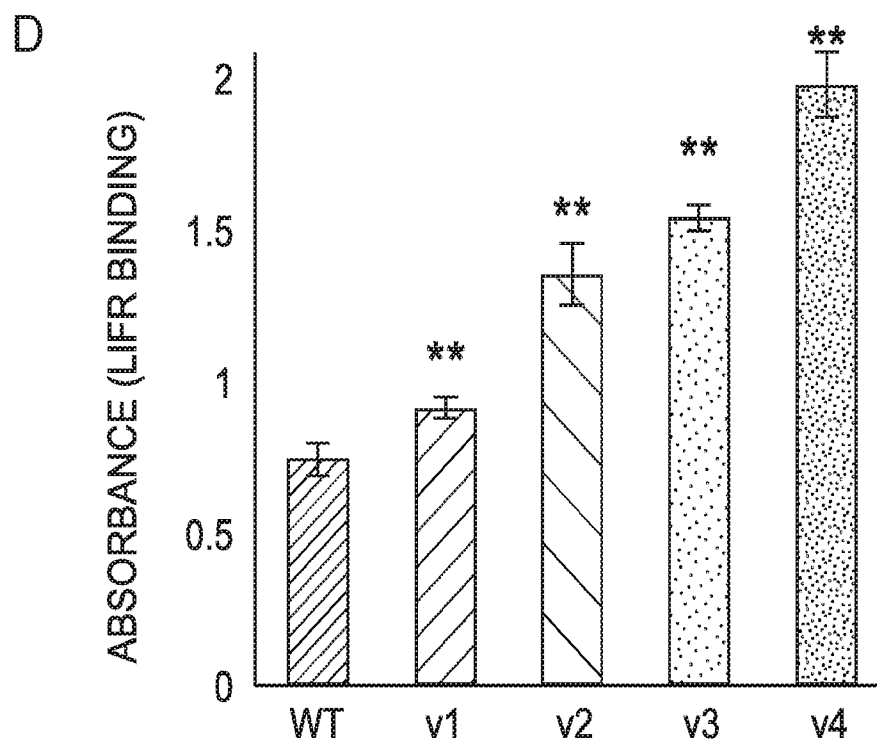
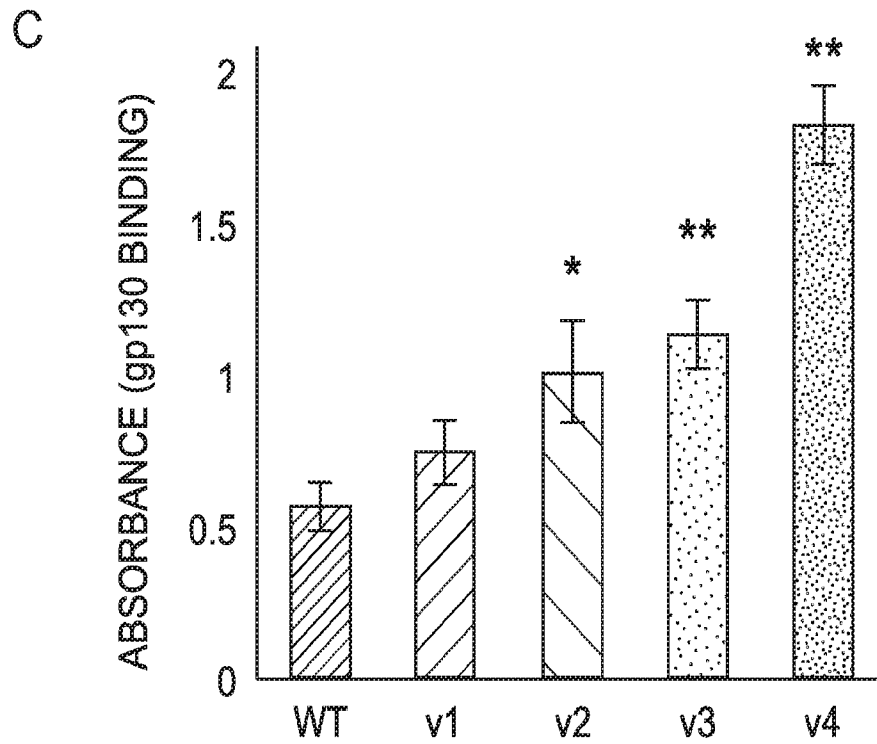


B

	RESIDUE NO.			CNTFR AFFINITY
	86	96	148	
WT	L	Q	H	K _d (nM)
v1		R		6.6 ± 4.1
v2	F	R		2.3 ± 0.3
v3		R	R	2.5 ± 1.0
v4	F	R	R	0.86 ± 0.26

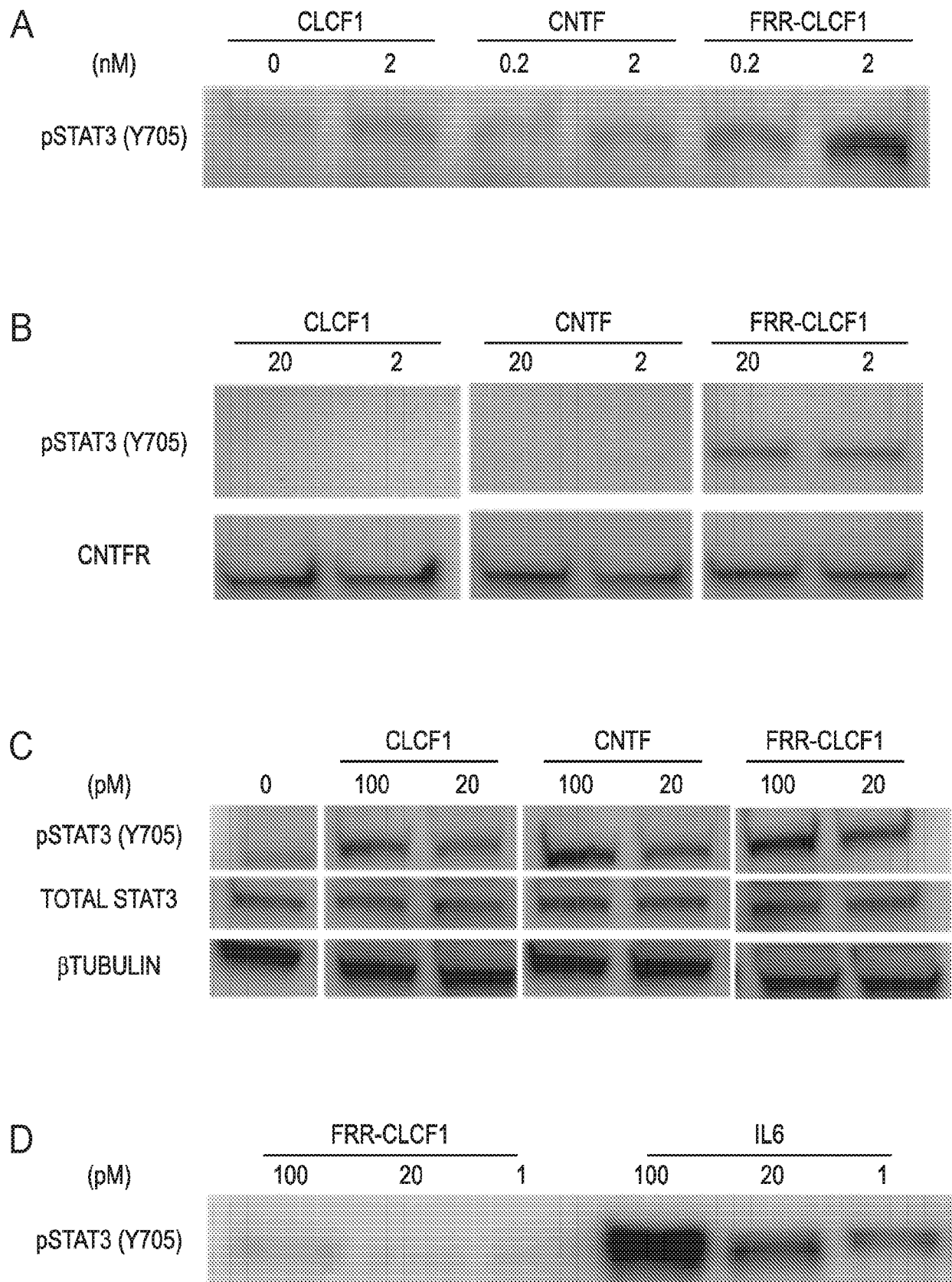
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FIG. 8 (Cont.)



