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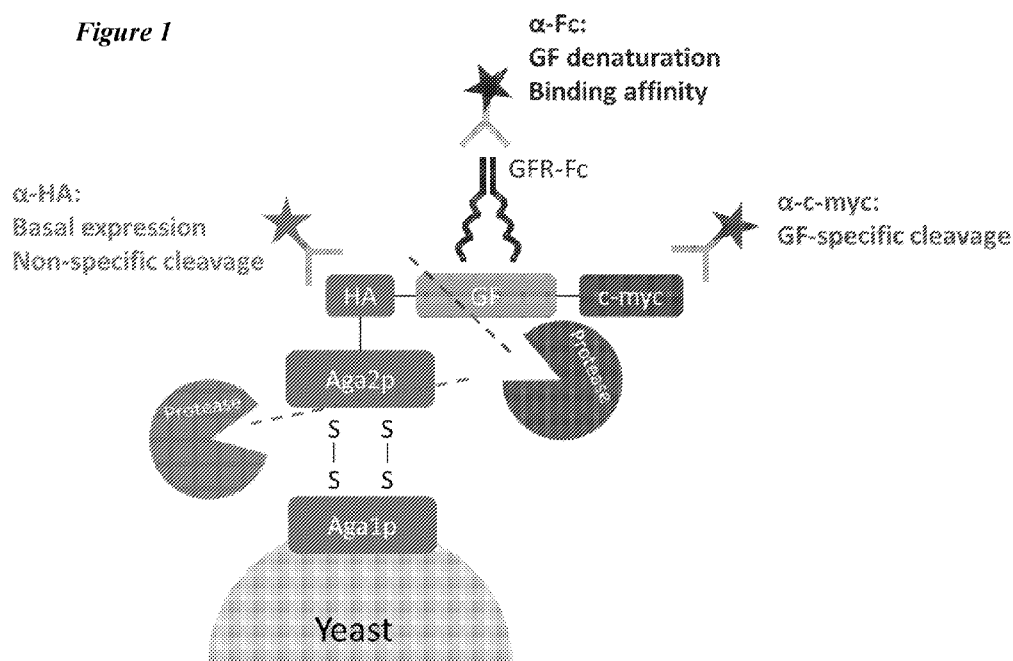
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(54) Title: ENGINEERED FIBROBLAST GROWTH FACTOR VARIANTS AS RECEPTOR ANTAGONISTS

Figure 1



(57) Abstract: The present invention provides methods of screening for proteolytically stable growth factor variants, including, for example variants of human fibroblast growth factor 1 (FGF1). The present invention also provides for FGF1 variants comprising at least one amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof, wherein the resulting FGF1 variant exhibits increased proteolytic stability as compared to wild-type FGF1, as well as related uses.

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ENGINEERED FIBROBLAST GROWTH FACTOR VARIANTS AS RECEPTOR ANTAGONISTS

CROSS REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to United States Provisional Patent

5 Application No. 62/743,414, filed on October 09, 2018, entitled “Engineered Fibroblast Growth Factor Variants As Receptor Antagonists”, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention pertains to the field of polypeptide variants, in particular variants of
10 fibroblast growth factor (FGF).

BACKGROUND OF THE INVENTION

[0003] Human growth factors play a pivotal role in orchestrating many complex processes, such as wound healing, tissue regeneration, angiogenesis, and tumor formation¹⁻⁴. Thus, there is immense interest in utilizing growth factors as protein therapeutics for accelerating wound
15 healing and regenerative processes, or inhibiting cancer growth and angiogenesis in a variety of diseases and conditions⁵⁻⁷. However, even though numerous recombinant growth factors have been developed as therapeutics, only a few candidates have been effective enough to receive clinical approval^{8,9}. This is due, in large part, to the short effective half-life of growth factors *in vivo*, stemming from their generally poor stability and fast blood clearance^{5,10}.
20 Therapeutic growth factors must remain active in the wound area for an extended period to be efficacious. However, growth factors can become denatured or degraded upon exposure to physiological temperatures and proteases^{11,12}. Resistance to protease-mediated degradation can be particularly important, as proteases such as plasmin and metalloproteinases are especially active in tissue remodeling¹³.

25 [0004] Various growth factors have previously been modified to improve their thermal and proteolytic stability, and this has been shown to enhance their biological activity in both *in vitro* functional assays and *in vivo* experiments¹⁴⁻¹⁷. For example, a growth hormone-releasing hormone (GHRH) designed to be more resistant to serine proteases, oxidation, and dipeptidyl peptidase IV was found to be more potent than wild type GHRH in inducing

weight gain in pigs¹⁸. However, many of these constructs have been rationally designed based on protein-specific hypotheses and tested in a low-throughput manner. Thus, it can be a difficult and slow process to generate new growth factor variants with improved stability. For engineering proteins with increased proteolytic stability, single mutations are often made directly adjacent to the predicted cleavage site, which is based on primary sequence specificity or mass spectroscopy after proteolysis^{17,19}. However, this method is far from reliable, as proteolytic stability against a specific protease is multifactorial. First, the activity of a protease in cleaving a specific amino acid sequence is greatly affected by multiple amino acids near the cleavage site. Up to 8 amino acids can determine whether cleavage occurs at a specific site and at what rate cleavage occurs, even if the amino acid directly upstream of the predicted cleavage site (P1) matches the primary specificity of the protease. In a publication by *Gosalia et al.*, they find that even combinatorially varying the two amino acids (P2, P3) upstream of a correct P1 site will drastically affect the proteolytic cleavage rate of a peptide substrate²⁰. Second, the proteolytic cleavage of a protein substrate is also determined by the steric accessibility of the cleavage site by the protease's enzymatic binding pocket. Although computational methods have improved for predicting how mutations may affect protein structure, it remains computationally expensive and technically challenging to predict how mutations may change the accessibility of a potential cleavage site by a protease. Thus, there are severe limitations in predicting mutations which would increase proteolytic stability through rational design. The lack of easily adaptable methods for engineering proteolytic stability may partially explain the limited development of proteolytically stable growth factors to date.

[0005] The present invention meets this need by providing methods for engineering proteolytically stable growth factors. The present invention also provides proteolytically stable FGF peptide variants generated by the described method, as well as uses of such FGF peptide variants.

BRIEF SUMMARY OF THE INVENTION

[0006] The present invention provides a variant of human fibroblast growth factor 1 (FGF1) comprising at least one member selected from an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof, wherein the resulting FGF1 variant exhibits increased proteolytic stability as compared to wild-type FGF1 of SEQ ID NO:1.

[0007] In some embodiments, the FGF1 variant comprises an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof in the β -loop or near the C-terminus.

[0008] In some embodiments, the FGF1 variant is a fibroblast growth factor receptor (FGFR) antagonist.

[0009] In some embodiments, the FGF1 variant comprises at least one amino acid substitution at position 28, 40, 47, 93 or 131.

[0010] In some embodiments, the FGF1 variant comprise at least one amino acid substitution selected from the group consisting of D28N, Q40P, S47I, H93G, L131R, and L131K.

[0011] In some embodiments, the FGF1 variant comprises amino acid substitution L131R.

[0012] In some embodiments, the FGF1 variant comprises amino acid substitution L131K.

[0013] In some embodiments, the FGF1 variant comprises amino acid substitutions D28N and L131R.

[0014] In some embodiments, the FGF1 variant comprises amino acid substitutions D28N and L131K.

[0015] In some embodiments, the FGF1 variant comprises amino acid substitutions Q40P, S47I, H93G, and L131R.

[0016] In some embodiments, the FGF1 variant comprises amino acid substitutions Q40P, S47I, H93G, and L131K.

[0017] In some embodiments, the FGF1 variant comprises amino acid substitutions D28N, Q40P, S47I, H93G, and L131R.

[0018] In some embodiments, the FGF1 variant comprises amino acid substitutions D28N, Q40P, S47I, H93G, and L131K.

[0019] In some embodiments, the FGF1 variant does not comprise the amino acid substitution L131A.

[0020] In some embodiments, the FGF1 variant is conjugated to a member selected from a detectable moiety, a water-soluble polymer, a water-insoluble polymer, a therapeutic moiety, a targeting moiety and a combination thereof.

[0021] In some embodiments, the FGF1 variant is conjugated to a detectable moiety selected from a radioisotope, a paramagnet, a fluorophore and combinations thereof.

[0022] In some embodiments, the FGF1 variant is a diagnostic imaging agent.

5 [0023] The present invention also provides a pharmaceutical formulation comprising a FGF1 variant according to claim 1, wherein said variant is in combination with a pharmaceutically acceptable carrier. A method of inhibiting or preventing angiogenesis a subject in need thereof, comprising administering a variant according to claim 1 to the subject in need thereof, thereby preventing or inhibiting angiogenesis.

[0024] In some embodiments, the subject has cancer.

10 [0025] In some embodiments, the subject is treated to prevent neovascularization in the eye.

[0026] The present invention also provides a method of treating cancer in a subject in need of the treatment, the method comprising administering to the subject a therapeutically effective amount of an FGF1 variant as provided herein, thereby treating the cancer.

15 [0027] The present invention also provides a method of reducing a process which is a member selected from tumor progression, angiogenesis, metastasis and combinations thereof in a subject, the method comprising administering to said subject an amount of a variant according to claim 1 sufficient to reduce the process.

20 [0028] In some embodiments, the cancer is a member selected from colorectal, oral, hepatocellular, renal, breast, lung, ovarian, stomach, brain, prostate, and combinations thereof.

[0029] The present invention also provides nucleic acids encoding the FGF1 variant polypeptides as described herein.

25 [0030] The present invention also provides isolated cells comprising the nucleic acids encoding the FGF1 variant polypeptides as described herein, and which are capable of expressing the FGF1 variant polypeptides as described herein.

[0031] The present invention also provides a method of screening for proteolytically stable growth factor variants, said method comprising:

- i. expressing a library of growth factor variants in a yeast display system;

- ii. testing the yeast displayed growth factor variants from i) for proper folding by measuring binding activity of the yeast displayed growth factor variants to the relevant growth factor receptor;
- iii. incubating the yeast displayed growth factor variants from ii) with at least one protease;
- iv. determining the protease cleavage of the yeast displayed growth factor variants from iii) as compared to the protease cleavage of the wild-type growth factor; and
- v. selecting the variants from iv) that exhibit reduced protease cleavage by and/or increased proteolytic stability to at least one protease as compared to the protease cleavage of the wild-type growth factor by the same protease, wherein said selected growth factor variants are proteolytically stable growth factor variants.

[0032] In some embodiments, the at least one protease is a protease capable of cleaving the wild-type growth factor.

[0033] In some embodiments, the at least one protease is capable of selectively cleaving the growth factor and which exhibits minimal and/or no non-specific cleavage of the yeast display proteins

[0034] In some embodiments, the at least one protease is selected from the group consisting of serum, trypsin, chymotrypsin, and plasmin.

[0035] In some embodiments, the at least one protease is serum.

[0036] In some embodiments, the at least one protease is trypsin.

[0037] In some embodiments, the at least one protease is chymotrypsin.

[0038] In some embodiments, the at least one protease is plasmin.

BRIEF SUMMARY OF THE DRAWINGS

[0039] FIG. 1. Yeast display of growth factor for engineering proteolytic stability. The growth factor (GF) of interest is expressed as a fusion to adhesion protein agglutinin Aga2p, which is attached by two disulfide bonds to the cell wall protein Aga1p. Upon incubation with protease, cleavage can either occur within the growth factor (growth-factor-specific

cleavage) or within the yeast display proteins Aga1p or Aga2p (non-specific cleavage). After incubation with the soluble Fc fusion of the growth factor receptor (GFR-Fc), fluorescent antibodies can be used to stain for the HA tag, the c-myc tag, and the Fc domain. The HA signal is used to measure basal expression level of the growth factor and non-specific cleavage by the protease. The c-myc signal is in conjunction with the HA signal to measure GF-specific cleavage. The Fc signal is used to measure the level of GF denaturation and the binding affinity of the GF for its receptor.

[0040] FIG. 2. FACS-based screening method for proteolytically stable growth factor

mutants. A library of growth factor mutants is transformed into EBY100 yeast cells and induced to display growth factors by yeast display. Cells are incubated with protease, washed, then incubated with soluble Fc-fusion of the receptor. After labeling with appropriate fluorescent antibodies, flow activated cell sorting (FACS) is used to gate and collect cells that express mutants with low level of proteolytic cleavage and high levels of binding to the soluble receptor. This process of incubation and cell sorting is cycled multiple times to identify the mutants with greatest level of proteolytic stability.

[0041] FIG. 3. Yeast display of FGF1. (A) FGF1 is expressed as a fusion to adhesion protein agglutinin Aga2p, which is attached by two disulfide bonds to the cell wall protein Aga1p. FGFR1-Fc is the corresponding soluble receptor that binds to FGF1. (B) Fluorescent labeling of the c-myc tag shows that FGF1 is successfully expressed on the surface of yeast. (C) Fc fusion of FGFR1 shows specific binding to yeast-displayed FGF1. Yeast expressing surface-displayed FGF1 were incubated with soluble FGFR1-Fc for 3 hours at various concentrations. Cells were washed and stained with anti-Fc AlexaFluor488 for soluble FGFR1-Fc. Fluorescence associated with binding to yeast cells were measured by flow cytometry and plotted.

[0042] FIG. 4. Proteolytic stability assay with fetal bovine serum. Yeast cells displaying an FGF1 mutant library were incubated with different concentrations of fetal bovine serum. After washing cells and incubation with 10 nM FGFR1-Fc, cells were stained with fluorescent antibodies for c-myc and the Fc domain of the soluble receptor. Analysis by flow cytometry shows that increasing the concentration of FBS has relatively little effect on the FGF1-specific cleavage signal as well as the FGFR1-Fc binding signal.

[0043] FIG. 5. Proteolytic stability assay with trypsin. Yeast cells displaying FGF1 were incubated with different concentrations of trypsin. After washing cells and incubation with 10

nM FGFR1-Fc, cells were stained with fluorescent antibodies for c-myc and the Fc domain of the soluble receptor. Analysis by flow cytometry shows that increasing the concentration of trypsin leads to cleavage of the yeast displayed proteins (decreased c-myc) and loss of binding to FGFR1-Fc.

5 **[0044] FIG. 6. Proteolytic stability assay with chymotrypsin.** Yeast cells displaying FGF1 were incubated with different concentrations of chymotrypsin. After washing cells and incubation with 10 nM FGFR1-Fc, cells were stained with fluorescent antibodies for c-myc and the Fc domain of the soluble receptor. Analysis by flow cytometry shows that increasing the concentration of chymotrypsin leads to cleavage of the yeast displayed proteins
10 (decreased c-myc) and loss of binding to FGFR1-Fc.

[0045] FIG. 7A-FIG. 7B. Non-specific cleavage of yeast display proteins Aga1 and Aga2 by trypsin. Yeast cells displaying FGF1 were incubated with different concentrations of trypsin. After washing, cells were stained with fluorescent antibodies for HA and c-myc. Analysis by flow cytometry shows that increasing the concentration of trypsin leads to loss of
15 HA signal, indicating non-specific cleavage of yeast display proteins Aga1 and Aga2.

[0046] FIG. 8A-FIG. 8B. FGF1-specific cleavage by chymotrypsin. Yeast cells displaying FGF1 were incubated with different concentrations of trypsin. After washing, cells were stained with fluorescent antibodies for HA and c-myc. Analysis by flow cytometry shows that increasing the concentration of chymotrypsin leads to loss of c-myc signal but not of HA
20 signal, indicating that FGF1-specific cleavage occurs.

[0047] FIG. 9. Proteolytic stability assay with plasmin. Yeast cells displaying FGF1 were incubated with different concentrations of plasmin. After washing, cells were stained with fluorescent antibodies for HA and c-myc. Analysis by flow cytometry shows that there is a concentration-dependent cleavage of FGF1.

25 **[0048] FIG. 10. FGF1-specific cleavage by plasmin.** Yeast cells displaying FGF1 and an empty control expressing only the yeast display proteins Aga1 and Aga2 were incubated with 125 nM plasmin. After washing, cells were stained with fluorescent antibodies for HA and c-myc. Analysis by flow cytometry shows that increasing the concentration of plasmin leads to loss of c-myc signal for yeast cells displaying FGF1 but not for yeast cells displaying the
30 empty control. This confirms that cleavage of yeast displayed proteins by plasmin is FGF1-specific.

[0049] FIG. 11. Validation of proteolytic stability assay by differentiation of wild type FGF1 and proteolytically stable PM2. Plasmin enables differentiation between wild type FGF1 and proteolytically stable mutant (PM2) by yeast surface display after 2-day incubation at various plasmin concentrations. This demonstrates the ability of the plasmin-based screen to identify new proteolytically stable mutants.

[0050] FIG. 12. Sort 1: Selection for FGFR1-Fc binders. (A) Schematic of screening method for binders to FGFR1-Fc. Random mutagenesis libraries were induced for expression of FGF mutants on the surface of yeast. Cells were incubated with 10 nM FGFR1-Fc, washed, then stained with fluorescent antibodies for expression (α -c-myc) and FGFR1 binding (α -FGFR1-Fc). Fluorescence activated cell sorting (FACS) was used to analyze and gate for cells that exhibited high c-myc signal and high FGFR1-Fc signal. (B) The FACS dot plots are shown for FGF1. The percentage of cells that were collected from the total population is shown next to the drawn gates on the dot plots.

[0051] FIG. 13. Sort 2: Selection for resistance to FGF1-specific cleavage. (A) Schematic of screening method for Sort 2. Cells from Sort 1 were induced for expression and incubated with plasmin. Cells were washed, then stained with fluorescent antibodies for expression (α -HA) and resistance to FGF1-specific cleavage (α -c-myc). Fluorescence activated cell sorting (FACS) was used to analyze and gate for cells that exhibited high c-myc signal normalized by the HA expression signal. (B) The FACS dot plots is shown for FGF1. Cells from Sort 1 of each library were incubated in various concentrations of plasmin for varying incubation times as detailed. The final conditions used for gating and collection of cells for enrichment are highlighted in red. The same gate is drawn for all tested conditions.

[0052] FIG. 14. Isolation of peptide artifacts. (A) The FACS dot plot is shown for the sorting of the FGF1 Sort 2 library. A selection for resistance to FGF1-specific cleavage was applied in the same manner as Sort 2. A collection gate was drawn around a subpopulation of cells that exhibited significantly higher resistance to proteolytic cleavage (c-myc). (B) The protein sequence of mutants collected from the gate are shown. Most consist of short peptides that are artifacts of random mutagenesis and not derived from FGF1.

[0053] FIG. 15. Non-binding of peptide artifacts to FGFR1-Fc. Yeast cells expressing RTTTS or HTTS peptides on their cell surface were incubated with 10 nM FGFR1-Fc. Cells were stained with fluorescent antibodies for expression (α -c-myc) and binding (α -FGFR1-

Fc). No significant binding signal was detected, indicating that the peptides do not bind to FGFR1-Fc.

[0054] FIG. 16. Schematic for Sorts 3 and 4. Cells from the previous were induced for expression and incubated with varying concentrations of plasmin, washed, and incubated with FGFR1-Fc. After final wash, cells were then stained with fluorescent antibodies for expression (α -HA), resistance to FGF1-specific cleavage (α -c-myc), and FGFR1 binding (α -FGFR1-Fc). Fluorescence activated cell sorting (FACS) was used to analyze and gate for cells that exhibited high c-myc signal normalized by the HA expression signal and/or high FGFR1-Fc binding signal.

[0055] FIG. 17. Sort 3: Selection for protease-resistant, FGFR1-Fc binders. Induced cells from Sort 2 were incubated in the indicated concentrations of plasmin for 12 hours. After washing, cells were incubated with 10 nM FGFR1-Fc. After a final wash, cells were stained with fluorescent antibodies for expression (α -HA) and FGFR1 binding (α -FGFR1-Fc). Fluorescence activated cell sorting (FACS) was used to analyze and gate for cells that exhibited high HA signal and high FGFR1-Fc signal. The FACS dot plots are shown for FGF1. The percentage of cells that were collected from the total population is shown next to the drawn gates on the dot plots. Bottom panel: Retain binding to FGFR1-Fc after incubation with 1.25 μ M plasmin for 24 hours.

[0056] FIG. 18. Sort 4: Selection for protease-resistant, FGFR1-Fc binders. Induced cells from Sort 3 were incubated in various concentrations of plasmin for 36 hours. After washing, cells were incubated with 10 nM FGFR1-Fc. After a final wash, cells were stained with fluorescent antibodies for expression (α -c-myc) and FGFR1 binding (α -FGFR1-Fc). Fluorescence activated cell sorting (FACS) was used to analyze and gate for cells that exhibited high c-myc signal and high FGFR1-Fc signal. The FACS dot plots are shown for FGF1. The final conditions used for gating and collection of cells for enrichment are highlighted in red. The same gate is drawn for all conditions of a given FGF. The percentage of cells that were collected from the total population is shown next to the drawn gates on the dot plots. Bottom Panel: Retain binding to FGFR1-Fc after incubation with 3.75 μ M plasmin for 36 hours.

[0057] FIG. 19. BS4M1 mutations on FGF1 structure (PDB code 1E0O). Enriched mutations identified by screen for proteolytic stability are highlighted in blue. D28N mutation is located in one of three β -hairpins (highlighted in red) that stabilize six-stranded β -barrel

structure. L131R mutation is located near the C-terminus of the protein, where there is a lack of a stabilizing β -hairpin between the N- and C- termini.

[0058] FIG. 20. Recombinant expression of soluble wild-type FGF1. (A) Purified wild-type FGF1 was analyzed by non-reduced Coomassie-stained gel (left) and Western blot against FGF1 (right). Two significant bands indicate the presence of FGF1 monomer (19.7 kDa) and dimer (39.4 kDa). (B) Proper folding of FGF1 is confirmed by observing specific binding to yeast-displayed FGFR3 construct.

[0059] FIG. 21. Recombinant expression of FGF2 in pBAD vector. (A) Wild-type FGF2-His expressed in pBAD and purified was analyzed by reduced Coomassie-stained gel (left) and Western blot against FGF2 (right). Both indicate aggregation by the expressed FGF2. (B) FGF2-His expressed in pBAD is unable to bind to yeast-displayed FGFR3 construct.

[0060] FIG. 22. Recombinant expression of FGF2 in pET28b vector. Wild-type FGF2 and FGF2 mutants (BS5M1, BS5M3, BS5M5) were expressed as fusions to superfolder GFP in the pET28b vector. Wild-type FGF2-His expressed in pBAD and purified was analyzed by reduced Coomassie-stained gel (left) and Western blot against FGF2 (right). Wild type FGF2 is poorly expressed, while the FGF2 mutants shows signs of aggregation and/or oligomerization.

[0061] FIG. 23. Recombinant expression of wild-type FGF2 in pET32a vector. (A) FGF2 was expressed as a fusion to thioredoxin in the pET32a vector. After cleavage with TEV and purification by Ni-NTA and size exclusion chromatography, we analyzed the protein by Western blot against FGF2. We confirmed successful purification of FGF2 (19.3 kDa).

[0062] FIG. 24. Proteolytic stability assay of FGF1 WT and BS4M1 in plasmin. The FGF1 BS4M1 (D28N/L131R) mutant shows greater proteolytic stability in plasmin as compared to wild-type FGF1. 100 ng of FGF1 was incubated with 600 nM plasmin for various incubation times at 37°C. The incubated samples were run on separate lanes of a Western blot against FGF1 to measure the extent of protein degradation at each time point. The band intensities of the protein bands indicated by the red arrow were quantified by image analysis to measure the amount of remaining protein. The band intensities were normalized by the time point $t = 0$ for each protein and plotted.

[0063] FIG. 25. Proteolytic stability assay of FGF1 WT, BS4M1, PM2, and PM3 in plasmin. The mutations from BS4M1 (D28N, L131R) are combined with those from PM2 (Q40P, S47I, H93G) to create PM3. PM3 shows greater proteolytic stability in plasmin as

compared to either BS4M1 or PM2. 125 ng of FGF1 was incubated for 48 hours at 37°C with various concentrations of plasmin. The incubated samples were run on separate lanes of a Western blot against FGF1 to measure the extent of protein degradation at each time point. The band intensities of the protein bands indicated by the red arrow were quantified by image analysis to measure the amount of remaining protein. The band intensities were normalized by the amount of protein for each construct when incubated with 0 μ M plasmin and plotted.

[0064] FIG. 26. Proteolytic stability assay of FGF1 WT and BS4M1 in trypsin. The FGF1 BS4M1 (D28N/L131R) mutant shows greater proteolytic stability in trypsin as compared to wild-type FGF1. 100 ng of FGF1 was incubated with 1:20 molar ratio of trypsin to FGF1 for various incubation times at 37°C. The incubated samples were run on separate lanes of a Western blot against FGF1 to measure the extent of protein degradation at each time point. The band intensities of the protein bands indicated by the red arrow were quantified by image analysis to measure the amount of remaining protein. The band intensities were normalized by the amount of protein for each construct at the time point $t = 0$ and plotted.

[0065] FIG. 27. Proteolytic stability assay of FGF1 WT, BS4M1, D28N, and L131R in plasmin. The FGF1 L131R single mutant retains most of its proteolytic stability as compared to BS4M1. The FGF1 D28N single mutant has a lower proteolytic stability even as compared to wild-type FGF1. 100 ng of FGF1 was incubated for 48 hours at 37°C with various concentrations of plasmin. The incubated samples were run on separate lanes of a Western blot against FGF1 to measure the extent of protein degradation at each time point. The band intensities of the protein bands indicated by the red arrow were quantified by image analysis to measure the amount of remaining protein. The band intensities were normalized by the amount of protein for each construct when incubated with 0 μ M plasmin and plotted.

[0066] FIG. 28. Proteolytic stability assay of FGF1 WT, L131R, L131A, and L131K in plasmin. The FGF1 L131K single mutant retains most of its proteolytic stability as compared to FGF1 L131R. The FGF1 L131A single mutant has a lower proteolytic stability even as compared to wild-type FGF1. 100 ng of FGF1 was incubated for 48 hours at 37°C with various concentrations of plasmin. The incubated samples were run on separate lanes of a Western blot against FGF1 to measure the extent of protein degradation at each time point. The band intensities of the protein bands indicated by the red arrow were quantified by image analysis to measure the amount of remaining protein. The band intensities were normalized by the amount of protein for each construct when incubated with 0 μ M plasmin and plotted.

[0067] **FIG. 29. ThermoFluor assay of FGF1 wild-type and L131R mutant.** The melting temperatures of FGF1 wild-type and the L131R mutant were measured in triplicate and plotted. There was no statistically significant difference between the melting temperatures of the two proteins

5 [0068] **FIG. 30. Stability of FGF1 wild-type and L131R mutant in MDA-MB-231 culture.** The FGF1 L131R mutant shows greater stability in culture with MDA-MB-231 as compared to wild-type FGF1. 500 ng of FGF1 was incubated with MDA-Mb-231 cells for various incubation times at 37°C. The incubated samples were concentrated and run on separate lanes of a Western blot against FGF1 to measure the extent of protein degradation at
10 each time point. The band intensities of the protein bands indicated by the red arrow were quantified by image analysis to measure the amount of remaining protein. The band intensities were normalized by the time point $t = 0$ for each protein and plotted.

[0069] **FIG. 31. NIH3T3 ERK Phosphorylation assay.** The FGF1 L131R mutant inhibits NIH3T3 ERK phosphorylation by wild-type FGF1. NIH3T3 cells were stimulated for 15
15 hours with FGF1 wild-type and/or various concentrations of FGF1 L131R mutant. Cells were lysed and the lysate was probed with anti-phosphoERK on a Western blot. The band intensities were quantified by image analysis to measure the extent of FGF pathway activation. Bottom panel: NIH3T3 cells were stimulated for 10 hours with FGF1 wild-type and/or various concentrations of FGF1 L131R mutant.

20 [0070] **FIG. 32. Inhibition of FGF1-stimulated ERK phosphorylation by FGF1 L131R mutant in NIH3T3 cells.** NIH3T3 cells were incubated with 1 nM FGF1 and various concentrations of FGF1 L131R. The extent of ERK phosphorylation for each condition is measured by Western blot against phosphoERK. The band intensities were quantified by image analysis and plotted to obtain an IC50 value.

25 [0071] **FIG. 33. Binding of FGF1 wild-type and L131R mutant to NIH3T3 cells.** Equilibrium binding titrations of His-tagged FGF1 WT and L131R mutant to FGFR-expressing NIH3T3 cells. Cells were incubated at 4°C with varying concentrations of each protein, and stained with fluorescent antibody against His to quantify binding to the cells.

[0072] **FIG. 34A- FIG. 34B.** Provides examples of IgG1, IgG2, IgG3, and IgG4 sequences.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0073] The fibroblast growth factors are a family of cell signaling proteins that are involved in a wide variety of processes, most notably as crucial elements for normal development.

5 These growth factors generally act as systemic or locally circulating molecules of extracellular origin that activate cell surface receptors. The mammalian fibroblast growth factor receptor family has 4 members, FGFR1, FGFR2, FGFR3, and FGFR4. The FGFRs consist of three extracellular immunoglobulin-type domains (D1-D3), a single-span trans-membrane domain and an intracellular split tyrosine kinase domain. FGFs interact with the
10 D2 and D3 domains, with the D3 interactions primarily responsible for ligand-binding specificity (see below). Heparan sulfate binding is mediated through the D3 domain. A short stretch of acidic amino acids located between the D1 and D2 domains has auto-inhibitory functions. This 'acid box' motif interacts with the heparan sulfate binding site to prevent receptor activation in the absence of FGFs. Each FGFR binds to a specific subset of the
15 FGFs. Similarly, most FGFs can bind to several different FGFR subtypes. FGF1 is sometimes referred to as the 'universal ligand' as it is capable of activating all 7 different FGFRs. In contrast, FGF7 (keratinocyte growth factor, KGF) binds only to FGFR2b (KGFR).

[0074] The present invention provides methods for a combinatorial approach to engineering proteolytically stable growth factors using the yeast display platform and flow-activated cell
20 sorting (FACS) for screening. The process of setting up the screening method using FGF1 as a model example is described and methods for engineering an exemplary proteolytically stable growth factor are provided. The present invention also provides the characterization of a proteolytically stable FGF1 mutant.

25 II. Definitions

[0075] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and
30 hybridization are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally

performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures of analytical and synthetic organic chemistry described below are those well-known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

[0076] The terms “BS4M1” and “PM2”, and “PM3” refer to variants of SEQ ID NO:1 having the following substitutions: (i) BS4M1 (D28N and L131R), (ii) PM2 (Q40P, S47I, H93G), and (iii) PM3 (D28N, Q40P, S47I, H93G, L131R). FGF1:

FNLPNGNYKKPKLLYCSNGGHFLRLP~~PT~~GTVDGTRDRSDQHIQLQLSAESVGEVYIKS
TETGQYLA~~MD~~TDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWVGLKKN
GSCKRGPRTHYGQKA~~IT~~FLPLVSSD (SEQ ID NO:1). SEQ ID NO:1 is the FGF1

sequence without the propeptide ([https://www.uniprot.org/blast/?about=P05230\[16-155\]&key=Chain&id=PRO_0000008908](https://www.uniprot.org/blast/?about=P05230[16-155]&key=Chain&id=PRO_0000008908)). The numbering described herein is based on the first amino acid of the sequence above being position 1 (ex: F1, N2, etc.). Other numbering for FGF1 can include the propeptide sequence in the numbering, which would cause the numbering to be larger by 14. However, the numbering herein is based on SEQ ID NO:1 and does not include the FGF1 propeptide.

[0077] The term “nucleic acid” or “polynucleotide” refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene. Moreover, as used herein, a nucleic acid encoding a polypeptide variant of the

invention is defined to include the nucleic acid sequence complementary to this nucleic acid sequence.

[0078] The term “gene” means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0079] The term “isolated,” when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term “purified” denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure. An isolated nucleic acid can be a component of an expression vector.

[0080] Typically, isolated polypeptides of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the polypeptide is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90%, about 95%, or more than about 95%. When the polypeptides are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

[0081] Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, mass-spectroscopy, or a similar means).

[0082] The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have

the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. “Amino acid mimetics” refers to chemical compounds having a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0083] “Hydrophilic Amino Acid” refers to an amino acid exhibiting a hydrophobicity of less than zero according to the normalized consensus hydrophobicity scale of Eisenberg et al., 1984, J. Mol. Biol. 179: 125-142. Genetically encoded hydrophilic amino acids include Thr (T), Ser (S), His (H), Glu (E), Asn (N), Gln (Q), Asp (D), Lys (K) and Arg (R).

[0084] “Acidic Amino Acid” refers to a hydrophilic amino acid having a side chain pK value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Genetically encoded acidic amino acids include Glu (E) and Asp (D).

[0085] “Basic Amino Acid” refers to a hydrophilic amino acid having a side chain pK value of greater than 7. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Genetically encoded basic amino acids include His (H), Arg (R) and Lys (K).

[0086] “Polar Amino Acid” refers to a hydrophilic amino acid having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Genetically encoded polar amino acids include Asn (N), Gln (Q), Ser (S) and Thr (T).

[0087] “Hydrophobic Amino Acid” refers to an amino acid exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg, 1984, J. Mol. Biol. 179:125-142. Exemplary hydrophobic amino acids include Ile (I), Phe (F), Val (V), Leu (L), Trp (W), Met (M), Ala (A), Gly (G), Tyr (Y), Pro (P), and proline analogues.

[0088] “Aromatic Amino Acid” refers to a hydrophobic amino acid with a side chain having at least one aromatic or heteroaromatic ring. The aromatic or heteroaromatic ring may contain one or more substituents such as -OH, -SH, -CN, -F, -Cl, -Br, -I, -NO₂, -NO, -NH₂, -NHR, -NRR, -C(O)R, -C(O)OH, -C(O)OR, -C(O)NH₂, -C(O)NHR, -C(O)NRR and the like where

each R is independently (C₁-C₆) alkyl, substituted (C₁-C₆) alkyl, (C₁-C₆) alkenyl, substituted (C₁-C₆) alkenyl, (C₁-C₆) alkynyl, substituted (C₁-C₆) alkynyl, (C₁-C₂₁) aryl, substituted (C₅-C₂₀) aryl, (C₆-C₂₆) alkaryl, substituted (C₆-C₂₆) alkaryl, 5-20 membered heteroaryl, substituted 5-20 membered heteroaryl, 6-26 membered alkheteroaryl or substituted 6-26 membered alkheteroaryl. Genetically encoded aromatic amino acids include Phe (F), Tyr (Y) and Trp (W).

[0089] “Nonpolar Amino Acid” refers to a hydrophobic amino acid having a side chain that is uncharged at physiological pH and which has bonds in which the pair of electrons shared in common by two atoms is generally held equally by each of the two atoms (*i.e.*, the side chain is not polar). Genetically encoded apolar amino acids include Leu (L), Val (V), Ile (I), Met (M), Gly (G) and Ala (A).

[0090] “Aliphatic Amino Acid” refers to a hydrophobic amino acid having an aliphatic hydrocarbon side chain. Genetically encoded aliphatic amino acids include Ala (A), Val (V), Leu (L) and Ile (I).

[0091] The amino acid residue Cys (C) is unusual in that it can form disulfide bridges with other Cys (C) residues or other sulfonyl-containing amino acids. The ability of Cys (C) residues (and other amino acids with –SH containing side chains) to exist in a peptide in either the reduced free-SH or oxidized disulfide-bridged form affects whether Cys (C) residues contribute net hydrophobic or hydrophilic character to a peptide. While Cys (C) exhibits a hydrophobicity of 0.29 according to the normalized consensus scale of Eisenberg (Eisenberg, 1984, *supra*), it is to be understood that for purposes of the present invention Cys (C) is categorized as a polar hydrophilic amino acid, notwithstanding the general classifications defined above.

[0092] The term “linker” refers to an amino-acid polypeptide spacer that covalently links two or more polypeptides. The linker can be 1-15 amino acid residues. Preferably the linker is a single cysteine residue. The linker can also have the amino acid sequence SEQ ID NO:1 KESCAKKQRQHMDS.

[0093] As will be appreciated by those of skill in the art, the above-defined categories are not mutually exclusive. Thus, amino acids having side chains exhibiting two or more physical-chemical properties can be included in multiple categories. For example, amino acid side chains having aromatic moieties that are further substituted with polar substituents, such as Tyr (Y), may exhibit both aromatic hydrophobic properties and polar or hydrophilic

properties, and can therefore be included in both the aromatic and polar categories. The appropriate categorization of any amino acid will be apparent to those of skill in the art, especially in light of the detailed disclosure provided herein.

[0094] Certain amino acid residues, called “helix breaking” amino acids, have a propensity to disrupt the structure of α -helices when contained at internal positions within the helix. Amino acid residues exhibiting such helix-breaking properties are well-known in the art (see, *e.g.*, Chou and Fasman, *Ann. Rev. Biochem.* 47:251-276) and include Pro (P), Gly (G) and potentially all D-amino acids (when contained in an L-peptide; conversely, L-amino acids disrupt helical structure when contained in a D-peptide) as well as a proline analogue. While these helix-breaking amino acid residues fall into the categories defined above, with the exception of Gly (G) (discussed *infra*), these residues should not be used to substitute amino acid residues at internal positions within the helix – they should only be used to substitute 1-3 amino acid residues at the N-terminus and/or C-terminus of the peptide.

[0095] While the above-defined categories have been exemplified in terms of the genetically encoded amino acids, the amino acid substitutions need not be, and in certain embodiments preferably are not, restricted to the genetically encoded amino acids. Indeed, many of the preferred peptides of formula (I) contain genetically non-encoded amino acids. Thus, in addition to the naturally occurring genetically encoded amino acids, amino acid residues in the core peptides of formula (I) may be substituted with naturally occurring non-encoded amino acids and synthetic amino acids.

[0096] Certain commonly encountered amino acids which provide useful substitutions for the core peptides of formula (I) include, but are not limited to, β -alanine (β -Ala) and other omega-amino acids such as 3-aminopropionic acid, 2, 3-diaminopropionic acid (Dpr), 4-aminobutyric acid and so forth; α -aminoisobutyric acid (Aib); ϵ -aminohexanoic acid (Aha); δ -aminovaleric acid (Ava); N-methylglycine or sarcosine (MeGly); ornithine (Orn); citrulline (Cit); t-butylalanine (t-BuA); t-butylglycine (t-BuG); N-methylisoleucine (MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); naphthylalanine (Nal); 4-chlorophenylalanine (Phe (4-Cl)); 2-fluorophenylalanine (Phe (2-F)); 3-fluorophenylalanine (Phe (3-F)); 4-fluorophenylalanine (Phe (4-F)); penicillamine (Pen); 1/2/3/4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β -2-thienylalanine (Thi); methionine sulfoxide (MSO); homoarginine (hArg); N-acetyl lysine (AcLys); 2,4-diaminobutyric acid (Dbu); 2,3-diaminobutyric acid (Dab); *p*-aminophenylalanine (Phe (pNH₂)); N-methyl valine (MeVal); homocysteine (hCys), homophenylalanine (hPhe) and homoserine (hSer);

hydroxyproline (Hyp), homoproline (hPro), N-methylated amino acids and peptoids (N-substituted glycines). In addition, in some embodiments the amino acid proline in the core peptides of formula (I) is substituted with a proline analogue, including, but not limited to, azetidine-2-carboxylate (A2C), L-Thiazolidine-4-carboxylic Acid, cis-4-hydroxy-L-proline (CHP), 3,4-dehydropyrolidine, thioproline, and isonipecotic acid (Inp).

[0097] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0098] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0099] The following eight groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
 - 2) Aspartic acid (D), Glutamic acid (E);
 - 3) Asparagine (N), Glutamine (Q);
 - 4) Arginine (R), Lysine (K);
 - 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
 - 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
 - 7) Serine (S), Threonine (T); and
 - 8) Cysteine (C), Methionine (M)
- (see, e.g., Creighton, *Proteins* (1984)).