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FIG. 9



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FIG. 10

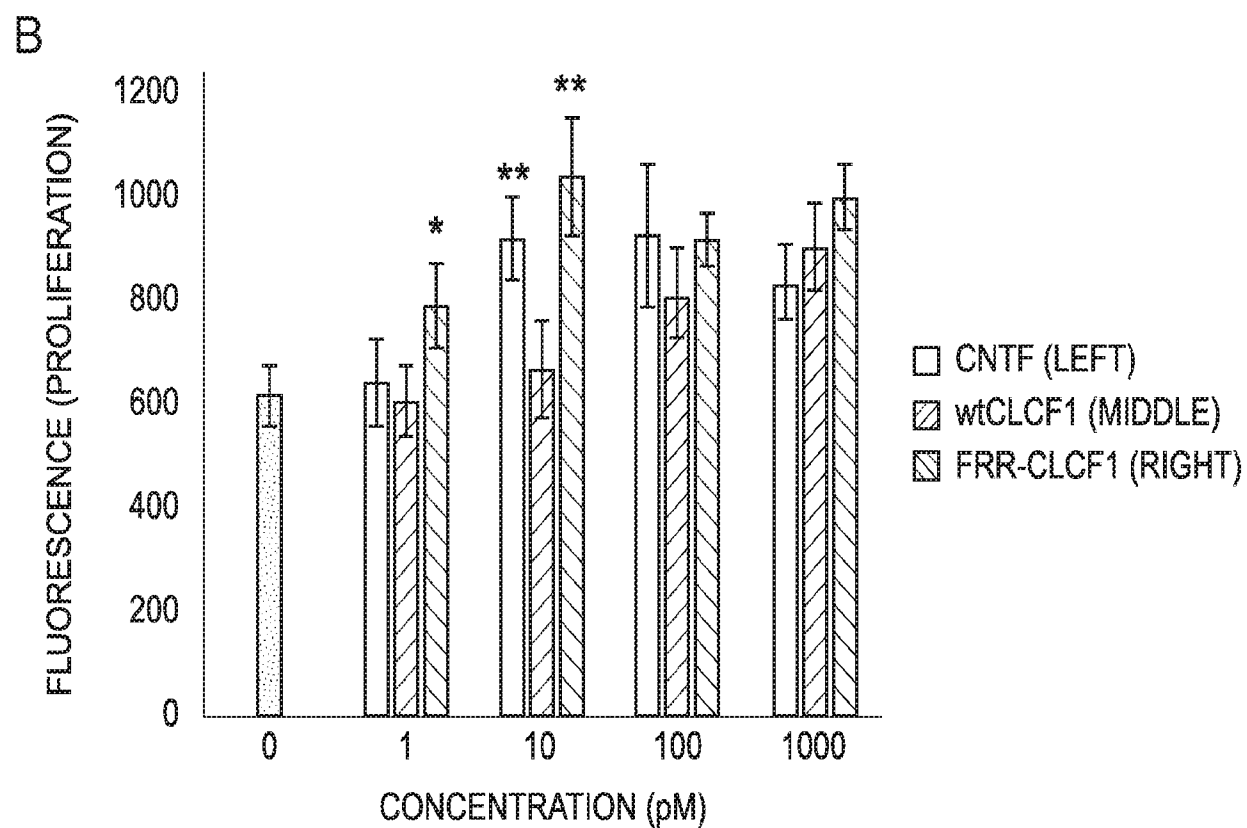
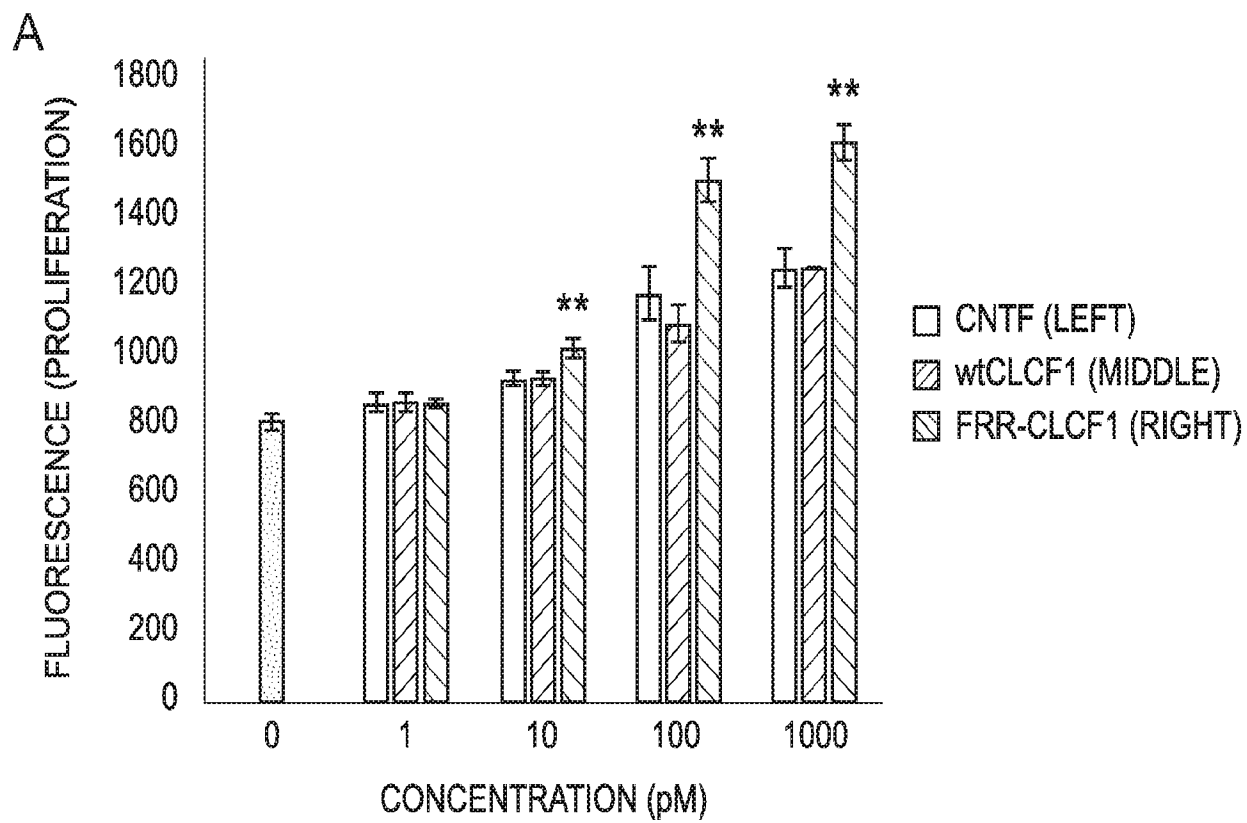


FIG. 11

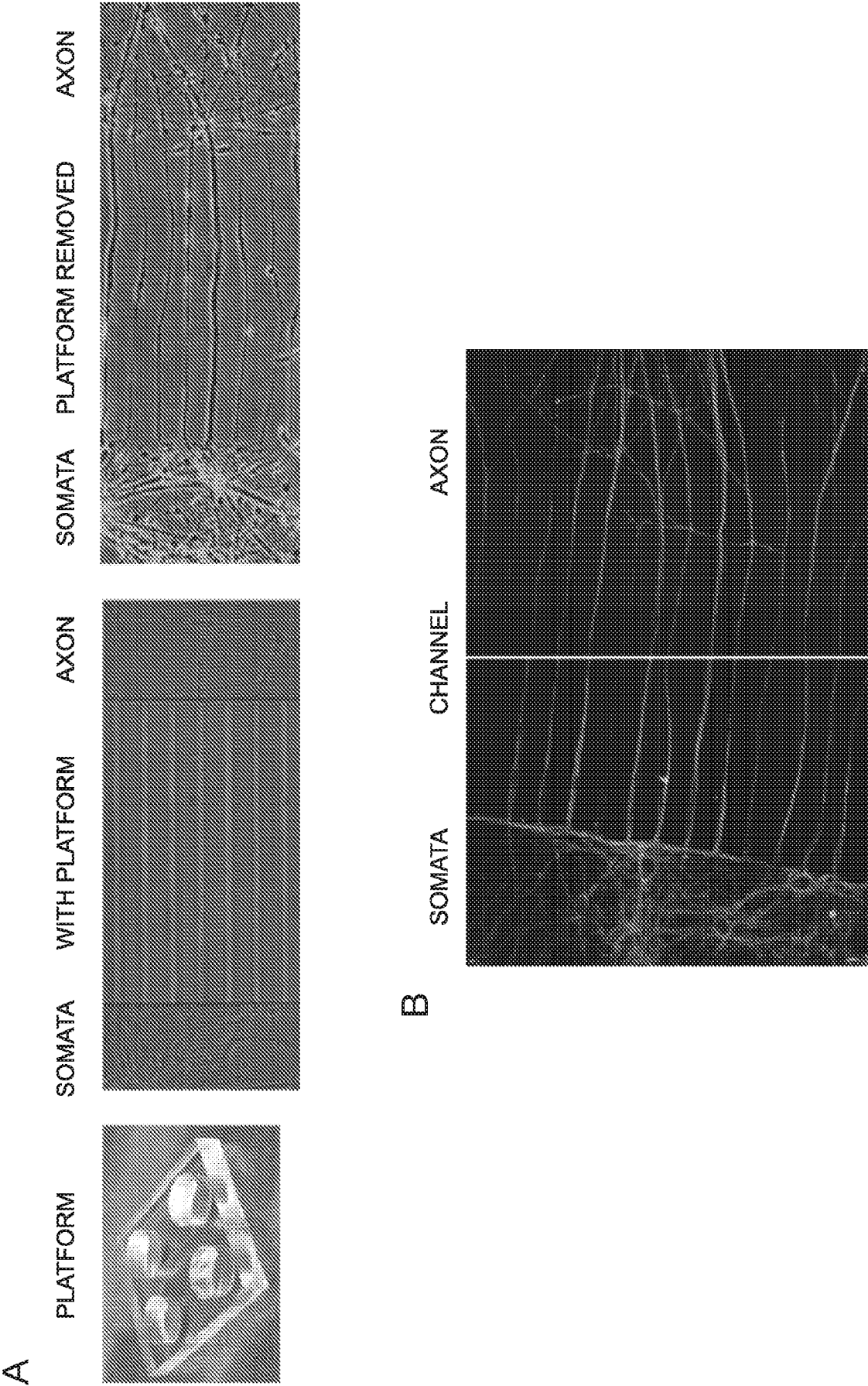
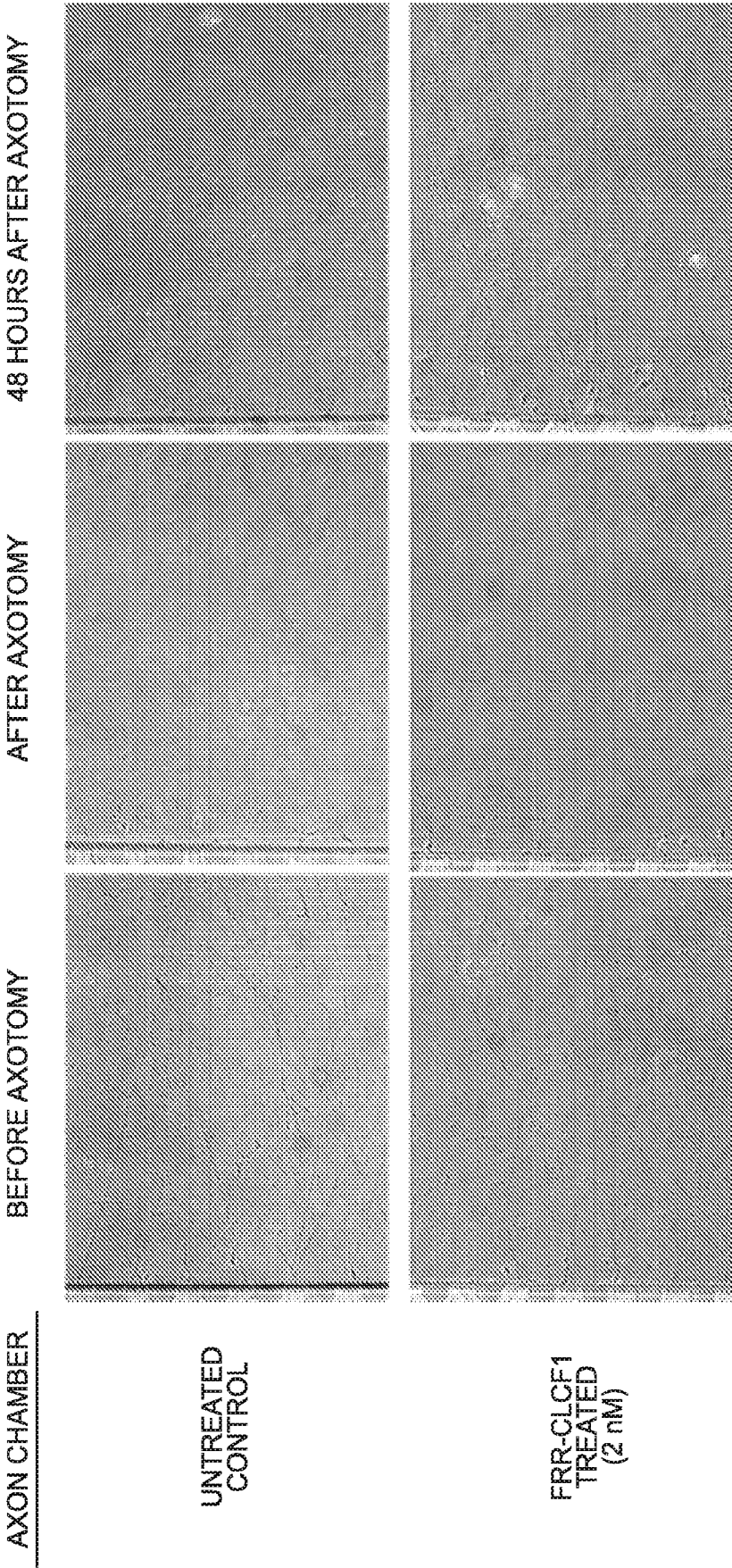
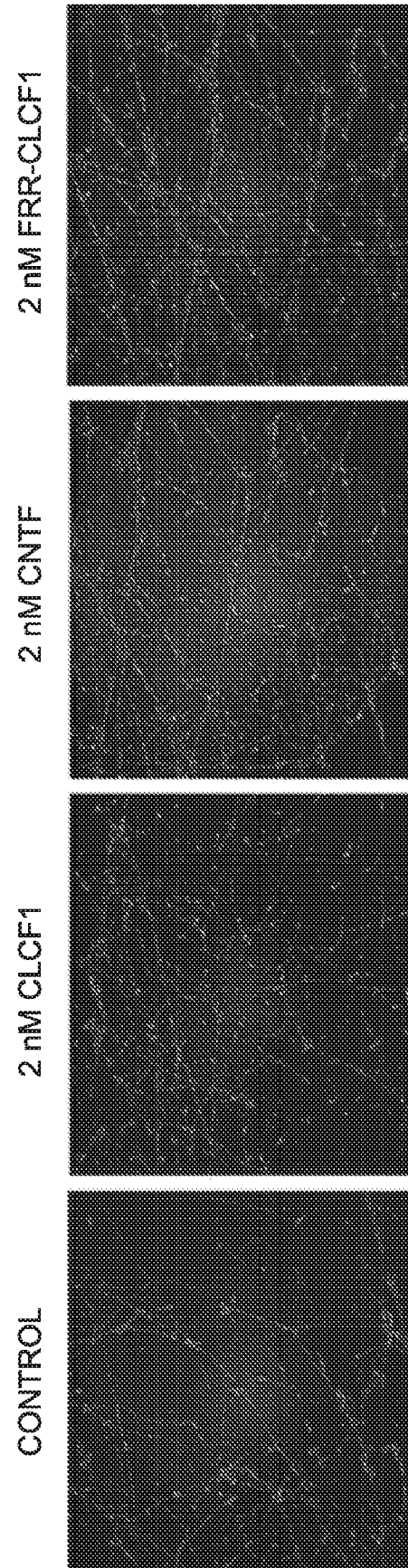


FIG. 12



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FIG. 13



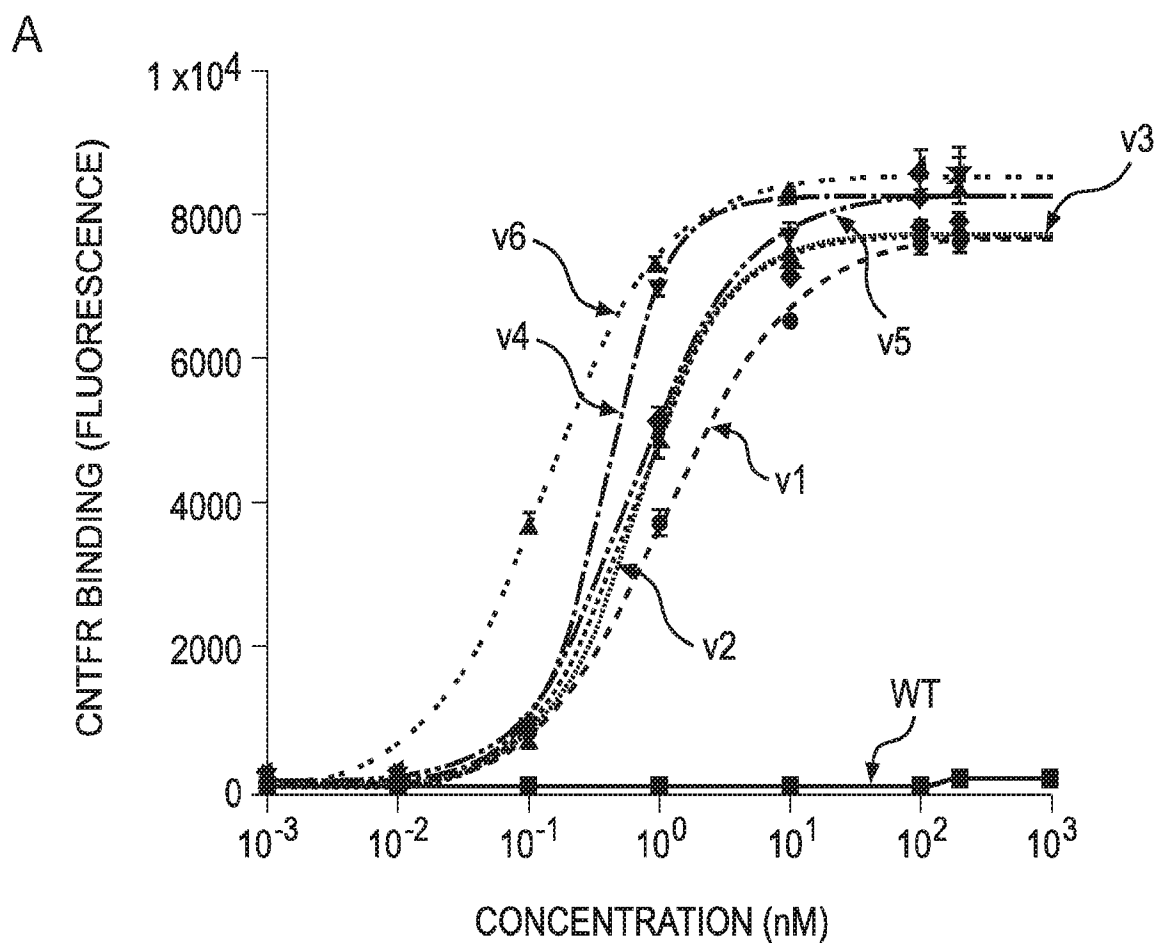
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FIG. 14