[00100] Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take one or more of the foregoing characteristics into consideration are well known to those of skill in the art and include, but

are not limited to (original residue: exemplary substitution): (Ala: Gly, Ser), (Arg: Lys), (Asn: Gln, His), (Asp: Glu, Cys, Ser), (Gln: Asn), (Glu: Asp), (Gly: Ala), (His: Asn, Gln), (Ile: Leu, Val), (Leu: Ile, Val), (Lys: Arg), (Met: Leu, Tyr), (Ser: Thr), (Thr: Ser), (Tyr: Trp, Phe), and (Val: Ile, Leu). Embodiments of this disclosure, therefore, consider functional or biological equivalents of a polypeptide or protein as set forth above. In particular, embodiments of the invention provide variants having about 50%, 60%, 70%, 80%, 90%, and 95% sequence identity to the parent polypeptide. In various embodiments, the invention provides variants having this level of identity to a portion of the parent polypeptide sequence, *e.g.*, the wild-type growth factor including for example wild-type FGF1 (SEQ ID NO:1). In various embodiments, the variant has at least about 95%, 96%, 97%, 98% or 99% sequence identity to the parent polypeptide or to a portion of the parent polypeptide sequence, *e.g.*, the wild-type growth factor including for example wild-type FGF1 (SEQ ID NO:1), as defined herein.

[00101] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, “conservatively modified variants” refers to those nucleic acids that encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[00102] “Identity,” as known in the art, is a relationship between two or more polypeptide or protein sequences, as determined by comparing the sequences. In the art, “identity” also refers to the degree of sequence relatedness between polypeptides or proteins,

as determined by the match between strings of such sequences. “Identity” can be readily calculated by known bioinformational methods.

[00103] “Peptide” refers to a polymer in which the monomers are amino acids and are joined together through amide bonds. Peptides of the present invention can vary in size, *e.g.*, from two amino acids to hundreds or thousands of amino acids. A larger peptide (*e.g.*, at least 10, at least 20, at least 30 or at least 50 amino acid residues) is alternatively referred to as a “polypeptide” or “protein”. Additionally, unnatural amino acids, for example, β -alanine, phenylglycine, homoarginine and homophenylalanine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sequences, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D – or L – isomer. The L – isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, “peptide” or “polypeptide” refers to both glycosylated and non-glycosylated peptides or “polypeptides”. Also included are polypeptides that are incompletely glycosylated by a system that expresses the polypeptide. For a general review, *see*, Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[00104] In the present application, amino acid residues are numbered (typically in the superscript) according to their relative positions from the N-terminal amino acid (*e.g.*, N-terminal methionine) of the polypeptide, which is numbered “1”. The N-terminal amino acid may be a methionine (M), numbered “1”. The numbers associated with each amino acid residue can be readily adjusted to reflect the absence of N-terminal methionine if the N-terminus of the polypeptide starts without a methionine. It is understood that the N-terminus of an exemplary polypeptide can start with or without a methionine. Accordingly, in instances in which an amino acid linker is added to the N-terminus of a wild-type polypeptide, the first linker amino acid adjoined to the N-terminal amino acid is number -1 and so forth. For example, if the linker has the amino acid sequence KESCAKKQRQHMDs, (SEQ ID NO:2) with the S residue adjoined to the N-terminal amino acid of the wild-type polypeptide, then the most N-terminal linker amino acid K would be -14, while the most C-terminal linker amino acid S would be -1. In this way, the numbering of amino acids in the wild type polypeptide and linker bound wild type polypeptide is preserved.

[00105] The term “parent polypeptide” refers to a wild-type polypeptide and the amino acid sequence or nucleotide sequence of the wild-type polypeptide is part of a publicly accessible protein database (*e.g.*, EMBL Nucleotide Sequence Database, NCBI Entrez, ExPasy, Protein Data Bank and the like).

5 **[00106]** The term “mutant polypeptide” or “polypeptide variant” or “mutein” or “variant polypeptide” refers to a form of a polypeptide, wherein its amino acid sequence differs from the amino acid sequence of its corresponding wild-type (parent) form, naturally existing form or any other parent form. A mutant polypeptide can contain one or more mutations, *e.g.*, replacement, insertion, deletion, etc. which result in the mutant polypeptide.

10 **[00107]** The term “corresponding to a parent polypeptide” (or grammatical variations of this term) is used to describe a polypeptide of the invention, wherein the amino acid sequence of the polypeptide differs from the amino acid sequence of the corresponding parent polypeptide only by the presence of at least amino acid variation. Typically, the amino acid sequences of the variant polypeptide and the parent polypeptide exhibit a high percentage of
15 identity. In one example, “corresponding to a parent polypeptide” means that the amino acid sequence of the variant polypeptide has at least about 50% identity, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or at least about 98% identity to the amino acid sequence of the parent polypeptide. In another example, the nucleic acid sequence that encodes the variant polypeptide has at least about 50% identity, at
20 least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or at least about 98% identity to the nucleic acid sequence encoding the parent polypeptide. In some embodiments, the parent polypeptide corresponds to the FGF1 of SEQ ID NO:1.

[00108] The term “introducing (or adding etc.) a variation into a parent polypeptide”
25 (or grammatical variations thereof), or “modifying a parent polypeptide” to include a variation (or grammatical variations thereof) do not necessarily mean that the parent polypeptide is a physical starting material for such conversion, but rather that the parent polypeptide provides the guiding amino acid sequence for the making of a variant polypeptide. In one example, “introducing a variant into a parent polypeptide” means that the
30 gene for the parent polypeptide is modified through appropriate mutations to create a nucleotide sequence that encodes a variant polypeptide. In another example, “introducing a variant into a parent polypeptide” means that the resulting polypeptide is theoretically

designed using the parent polypeptide sequence as a guide. The designed polypeptide may then be generated by chemical or other means.

[00109] The term “library” refers to a collection of different polypeptides each corresponding to a common parent polypeptide. Each polypeptide species in the library is referred to as a member of the library. Preferably, the library of the present invention represents a collection of polypeptides of sufficient number and diversity to afford a population from which to identify a lead polypeptide. A library includes at least two different polypeptides. In one embodiment, the library includes from about 2 to about 100,000,000 members. In another embodiment, the library includes from about 10,000 to about 100,000,000 members. In yet another embodiment, the library includes from about 100,000 to about 100,000,000 members. In a further embodiment, the library includes from about 1,000,000 to about 100,000,000 members. In another embodiment, the library includes from about 10,000,000 to about 100,000,000 members. In yet another embodiment, the library includes more than 100 members.

[00110] The members of the library may be part of a mixture or may be isolated from each other. In one example, the members of the library are part of a mixture that optionally includes other components. For example, at least two polypeptides are present in a volume of cell-culture broth. In another example, the members of the library are each expressed separately and are optionally isolated. The isolated polypeptides may optionally be contained in a multi-well container, in which each well contains a different type of polypeptide. In another example, the members of the library are each expressed as fusions to a yeast or bacteria cell or phage or viral particle.

[00111] As used herein, the term “polymeric modifying group” is a modifying group that includes at least one polymeric moiety (polymer). The polymeric modifying group added to a polypeptide can alter a property of such polypeptide, for example, its bioavailability, biological activity or its half-life in the body. Exemplary polymers include water soluble and water insoluble polymers. A polymeric modifying group can be linear or branched and can include one or more independently selected polymeric moieties, such as poly(alkylene glycol) and derivatives thereof. In one example, the polymer is non-naturally occurring. In an exemplary embodiment, the polymeric modifying group includes a water-soluble polymer, *e.g.*, poly(ethylene glycol) and derivatives thereof (PEG, m-PEG), poly(propylene glycol) and derivatives thereof (PPG, m-PPG) and the like. In a preferred embodiment, the poly(ethylene glycol) or poly(propylene glycol) has a molecular weight that

is essentially homodisperse. In one embodiment the polymeric modifying group is not a naturally occurring polysaccharide.

[00112] The term “targeting moiety,” as used herein, refers to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art. Exemplary targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins, β -glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

[00113] The term “Fc-fusion protein”, as used herein, is meant to encompass proteins, in particular therapeutic proteins, comprising an immunoglobulin-derived moiety, which will be called herein the “Fc-moiety”, and a moiety derived from a second, non-immunoglobulin protein, which will be called herein the “therapeutic moiety”, irrespective of whether or not treatment of disease is intended.

[00114] As used herein, “therapeutic moiety” means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. “Therapeutic moiety” includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is bound to a carrier, *e.g.*, multivalent agents.

[00115] Therapeutic moiety also includes proteins and constructs that include proteins.

[00116] As used herein, “anti-tumor drug” means any agent useful to combat cancer including.

[00117] As used herein, “a cytotoxin or cytotoxic agent” means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracenedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diptheria toxin, and snake venom (*e.g.*, cobra venom).

[00118] As used herein, “a radioactive agent” includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60, fluorine-18, copper-64, copper-67, lutetium-177, or technetium-99m. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety. The radioactive agent or radionuclide can be a component of an imaging agent.

[00119] Near-infrared dyes can also be conjugated using standard chemistries for optical imaging applications. “Near infrared” refers to radiation in the portion of the electromagnetic spectrum adjacent to that portion associated with visible light, for example, from about 0.7 μm to about 1 μm . The near infrared dye may include, for example, a cyanine or indocyanine derivative such as Cy5.5. The infrared dye may also include phosphoramidite dyes, for example, IRDye® 800 (LI-COR® Biosciences).

[00120] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (*e.g.*, EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc.*). *See*, for example, Pitt *et al.*, “The Design of Chelating Agents for the Treatment of Iron Overload,” In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge, 1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein. Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. *See*, for example, Meares *et al.*, “Properties of In Vivo Chelate-Tagged Proteins and Polypeptides.” In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS; Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al.*, *Bioconjugate Chem.*, 9: 108-117 (1998); Song *et al.*, *Bioconjugate Chem.*, 8: 249-255 (1997). These metal binding agents can be used to bind a metal ion detectable in an imaging modality.

[00121] As used herein, “pharmaceutically acceptable carrier” includes any material, which when combined with the conjugate retains the conjugates’ activity and is non-reactive with the subject’s immune systems. “Pharmaceutically acceptable carrier” includes solids and liquids, such as vehicles, diluents and solvents. Examples include, but are not limited to,

any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well-known conventional methods.

[00122] As used herein, “administering” means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intrathecal, intralesional, or subcutaneous administration, administration by inhalation, or the implantation of a slow-release device, *e.g.*, a mini-osmotic pump, to the subject. Administration is by any route including parenteral and transmucosal (*e.g.*, oral, nasal, vaginal, rectal, or transdermal), particularly by inhalation. Parenteral administration includes, *e.g.*, intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, *e.g.*, induce apoptosis, administration may be directly to the tumor and/or into tissues surrounding the tumor. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

[00123] The term “ameliorating” or “ameliorate” refers to any indicia of success in the treatment of a pathology or condition, including any objective or subjective parameter such as abatement, remission or diminishing of symptoms or an improvement in a patient’s physical or mental well-being. Amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination and/or a psychiatric evaluation.

[00124] The term “therapy” refers to “treating” or “treatment” of a disease or condition including preventing the disease or condition from occurring in a subject (*e.g.*, human) that may be predisposed to the disease but does not yet experience or exhibit symptoms of the disease (prophylactic treatment), inhibiting the disease (slowing or arresting its development), providing relief from the symptoms or side-effects of the disease (including palliative treatment), and relieving the disease (causing regression of the disease).

[00125] The term “effective amount” or “an amount effective to” or a “therapeutically effective amount” or any grammatically equivalent term means the amount that, when

administered to an animal or human for treating a disease, is sufficient to effect treatment for that disease. An effective amount can also refer to the amount necessary to cause a cellular response, including for example, apoptosis, cell cycle initiation, and/or signal transduction.

[00126] The term “pharmaceutically acceptable salts” includes salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (see, for example, Berge *et al.*, *Journal of Pharmaceutical Science*, **66**: 1-19 (1977)). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

[00127] The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

[00128] The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for

example tritium (^3H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present invention, whether radioactive or not, are intended to be encompassed within the scope of the present invention.

[00129] “Reactive functional group,” as used herein refers to groups including, but not limited to, olefins, acetylenes, alcohols, phenols, ethers, oxides, halides, aldehydes, ketones, carboxylic acids, esters, amides, cyanates, isocyanates, thiocyanates, isothiocyanates, amines, hydrazines, hydrazones, hydrazides, diazo, diazonium, nitro, nitriles, mercaptans, sulfides, disulfides, sulfoxides, sulfones, sulfonic acids, sulfinic acids, acetals, ketals, anhydrides, sulfates, sulfenic acids isonitriles, amidines, imides, imidates, nitrones, hydroxylamines, oximes, hydroxamic acids thiohydroxamic acids, allenes, ortho esters, sulfites, enamines, ynamines, ureas, pseudoureas, semicarbazides, carbodiimides, carbamates, imines, azides, azo compounds, azoxy compounds, and nitroso compounds. Reactive functional groups also include those used to prepare bioconjugates, *e.g.*, N-hydroxysuccinimide esters, maleimides and the like. Methods to prepare each of these functional groups are well known in the art and their application or modification for a particular purpose is within the ability of one of skill in the art (*see*, for example, Sandler and Karo, eds. ORGANIC FUNCTIONAL GROUP PREPARATIONS, Academic Press, San Diego, 1989).

III. The Variants

[00130] In some embodiments, the variant is a proteolutically stable variant as compared to the wild-type growth factor. In an exemplary embodiment, the variant exhibits increased proteolytic stability as compared to wild-type. In some embodiments, the variant is any variant of a wild-type growth factor. In some embodiments, the variant is an antagonist for the growth factor receptor to which the wild-type growth factor binds.

[00131] In some embodiments, the variant is a variant of FGF1. In some embodiments, a variant of human fibroblast growth factor 1 (FGF1) comprising at least one member selected from an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof is provided. In some embodiments, a variant of human fibroblast growth factor 1 (FGF1) comprising at least one member selected from an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof, wherein the resulting FGF1 variant exhibits increased proteolytic stability as compared to wild-type FGF1 of SEQ ID NO:1 is provided. In some embodiments, the FGF1 variant

comprises an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof in the β -loop or near the C-terminus. In some embodiments, the FGF1 variant is a fibroblast growth factor receptor (FGFR) antagonist. The present invention provides an FGF1 polypeptide including at least one amino acid in at least one position in which this amino acid is not found in the parent FGF1 polypeptide (wild type, SEQ ID NO:1).

FNLPNGNYKKPKLLYCSNGGHFLRILP~~Q~~GTVDGTRDRSDQHIQLQLSAESVGEVYIKS
TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWFVGLKKN
GSCKRGPRTHYGQKAI~~Q~~FLPLPVSSD (SEQ ID NO:1).

10 **[00132]** In some embodiments, the FGF1 variant of SEQ ID NO:1 having at least one amino acid substitution. In some embodiments, the FGF1 variant comprises at least one amino acid substitution at position 28, 40, 47, 93 or 131. In some embodiments, the FGF1 variant comprise at least one amino acid substitution selected from the group consisting of D28N, Q40P, S47I, H93G, L131R, and L131K. In some embodiments, the FGF1 variant
15 comprises amino acid substitution L131R. In some embodiments, the FGF1 variant comprises amino acid substitution L131K. In some embodiments, the variant comprises amino acid substitutions D28N and L131R. In some embodiments, the variant comprises amino acid substitutions D28N and L131K. In some embodiments, the variant comprises amino acid substitutions Q40P, S47I, H93G, and L131R. In some embodiments, the variant
20 comprises amino acid substitutions Q40P, S47I, H93G, and L131K. In some embodiments, the variant comprises amino acid substitutions D28N, Q40P, S47I, H93G, and L131R. In some embodiments, the variant comprises amino acid substitutions D28N, Q40P, S47I, H93G, and L131K. In some embodiments, the FGF1 variant does not comprise the amino acid substitution L131A.

25 **[00133]** In some embodiments, the variant FGF1 is the variant referred to as BS4M1 (D28N and L131R) variant. In some embodiments, BS4M1 comprises the sequence
FNLPNGNYKKPKLLYCSNGGHFLRILP~~Q~~GTVDGTRDRSDQHIQLQLSAESVGEVYIKS
TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWFVGLKKN
GSCKRGPRTHYGQKAI~~Q~~FLPLPVSSD (SEQ ID NO:2).

30 **[00134]** In some embodiments, the variant FGF1 is the variant referred to as PM2 (Q40P, S47I, H93G). In some embodiments, PM2 comprises the sequence
FNLPNGNYKKPKLLYCSNGGHFLRILP~~Q~~GTVDGTRDRSDPHIQLQLIAESVGEVYIKS

TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENGYNTYISKKHAEKNWFVGLKKN
GSKCRGPRTHYGQKAIIFLPLPVSSD (SEQ ID NO:3).

[00135] In some embodiments, the variant FGF1 is the variant referred to as PM3 (D28N, Q40P, S47I, H93G, L131R). In some embodiments, PM3 comprises the sequence

5 FNLPPGNYKKPKLLYCSNGGHFLRILPITGTVDGTRDRSDPHIQLQLIAESVGEVYIKS
TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENGYNTYISKKHAEKNWFVGLKKN
GSKCRGPRTHYGQKAIIFLPLPVSSD (SEQ ID NO:4).

[00136] In some embodiments, variant FGF1 comprises the sequence

FNLPPGNYKKPKLLYCSNGGHFLRILPITGTVDGTRDRSDQHIQLQLSAESVGEVYIKS
10 TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWFVGLKKN
GSKCRGPRTHYGQKAIIFLPLPVSSD (SEQ ID NO:5).

[00137] In some embodiments, variant FGF1 comprises the sequence

FNLPPGNYKKPKLLYCSNGGHFLRILPITGTVDGTRDRSDQHIQLQLSAESVGEVYIKS
TETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWFVGLKKN
15 GSKCRGPRTHYGQKAIIFLPLPVSSD (SEQ ID NO:6).

[00138] In some embodiments, the variant is an isolated variant. In some embodiments, the variant exhibits at least one desirable characteristic not present in the present polypeptide. Exemplary characteristics include, but are not limited to, an increase in proteolytic stability, an increase in thermal stability, an increase or decrease in
20 conformational flexibility and increased antagonistic activity. As will be appreciated by those of skill in the art, the variant may exhibit any combination of two or more of these improved characteristics.

[00139] In some embodiments, the variant FGF1 is an antagonist for the FGFR receptor. In some embodiments, the FGF1 variant has a sequence selected from the group consisting of
25 SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6,

[00140] In some embodiments, the growth factor variants have a sequence identity with the parent polypeptide of at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 96%, 97%, 98% or 99%. In some embodiments, the growth factor variants of the invention have a sequence identity with the parent polypeptide of at
30 least about 99.2%, at least about 99.4%, at least about 99.6% or at least about 99.8%.

[00141] In some embodiments, the FGF1 variants have a sequence identity with the parent polypeptide of at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 96%, 97%, 98% or 99%. In some embodiments, the FGF1 variants of the invention have a sequence identity with the parent poly peptide of at least about 99.2%, at least about 99.4%, at least about 99.6% or at least about 99.8%.

[00142] In some embodiments, the positions of SEQ ID NO:1, which are mutated include one or more of 28, 40, 47, 93 or 131. As those of skill will realize, any combination of these positions can be mutated.

[00143] In some embodiments, an amino acid of the parent polypeptide at position 28 is altered to N, as compared to the wild-type FGF1 (*e.g.*, SEQ ID NO:

[00144] In some embodiments, an amino acid of the parent polypeptide at position 40 is altered to P.

[00145] In some embodiments, an amino acid of the parent polypeptide at position 47 is altered to I.

[00146] In some embodiments, an amino acid of the parent polypeptide at position 93 is altered to G.

[00147] In some embodiments, an amino acid of the parent polypeptide at position 131 is altered to R. In some embodiments, an amino acid of the parent polypeptide at position 131 is altered to K.

a. Conjugates

[00148] The present invention provides conjugates of the variants of the invention with one or more conjugation partner. Exemplary conjugation partners include polymers, targeting agents, therapeutic agents, cytotoxic agents, chelating agents and detectable agents. Those of skill will recognize that there is overlap between these non-limiting agent categories.

[00149] The conjugation partner or “modifying group” can be any conjugatable moiety. Exemplary modifying groups are discussed below. The modifying groups can be selected for their ability to alter the properties (*e.g.*, biological or physicochemical properties) of a given polypeptide. Exemplary polypeptide properties that may be altered by the use of modifying

groups include, but are not limited to, pharmacokinetics, pharmacodynamics, metabolic stability, biodistribution, water solubility, lipophilicity, tissue targeting capabilities and the therapeutic activity profile. Modifying groups are useful for the modification of polypeptides of use in diagnostic applications or in *in vitro* biological assay systems.