CLCF1	7	22	25	32	62	71	74	86	88	96	111	112	133	139	142	148	151	152	156	158	162	169	180	183
wt	P	Y	H	H	V	N	L	L	C	Q	T	S	Q	E	W	H	F	S	D	F	K	W	K	Q
1					I					R			R			R					L			
2		C					Q									R					L		R	
3										R		G		S		S					L		R	
4										R			R								R			
5						S				R						R					L		R	
6			Y					F		R		G			S				Y				R	
7						S				R													R	
8			R	R				F		R	A					R					L			
9					I				R	R					S						L		R	
10		C						F		R				G									R	
11					I		Q			R											R		R	L
12						S		F		R														
13					I					R								G			L			
14				R				F		R											L			
15					I					R						R					L			
16								F		R											R		R	R
18						Q		F		R						R					L		R	L
19								F		R						R					L			
20								F	R	R		G							E		L			
21	T					S		F		R						R					L			
22		R						F		R											R		L	

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FIG. 15

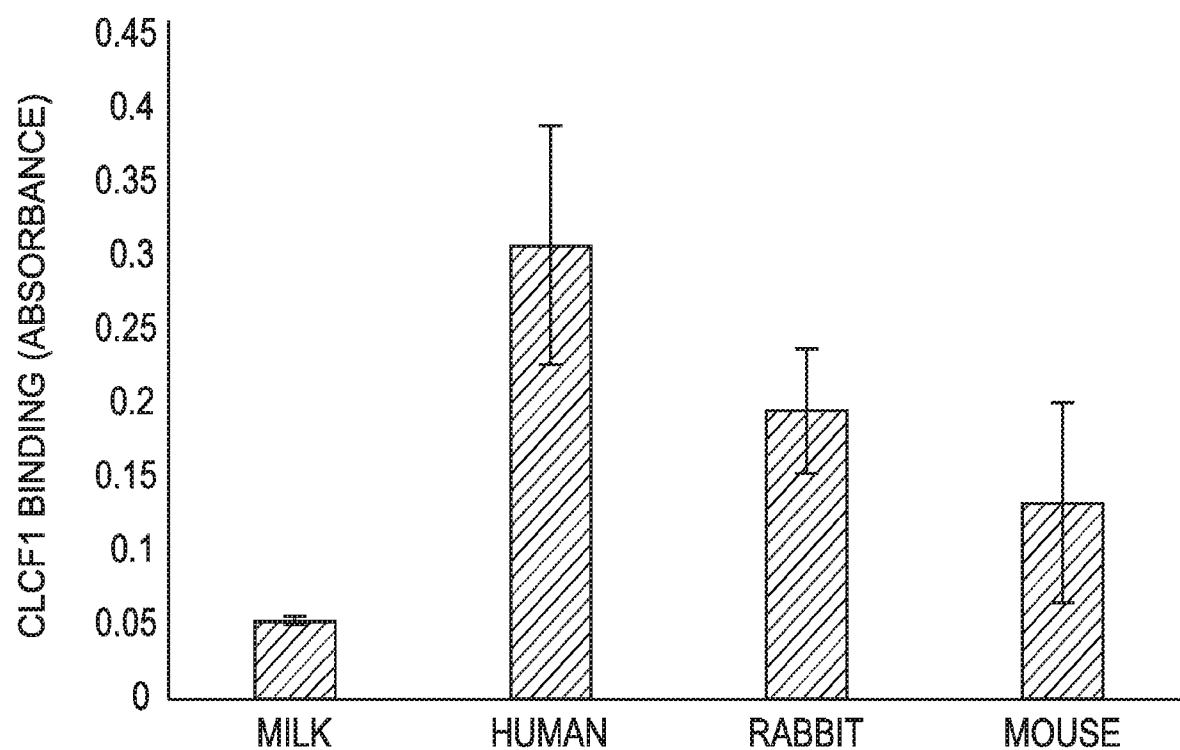


B

	RESIDUE NO.						CNTFR AFFINITY
	22	86	96	148	169	180	
WT	Y	L	Q	H	W	K	K _d (pM)
v1		F	R	R			830 ₊₃₀
v2		F	R	R	L		220 ₊₂₀
v3		F	R	R		R	280 ₊₃₀
v4		F	R	R	L	R	80 ₊₂₀
v5	C	F	R	R			180 ₊₇₀
v6	C	F	R	R	L	R	60 ₊₁₀

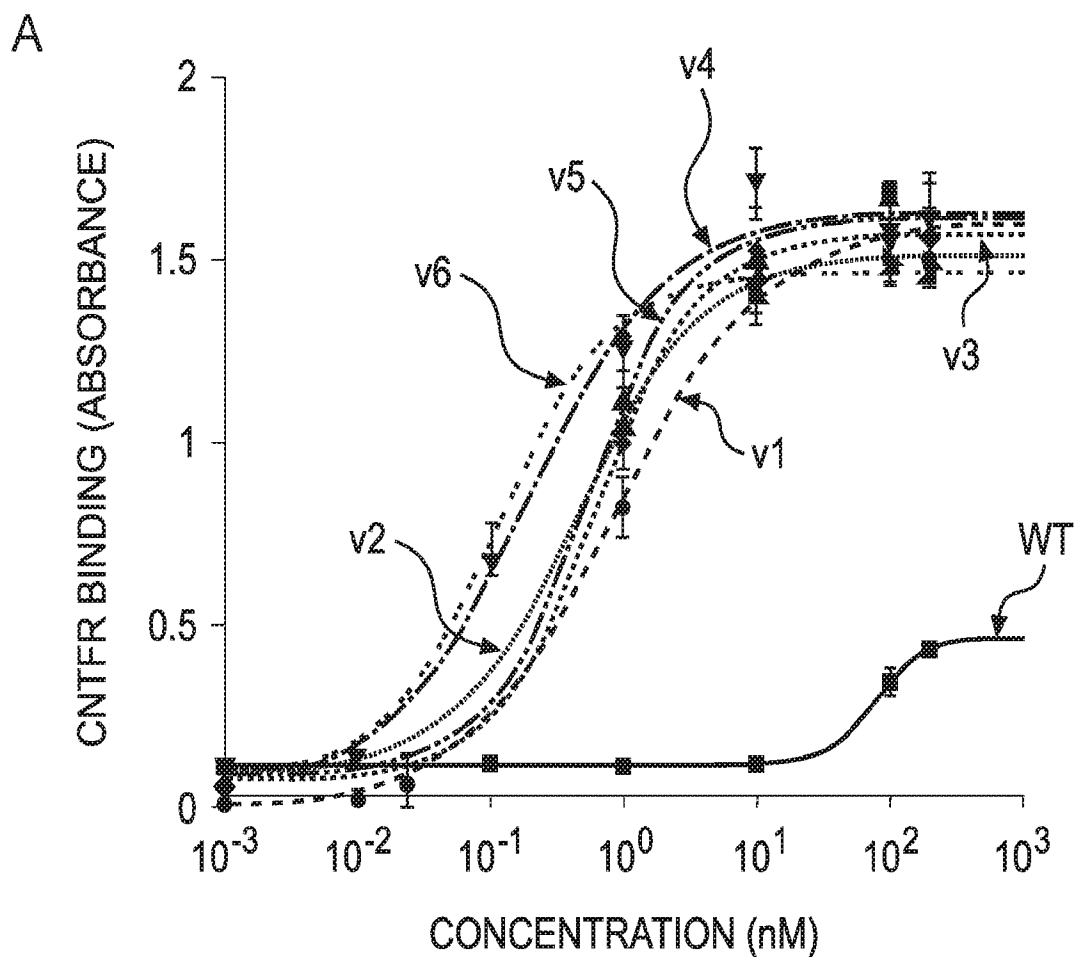
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FIG. 16