- 5 **[00150]** In some embodiments, a growth factor variant, including for example, an FGF1 variant as described herein is combined with an Fc moiety. The Fc-moiety may be derived from a human or animal immunoglobulin (Ig) that is preferably an IgG. The IgG may be an IgG1, IgG2, IgG3 or IgG4 (*see*, for example Figure 34). It is also preferred that the Fc-moiety is derived from the heavy chain of an immunoglobulin, preferably an IgG. More preferably,
- 10 the Fc-moiety comprises a portion, such as *e.g.*, a domain, of an immunoglobulin heavy chain constant region. Such Ig constant region preferably comprises at least one Ig constant domain selected from any of the hinge, CH2, CH3 domain, or any combination thereof. In some embodiments, the Fc-moiety comprises at least a CH2 and CH3 domain. It is further preferred that the Fc-moiety comprises the IgG hinge region, the CH2 and the CH3 domain.

15 Table 1: Exemplary IgG sequences:

SEQ ID NO:	Name	Sequence
126	IgG1	ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 60 GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG 120 PSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNA KTKPREEQYN 180 STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE 240 LTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTTPPV LDSDGSFFLY SKLTVDKSRW 300 QQGNVFSCSV MHEALHNHYT QKSLSLSPGK 330
127	IgG2	ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 60 GLYSLSSVVT VPSSNFGTQT YTCNVNHNKPS NTKVDKTVET KCCVECPPCP APPVAGPSVF 120 LFPPKPKDTL MISRTPEVTC VVVDVSHEDPEVQFNWYVDG VEVHNAKTKP REEQFNSTFR 180 VVSVLTIVHQ DWLNGKEYKCKVSNKGLPAP IEKTIKTKG QPREPQVYTL PPSREEMTKN 240 QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPMPLDSD GSFFLYSKLT VDKSRWQQGN 300 VFSCSVMEHA LHNHYTQKSL SLSPGK 326
128	IgG3	ASTKGPSVFP LAPCSRSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 60 GLYSLSSVVT VPSSSLGTQT YTCNVNHNKPS NTKVDKRVEL KTPLGDTTHT CPRCPEPKSC 120 DTPPPCPRCP EPKSCDTPPP CPRCPEPKSC DTPPPCPRCP APELLGGPSV FLFPPKPKDT 180 LMISRTPEVT CVVVDVSHEDPEVQFKWYVD GVEVHNAKTK PREEQYNSTF RVVSVLTVLH 240 QDWLNGKEYKCKVSNKALPAPIEKTIKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVK 300 GFYPSDIAVEWESNGQPENNYNTTTPMPLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVME 360 ALHNRTQKSLSLSPGK 377
129	IgG4	ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 60 GLYSLSSVVT VPSSSLGTQT YTCNVNHNKPS NTKVDKRVES KYGPPCPCP APEFLGGPSV 120 FLFPPKPKDT LMISRTPEVT CVVVDVSHEDPEVQFNWYVD GVEVHNAKTK PREEQFNSTY 180 RVVSVLTVLH QDWLNGKEYKCKVSNKGLPAPIEKTIKAKGQPREPQVYTLPPSREEMTK 240 NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQEG 300 NVFSCSVMEALHNHYTQKSLSLSPGK 327

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[00151] Fc domains of the IgG1 subclass are often used as the Fc moiety, because IgG1 has the longest serum half-life of any of the serum proteins. Lengthy serum half-life can be a desirable protein characteristic for animal studies and potential human therapeutic use. In addition, the IgG1 subclass possesses the strongest ability to carry out antibody mediated effector functions.

[00152] The primary effector function that may be most useful in a fusion protein is the ability for an IgG1 antibody to mediate antibody dependent cellular cytotoxicity. On the other hand, this could be an undesirable function for a fusion protein that functions primarily as an antagonist. Several of the specific amino acid residues that are important for antibody constant region-mediated activity in the IgG1 subclass have been identified. Inclusion or exclusion of these specific amino acids therefore allows for inclusion or exclusion of specific immunoglobulin constant region-mediated activity.

[00153] In accordance with the present invention, the Fc-moiety may also be modified in order to modulate effector functions. For instance, the following Fc mutations, according to EU index positions (Kabat et al., 1991), can be introduced if the Fc-moiety is derived from IgG1: T250Q/M428L; M252Y/S254T/T256E+H433K/N434F; E233P/L234V/L235A/AΔ236+A327G/A330S/P331S; E333A; K322A.

[00154] Further Fc mutations may *e.g.* be the substitutions at EU index positions selected from 330, 331 234, or 235, or combinations thereof. An amino acid substitution at EU index position 297 located in the CH2 domain may also be introduced into the Fc-moiety in the context of the present invention, eliminating a potential site of N-linked carbohydrate attachment. The cysteine residue at EU index position 220 may also be replaced.

[00155] The Fc-fusion protein of the invention may be a monomer or dimer. The Fc-fusion protein may also be a “pseudo-dimer”, containing a dimeric Fc-moiety (*e.g.* a dimer of two disulfide-bridged hinge-CH2-CH3 constructs), of which only one is fused to a therapeutic moiety.

[00156] The Fc-fusion protein may be a heterodimer, containing two different therapeutic moieties, or a homodimer, containing two copies of a single therapeutic moiety.

[00157] In some embodiments, the *in vivo* half-life of the growth factor variant, including for example, an FGF1 variant, as described herein can be enhanced with polyethylene glycol (PEG) moieties. Chemical modification of polypeptides with PEG (PEGylation) increases their molecular size and typically decreases surface- and functional group-accessibility, each of which are dependent on the number and size of the PEG moieties attached to the polypeptide. Frequently, this modification results in an improvement of plasma half-life and in proteolytic-stability, as well as a decrease in immunogenicity and hepatic uptake (Chaffee *et al. J. Clin. Invest.* 89: 1643-1651 (1992); Pyatak *et al. Res. Commun. Chem. Pathol Pharmacol.* 29: 113-127 (1980)). For example, PEGylation of interleukin-2 has been reported to increase its antitumor potency *in vivo* (Katre *et al. Proc. Natl. Acad. Sci. USA.* 84: 1487-1491 (1987)) and PEGylation of a F(ab')₂ derived from the monoclonal antibody A7 has improved its tumor localization (Kitamura *et al. Biochem. Biophys. Res. Commun.* 28: 1387-1394 (1990)). Thus, in another embodiment, the *in vivo* half-life of a polypeptide derivatized with a PEG moiety by a method of the invention is increased relative to the *in vivo* half-life of the non-derivatized parent polypeptide.

[00158] The increase in polypeptide *in vivo* half-life is best expressed as a range of percent increase relative to the parent polypeptide. The lower end of the range of percent increase is about 40%, about 60%, about 80%, about 100%, about 150% or about 200%. The upper end of the range is about 60%, about 80%, about 100%, about 150%, or more than about 250%.

[00159] Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (*e.g.*, dextran, amylose, hyaluronic acid, poly(sialic acid), heparans, heparins, etc.); poly(amino acids), *e.g.*, poly(aspartic acid) and poly(glutamic acid); nucleic acids; synthetic polymers (*e.g.*, poly(acrylic acid), poly(ethers), *e.g.*, poly(ethylene glycol)); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached. See, for example, Harris, *Macromol. Chem. Phys.* C25: 325-373 (1985); Scouten, *Methods in Enzymology* 135: 30-65 (1987); Wong *et al., Enzyme Microb. Technol.* 14: 866-874 (1992); Delgado *et al., Critical Reviews in Therapeutic Drug Carrier Systems* 9: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* 6: 150-165 (1995); and Bhadra, *et al., Pharmazie*, 57:5-29 (2002).

[00160] In another embodiment, analogous to those discussed above, the modified sugars include a water-insoluble polymer, rather than a water-soluble polymer. The conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic polypeptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See*, for example, Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

[00161] Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

[00162] Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those including collagen, pluronics and the like.

[00163] Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly(α -hydroxy-carboxylic acid)/poly(oxyalkylene, (*see*, Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See*,

Younes *et al.*, *J Biomed. Mater. Res.* **21**: 1301-1316 (1987); and Cohn *et al.*, *J Biomed. Mater. Res.* **22**: 993-1009 (1988).

[00164] Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

[00165] In another embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronics, collagen, gelatin, hyaluronic acid, polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

[00166] In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811, which issued on June 11, 1985. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearyl phosphatidyl ethanolamine, stearyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

[00167] The present invention also provides conjugates analogous to those described above in which the polypeptide is conjugated to a therapeutic moiety, diagnostic moiety, targeting moiety, toxin moiety or the like. Each of the above-recited moieties can be a small molecule, natural polymer (*e.g.*, polypeptide) or a synthetic polymer.

[00168] In various embodiments, the variant is conjugated to a component of a matrix for tissue regeneration. Exemplary matrices are known in the art and it is within the ability of a skilled worker to select and modify an appropriate matrix with of the growth factor variant, including for example, an FGF1 variant, of the invention. The growth factor variant, including for example, an FGF1 variant, of the invention are generally of use in regenerative medicine applications, including the regeneration of, *e.g.*, eye, liver, muscle, nerve and cardiac tissue.

[00169] In some embodiments, the invention provides conjugates that localize selectively in a particular tissue due to the presence of a targeting agent as a component of the conjugate.

In an exemplary embodiment, the targeting agent is a protein. Exemplary proteins include transferrin (brain, blood pool), HS-glycoprotein (bone, brain, blood pool), antibodies (brain, tissue with antibody-specific antigen, blood pool), coagulation factors V-XII (damaged tissue, clots, cancer, blood pool), serum proteins, *e.g.*, α -acid glycoprotein, fetuin, α -fetal protein (brain, blood pool), β 2-glycoprotein (liver, atherosclerosis plaques, brain, blood pool), G-CSF, GM-CSF, M-CSF, and EPO (immune stimulation, cancers, blood pool, red blood cell overproduction, neuroprotection), albumin (increase in half-life), IL-2 and IFN- α .

[00170] In another embodiment, the invention provides a conjugate between the growth factor variant, including for example, an FGF1 variant, of the invention and a therapeutic moiety. Therapeutic moieties, which are useful in practicing the instant invention include drugs from a broad range of drug classes having a variety of pharmacological activities. Methods of conjugating therapeutic and diagnostic agents to various other species are well known to those of skill in the art. *See*, for example Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.

[00171] Classes of useful therapeutic moieties include, for example, antineoplastic drugs (*e.g.*, antiandrogens (*e.g.*, leuprolide or flutamide), cytocidal agents (*e.g.*, adriamycin, doxorubicin, taxol, cyclophosphamide, busulfan, cisplatin, β -2-interferon) anti-estrogens (*e.g.*, tamoxifen), antimetabolites (*e.g.*, fluorouracil, methotrexate, mercaptopurine, thioguanine). Also included within this class are radioisotope-based agents for both

diagnosis and therapy, and conjugated toxins, such as ricin, geldanamycin, mytansin, CC-1065, the duocarmycins, Chlicheamycin and related structures and analogues thereof.

[00172] The therapeutic moiety can also be a hormone (*e.g.*, medroxyprogesterone, estradiol, leuprolide, megestrol, octreotide or somatostatin); endocrine modulating drugs
5 (*e.g.*, contraceptives (*e.g.*, ethinodiol, ethinyl estradiol, norethindrone, mestranol, desogestrel, medroxyprogesterone). Of use in various embodiments of the invention are conjugates with estrogens (*e.g.*, diethylstilbesterol), glucocorticoids (*e.g.*, triamcinolone, betamethasone, etc.) and progestogens, such as norethindrone, ethynodiol, norethindrone, levonorgestrel; thyroid agents (*e.g.*, liothyronine or levothyroxine) or anti-thyroid agents (*e.g.*, methimazole);
10 antihyperprolactinemic drugs (*e.g.*, cabergoline); hormone suppressors (*e.g.*, danazol or goserelin), oxytocics (*e.g.*, methylergonovine or oxytocin) and prostaglandins, such as mioprostonol, alprostadil or dinoprostone, can also be employed.

[00173] Other useful modifying groups include immunomodulating drugs (*e.g.*, antihistamines, mast cell stabilizers, such as lodoxamide and/or cromolyn, steroids (*e.g.*,
15 triamcinolone, beclomethazone, cortisone, dexamethasone, prednisolone, methylprednisolone, beclomethasone, or clobetasol), histamine H2 antagonists (*e.g.*, famotidine, cimetidine, ranitidine), immunosuppressants (*e.g.*, azathioprine, cyclosporin), etc. Groups with anti-inflammatory activity, such as sulindac, etodolac, ketoprofen and ketorolac, are also of use. Other drugs of use in conjunction with the present invention will be apparent
20 to those of skill in the art.

[00174] In some embodiments, the conjugate is formed by reaction between a reactive amino acid and a reactive conjugation partner for the reactive amino acid. Both the reactive amino acid and the reactive conjugation partner include within their framework one or more reactive functional group. One of the two binding species may include a "leaving group" (or
25 activating group) refers to those moieties, which are easily displaced in enzyme-regulated nucleophilic substitution reactions or alternatively, are replaced in a chemical reaction utilizing a nucleophilic reaction partner (*e.g.*, an amino acid moiety carrying a sulfhydryl group). It is within the abilities of a skilled person to select a suitable leaving group for each type of reaction. Many activated sugars are known in the art. See, for example, Vocadlo et
30 al., In CARBOHYDRATE CHEMISTRY AND BIOLOGY, Vol. 2, Ernst *et al.* Ed., Wiley-VCH Verlag: Weinheim, Germany, 2000; Kodama *et al.*, *Tetrahedron Lett.* 34: 6419 (1993); Loughheed, *et al.*, *J. Biol. Chem.* 274: 37717 (1999)).

[00175] In various embodiments, the amino acid substitution, which is the variant (or a variant) of naturally occurring FGF1, is the locus for attachment of the conjugation partner, *e.g.*, a side-chain amino acid, *e.g.*, cysteine, lysine, serine, etc.

[00176] Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently
5 favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and
10 carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society,
15 Washington, D.C., 1982.

b. Reactive Functional Groups

[00177] Useful reactive functional groups on a reactive amino acid or reactive conjugation partner include, but are not limited to:

- (a) carboxyl groups and various derivatives thereof including, but not limited to,
20 N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;
- (b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*
- (c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic
25 group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;
- (d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

5 (f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with acyl halides;

10 (h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

[00178] The reactive functional groups can be chosen such that they do not participate in, or
15 interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, *see*, for example, Greene *et*
20 *al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

[00179] The group linking the polypeptide and conjugation partner can also be a cross-linking group, *e.g.*, a zero- or higher-order cross-linking group (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* 25: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.)
25 pp. 395-442, Wiley, New York, 1981; Ji, T. H., *Meth. Enzymol.* 91: 580-609, 1983; Mattson *et al.*, *Mol. Biol. Rep.* 17: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material.
30 Agents that catalyze formation of a disulfide bond belong to this category. Another example

is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide γ -glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-bound glutamyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

[00180] Exemplary conjugation partners attached to the polypeptides of the invention include, but are not limited to, PEG derivatives (*e.g.*, alkyl-PEG, acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (*e.g.*, alkyl-PPG, acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, S_{Le^x} , mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins, chondroitin, keratan, dermatan, albumin, integrins, antennary oligosaccharides, peptides and the like.

[00181] In addition to covalent attachments, the growth factor variant, including for example, an FGF1 variant, of the instant invention can be attached onto the surface of a biomaterial through non-covalent interactions. Non covalent protein incorporation can be done, for example, through encapsulation or absorption. Attachment of the polypeptides of the instant invention to a biomaterial may be mediated through heparin. In some embodiments, the polypeptides of the instant invention are attached to a heparin-alginate polymer and alginate as described in Harada et al., J. Clin. Invest. (1994) 94:623-630; Laham et al., Circulation (1999) 1865-1871 and references cited therein. In other embodiments, the polypeptides of the instant invention are attached to a collagen based biomaterial.

c. Imaging Agents

[00182] An exemplary conjugate of the invention is an imaging agent comprising a variant of the invention and a detectable moiety, which is detectable in an imaging modality. There is a critical need for molecular imaging probes that will specifically target Met receptors in living subjects and allow noninvasive characterization of tumors for patient-specific cancer

treatment and disease management. The ability to detect Met-expressing tumors through non-invasive imaging could also serve as an indicator of metastatic risk.

[00183] Exemplary imaging modalities in which the conjugates of the invention find use include, without limitation, positron emission tomography (PET) in which a variant of the invention is tagged with a positron emitting isotope. Typical isotopes include ^{11}C , ^{13}N , ^{15}O , ^{18}F , ^{64}Cu , ^{62}Cu , ^{124}I , ^{76}Br , ^{82}Rb and ^{68}Ga , with ^{18}F being the most clinically utilized. The variants can also be incorporated into ultrasound agents, magnetic resonance imaging agents, X-ray agents, CT agents, gamma camera scintigraphy agents and fluorescent imaging agents. Additional detectable moieties and methods of imaging are set forth in the Methods section herein below.

[00184] In an exemplary embodiment, the conjugation partner is attached to a polypeptide variant of the invention via a linkage that is cleaved under selected conditions. Exemplary conditions include, but are not limited to, a selected pH (*e.g.*, stomach, intestine, endocytotic vacuole), the presence of an active enzyme (*e.g.*, esterase, reductase, oxidase), light, heat and the like. Many cleavable groups are known in the art. *See*, for example, Jung *et al.*, *Biochem. Biophys. Acta*, 761: 152-162 (1983); Joshi *et al.*, *J. Biol. Chem.*, 265: 14518-14525 (1990); Zarling *et al.*, *J. Immunol.*, 124: 913-920 (1980); Bouizar *et al.*, *Eur. J. Biochem.*, 155: 141-147 (1986); Park *et al.*, *J. Biol. Chem.*, 261: 205-210 (1986); Browning *et al.*, *J. Immunol.*, 143: 1859-1867 (1989).

IV. Pharmaceutical Compositions

[00185] The growth factor variants, including for example, the FGF1 variants, and their conjugates of the invention have a broad range of pharmaceutical applications.

[00186] Thus, in another aspect, the invention provides a pharmaceutical composition including at least one polypeptide or polypeptide conjugate of the invention and a pharmaceutically acceptable diluent, carrier, vehicle, additive or combinations thereof. Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* 249:1527-1533 (1990).

[00187] The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable matrices, such as microspheres (*e.g.*, polylactate polyglycolate), may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

[00188] Commonly, the pharmaceutical compositions are administered subcutaneously or parenterally, *e.g.*, intravenously. Thus, the invention provides compositions for parenteral administration, which include the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, *e.g.*, water, buffered water, saline, PBS and the like. The compositions may also contain detergents such as Tween 20 and Tween 80; stabilizers such as mannitol, sorbitol, sucrose, and trehalose; and preservatives such as EDTA and meta-cresol. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

[00189] These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9 and most preferably from 7 and 8.

[00190] In some embodiments the glycopeptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using a variety of targeting agents (*e.g.*, the sialyl galactosides of the invention) is well known in the art (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044).

[00191] Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid-derivatized glycopeptides of the invention.

5 [00192] Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those of skill in the art (*e.g.*, alkylation or acylation of a hydroxyl group present on
10 the carbohydrate with a long chain alkyl halide or with a fatty acid, respectively).

[00193] Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also have a reactive portion, which is chemically available on the
15 aqueous surface of the liposome. The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some embodiments, it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the
20 target agent or carbohydrate which is extended, three dimensionally, off of the vesicle surface.

[00194] The growth factor variants, including for example, the FGF1 variants, prepared by the methods of the invention may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient
25 suspected of having an inflammation. For this use, the compounds can be labeled with ^{125}I , ^{14}C , or tritium.

V. Nucleic Acids

[00195] In some embodiments, the invention provides an isolated nucleic acid encoding the
30 growth factor variant, including for example, the FGF1 variant, according to any of the

embodiments set forth hereinabove. In some embodiments, the invention provides a nucleic acid complementary to this nucleic acid.

[00196] In some embodiments, the invention provides an expression vector including a nucleic acid encoding a polypeptide variant according to any of the embodiments set forth
5 hereinabove operatively linked to a promoter.