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FIG. 17



B

	RESIDUE NO.						CNTFR AFFINITY
	22	86	96	148	169	180	
WT	Y	L	Q	H	W	K	K _d (pM)
v1		F	R	R			870 ⁺³⁰
v2		F	R	R	L		640 ⁺¹⁰
v3		F	R	R		R	560 ⁺²⁰
v4		F	R	R	L	R	200 ⁺⁴⁰
v5	C	F	R	R			460 ⁺⁶⁰
v6	C	F	R	R	L	R	120 ⁺¹⁰

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FIG. 18

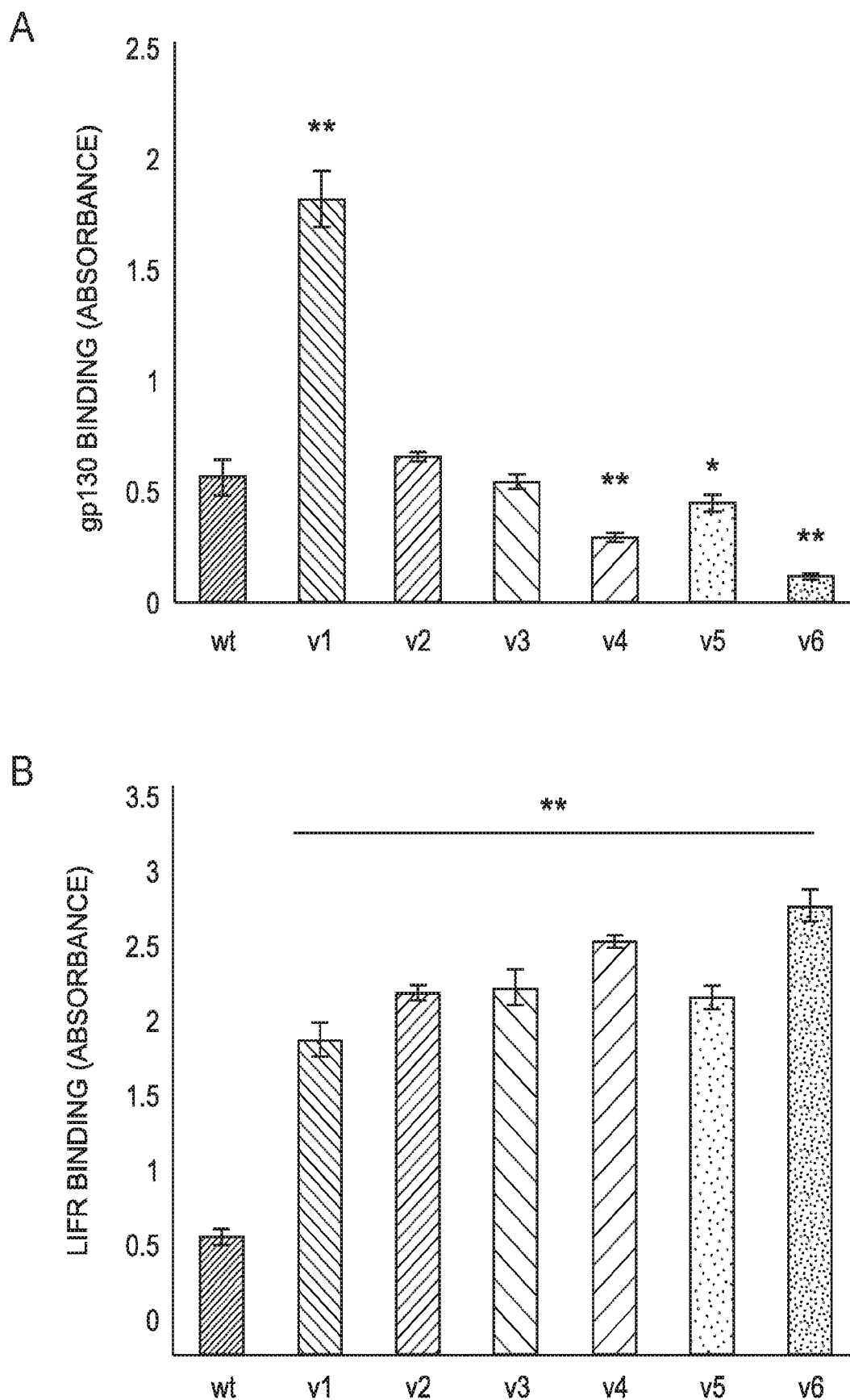


FIG. 19

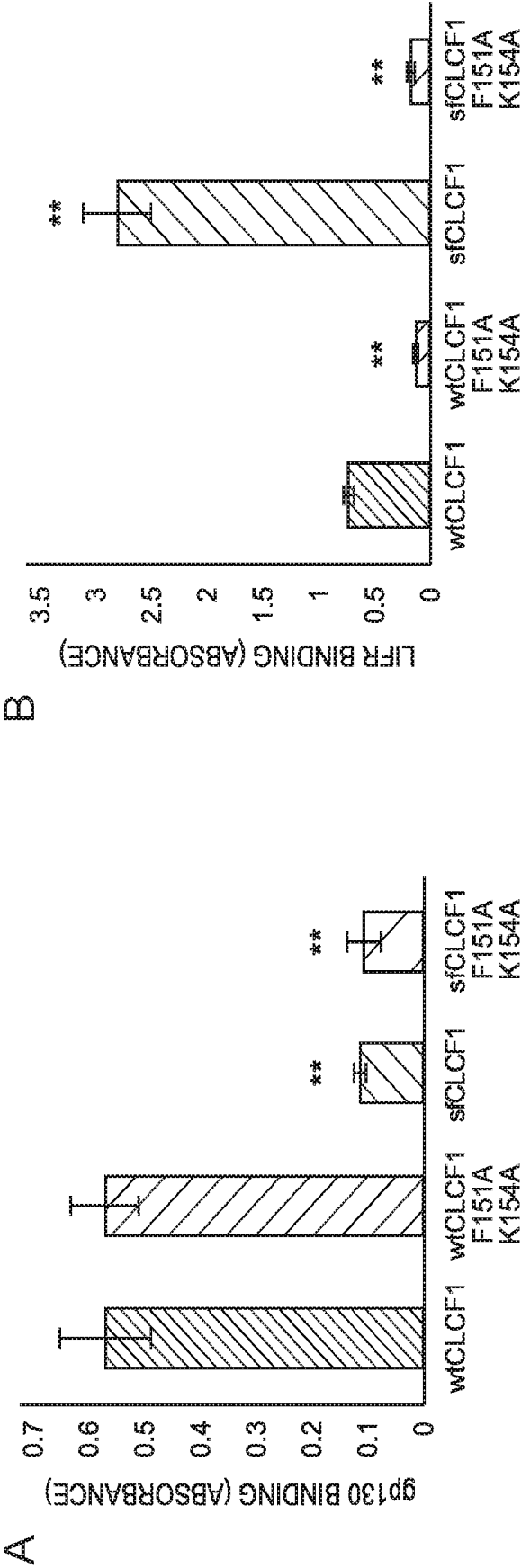
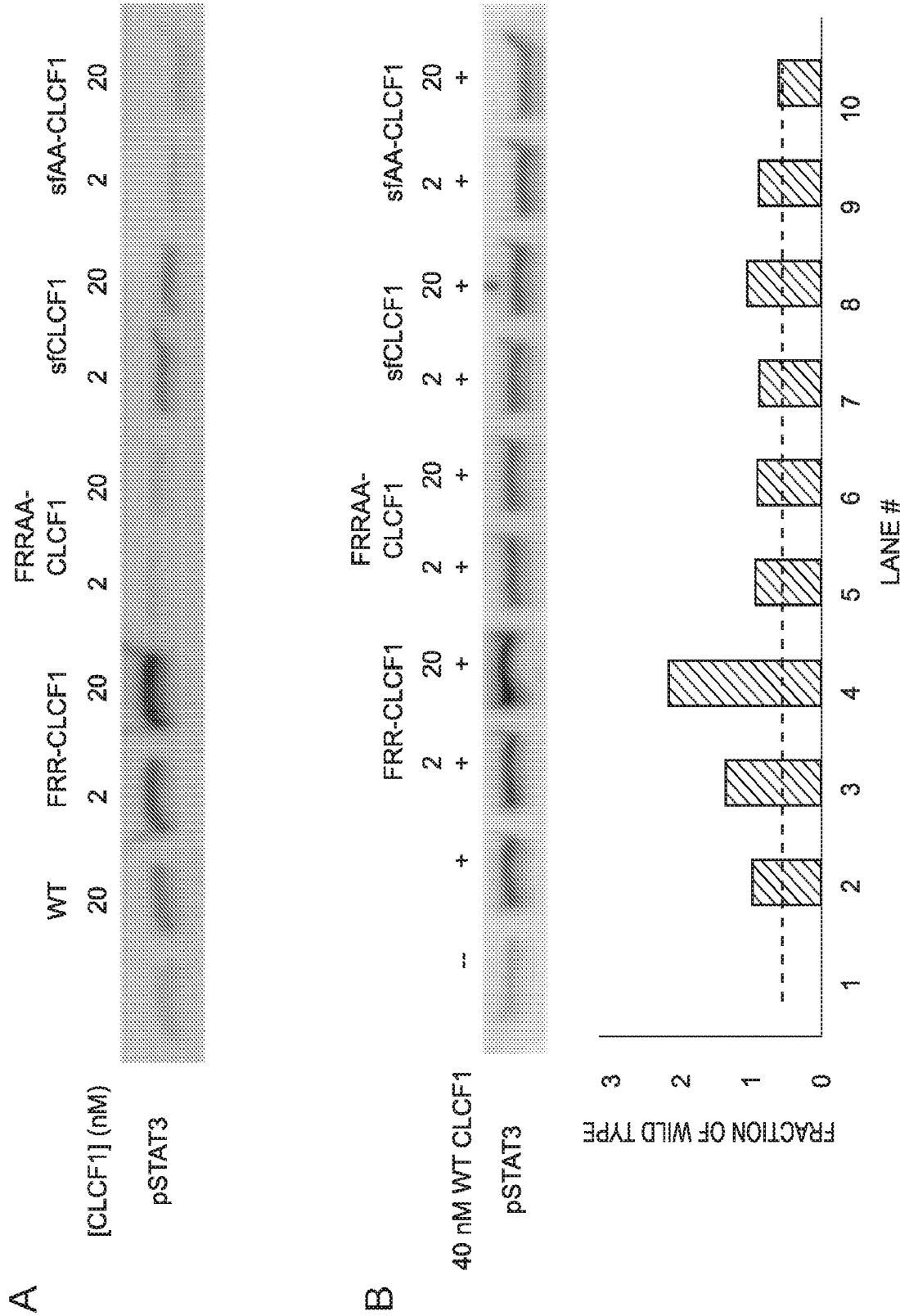


FIG. 20

RESIDUE NO.										
	22	86	96	148	151	154	169	180	Kd (nM)	BINDING
wICLCF1	Y	L	Q	H	F	K	W	K	CNTR	LIFR
FRR-CLCF1		F	R	R					0.87±0.03	+++
FRRAA-CLCF1		F	R	R	A	A			0.93±0.11	+++
sfICLCF1	C	F	R	R			L	R	0.12±0.01	+++
sfAA-CLCF1	C	F	R	R	A	A	L	R	0.12±0.05	-

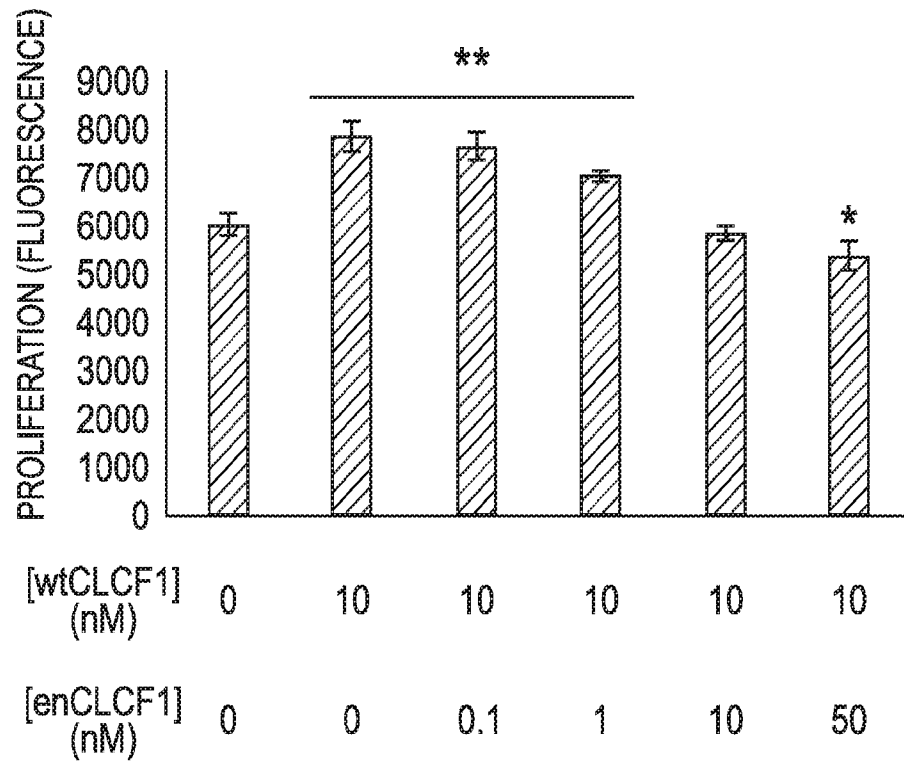
FIG. 21



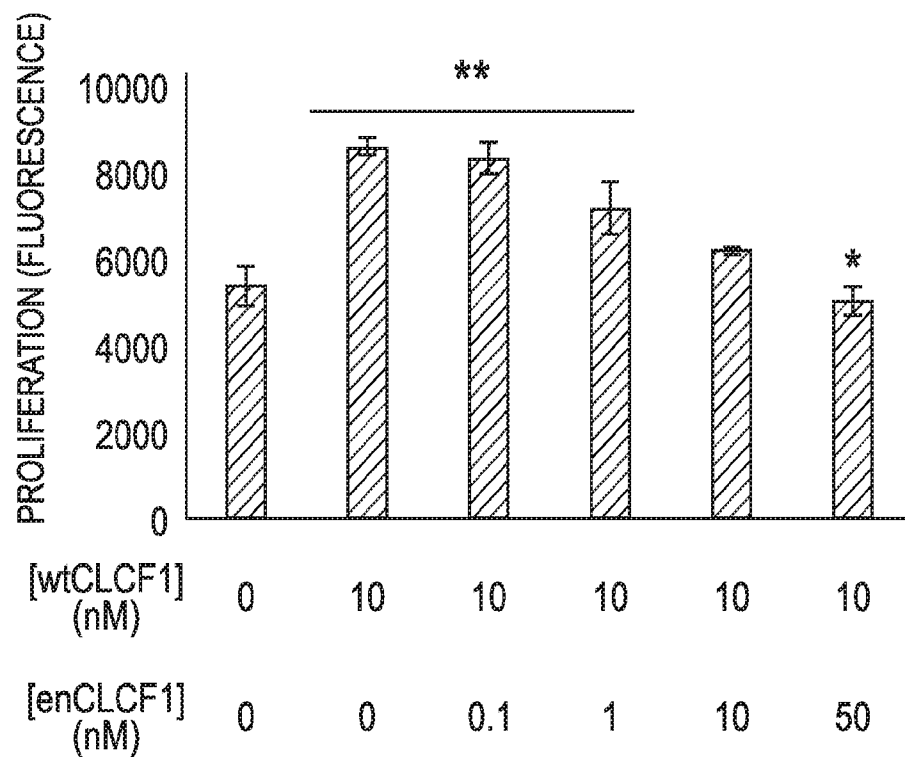
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FIG. 22