VI. Libraries and methods of screening

[00197] Also provided in various embodiments is a library of the growth factor variant polypeptides, including for example, an FGF1 variant polypeptides, comprising a plurality of
10 different members, wherein each member of the library corresponds to a common parent growth factor polypeptide or FGF1 parent polypeptide, and wherein each member of the library comprises an amino acid at a position at which the amino acid is not found in the parent polypeptide.

a. Library Creation

[00198] In order to generate a randomized library of FGF1 or other growth factors, oligonucleotides were prepared which coded for various FGF1 or other growth factor sequences. The DNA used to express growth factor variant polypeptide, including for example, an FGF1 variant polypeptides in yeast was prepared synthetically or by standard recombinant techniques. Where an amino acid was to be varied, twenty different codons,
20 each coding for a different amino acid, were synthesized for a given position. Randomized oligonucleotide synthesis has been used to create a coding cassette in which about 5 to about 15 amino acids are randomized (see, *e.g.*, Burritt et al., (1996) Anal. Biochem. 238:1 13; Lowman (1997) Annu. Rev. Biophys. Biomol. Struct. 26:410 24; Wilson (1998) Can. J. Microbiol. 44:313 329).

[00199] The yeast display vector typically used for evolution of improved mutants is called "pCT". The vector is further described in US 2004/0146976 to Wittrup, et al., published Jul. 29, 2004, entitled "Yeast cell surface display of proteins and uses thereof." As described there, the vector provides a genetic fusion of the N terminus of a polypeptide of interest to the C-terminus of the yeast Aga2p cell wall protein. The outer wall of each yeast cell can display
30 approximately 10^4 - 10^5 protein agglutinins. The vector contains the specific restriction sites

and illustrates the transcriptional regulation by galactose, the N-terminal HA and C-terminal c-myc epitope tags and the Factor Xa protease cleavage site.

[00200] In some embodiments of the present invention, the yeast display platform, which is commonly used to engineer high affinity binders, is also utilized to engineer proteins with greater proteolytic stability (see, for example, Figure 1). In some embodiments, several thousand copies of a single growth factor variant are displayed on the surface of yeast as tethered fusions. In some embodiments, the hemagglutinin (HA) tag is expressed upstream of the growth factor while the c-myc tag is expressed downstream of the growth factor. In some embodiments, cells can be incubated with soluble Fc fusions of the corresponding receptor, which can bind to the yeast displayed growth factor.

[00201] In some embodiments, the yeast display platform is combined with flow-activated cell sorting (FACS) to engineer growth factors with higher proteolytic stability (see, for example, Figure 2). In some embodiments, a library of growth factor mutants can be generated by random mutagenesis, directed mutagenesis, or DNA shuffling, or other recombinant techniques as discussed above or known in the art. In some embodiments, the library of yeast cells is incubated with a protease of interest, during which cleavage of the yeast surface displayed proteins occurs. In some embodiments, the growth factor mutants with greater proteolytic stability are more resistant to cleavage on the yeast cell surface. In some embodiments, after protease incubation, the cells are washed and incubated with soluble Fc fusions of the functional receptor that bind to properly folded growth factor mutants with retained receptor binding affinity. In some embodiments, FACS is used to sort for properly folded, uncleaved growth factor mutants, which are expanded and induced for the next round of sorting.

[00202] In some embodiments, fluorescent antibody markers against the Fc domain, the c-myc domain, and the HA tag are used to measure receptor binding, growth factor-specific cleavage, and non-specific cleavage (see, for example, Table 2 below). In some embodiments, detection of the bound Fc-fusion receptor allows for confirming that mutations in the growth factor do not severely reduce the binding affinity for the receptor or lead to improper protein folding. In some embodiments, growth factor-specific cleavage is a direct measure of a growth factor's proteolytic stability. In some embodiments, growth factor-specific cleavage is detected by the c-myc signal, as a cleaved growth factor will have the C-terminal c-myc tag removed. In some embodiments, non-specific cleavage occurs when the

protease cleaves within the yeast surface display proteins, for example, the yeast display proteins Aga1p and Aga2p. In some embodiments, during non-specific cleavage, the fluorescent signals for all three markers are decreased. In some embodiments, this is undesirable, as the dynamic range for detecting growth factor cleavage and binding activity are decreased. In some embodiments, the HA signal is used to ensure that non-specific cleavage by the protease of interest is minimal.

Table 2: Effect of different events on the observed signal from fluorescent antibody markers.

	HA	c-myc	Fc
Denaturation/loss of binding affinity			↓↓↓
Growth factor-specific cleavage		↓↓↓	↓↓↓
Non-specific cleavage	↓↓↓	↓↓↓	↓↓↓

[00203] In some embodiments, a wild-type growth factor and variants thereof can be cloned into the pCT vector. In some embodiments, the wild-type growth factor and variants thereof can be expressed on the surface of *S. cerevisiae* yeast cells as a fusion to the Aga2p mating protein. In some embodiments, successful expression of the wild-type growth factor and variants thereof on the yeast cell surface can be confirmed by detection of the c-myc tag on the C-terminus of the protein. In some embodiments, proper folding of yeast-displayed wild-type growth factor and variants thereof can be confirmed by measuring specific binding activity to wild-type growth factor-Fc.

[00204] In some embodiments, the FGF1 polypeptide of SEQ ID NO:1 was employed as a model for demonstrating the setup of the proteolytic stability screen. In some embodiments, the wild type FGF1 was cloned into the pCT vector. In some embodiments, this FGF1 polypeptide and FGF1 variants thereof can be expressed on the surface of *S. cerevisiae* yeast cells as a fusion to the Aga2p mating protein (see, for example, Figure 3A). In some embodiments, successful expression of FGF1 on the yeast cell surface can be confirmed by detection of the c-myc tag on the C-terminus of the protein (see, for example, Figure 3B). In some embodiments, proper folding of yeast-displayed FGF can be confirmed by measuring specific binding activity to FGFR1-Fc (see, for example, Figure 3C).

[00205] In some embodiments, serum, trypsin, chymotrypsin, and plasmin can be used for developing a proteolytic stability screen for growth factor variant polypeptides, including for example, an FGF1 variant polypeptides. In some embodiments, these proteases were selected, based on their scientific and biological relevance to for growth factor variant

polypeptides, including for example, an FGF1 variant polypeptides. In some embodiments, the suitability of the protease for the screen was determined by its ability to cleave the growth factor at a reasonable rate with minimal non-specific cleavage of the yeast display proteins.

In some embodiments, serum can be used for developing a proteolytic stability screen for growth factor variant polypeptides, including for example, an FGF1 variant polypeptides. In some embodiments, trypsin can be used for developing a proteolytic stability screen for growth factor variant polypeptides, including for example, an FGF1 variant polypeptides. In some embodiments, chymotrypsin can be used for developing a proteolytic stability screen for growth factor variant polypeptides, including for example, an FGF1 variant polypeptides.

In some embodiments, plasmin can be used for developing a proteolytic stability screen for growth factor variant polypeptides, including for example, an FGF1 variant polypeptides.

[00206] In some embodiments, the stability is determined by comparing proteolytic cleavage of the wild-type growth factor to proteolytic cleavage of the variant growth factor. In some embodiments, the stability is determined by comparing proteolytic cleavage of the wild-type FGF1 to proteolytic cleavage of the FGF1 variant.

[00207] In some embodiments, stability of the growth factor variant is increased by at least 5% to at least 95%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 10% to at least 90%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 5% to at least 90%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 5% to at least 85%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 5% to at least 80%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 5% to at least 75%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 5% to at least 70%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 10% to at least 70%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 5%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 10%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 15%, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 20%, as

increased by at least 10% to at least 70%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 5%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 10%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 15%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 20%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 25%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 30%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 35%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 40%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 45%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 50%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 55%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 60%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 65%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 70%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 75%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 80%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 85%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 90%, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 95%, as compared to wild-type FGF1.

[00209] In some embodiments, stability of the growth factor variant is increased by at least 1-fold to at least 10-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 1-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 2-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 3-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 4-fold, as compared to wild-type growth factor. In some

embodiments, stability of the growth factor variant is increased by at least 5-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 6-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 7-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 8-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 9-fold, as compared to wild-type growth factor. In some embodiments, stability of the growth factor variant is increased by at least 10-fold, as compared to wild-type growth factor.

[00210] In some embodiments, stability of the FGF1 variant is increased by at least 1-fold to at least 10-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 1-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 2-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 3-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 4-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 5-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 6-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 7-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 8-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 9-fold, as compared to wild-type FGF1. In some embodiments, stability of the FGF1 variant is increased by at least 10-fold, as compared to wild-type FGF1.

b. Fluorescent Cell Sorting

[00211] In some embodiments, screening can include the use of a cell sorter. Commercially available flow cytometers can measure fluorescence emissions at the single-cell level at four or more wavelengths, at a rate of approximately 50,000 cells per second (Ashcroft and Lopez, 2000). Typical flow cytometry data can be shown in which yeast have been labeled with two different color fluorescent probes to measure protein expression levels and bound soluble ligand (for example, a growth factor receptor). A “diagonal” population of

cells results due to variation in protein expression levels on a per cell basis: cells that express more protein will bind more ligand. The equilibrium binding constant (K_D) can be determined by titration of soluble ligand, and the dissociation rate constant (k_{off}) can be measured through competition binding of unlabeled ligand. With yeast, a monodispersity of tethered proteins exists over the cell surface, and soluble ligand are used for binding and testing, such that avidity effects are not observed, unlike other display methods using immobilized ligands. To date, the properties of most proteins expressed on the yeast cell surface mimic what is seen in solution in terms of stability and binding affinity (Bader et al., 2000; Feldhaus et al., 2003; Holler et al., 2000; VanAntwerp and Wittrup, 2000). See, also, Weaver-Feldhaus et al., "Directed evolution for the development of conformation-specific affinity reagents using yeast display," Protein Engineering Design and Selection Sep. 26, 2005 18(11):527-536.

[00212] Cell sorting can be carried out on a FACS Vantage (BD Biosciences) multiparameter laser flow cytometer and cell sorter. Before sorting, fluorescent staining was carried out as described above, so that analysis of various polypeptide levels were detected, as described above.

VII. Methods

a. Chemical Synthesis

[00213] Polypeptide variants of the invention may be prepared using conventional step-wise solution or solid phase synthesis (see, *e.g.*, Chemical Approaches to the Synthesis of Peptides and Proteins, Williams et al., Eds., 1997, CRC Press, Boca Raton Florida, and references cited therein; Solid Phase Peptide Synthesis: A Practical Approach, Atherton & Sheppard, Eds., 1989, IRL Press, Oxford, England, and references cited therein).

[00214] Alternatively, the peptides of the invention may be prepared by way of segment condensation, as described, for example, in Liu et al., 1996, Tetrahedron Lett. 37(7):933-936; Baca, et al., 1995, J. Am. Chem. Soc. 117:1881-1887; Tam et al., 1995, Int. J. Peptide Protein Res. 45:209-216; Schnölzer and Kent, 1992, Science 256:221-225; Liu and Tam, 1994, J. Am. Chem. Soc. 116(10):4149-4153; Liu and Tam, 1994, Proc. Natl. Acad. Sci. USA 91:6584-6588; Yamashiro and Li, 1988, Int. J. Peptide Protein Res. 31:322-334). Segment condensation is a particularly useful method for synthesizing embodiments containing internal glycine residues. Other methods useful for synthesizing the peptides of the invention are described in Nakagawa et al., 1985, J. Am. Chem. Soc. 107:7087-7092.

[00215] Polypeptide variants containing N-and/or C-terminal blocking groups can be prepared using standard techniques of organic chemistry. For example, methods for acylating the N-terminus of a peptide or amidating or esterifying the C-terminus of a peptide are well-known in the art. Modes of carrying other modifications at the N-and/or C-terminus will be apparent to those of skill in the art, as will modes of protecting any side-chain functionalities as may be necessary to attach terminal blocking groups. Pharmaceutically acceptable salts (counter ions) can be conveniently prepared by ion-exchange chromatography or other methods as are well known in the art.

[00216] Compounds of the invention which are in the form of tandem multimers can be conveniently synthesized by adding the linker(s) to the peptide chain at the appropriate step in the synthesis. Alternatively, the helical segments can be synthesized and each segment reacted with the linker. Of course, the actual method of synthesis will depend on the composition of the linker. Suitable protecting schemes and chemistries are well known, and will be apparent to those of skill in the art.

[00217] Compounds of the invention which are in the form of branched networks can be conveniently synthesized using the trimeric and tetrameric resins and chemistries described in Tam, 1988, Proc. Natl. Acad. Sci. USA 85:5409-5413 and Demoor et al., 1996, Eur. J. Biochem. 239:74-84. Modifying the synthetic resins and strategies to synthesize branched networks of higher or lower order, or which contain combinations of different core peptide helical segments, is well within the capabilities of those of skill in the art of peptide chemistry and/or organic chemistry. Formation of disulfide linkages, if desired, is generally conducted in the presence of mild oxidizing agents.

[00218] Chemical oxidizing agents may be used, or the compounds may simply be exposed to atmospheric oxygen to effect these linkages. Various methods are known in the art, including those described, for example, by Tam et al., 1979, Synthesis 955-957; Stewart et al., 1984, Solid Phase Peptide Synthesis, 2d Ed., Pierce Chemical Company Rockford, IL; Ahmed et al., 1975, J. Biol. Chem. 250:8477-8482; and Pennington et al., 1991 Peptides 1990 164-166, Giralt and Andreu, Eds., ESCOM Leiden, The Netherlands. An additional alternative is described by Kamber et al., 1980, Helv. Chim. Acta 63:899-915. A method conducted on solid supports is described by Albericio, 1985, Int. J. Peptide Protein Res. 26:92-97. Any of these methods may be used to form disulfide linkages in the peptides of the invention.

VIII. Acquisition of Polypeptide Coding Sequences

a. General Recombinant Technology

[00219] The creation of variant and/or mutant polypeptides, which incorporate an O-linked glycosylation sequence of the invention can be accomplished by altering the amino acid sequence of a corresponding parent polypeptide, by either mutation or by full chemical synthesis of the polypeptide. The polypeptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA sequence encoding the polypeptide at preselected bases to generate codons that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

[00220] This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel *et al.*, eds., *Current Protocols in Molecular Biology* (1994).

[00221] Nucleic acid sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[00222] Oligonucleotides that are not commercially available can be chemically synthesized, *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* 22: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, *Nucleic Acids Res.* 12: 6159-6168 (1984). Entire genes can also be chemically synthesized. Purification of oligonucleotides is performed using any art-recognized strategy, *e.g.*, native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255: 137-149 (1983).

[00223] The sequence of the cloned wild-type polypeptide genes, polynucleotide encoding mutant polypeptides, and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16: 21-26 (1981).

[00224] In an exemplary embodiment, the glycosylation sequence is added by shuffling polynucleotides. Polynucleotides encoding a candidate polypeptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g.,* Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751 (1994); Stemmer, *Nature* 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

b. Cloning and Subcloning of a Wild-Type Peptide Coding Sequence

[00225] Numerous polynucleotide sequences encoding wild-type polypeptides have been determined and are available from a commercial supplier, *e.g.,* human growth hormone, *e.g.,* GenBank Accession Nos. NM 000515, NM 002059, NM 022556, NM 022557, NM 022558, NM 022559, NM 022560, NM 022561, and NM 022562.

[00226] The rapid progress in the studies of human genome has made possible a cloning approach where a human DNA sequence database can be searched for any gene segment that has a certain percentage of sequence homology to a known nucleotide sequence, such as one encoding a previously identified polypeptide. Any DNA sequence so identified can be subsequently obtained by chemical synthesis and/or a polymerase chain reaction (PCR) technique such as overlap extension method. For a short sequence, completely *de novo* synthesis may be sufficient; whereas further isolation of full length coding sequence from a human cDNA or genomic library using a synthetic probe may be necessary to obtain a larger gene.

[00227] Alternatively, a nucleic acid sequence encoding a polypeptide can be isolated from a human cDNA or genomic DNA library using standard cloning techniques such as polymerase chain reaction (PCR), where homology-based primers can often be derived from a known nucleic acid sequence encoding a polypeptide. Most commonly used techniques for this purpose are described in standard texts, *e.g.,* Sambrook and Russell, *supra*.

[00228] cDNA libraries suitable for obtaining a coding sequence for a wild-type polypeptide may be commercially available or can be constructed. The general methods of isolating mRNA, making cDNA by reverse transcription, ligating cDNA into a recombinant vector, transfecting into a recombinant host for propagation, screening, and cloning are well known (*see, e.g.,* Gubler and Hoffman, *Gene*, 25: 263-269 (1983); Ausubel *et al., supra*). Upon

obtaining an amplified segment of nucleotide sequence by PCR, the segment can be further used as a probe to isolate the full-length polynucleotide sequence encoding the wild-type polypeptide from the cDNA library. A general description of appropriate procedures can be found in Sambrook and Russell, *supra*.

5 **[00229]** A similar procedure can be followed to obtain a full length sequence encoding a wild-type polypeptide, *e.g.*, any one of the GenBank Accession Nos mentioned above, from a human genomic library. Human genomic libraries are commercially available or can be constructed according to various art-recognized methods. In general, to construct a genomic library, the DNA is first extracted from an tissue where a polypeptide is likely found. The DNA
10 is then either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb in length. The fragments are subsequently separated by gradient centrifugation from polynucleotide fragments of undesired sizes and are inserted in bacteriophage λ vectors. These vectors and phages are packaged *in vitro*. Recombinant phages are analyzed by plaque hybridization as described in Benton and Davis, *Science*, 196: 180-182 (1977). Colony
15 hybridization is carried out as described by Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 72: 3961-3965 (1975).

[00230] Based on sequence homology, degenerate oligonucleotides can be designed as primer sets and PCR can be performed under suitable conditions (*see, e.g.*, White *et al.*, *PCR Protocols: Current Methods and Applications*, 1993; Griffin and Griffin, *PCR Technology*,
20 CRC Press Inc. 1994) to amplify a segment of nucleotide sequence from a cDNA or genomic library. Using the amplified segment as a probe, the full-length nucleic acid encoding a wild-type polypeptide is obtained.

[00231] Upon acquiring a nucleic acid sequence encoding a wild-type polypeptide, the coding sequence can be subcloned into a vector, for instance, an expression vector, so that a
25 recombinant wild-type polypeptide can be produced from the resulting construct. Further modifications to the wild-type polypeptide coding sequence, *e.g.*, nucleotide substitutions, may be subsequently made to alter the characteristics of the molecule.

c. Introducing Mutations into a Polypeptide Sequence

30 **[00232]** From an encoding polynucleotide sequence, the amino acid sequence of a wild-type polypeptide can be determined. Subsequently, this amino acid sequence may be

modified to alter the protein's glycosylation pattern, by introducing additional glycosylation sequence(s) at various locations in the amino acid sequence.

[00233] A variety of mutation-generating protocols are established and described in the art. *See, e.g.,* Zhang et al., *Proc. Natl. Acad. Sci. USA*, 94: 4504-4509 (1997); and Stemmer, *Nature*, 370: 389-391 (1994). The procedures can be used separately or in combination to produce variants of a set of nucleic acids, and hence variants of encoded polypeptides. Kits for mutagenesis, library construction, and other diversity-generating methods are commercially available.

[00234] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Botstein and Shortle, *Science*, 229: 1193-1201 (1985)), mutagenesis using uracil-containing templates (Kunkel, *Proc. Natl. Acad. Sci. USA*, 82: 488-492 (1985)), oligonucleotide-directed mutagenesis (Zoller and Smith, *Nucl. Acids Res.*, 10: 6487-6500 (1982)), phosphorothioate-modified DNA mutagenesis (Taylor et al., *Nucl. Acids Res.*, 13: 8749-8764 and 8765-8787 (1985)), and mutagenesis using gapped duplex DNA (Kramer et al., *Nucl. Acids Res.*, 12: 9441-9456 (1984)).

[00235] Other methods for generating mutations include point mismatch repair (Kramer et al., *Cell*, 38: 879-887 (1984)), mutagenesis using repair-deficient host strains (Carter et al., *Nucl. Acids Res.*, 13: 4431-4443 (1985)), deletion mutagenesis (Eghtedarzadeh and Henikoff, *Nucl. Acids Res.*, 14: 5115 (1986)), restriction-selection and restriction-purification (Wells et al., *Phil. Trans. R. Soc. Lond. A*, 317: 415-423 (1986)), mutagenesis by total gene synthesis (Nambiar et al., *Science*, 223: 1299-1301 (1984)), double-strand break repair (Mandecki, *Proc. Natl. Acad. Sci. USA*, 83: 7177-7181 (1986)), mutagenesis by polynucleotide chain termination methods (U.S. Patent No. 5,965,408), and error-prone PCR (Leung et al., *Biotechniques*, 1: 11-15 (1989)).

d. Modification of Nucleic Acids for Preferred Codon Usage in a Host Organism

[00236] The polynucleotide sequence encoding a polypeptide variant can be further altered to coincide with the preferred codon usage of a particular host. For example, the preferred codon usage of one strain of bacterial cells can be used to derive a polynucleotide that encodes a polypeptide variant of the invention and includes the codons favored by this strain. The frequency of preferred codon usage exhibited by a host cell can be calculated by

averaging frequency of preferred codon usage in a large number of genes expressed by the host cell (e.g., calculation service is available from web site of the Kazusa DNA Research Institute, Japan). This analysis is preferably limited to genes that are highly expressed by the host cell. U.S. Patent No. 5,824,864, for example, provides the frequency of codon usage by highly expressed genes exhibited by dicotyledonous plants and monocotyledonous plants.

[00237] At the completion of modification, the polypeptide variant coding sequences are verified by sequencing and are then subcloned into an appropriate expression vector for recombinant production in the same manner as the wild-type polypeptides.

IX. Expression of Mutant Polypeptides

[00238] Following sequence verification, the polypeptide variant of the present invention can be produced using routine techniques in the field of recombinant genetics, relying on the polynucleotide sequences encoding the polypeptide disclosed herein.

a. Expression Systems

[00239] To obtain high-level expression of a nucleic acid encoding a mutant polypeptide of the present invention, one typically subclones a polynucleotide encoding the mutant polypeptide into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook and Russell, *supra*, and Ausubel *et al.*, *supra*. Bacterial expression systems for expressing the wild-type or mutant polypeptide are available in, e.g., *E. coli*, *Bacillus sp.*, *Salmonella*, and *Caulobacter*. Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[00240] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[00241] In addition to the promoter, the expression vector typically includes a transcription unit or expression cassette that contains all the additional elements required for the expression of the mutant polypeptide in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding the mutant polypeptide and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the polypeptide is typically linked to a cleavable signal peptide sequence to promote secretion of the polypeptide by the transformed cell. Such signal peptides include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[00242] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[00243] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322-based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

[00244] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[00245] In some exemplary embodiments the expression vector is chosen from pCWin1, pCWin2, pCWin2/MBP, pCWin2-MBP-SBD (pMS₃₉), and pCWin2-MBP-MCS-

SBD (pMXS₃₉) as disclosed in co-owned U.S. Patent application filed April 9, 2004 which is incorporated herein by reference.

[00246] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase.

5 Alternatively, high yield expression systems not involving gene amplification are also suitable, such as a baculovirus vector in insect cells, with a polynucleotide sequence encoding the mutant polypeptide under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[00247] The elements that are typically included in expression vectors also include a
10 replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable.

[00248] The prokaryotic sequences are optionally chosen such that they do not
15 interfere with the replication of the DNA in eukaryotic cells, if necessary.

[00249] When periplasmic expression of a recombinant protein (*e.g.*, a hgh mutant of the present invention) is desired, the expression vector further comprises a sequence encoding a secretion signal, such as the *E. coli* OppA (Periplasmic Oligopeptide Binding Protein) secretion signal or a modified version thereof, which is directly connected to 5' of the coding
20 sequence of the protein to be expressed. This signal sequence directs the recombinant protein produced in cytoplasm through the cell membrane into the periplasmic space. The expression vector may further comprise a coding sequence for signal peptidase 1, which is capable of enzymatically cleaving the signal sequence when the recombinant protein is entering the periplasmic space. More detailed description for periplasmic production of a recombinant
25 protein can be found in, *e.g.*, Gray *et al.*, *Gene* 39: 247-254 (1985), U.S. Patent Nos. 6,160,089 and 6,436,674.

[00250] As discussed above, a person skilled in the art will recognize that various conservative substitutions can be made to any wild-type or mutant polypeptide or its coding sequence while still retaining the biological activity of the polypeptide. Moreover,
30 modifications of a polynucleotide coding sequence may also be made to accommodate preferred codon usage in a particular expression host without altering the resulting amino acid sequence.

b. Transfection Methods

[00251] Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of the mutant polypeptide, which are then purified using standard techniques (*see, e.g., Colley et al., J. Biol. Chem.* 264: 17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g., Morrison, J. Bact.* 132: 349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101: 347-362 (Wu *et al.*, eds, 1983).

[00252] Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well-known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, or other foreign genetic material into a host cell (*see, e.g., Sambrook and Russell, supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the mutant polypeptide.

c. Detection of Expression of Mutant Polypeptides in Host Cells

[00253] After the expression vector is introduced into appropriate host cells, the transfected cells are cultured under conditions favoring expression of the mutant polypeptide. The cells are then screened for the expression of the recombinant polypeptide, which is subsequently recovered from the culture using standard techniques (*see, e.g., Scopes, Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra*; and Sambrook and Russell, *supra*).

[00254] Several general methods for screening gene expression are well known among those skilled in the art. First, gene expression can be detected at the nucleic acid level. A variety of methods of specific DNA and RNA measurement using nucleic acid hybridization techniques are commonly used (*e.g., Sambrook and Russell, supra*). Some methods involve an electrophoretic separation (*e.g., Southern blot for detecting DNA and Northern blot for detecting RNA*), but detection of DNA or RNA can be carried out without electrophoresis as well (such as by dot blot). The presence of nucleic acid encoding a mutant polypeptide in transfected cells can also be detected by PCR or RT-PCR using sequence-specific primers.

[00255] Second, gene expression can be detected at the polypeptide level. Various immunological assays are routinely used by those skilled in the art to measure the level of a gene product, particularly using polyclonal or monoclonal antibodies that react specifically with a mutant polypeptide of the present invention (*e.g.*, Harlow and Lane, *Antibodies, A Laboratory Manual*, Chapter 14, Cold Spring Harbor, 1988; Kohler and Milstein, *Nature*, 256: 495-497 (1975)). Such techniques require antibody preparation by selecting antibodies with high specificity against the mutant polypeptide or an antigenic portion thereof. The methods of raising polyclonal and monoclonal antibodies are well established and their descriptions can be found in the literature, *see, e.g.*, Harlow and Lane, *supra*; Kohler and Milstein, *Eur. J. Immunol.*, 6: 511-519 (1976). More detailed descriptions of preparing antibody against the mutant polypeptide of the present invention and conducting immunological assays detecting the mutant polypeptide are provided in a later section.

X. Purification of Recombinantly Produced Mutant Polypeptides

[00256] Once the expression of a recombinant mutant polypeptide in transfected host cells is confirmed, the host cells are then cultured in an appropriate scale for the purpose of purifying the recombinant polypeptide.

a. Purification from Bacteria

[00257] When the mutant polypeptides of the present invention are produced recombinantly by transformed bacteria in large amounts, typically after promoter induction, although expression can be constitutive, the proteins may form insoluble aggregates. There are several protocols that are suitable for purification of protein inclusion bodies. For example, purification of aggregate proteins (hereinafter referred to as inclusion bodies) typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.*, by incubation in a buffer of about 100-150 µg/ml lysozyme and 0.1% Nonidet P40, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, NY). Alternatively, the cells can be sonicated on ice. Alternate methods of lysing bacteria are described in Ausubel *et al.* and Sambrook and Russell, both *supra*, and will be apparent to those of skill in the art. For further description of purifying recombinant polypeptides from bacterial inclusion body, *see, e.g.*, Patra *et al.*, *Protein Expression and Purification* 18: 182-190 (2000).

[00258] The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

b. Immunoassays for Detection of Mutant Polypeptide Expression

5 [00259] To confirm the production of a recombinant mutant polypeptide, immunological assays may be useful to detect in a sample the expression of the polypeptide. Immunological assays are also useful for quantifying the expression level of the recombinant hormone. Antibodies against a mutant polypeptide are necessary for carrying out these immunological assays.

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c. Production of Antibodies against Mutant Polypeptides

[00260] Methods for producing polyclonal and monoclonal antibodies that react specifically with an immunogen of interest are known to those of skill in the art (*see, e.g.,* Coligan, *Current Protocols in Immunology* Wiley/Greene, NY, 1991; Harlow and Lane, 15 *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, 1989; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY, 1986; and Kohler and Milstein *Nature* **256**: 495-497, 1975). Such techniques include antibody preparation by selection of antibodies from libraries of 20 recombinant antibodies in phage or similar vectors (*see, Huse et al., Science* 246: 1275-1281, 1989; and Ward et al., *Nature* 341: 544-546, 1989).

[00261] In order to produce antisera containing antibodies with desired specificity, the polypeptide of interest (*e.g.,* a mutant polypeptide of the present invention) or an antigenic fragment thereof can be used to immunize suitable animals, *e.g.,* mice, rabbits, or primates. 25 A standard adjuvant, such as Freund's adjuvant, can be used in accordance with a standard immunization protocol. Alternatively, a synthetic antigenic peptide derived from that particular polypeptide can be conjugated to a carrier protein and subsequently used as an immunogen.

[00262] The animal's immune response to the immunogen preparation is monitored by 30 taking test bleeds and determining the titer of reactivity to the antigen of interest. When appropriately high titers of antibody to the antigen are obtained, blood is collected from the

animal and antisera are prepared. Further fractionation of the antisera to enrich antibodies specifically reactive to the antigen and purification of the antibodies can be performed subsequently, *see*, Harlow and Lane, *supra*, and the general descriptions of protein purification provided above.

5 **[00263]** Monoclonal antibodies are obtained using various techniques familiar to those of skill in the art. Typically, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see*, Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976). Alternative methods of immortalization include, *e.g.*, transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well
10 known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and the yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host.

[00264] Additionally, monoclonal antibodies may also be recombinantly produced
15 upon identification of nucleic acid sequences encoding an antibody with desired specificity or a binding fragment of such antibody by screening a human B cell cDNA library according to the general protocol outlined by Huse *et al.*, *supra*. The general principles and methods of recombinant polypeptide production discussed above are applicable for antibody production by recombinant methods.

20 **[00265]** When desired, antibodies capable of specifically recognizing a mutant polypeptide of the present invention can be tested for their cross-reactivity against the wild-type polypeptide and thus distinguished from the antibodies against the wild-type protein. For instance, antisera obtained from an animal immunized with a mutant polypeptide can be run through a column on which a wild-type polypeptide is immobilized. The portion of the
25 antisera that passes through the column recognizes only the mutant polypeptide and not the wild-type polypeptide. Similarly, monoclonal antibodies against a mutant polypeptide can also be screened for their exclusivity in recognizing only the mutant but not the wild-type polypeptide.

[00266] Polyclonal or monoclonal antibodies that specifically recognize only the
30 mutant polypeptide of the present invention but not the wild-type polypeptide are useful for isolating the mutant protein from the wild-type protein, for example, by incubating a sample

with a mutant peptide-specific polyclonal or monoclonal antibody immobilized on a solid support.

XI. Methods of Treatment and Diagnosis

5 **[00267]** In various embodiments, the invention provides a method of preventing, ameliorating or treating a disease state. In these embodiments, the invention provides a method that comprises administering to a subject in need thereof an amount of a polypeptide variant of the invention sufficient to prevent, ameliorate or treat the disease state. An exemplary disease state is cancer. The disclosed agonist variants can be useful for the
10 promotion of cell growth, particularly for angiogenesis, and the treatment of cardiovascular, hepatic, musculoskeletal and neuronal diseases. For example, certain polypeptide variants of the invention are useful in the prevention or treatment of hyperproliferative diseases or disorders, *e.g.*, various forms of cancer.

[00268] In an exemplary embodiment, the invention provides a method of treating
15 cancer in a subject in need of such treatment. The method includes administering to the subject a therapeutically effective amount of a polypeptide variant of the invention.

[00269] It is contemplated that the polypeptide variants of the invention can be used in the treatment of a variety of FGF responsive disorders, including, for example, various eye disorders, FGF responsive tumor cells in lung cancer, breast cancer, colon cancer, prostate
20 cancer, ovarian cancer, head and neck cancer, ovarian cancer, multiple myeloma, liver cancer, gastric cancer, esophageal cancer, kidney cancer, nasopharyngeal cancer, pancreatic cancer, mesothelioma, melanoma and glioblastoma.

[00270] In exemplary embodiments, the cancer is a carcinoma, *e.g.*, colorectal, squamous cell, hepatocellular, renal, breast or lung.

25 **[00271]** The polypeptide variants can be used to inhibit or reduce the proliferation of tumor cells. In such an approach, the tumor cells are exposed to a therapeutically effective amount of the polypeptide variant so as to inhibit or reduce proliferation of the tumor cell. In certain embodiments, the polypeptide variants inhibit tumor cell proliferation by at least 50%, 60%, 70%, 80%, 90%, 95% or 100%.

30 **[00272]** In certain embodiments, the polypeptide variant is used to inhibit or reduce proliferation of a tumor cell wherein the variant reduces the ability of FGF1 to bind to FGFR.

In certain embodiments, the FGF1 polypeptide variant is used to inhibit or promote wound healing.

[00273] In addition, the polypeptide variant can be used to inhibit, or slow down tumor growth or development in a mammal. In such a method, an effective amount of the polypeptide variant is administered to the mammal so as to inhibit or slow down tumor growth in the mammal. Accordingly, the polypeptide variants can be used to treat tumors, for example, in a mammal. The method comprises administering to the mammal a therapeutically effective amount of the polypeptide variant. The polypeptide variant can be administered alone or in combination with another pharmaceutically active molecule, so as to treat the tumor.

[00274] Generally, a therapeutically effective amount of polypeptide variant will be in the range of from about 0.1 mg/kg to about 100 mg/kg, optionally from about 1 mg/kg to about 100 mg/kg, optionally from about 1 mg/kg to 10 mg/kg. The amount administered will depend on variables such as the type and extent of disease or indication to be treated, the overall health status of the particular patient, the relative biological efficacy of the polypeptide variant delivered, the formulation of the polypeptide variant, the presence and types of excipients in the formulation, and the route of administration. The initial dosage administered may be increased beyond the upper level in order to rapidly achieve the desired blood-level or tissue level, or the initial dosage may be smaller than the optimum and the daily dosage may be progressively increased during the course of treatment depending on the particular situation. Human dosage can be optimized, *e.g.*, in a conventional Phase I dose escalation study designed to run from 0.5 mg/kg to 20 mg/kg. Dosing frequency can vary, depending on factors such as route of administration, dosage amount and the disease condition being treated. Exemplary dosing frequencies are once per day, once per week and once every two weeks. A preferred route of administration is parenteral, *e.g.*, intravenous infusion. Formulation of protein-based drugs is within ordinary skill in the art. In some embodiments of the invention, the polypeptide variant, *e.g.*, protein-based, is lyophilized and reconstituted in buffered saline at the time of administration.

[00275] The polypeptide variants may be administered either alone or in combination with other pharmaceutically active ingredients. The other active ingredients, *e.g.*, immunomodulators, can be administered together with the polypeptide variant, or can be administered before or after the polypeptide variant.

[00276] Formulations containing the polypeptide variants for therapeutic use, typically include the polypeptide variants combined with a pharmaceutically acceptable carrier. As used herein, “pharmaceutically acceptable carrier” means buffers, carriers, and excipients, that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The carrier(s) should be “acceptable” in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient. Pharmaceutically acceptable carriers, in this regard, are intended to include any and all buffers, solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is known in the art.

[00277] The formulations can be conveniently presented in a dosage unit form and can be prepared by any suitable method, including any of the methods well known in the pharmacy art. Remington’s Pharmaceutical Sciences, 18th ed. (Mack Publishing Company, 1990).

[00278] In exemplary embodiments, the polypeptide variants are used for diagnostic purposes, either *in vitro* or *in vivo*, the polypeptide variants typically are labeled either directly or indirectly with a detectable moiety. The detectable moiety can be any moiety which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, Cy5.5 (GE Healthcare), Alexa Fluro® dyes (Invitrogen), IRDye® infrared dyes (LI-COR® Biosciences), rhodamine, or luciferin; an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase; a spin probe, such as a spin label; or a colored particle, for example, a latex or gold particle. It is understood that the polypeptide variant can be conjugated to the detectable moiety using a number of approaches known in the art, for example, as described in Hunter et al. (1962) Nature 144: 945; David et al. (1974) Biochemistry 13: 1014; Pain et al. (1981) J. Immunol Meth 40: 219; and Nygren (1982) J. Histochem and Cytochem. 30: 407. The labels may be detected, *e.g.*, visually or with the aid of a spectrophotometer or other detector or other appropriate imaging system.

[00279] The polypeptide variants can be employed in a wide range of immunoassay techniques available in the art. Exemplary immunoassays include, for example, sandwich immunoassays, competitive immunoassays, immunohistochemical procedures.

[00280] In a sandwich immunoassay, two antibodies that bind an analyte or antigen of interest are used, *e.g.*, one immobilized onto a solid support, and one free in solution and labeled with a detectable moiety. When a sample containing the antigen is introduced into this system, the antigen binds to both the immobilized antibody and the labeled antibody, to form a “sandwich” immune complex on the surface of the support. The complexed protein is detected by washing away non-bound sample components and excess labeled antibody, and measuring the amount of labeled antibody complexed to protein on the support’s surface. Alternatively, the antibody free in solution can be detected by a third antibody labeled with a detectable moiety which binds the free antibody. A detailed review of immunological assay design, theory and protocols can be found in numerous texts, including Butt, ed., (1984) *Practical Immunology*, Marcel Dekker, New York; Harlow et al. eds. (1988) *Antibodies, A Laboratory Approach*, Cold Spring Harbor Laboratory; and Diamandis et al., eds. (1996) *Immunoassay*, Academic Press, Boston.

[00281] It is contemplated that the labeled polypeptide variants are useful as *in vivo* imaging agents, whereby the polypeptide variants can target the imaging agents to particular tissues of interest in the recipient. A remotely detectable moiety for *in vivo* imaging includes the radioactive atom ^{99m}Tc , a gamma emitter with a half-life of about six hours. Non-limiting examples of radionuclide diagnostic agents include, for example ^{110}In , ^{111}In , ^{177}Lu , ^{18}F , ^{52}Fe , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{86}Y , ^{90}Y , ^{89}Zr , ^{94m}Tc , ^{94}Tc , ^{99m}Tc , ^{120}I , ^{123}I , ^{124}I , ^{125}I , ^{131}I , $^{154-158}\text{Gd}$, ^{32}P , ^{11}C , ^{13}N , ^{15}O , ^{186}Re , ^{188}Re , ^{51}Mn , ^{52m}Mn , ^{55}Co , ^{72}As , ^{75}Br , ^{76}Br , ^{82m}Rb , ^{83}Sr , or other γ -, β -, or positron-emitters.

[00282] Non-radioactive moieties also useful in *in vivo* imaging include nitroxide spin labels as well as lanthanide and transition metal ions all of which induce proton relaxation *in situ*. In addition to imaging the complexed radioactive moieties may be used in standard radioimmunotherapy protocols to destroy the targeted cell.

[00283] A wide variety of fluorescent labels are known in the art, including but not limited to fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Chemiluminescent labels of use may

include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt or an oxalate ester.

[00284] The disclosed polypeptide variants may also be labeled with a fluorescent marker so as to allow detection in vivo. In some embodiments, the fluorescent label is Cy5.5 (GE Healthcare). In other embodiments, the fluorescent label is an Alexa Fluoro® dye (Invitrogen). In some embodiments, the fluorescent label is an IRDye® infrared dye (LI-COR® Biosciences).

[00285] Exemplary nucleotides for high dose radiotherapy include the radioactive atoms ^{90}Yt , ^{131}I and ^{111}In . The polypeptide variant can be labeled with ^{131}I , ^{111}In and $^{99\text{m}}\text{Tc}$ using coupling techniques known in the imaging arts. Similarly, procedures for preparing and administering the imaging agent as well as capturing and processing images are well known in the imaging art and so are not discussed in detail herein. Similarly, methods for performing antibody-based immunotherapies are well known in the art. See, for example, U.S. Pat. No. 5,534,254.