A

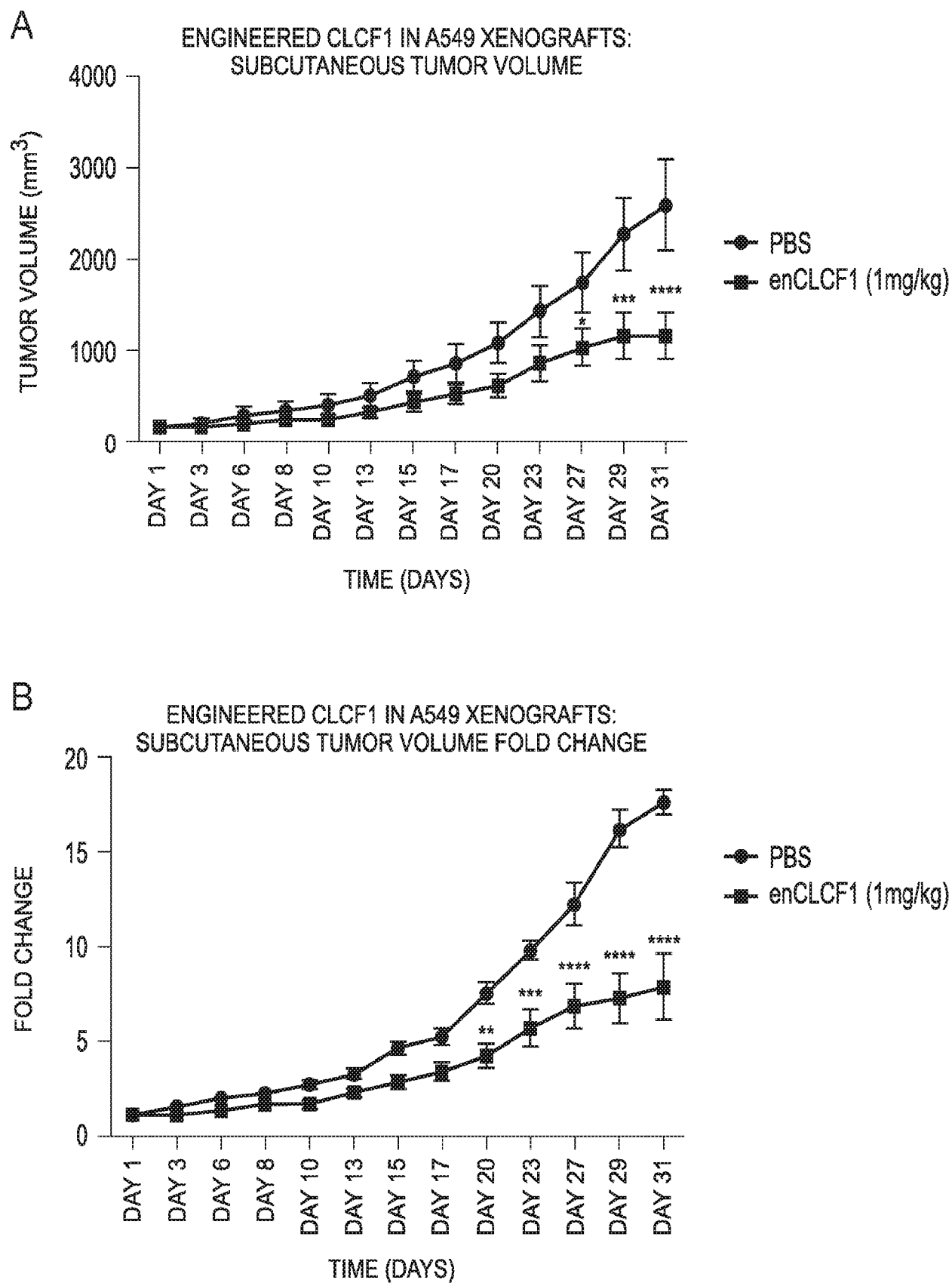


B



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FIG. 23



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FIG. 24

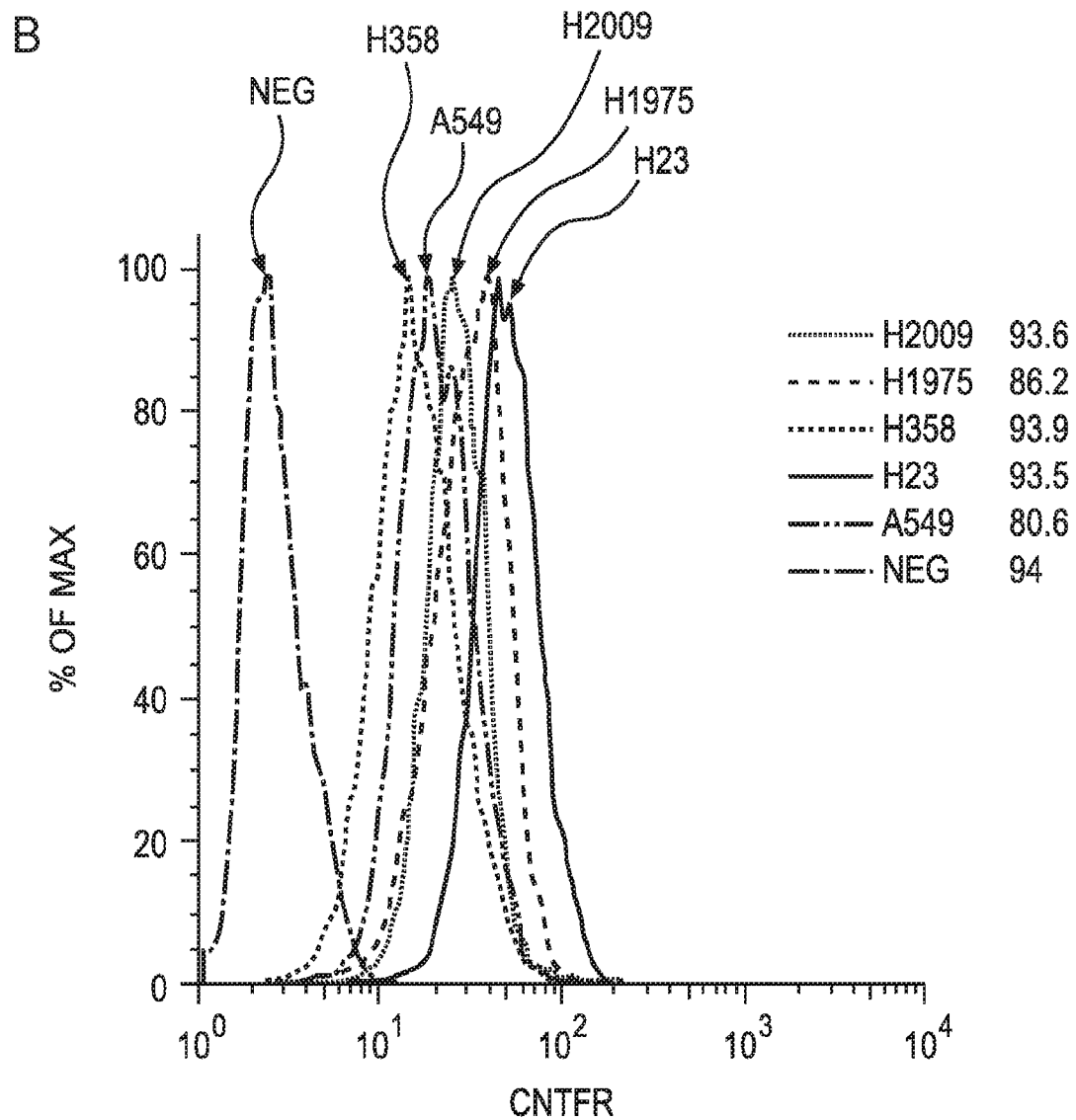
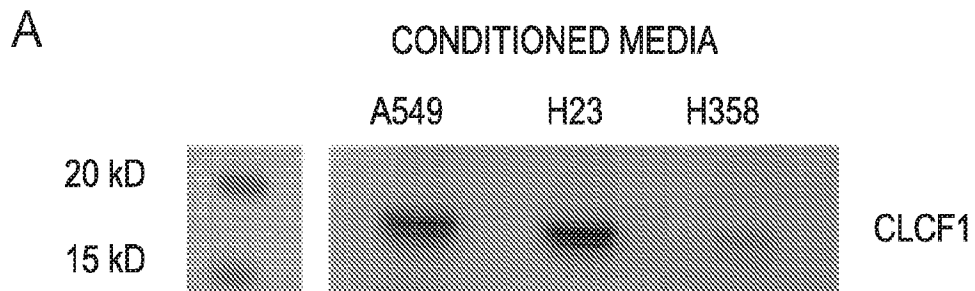


FIG. 24 (Cont.)