EXAMPLES

EXAMPLE 1: A HIGH-THROUGHPUT SCREENING METHOD FOR ENGINEERING PROTEOLYTICALLY STABLE GROWTH FACTORS

ABSTRACT

[00286] Growth factors are an important class of regulatory proteins which have great potential to be developed as therapeutic molecules for regenerative medicine and cancer treatment. However, the activity and efficacy of growth factors as therapeutic molecules are greatly limited by their poor thermal and proteolytic stability. While numerous methods have been developed to engineer growth factors with increased thermal stability, there has been a lack of focus and methods development for engineering growth factors with increased proteolytic stability. Proteases such as plasmin, elastase, uPA, cathepsins, and MMPs play critical roles in extracellular matrix degradation and signal transduction, particularly in wound healing and tumor formation. These proteases have been reported to commonly degrade growth factors as well. In this work, we describe a generalizable method for engineering growth factors for increased proteolytic stability. We utilize the yeast display platform and FACS screening as a combinatorial approach to selecting for mutants with

increased proteolytic stability. This method was validated by demonstrating the ability of the screen to differentiate between wild type FGF1 and a proteolytically stable FGF1 mutant reported in literature.

INTRODUCTION

5 **[00287]** This example describes a combinatorial approach to engineering proteolytically stable growth factors using the yeast display platform and flow-activated cell sorting (FACS) for screening. The process of setting up the screening method using FGF1 as a model example is demonstrated. The screen was set up for FGF1 because of its extremely poor thermal and proteolytic stability^{14,21}. Wild type growth factors with the poorest stability
10 have the greatest need for engineering stable versions for use in therapeutics. Thus, it was important for us to demonstrate the utility of the method for engineering growth factors by selecting a model growth factor that was poorly stable. In this example, the use of serum or several different proteases as the selective pressure for screening was explored. Finally, the ability of the screen to differentiate between FGF variants of different proteolytic stabilities
15 was validated. In Example 2, the capability of the combinatorial screen through the engineering and characterization of a proteolytically stable FGF1 mutant is exhibited.

RESULTS

[00288] Workflow of the combinatorial screening method for engineering proteolytically stable proteins

20 **[00289]** The yeast display platform, which is commonly used to engineer high affinity binders, is also utilized to engineer proteins with greater proteolytic stability (Figure 1). Several thousand copies of a single growth factor variant are displayed on the surface of yeast as tethered fusions. The hemagglutinin (HA) tag is expressed upstream of the growth factor while the c-myc tag is expressed downstream of the growth factor. Cells can be incubated
25 with soluble Fc fusions of the corresponding receptor, which can bind to the yeast displayed growth factor.

[00290] The yeast display platform is combined with flow-activated cell sorting (FACS) to engineer growth factors with higher proteolytic stability (Figure 2). A library of growth factor mutants is generated by random mutagenesis, directed mutagenesis, or DNA
30 shuffling. The library of yeast cells is incubated with a protease of interest, during which cleavage of the yeast surface displayed proteins occurs. Growth factor mutants with greater proteolytic stability are more resistant to cleavage on the yeast cell surface. After protease

incubation, the cells are washed and incubated with soluble Fc fusions of the functional receptor that bind to properly folded growth factor mutants with retained receptor binding affinity. FACS is used to sort for properly folded, uncleaved growth factor mutants, which are expanded and induced for the next round of sorting.

5 **[00291]** Fluorescent antibody markers against the Fc domain, the c-myc domain, and the HA tag are used to measure receptor binding, growth factor-specific cleavage, and non-specific cleavage (Table 2.1). Detection of the bound Fc-fusion receptor is important to ensure that mutations in the growth factor do not severely reduce the binding affinity for the receptor or lead to improper protein folding. Growth factor-specific cleavage is a direct
10 measure of a growth factor's proteolytic stability. It is detected by the c-myc signal, as a cleaved growth factor will have the C-terminal c-myc tag removed. Non-specific cleavage occurs when the protease cleaves within the yeast surface display proteins Aga1p and Aga2p. During non-specific cleavage, the fluorescent signals for all three markers are decreased. This is undesirable, as the dynamic range for detecting growth factor cleavage and binding activity
15 are decreased. Thus, the HA signal is used to ensure that non-specific cleavage by the protease of interest is minimal.

Yeast display of FGF1

[00292] FGF1 was chosen as a model for demonstrating the setup of the proteolytic stability screen. Wild-type FGF1 was cloned into the pCT vector, to be expressed on the
20 surface of *S. cerevisiae* yeast cells as a fusion to the Aga2p mating protein (Figure 3A). Successful expression of FGF1 on the yeast cell surface was confirmed by detection of the c-myc tag on the C-terminus of the protein (Figure 3B). Finally, we confirmed proper folding of yeast-displayed FGF by measuring specific binding activity to FGFR1-Fc (Figure 3C).

Selection of protease for engineering proteolytically stable FGF1

25 **[00293]** We tested the use of serum, trypsin, chymotrypsin, and plasmin for developing a proteolytic stability screen for FGF1. These proteases were selected, based on their scientific and biological relevance to FGF1. The suitability of the protease for the screen was determined by its ability to cleave the growth factor at a reasonable rate with minimal non-specific cleavage of the yeast display proteins.

30 **[00294]** We first attempted to develop the screen using serum, a natural blood product consisting of numerous proteases that might be encountered by growth factors in the body²²⁻²⁴. We incubated a library of FGF1 mutants with various concentrations of fetal bovine serum

(FBS) to see if we could observe FGF1 cleavage and a decrease in FGFR1-Fc binding signal (Figure 4). We found that even when the concentration of FBS was increased to 100%, we only observed a minimal decrease in the FGF1 cleavage signal (α -c-myc) and the FGFR1-Fc binding signal. Thus, we concluded that serum did not provide sufficiently stringent selective pressure to cleave yeast-displayed FGF1 mutants with low proteolytic stability.

[00295] Next, we tested the development of the screen using trypsin and chymotrypsin, two proteases that are commonly used to measure and report the proteolytic stability of proteins. We incubated yeast-displayed wild-type FGF1 with various concentrations of trypsin (Figure 5) and chymotrypsin (Figure 6), then measured the extent of protein cleavage (α -c-myc) and binding to FGFR1-Fc. We found that both trypsin and chymotrypsin had a concentration-dependent effect on the extent of observed protein cleavage and binding to FGFR1-Fc. We then determined whether the observed protein cleavage was due to non-specific cleavage (α -HA) or FGF1-specific cleavage (α -c-myc). We found that the HA signal was significantly decreased upon incubation with higher trypsin concentrations, indicating that much of the observed protein cleavage by trypsin was due to non-specific cleavage (Figure 7). Thus, we concluded that trypsin could not be used for a proteolytic stability screen. Meanwhile, we found that only the c-myc signal decreased while HA signal was relatively unaffected by incubation with higher chymotrypsin concentrations, indicating that the protein cleavage by chymotrypsin was primarily attributable to cleavage within FGF1 (Figure 8). Thus, we concluded that chymotrypsin was a reasonable candidate for use in the proteolytic stability screen.

[00296] Finally, we evaluated the development of the proteolytic stability screen using plasmin, a protease that degrades extracellular matrix proteins and that has been reported to degrade FGF1²⁵. We incubated yeast-displayed wild-type FGF1 with various concentrations of plasmin and found that yeast-displayed protein was cleaved in a concentration-dependent manner (Figure 9). To confirmed that the observed cleavage was FGF1-specific, rather than non-specific, we compared the cleavage of yeast-displayed FGF1 to an empty control expressing only the yeast display proteins, Aga1 and Aga2, as well as the HA and c-myc tags (Figure 10). During incubation with plasmin over the course of 96 hours, we found that yeast-displayed FGF1 was being cleaved while the empty control was not. Thus, we concluded that the observed cleavage was FGF1-specific.

[00297] Validation of screening method by differentiating between wild type FGF1 and a proteolytically stable FGF1 mutant

[00298] To test the ability of a plasmin-based screen to differentiate between FGFs of different proteolytic stabilities, we compared wild type (WT) FGF1 to a thermally stabilized FGF1 mutant (PM2) developed in literature by rational design¹⁴. PM2 was characterized by *Zakrzewska et al.* to be more stable in the presence of trypsin. We hypothesized that because plasmin shares primary sequence specificity with trypsin, PM2 would be more resistant to cleavage by plasmin. Thus, we expected that a functional proteolytic stability screening method using plasmin would enable us to observe less FGF1-specific cleavage in PM2 as compared to WT FGF1.

[00299] Yeast cells displaying PM2 or WT FGF1 were incubated with varying concentrations of plasmin for 48 hours and stained for non-specific cleavage (anti-HA) and FGF1-specific cleavage (anti-c-myc) (Figure 11). It was found that clean separation of the populations was obtainable by the difference in c-myc signal, with relatively little effect on the HA signal. This difference in cleavage signal confirmed that using plasmin would enable the screen to properly identify new FGF mutants with greater proteolytic stability, and to sort for these populations by FACS.

DISCUSSION

[00300] In this example, describe the development of a high-throughput, generalizable screening method for engineering proteolytically stable growth factors using the yeast display platform and flow-activated cell sorting is described. As an example, the setup of the screen for FGF1, a highly unstable growth factor is provided.

[00301] In establishing the screen for a growth factor of interest, the first step is to ensure that the growth factor can be expressed on the surface of yeast and that it is able to bind to a soluble version of its receptor. It was confirmed that FGF1 can be expressed in the pCT vector as a C-terminal fusion to the Aga2 yeast display protein, and that it binds specifically to FGFR1-Fc. In the past, VEGF, EGF, and HGF have successfully been expressed by yeast display^{6,26,27}. This suggests that the yeast-display-based proteolytic screening method can be applied more generally to other growth factors as well. If the growth factor cannot be expressed in the pCT vector, the pTMY vector could be used to successfully express the growth factor as a N-terminal fusion to Aga2 instead. In the case of HGF, it could not be expressed in pCT vector, but was successfully expressed in pTMY.

[00302] The second step was to determine the protease to be used for the proteolytic screen. We tested the use of serum, trypsin, chymotrypsin, and plasmin for engineering yeast-

displayed FGF1. We found that fetal bovine serum (FBS) provided too weak of a selective pressure even at high concentrations. Although proteases are found in FBS, protease inhibitors found in FBS such as α -1-antiproteinase and α -1-antichymotrypsin may cause their activity to be low²⁸. Given that FGF1 is a particularly unstable growth factor, it is likely that FBS would not be an appropriate selective proteolytic pressure for engineering other growth factors as well. However, other types of serum with different compositions such as newborn calf serum, adult bovine serum, or human serum could be considered. We tested the use of trypsin and chymotrypsin, which are proteases commonly used to measure proteolytic stability of proteins in literature. This is likely because trypsin and chymotrypsin have high activity and low specificity, which allow them to cleave almost any protein at a certain degradation rate²⁹. However, these properties may make them unattractive for use in a proteolytic stability screen. We found that for trypsin, much of the loss in expression (c-myc) signal was due to non-specific cleavage of the yeast display proteins, making trypsin a poor candidate for the proteolytic stability screening of any growth factor. While chymotrypsin did not seem to demonstrate a significant level of non-specific cleavage, it is important to note that the protease is primarily found in the digestive tract and unlikely to be biologically relevant to growth factors in the bloodstream. Finally, we tested the use of plasmin, a protease that is found in virtually all tissues and that has been shown to degrade FGF1^{13,25}. Plasmin has also been implicated in the degradation of other growth factors, such as VEGF³⁰. We found that plasmin was able to cleave yeast displayed FGF1 specifically, with relatively little non-specific cleavage of yeast display proteins. Based on the proteases that we were able to test, we concluded that plasmin would be the most appropriate protease to use as the selective pressure for the screen. Other proteases that are biologically relevant to growth factors, such as elastase, uPA, cathepsins, and MMPs may also be validated by testing for their high growth-factor-specific cleavage and low non-specific cleavage of yeast displayed proteins as described.

[00303] The final step in the setup of the screen is to determine whether growth factor mutants with different proteolytic stabilities can be differentiated. This optional step provides an important benchmark that provides confidence in the ability of the screen to select for proteolytically stable mutants. For FGF1, we confirmed that PM2, a FGF1 mutant with increased thermal and proteolytic stability, could be differentiated from wild-type FGF1 when displayed on the surface of yeast. In the absence of available proteolytically stabilized growth factor mutants, the screen could still be performed as long as the protease

demonstrates high growth-factor-specific cleavage and low non-specific cleavage of yeast displayed proteins. In Example 2, we report the engineering of FGF1 for proteolytic stability using the method we have developed.

MATERIALS AND METHODS

5 Cloning of yeast display constructs

[00304] FGF1 was cloned from human FGF1 cDNA (MGC Clone: 9218, IMAGE: 3896359, Residues: Phe16 to Asp155) into pCT vector (restriction sites: NheI, BamHI) for yeast display. For the proteolytically stable FGF1 mutant, PM2, the mutations Q40P (CAA to CCA), S47I (TCC to ATC), and H93G (CAT to GGT) were made to FGF1 using site-
10 directed mutagenesis.

Binding assay for yeast-displayed FGF1

[00305] 50,000 induced yeast cells were incubated with varying concentrations of human FGFR1 beta (IIIc)-Fc (R&D Systems) in phosphate-buffered saline with 1 g/L BSA (PBSA) at room temperature. Cells were incubated in sufficiently large volumes to avoid
15 ligand depletion and long enough times (typically 3 to 24 hours) to reach equilibrium. During the last 30 minutes of incubation, yeast cells were incubated with 1:2500 dilution of chicken anti-c-Myc (Invitrogen) in PBSA. Yeast were pelleted, washed, then incubated with 1:200 dilution of secondary antibodies on ice for 10 min: anti-Human IgG-FITC (Sigma Aldrich) and anti-chicken-IgY-PE (Santa Cruz Biotechnology) against anti-c-myc. Yeast were
20 washed, pelleted, and resuspended in PBSA immediately before analysis by flow cytometry using EMD Millipore Guava EasyCyte. Flow cytometry data were analyzed using FlowJo (v7.6.1). Binding curves were plotted and K_d values were obtained using GraphPad Prism 6.

Proteolytic stability assays for screening

[00306] Fetal bovine serum (Gibco), trypsin from bovine pancreas (Sigma Aldrich),
25 chymotrypsin type VII from bovine pancreas (Sigma Aldrich), or plasmin from human plasma (Sigma Aldrich) was used as the protease or protease mix for incubation. Fetal bovine serum was diluted in Dulbecco's Modified Eagle Medium (Gibco). Trypsin and chymotrypsin were diluted in trypsin buffer (100 mM Tris-HCl (pH 8), 1 mM CaCl_2 , 1% BSA). Plasmin was diluted in plasmin buffer (100 mM Tris-HCl, 0.01% BSA, pH 8.5).

30 **[00307]** 1 million induced yeast cells were incubated with various concentrations of protease in the appropriate buffers. At the end of incubation, cells were washed once with

PBSA (PBS + 0.1% BSA) and resuspended in buffer protease inhibitor cocktail (Sigma Aldrich) to quench residual protease activity. After 5 minutes, cells were washed once more with PBSA. For only experiments that measured FGFR binding activity, cells were incubated in 10 nM human FGFR1 beta (IIIc)-Fc (R&D Systems) in pBSA for 1 hour. After the final wash, cells were incubated with appropriate fluorescent antibodies.

[00308] For experiments measuring FGFR1 binding activity and c-myc signal, cells were incubated with 1:2000 dilution of chicken anti-c-Myc (Invitrogen) in PBSA for 30 minutes. After washing, cells were then incubated in secondary antibodies for 10 minutes on ice: anti-Human IgG-FITC (Sigma Aldrich) and anti-chicken-IgY-PE (Santa Cruz Biotechnology) against anti-c-myc.

[00309] For experiments measuring HA and c-myc signal, cells were incubated with 1:1000 dilution of anti-HA-Tag (6E2) Mouse mAb (Cell Signaling) and 1:2000 dilution of chicken anti-c-Myc (Invitrogen) for 30 minutes. After washing, cells were then incubated in secondary antibodies for 10 minutes on ice: goat anti-mouse-PE (Invitrogen) and goat anti-chicken-IgY-AlexaFluor488 (Santa Cruz Biotechnology).

[00310] Yeast were washed, pelleted, and resuspended in PBSA immediately before analysis by flow cytometry using EMD Millipore Guava EasyCyte. Flow cytometry data were analyzed using FlowJo (v7.6.1). Binding curves were plotted and K_d values were obtained using GraphPad Prism 6.

Table 3. Effect of different events on the observed signal from fluorescent antibody markers.

	HA	c-myc	Fc
Denaturation/loss of binding affinity			↓↓↓
Growth factor-specific cleavage		↓↓↓	↓↓↓
Non-specific cleavage	↓↓↓	↓↓↓	↓↓↓

EXAMPLE 2: ENGINEERING PROTEOLYTICALLY STABILIZED FIBROBLAST GROWTH FACTOR

ABSTRACT

[00311] FGF1 plays a significant role in cell differentiation and the induction of angiogenesis during wound healing, tissue regeneration, tumor formation, and other angiogenesis-dependent diseases. Thus, agonists and antagonists based on FGF1 can have important applications for cell culture and protein therapeutics. However, FGF1 have been reported to exhibit susceptibility to degradation when exposed to proteases in culture. Its poor proteolytic stability can hinder their activity and efficacy in cell culture or when developed as therapeutic molecules. In this example, FGF1 peptides were engineered for proteolytic stability using the yeast display-based screening method described in Example 1. Gating strategies for selection of proteolytically stable FGFs and successfully identify candidates for characterization were explored.

INTRODUCTION

[00312] Fibroblast growth factors (FGFs) are part of an important family of growth factors that regulate biological activities including embryonic development, cell differentiation, cell proliferation, cell migration, angiogenesis, metabolism, and wound healing^{1,31-35}. Thus, FGF-based therapeutics have been of interest for applications in cancer therapy, wound healing, tissue regeneration, and treatment of metabolic disorders^{32,36,37}. Of the many FGF family members, FGF1 is of particular interest as one of the most significant FGF ligands known to induce a pro-angiogenic phenotype in endothelial cells by signaling through FGFR1 and FGFR2³⁸.

[00313] FGF1 has been reported to protect functional vessels from regression, to induce arterial growth, and to promote capillary proliferation^{39,40}. It was found to induce tube formation in human umbilical vascular endothelial cells (HUVECs) and the formation of blood vessels in Matrigel plug assays⁴¹.

[00314] Despite the potency of FGF1 in the induction of angiogenesis for wound healing and tissue regeneration, efforts to utilize FGF1 as a therapeutic agent have been largely unsuccessful. Gene therapy in the form of an injectable intramuscular plasmid encoding FGF1 was shown in Phase I and II clinical studies to improve perfusion and reduce the need for amputation in patients with end-stage lower-extremity ischemia^{42,43}. However, it failed to show clinical efficacy in Phase III clinical studies for the reduction of amputation or mortality in patients with critical limb ischemia⁴⁴. CardioVascular BioTherapeutics has also developed a recombinant wild-type FGF1 (CVBT-141) for the treatment of ulcers, coronary

heart disease, and peripheral arterial disease, but clinical trials have remained unsuccessful for almost two decades⁴⁵.

[00315] The failure of recombinant FGF1 to be effective in many clinical applications likely stems, in part, from its poor stability. Wild type FGF1 is rapidly degraded upon incubation at 37°C in conditioned media or in culture, with a degradation half-life of approximately 25 minutes^{14,21}. It has specifically been shown that plasmin, a key protease found in areas of wound healing, can degrade both FGF1 and FGF2^{25,46,47}.

[00316] This example describes the engineering of FGF1 for improved proteolytic stability against plasmin using the developed screening method described in Example 1.

FGF1 was engineered using the yeast surface display platform to establish a gene-to-protein linkage. Random mutagenesis libraries for each growth factor were screened for FGFR1 binders, then mutants that remained uncleaved after incubation with protease, and finally, mutants that remained uncleaved and retained FGFR1 binding after incubation with protease. Several promising mutations were identified that appeared to increase proteolytic stability for each growth factor, and generated candidates for characterization as described in Example 3.