C

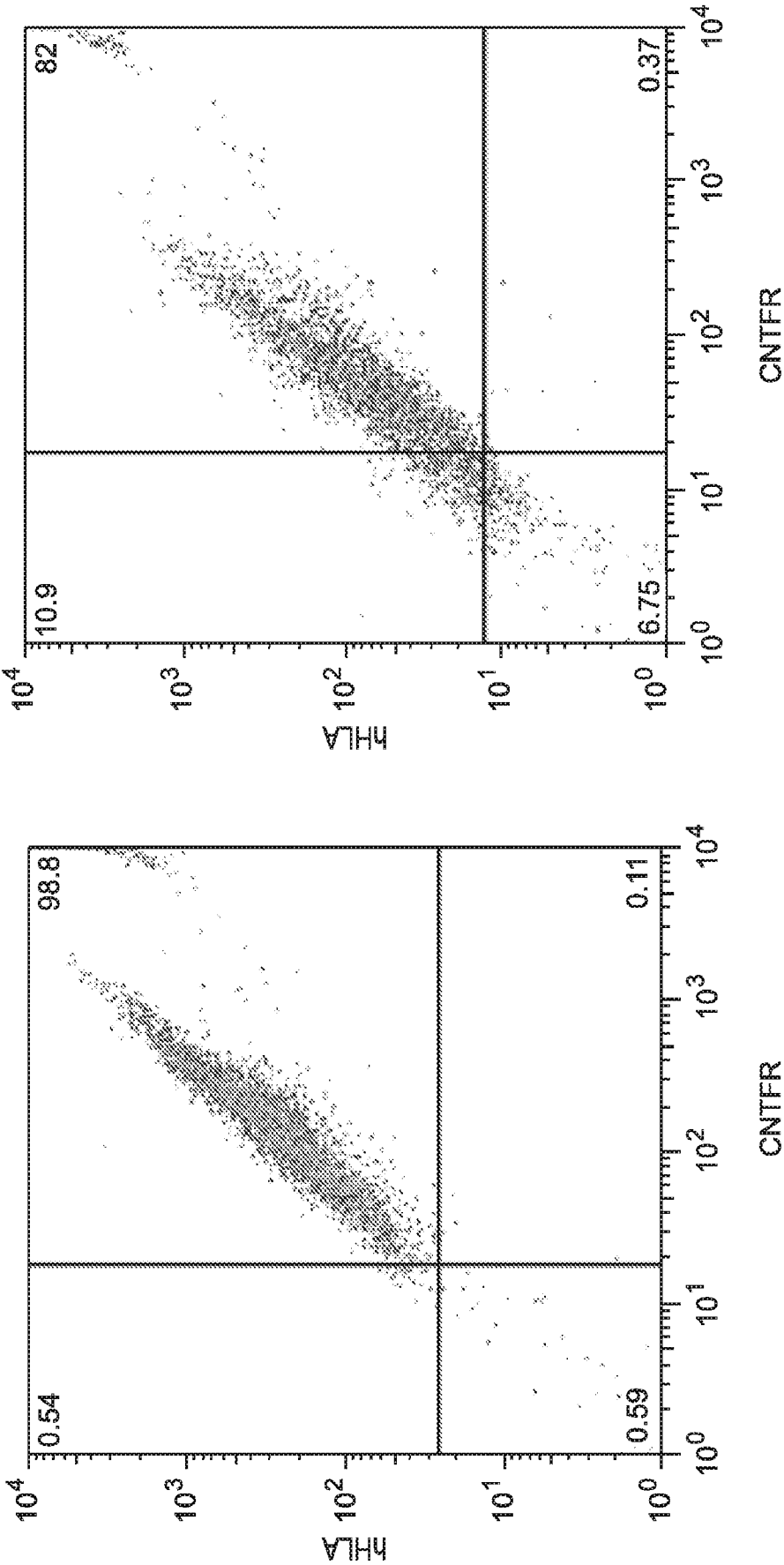
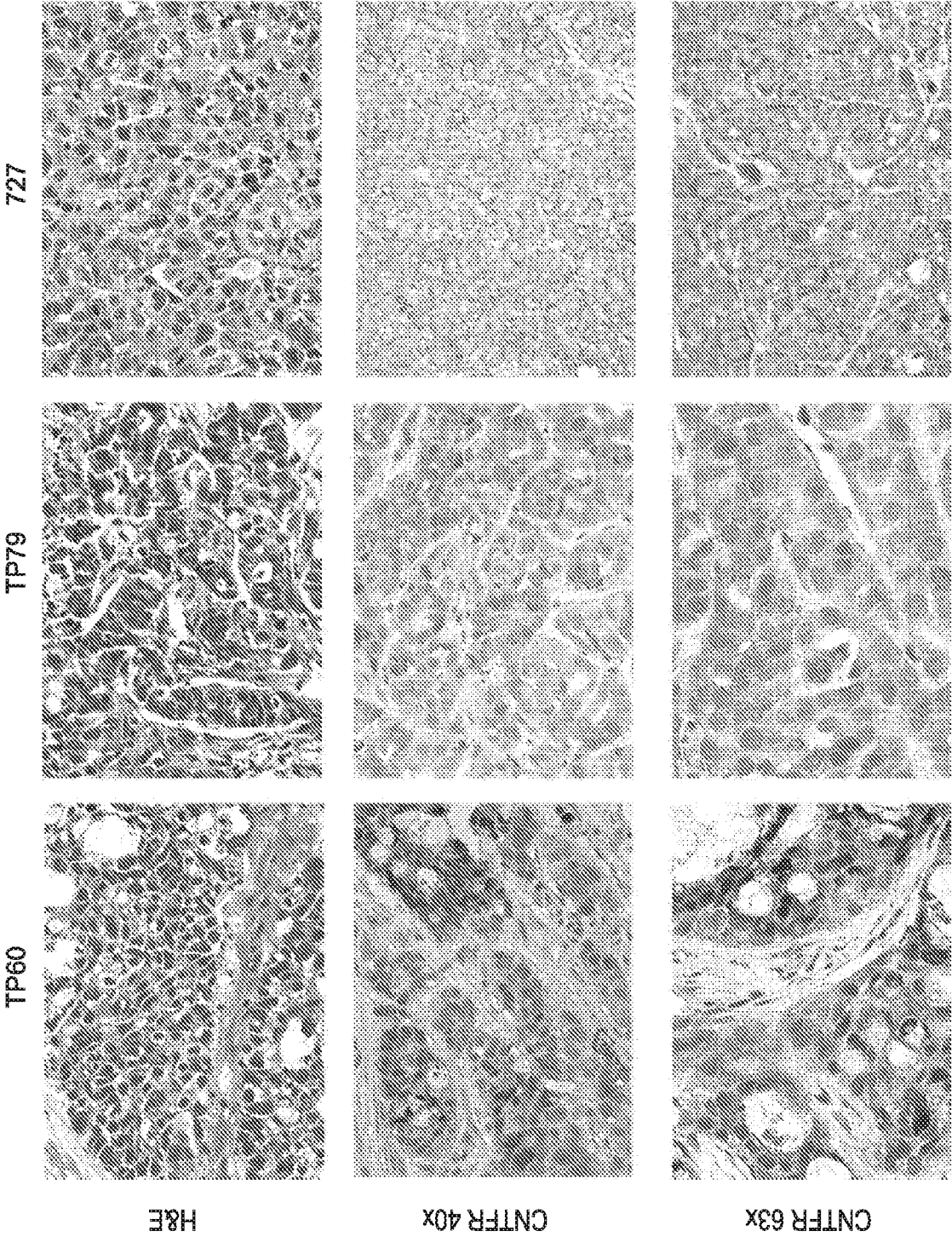


FIG. 24 (Cont.)

D



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/64934

Box No. 1 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:
- a. ☒ forming part of the international application as filed:
☒ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
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 forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/64934

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.: 6-44, 51-53
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-5, drawn to a ciliary neurotrophic factor receptor (CNTFR) ligand variant. The CNTFR will be searched to the extent that the CNTFR ligand variant encompasses a ciliary neurotrophic factor (CNTF) ligand. It is believed that claims 1-3 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass a CNTF variant. Additional CNTFR ligand variant(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected CNTFR ligand variant(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a CLCF1 (SEQ ID NO: 3) ligand having a mutation at L86 (claims 1-5).

(continued in supplemental box)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3 limited to a CNTF variant

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/64934

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - C07K 14/48, C07K 14/475, A61K 38/00 (2018.01)
 CPC - C07K 14/48, C07K 14/475, A61K 38/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2003/0176346 A1 (CILIBERTO ET AL.) 18 September 2003 (18.09.2003) abstract; Table 2; para [0001], [0064].	1-3
A	US 6,756,357 B1 (LAUFER ET AL.) 29 June 2004 (29.06.2004) abstract; col 2, ln 59-60; col 7, ln 7-9.	1-3

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

☐ See patent family annex.

* Special categories of cited documents:

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

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

"I," 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

03 April 2018

Date of mailing of the international search report

24 APR 2018

Name and mailing address of the ISA/US

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 Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
 PCT OSF: 571-272-7774

Continuation of: Box No. III Observations where unity of invention is lacking

Group II: claims 45-50, drawn to a nucleic acid that encodes a ciliary neurotrophic factor receptor (CNTFR) or CNTFR ligand fused to a cell surface display protein.

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

Special Technical Features

Each of the inventions of Group I+ include the special technical feature of CNTFR ligand variants, recited therein. Each of the inventions of Group I+ requires a specific CNTFR ligand variant, not required by the other inventions in Group I+ and Group II.

Group II includes the special technical feature of a polynucleotide fused to a cell surface protein, not required by Group I+.

Common Technical Features

The inventions of Groups I+ and II share the technical feature of CNTFR ligand.
The inventions of Groups I+ share the technical feature of Claim 1.

However, these shared technical features do not represent a contribution over prior art in view of US 2003/0176346 A1 to Ciliberto et al (hereinafter 'Ciliberto'). Ciliberto teaches a ciliary neurotrophic factor (CNTFR) ligand that exhibits increased binding affinity for CNTFR relative to the corresponding wild-type CNTFR ligand (abstract, "The present invention refers to the use of hCNTF (human ciliary neurotrophic factor), mutants thereof or other molecules that activate the CNTF receptor"; para [0001]. The subject of the present invention is the use of molecules that activate the CNTF (ciliary neurotrophic factor) receptor?such as hCNTF (human CNTF) or mutants of hCNTF- as active principles in the formulation of pharmaceutical compositions suitable for the treatment of obesity and of related diseases. The term hCNTF mutant is intended to mean an amino acid sequence that can in theory be derived from hCNTF by substitution of one or more amino acids.; [0064] - "The relative binding affinities to CNTF receptor-alpha (CNTFRalpha) of hCNTF and different hCNTF variants were determined by solid phase binding assay as previously described. As shown in Table 2, a number of hCNTF variants possessed greater affinity for CNTFRa than wild-type hCNTF. These variants, like DH-CNTF, have increased utility for treatment of obesity and associated diseases, such as diabetes." Table 2, SEQ ID NO: 1, hCNTF (wild type); Rrelative binding 1.1 +/- 0.3; SEQ ID NO: 5, (Ser166Asp/Gln167His) hCNTF, DH-CNTF ; Relative binding 22.8 +/- 3.5).

The inventions of Groups I+ further share the technical feature of CLCF1 having the amino acid sequence set forth in SEQ ID NO: 3 and variant thereof; which is disclosed by US 2003/0087395 A1 to Shi et al. (hereinafter 'Shi') (para [0063], SEQ ID NO: 2 is 100% match to SEQ ID NO: 3 of instant application.; [0061], The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the CLC protein.).

As said technical features were known in the art at the time of the invention, these cannot be considered special technical feature that would otherwise unify the groups.

Groups I+ and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.