RESULTS

Yeast display of wild type FGF and generation of random mutagenesis library

[00317] Successful yeast display of properly folded wild-type FGF1 is described in below. Error prone PCR was used with nucleotide analogues to randomly generate mutations within wild-type FGF1. We generated a library with 3.3×10^7 mutants. By varying the concentration of nucleotide analogues, it was possible to generate an average of 3.1 mutations per mutant (2.1% mutation rate), which was hypothesized to be a diversity that is high enough to generate mutants with improved proteolytic and low enough to avoid accumulating mutations that would impair proper protein folding or binding affinity for the FGFR1 receptor.

Sort 1: Selection for binders to FGFR1-Fc

[00318] For the first sort, FGF1 mutants were sorted for that retained binding affinity for FGFR1-Fc (Figure 12A). It was hypothesized that most random mutations would lead to a loss of binding affinity. After incubation with FGFR1-Fc, we gated for and collected cells that showed high expression (α -c-myc) and high binding signal (α -FGFR1-Fc). For the FGF1

library, a clear separation between mutants that were non-binders and those that retained FGFR binding affinity was observed (Figure 12B).

Sort 2: Selection for resistance to FGF1-specific cleavage

[00319] For the second sort, the cells from Sort 1 were expanded and sorted for FGF1 mutants that remained resistant to cleavage when incubated with plasmin (Figure 13A). To obtain an effective dynamic range and differentiate between mutants with different proteolytic stabilities, the incubation of the cells was tested with varying concentrations and durations of incubation. It was found that for the FGF1 library, incubation with 400 nM plasmin for 36 hours was necessary to achieve a clear separation between the populations of cleaved and uncleaved mutants (Figure 13B). The top 1-2% of cells exhibiting the highest level of protease resistance were collected (high α -c-myc) normalized by the expression level (α -HA).

Isolation of peptide artifacts during screening.

[00320] The second sort of the FGF1 library was expanded and subjected to another round of selection for resistance to FGF1-specific cleavage using HA and c-myc signals. Upon incubation with 200 nM plasmin for 36-hour incubation, it was clearly observed that a population of cells that showed much higher c-myc signal as compared to the rest of the library, indicating significantly greater resistance of yeast displayed protein to cleavage by plasmin (Figure 14A). This population was collected and sequenced individual clones for analysis (Figure 14B). It was found that the majority of cells did not express FGF2 mutants on their cell surface, but short peptide artifacts that may have been generated during random mutagenesis instead.

[00321] It was confirmed that these peptides did not exhibit any specific binding to FGFR1-Fc, indicating that sorting for resistance to FGF1-specific cleavage led to a rapid enrichment of a very small population of cells expressing these peptide artifacts (Figure 15). Thus, for all subsequent sorts, we proceeded to include a selective pressure for retaining FGFR1 binding affinity.

Sorts 3-4: Selection for protease-resistant, FGFR1-Fc binders

[00322] For the remaining sorts 3 and 4, the libraries were incubated with plasmin and, subsequently, FGFR1-Fc before selection (Figure 16). Different combinations of α -HA, α -c-myc, and α -FGFR1-Fc were used to select for FGF1 mutants that remained uncleaved and retained binding affinity for FGFR1

[00323] For the third sort of FGF1, we expanded the cells from Sort 2 and sorted for FGF mutants that retained FGFR1 binding after incubation with plasmin (Figure 17). 12-hour incubations with higher concentrations of plasmin were performed. 1.5 μ M plasmin was ultimately used for sorting the FGF1 library. We gated for and collected cells that showed high binding signal (α -FGFR1-Fc) and high expression (α -HA).

[00324] For the fourth sort of FGF1, we expanded the cells from Sort 3 and increased the time of plasmin incubation from 12 hours to 36 hours. The cells from Sort 3 were incubated in either 1.5 μ M plasmin or 500 nM plasmin. We ultimately sorted for cells incubated with 500 nM plasmin (Figure 18). We gated for and collected cells that showed high resistance to cleavage (α -c-myc) and high binding signal (α -FGFR1-Fc). By Sort 4, a completed consensus was reached for the FGF1 library and seized to perform additional rounds of sorting.

Sequence analysis of sorted FGF1 mutants

[00325] For the final sort 4, individual clones were randomly selected and sequence for analysis. We were able to reach complete consensus within four rounds of sorting. The mutant (BS4M1) contains two mutations: D28N and L131R (Figure 19). Aspartic acid 28 is part of the first (LPDG) of three key β -hairpins that close off the six-stranded β -barrel structure of FGF1. Leucine 131 is found within a β -strand pair between the N-terminus and C-terminus of FGF1.

DISCUSSION

[00326] In this example, the engineering of FGF1 for proteolytic stability against plasmin was described. As the first step, we were able to successfully express the wild-type FGF1 and wild-type FGF2. The proteins were shown to be successfully expressed and properly folded by detection of the c-myc tag testing for specific binding against FGFR1-Fc or sFGFR3-D2D3-Fc. It was observed that FGF1 exhibits a relatively high expression signal, which is interesting given that FGF1 is considered to be unstable with short half-life and a low melting temperature²¹. It is reported that yeast display expression and secretion efficiency is loosely correlated with protein stability for poorly stable proteins, but this is not always the case^{48,49}. Thus, this example demonstrated that yeast display was able to accommodate the expression of unstable wild-type growth factors for engineering.

[00327] The high expression of FGF1 by yeast display led to a good dynamic range of signals during the sorting of the FGF1 random mutagenesis library and subsequent sorts. The

FGF1 sorts could be subjected to high concentrations of plasmin and long incubation times for increasing stringency. This was particularly valuable in Sort 2, in which a clear separation between cleaved FGF1 mutants and non-cleaved FGF1 mutants was achieved.

[00328] Although an initial round of sorting was done to select for FGFR binders in Sort 1, we found that only using the HA and c-myc signals for measuring cleavage led to a rapid enrichment of non-FGFR-binding peptides in the sorts. In just two such sorts, a clear separation of the peptide-expressing population from the rest of the library was identified. Although the libraries were constructed by random mutagenesis with a wild type FGF as the scaffold, rare errors in the random mutagenesis process probably led to an extremely small population of cells expressing peptide artifacts. While these artifacts would be of little consequence for more traditional yeast display screens for affinity maturation, they became rapidly significant without a selective pressure for receptor binding. Thus, it was concluded that selective pressure for measuring binding affinity is essential, and that no more than one sort should be done by selecting mutants based on cleavage activity alone.

[00329] Through the screening process, we identified several enriched mutations that could be significant for improving proteolytic stability. Interestingly, the mutations are found in the β -loop region or near the C-terminus of the protein, which are implicated to be key regions for determining protein stability. For the FGF1 library, complete consensus within four rounds of sorting was reached. The FGF1 BS4M1 mutant contains two mutations: D28N and L131R. Aspartic acid 28 is part of the first (LPDG) of three key β -hairpins that close off the six-stranded β -barrel structure of FGF1. The importance of the Asx-Pro-Asx-Gly motifs in its contribution to the stability of FGF1 has previously been studied, and substituting Asx residues with alanines has been shown to greatly de-stabilized FGF1⁵⁰. However, it was shown that a substitution of D28N actually increases its Gibbs free energy by ~ 2.5 kJ/mol, suggesting that proteolytic stability may not always correlate with thermostability. Leucine 131 is found within a β -strand pair between the N-terminus and C-terminus of FGF1. Because there is no β -hairpin to stabilize the β -barrel structure adjacent to this β -strand pair, it has been hypothesized that the amino acids in this pair are important for stabilizing the barrel either by bonding strength between the two strands or by making it sterically favorable for the main chain to be positioned in a manner that closes the β -barrel structure. Indeed, the mutation of proline 134 to cysteine, threonine, or valine has been shown to increase stability of FGF1 by -6 to -8 kJ/mol⁵¹.

[00330] In conclusion, it was shown that the screen for proteolytic stability was able to successfully enrich for mutations in positions that are reported to be important for FGF1 protein stability in the literature. In Example 3, we characterize the mutations identified in the final FGF1 BS4M1 mutant for their effects on the stability of solubly expressed FGF1 and the ligand's ability to modulate the FGF pathway.

MATERIALS & METHODS

Yeast surface display of proteins

[00331] YPD medium consists of 20 g/L dextrose, 20 g/L peptone and 10 g/L yeast extract. Selective SD-CAA medium consists of 20 g/L dextrose, 6.7 g/L yeast nitrogenous base without amino acids (Difco), 5 g/L casamino acids (Bacto), 5.4 g/L Na₂HPO₄, and 8.56 g/L NaH₂PO₄·H₂O. SD-CAA plates contain the same components as the media, with the addition of 182 g/L sorbitol and 15 g/L of agar. SG-CAA induction medium is identical to SD-CAA but contains 20 g/L galactose instead of dextrose. Yeast were grown and induced at 30 °C with shaking at 235 rpm.

[00332] The pCT yeast display plasmids were transformed into *Saccharomyces cerevisiae* strain EBY100 by electroporation and recovered in YPD at 30 °C for 1 hr before plating on SD-CAA plates. After 3 days, yeast colonies were inoculated overnight in SD-CAA. Expression and yeast display of proteins were induced in SG-CAA at 30 °C for 24 hours according to established protocols⁵².

Library creation

[00333] FGF1 was cloned from human FGF1 cDNA (MGC Clone: 9218, IMAGE: 3896359, Residues: Phe16 to Asp155) into pCT vector (restriction sites: NheI, BamHI) for yeast display. The FGF1 random mutagenesis library was generated using error-prone PCR as described previously^{52,53}. FGF1 was used as the template, and mutations were introduced using Taq polymerase (New England Biolabs) and nucleotide analogs 8-oxo-dGTP and dPTP (TriLink Biotech). Primers that contained 50 bp overlaps with the pCT plasmid in the forward and reverse direction were used to enable the insertion of the mutant genes into the pCT vector through yeast homologous recombination. To obtain clones with a range of mutation frequencies, six PCRs were performed with varying concentrations of nucleotide analogs (40 μM, 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM) over 20 PCR cycles. PCR products were amplified in the absence of nucleotide analogs and purified using gel electrophoresis. The pCT plasmid was digested as the vector with NheI and BamHI. Eight transformations of

5 μ g purified DNA insert and 1 μ g restriction enzyme digested pCT were electroporated into electrocompetent EBY100 yeast cells. The transformed yeast cells were recovered in YPD at 30 °C for 1 hr, then grown in selective SD-CAA medium. Clones from each PCR were sampled to determine the mutagenic frequency. Clones from the 5 μ M and 2.5 μ M PCRs
5 were combined to create the final library with an average of 3 mutations per clone. After two passages, the cells were transferred to SG-CAA to induce protein expression. A library size of 2×10^7 transformants was obtained as estimated by dilution plating.

Library screening

[00334] Induced EBY100 yeast cells displaying FGF1 mutants were incubated with plasmin in plasmin digest buffer (100 mM Tris-HCl, 0.01% BSA, pH 8.5) at 37°C and/or FGFR1-Fc in PBS + 0.1% BSA (PBSA) at room temperature as described for each sort. After protease digestion steps, cells were washed with PBSA, incubated with 1:100 dilution of protease inhibitor cocktail (Sigma) in PBSA for 5 minutes, then washed again with PBSA. After FGFR incubation steps, cells were washed with PBSA. The number of yeast cells
15 incubated for each sort was ~10x the number of cells collected in the previous sort. Cells were incubated in volumes at a density of 2 million cells per mL. After all incubation steps, cells were stained with primary and secondary antibodies. For primary staining, cells were appropriately incubated with 1:1000 dilution of anti-HA-Tag (6E2) Mouse mAb (Cell Signaling) and/or 1:2000 dilution of chicken anti-c-Myc (Invitrogen) for 30 minutes. Cells
20 were washed with PBSA after primary staining. Secondary staining was done on ice for 10 minutes. For secondary staining, the following antibodies were used for each sort: Sort 1, 3, 4 - anti-chicken-IgY-PE (Santa Cruz Biotechnology) against anti-c-myc and anti-Human IgG-FITC (Sigma Aldrich) against FGFR1-Fc; Sort 2 - goat anti-mouse-PE (Invitrogen) and goat anti-chicken-IgY-AlexaFluor488 (Santa Cruz Biotechnology).

[00335] Labeled yeast cells were sorted by fluorescence activated cell sorting (FACS) using the BD FACS Aria II (Stanford Shared FACS Facility). In each sort, 0.5 to 10% of yeast cells were collected based on the criteria set for each sort. The cells collected in each sort were grown in SD-CAA (pH 5 to limit bacterial contamination) for several days until an OD of 5 to 8 was reach. Clones were induced for yeast display expression in SG-CAA for 24
30 hours at 30°C prior to the next round of sorting.

[00336] For sequencing and cloning, plasmid DNA was extracted from yeast cells using a Zymoprep Yeast Plasmid Miniprep I Kit (Zymo Research). The extracted DNA was

transformed into DH10B electrocompetent cells and plated. Single colonies were selected and grown in LB media (Fisher Scientific). Plasmid DNA was isolated from the single colony cultures using a plasmid miniprep kit (GeneJet). DNA sequencing was performed by MCLAB.

5 Binding affinity assays for yeast-displayed peptides RTTTS and HTTS

[00337] 50,000 induced yeast cells were incubated with various concentrations of FGFR1-Fc in phosphate-buffered saline with 1 g/L BSA (PBSA) at room temperature. Cells were incubated in sufficiently large volumes to avoid ligand depletion and long enough times (typically 3 to 24 hours) to reach equilibrium. During the last 30 minutes of incubation, yeast
10 cells were incubated with 1:2500 dilution of chicken anti-c-Myc (Invitrogen) in PBSA. Yeast were pelleted, washed, then incubated with 1:200 dilution of secondary antibodies on ice for 10 min: anti-Human IgG-FITC (Sigma Aldrich) against FGFR1-Fc and anti-chicken-IgY-PE (Santa Cruz Biotechnology) against anti-c-myc. Yeast were washed, pelleted, and resuspended in PBSA immediately before analysis by flow cytometry using EMD Millipore
15 Guava EasyCyte. Flow cytometry data were analyzed using FlowJo (v7.6.1). Binding curves were plotted and K_d values were obtained using GraphPad Prism 6.

**EXAMPLE 3: CHARACTERIZATION OF PROTEOLYTICALLY STABILIZED
FIBROBLAST GROWTH FACTORS**

20 ABSTRACT

[00338] Proteolytic stability can play an important role in improving the efficacy of unstable growth factors, such as FGF1. Studies have shown that FGF1 is rapidly degraded in culture, partially due to proteases that are found in serum or that are expressed by cells. In Example 2, the engineering of FGF1 for increased proteolytic stability is described. We
25 screened FGF1 random mutagenesis libraries for FGF1 mutants that exhibited enhanced proteolytic stability on the surface yeast. In this example, the recombinant expression of soluble FGF1 and the characterization of the mutations identified by the high-throughput screen to improve proteolytic stability in FGF1 are described. FGF1 and FGF2 were recombinantly express and purified in *E. coli* expression systems. It was confirmed that the
30 FGF1 BS4M1 (D28N, L131R) and L131R mutants are more proteolytically stable as

compared to wild-type FGF1, and that the FGF1 L131R mutant acts as a potent FGF pathway antagonist.

INTRODUCTION

[00339] FGF1 is a potent regulatory molecule for the induction of angiogenesis, but its poor stability limits its ability to sustain protein activity and achieve prolonged efficacy. In Example 2, the use of a high-throughput screen to select for FGF1 mutants that demonstrate increased proteolytic stability upon incubation with plasmin, an important protease found in areas of disease for ECM degradation is described. Complete consensus was achieved after four sorts of the FGF1 library and identified the FGF1 BS4M1 (D28N, L131R) mutant. The mutations were found in areas of the protein that have been reported to be important for the stability of FGFs.

[00340] In this example, it is described that the soluble expression and characterization of the mutant FGFs derived from the FGF1 BS4M1 mutant identified in the screen. The wild type and FGF1 BS4M1 mutant were cloned from the yeast display vector and inserted into *E. coli* expression vectors. After purification, the proper folding of recombinant FGF1 was tested for by detection of specific binding to a yeast-displayed FGFR3 construct.

[00341] For the FGF1 BS4M1 mutant and the wild type FGF1, their soluble stability and their ability to modulate the FGF pathway were characterized further. The proteolytic stability of the proteins in plasmin or trypsin was tested, and their extent of degradation at different time points was measured using Western blot and band intensity quantification. We probed into the significance of the mutations D28N and L131R and their contributions to protein stability. The thermal stability of FGF1's were measured to analyze their relationship to proteolytic stability. To test the stability of FGF1's in more biologically relevant conditions, their extent of degradation in MDA-MB-231 breast cancer cell culture was characterized. In addition, ERK phosphorylation assays in NIH3T3 cells to characterize the ability to modulate the activation of signaling molecules that are downstream of FGFR activation such as ERK were performed. The results from these characterization studies demonstrated the improved proteolytic stability and antagonistic activity of engineered FGF1 mutants, and their potential to be used for anti-angiogenesis therapy.

RESULTS

Recombinant expression of FGFs

[00342] In order to express the engineered FGF1 mutants in their soluble and compare them to the wild type protein, the proteins were recombinantly expressed in *E. coli* expression systems. FGF1 and FGF1 mutants were cloned into the pBAD vector for intracellular expression of recombinant proteins. The pBAD FGF1 expression vectors were transformed into the *E. coli* strain Rosetta that enhances the expression of eukaryotic proteins with codons rarely used in *E. coli*. Cells were lysed using a detergent-based solution. Proteins were then purified using Ni-NTA column chromatography and size exclusion chromatography. The identity and purity of wild-type FGF1 was confirmed by Coomassie-stained protein gel and Western blot (Figure 20A). Proper folding of FGF1 was confirmed by observation of specific binding to a yeast-displayed FGFR3 construct (Figure 20B).

Proteolytic stability of FGF1 mutant BS4M1 in plasmin

[00343] To measure the proteolytic stability of wild type FGF1 and the FGF1 mutant BS4M1 (D28N/L131R), 100 ng of soluble FGFs was incubated in plasmin for various incubation times. Then, their degradation rate was evaluated by running the samples on a Western blot and staining for anti-FGF. The amount of remaining FGF was calculated by measuring the band intensity for each condition and normalizing by the band intensity of protein without plasmin incubation. It was found that the BS4M1 (D28N, L131R) mutant exhibited lower levels of degradation at all incubation time points in plasmin, as compared to wild type FGF1 (Figure 21). Thus, it was confirmed that the screen for increasing the proteolytic stability of FGF1 against plasmin was successful.

[00344] The mutations from BS4M1 (D28N and L131R) were incorporated into the stabilized PM2 (Q40P, S47I, H93G) mutant to create PM3 (D28N, Q40P, S47I, H93G, L131R). We measured the degradation of each construct at different plasmin concentrations after a 48-hour incubation. It was found that introducing the mutations from BS4M1 to the mutations from PM2 led to a marked increase in the resistance to proteolytic degradation at all tested concentrations (Figure 22). Thus, it was concluded that the newly identified mutations in BS4M1 had an additive effect on proteolytic stability when combined with the mutations from PM2.

Proteolytic stability of engineered FGF mutants in trypsin

[00345] The proteolytic stability of wild type FGF1 and FGF1 mutant BS4M1 were measured in trypsin in a similar manner. It was hypothesized that engineering for proteolytic stability against plasmin could increase proteolytic stability in trypsin because plasmin and

trypsin share the same primary specificity of lysine and arginine. In addition, it was confirmed in that the FGF1 mutant PM2 (Q40P, S47I, H93G), which is more resistant to degradation by trypsin, is also more resistant to cleavage by plasmin. It found that the BS4M1 (D28N, L131R) mutant exhibited lower levels of degradation at all incubation time points in trypsin, as compared to wild type FGF1 (Figure 23). Thus, it was concluded that engineering for proteolytic stability of FGF1 in plasmin was successful in increasing proteolytic stability in trypsin as well.

Significance of mutations in FGF1 mutant BS4M1

[00346] In order to determine whether both the D28N and L131R mutations were important for conferring proteolytic stability to the BS4M1 mutant, versions of FGF1 with only the D28N or L131R mutation were created. The proteolytic stability of these mutants by evaluating their degradation rate in plasmin over time was measured. It was found that the L131R mutant exhibited comparable proteolytic stability as compared to the BS4M1 (D28N/L131R) mutant, but that the D28N mutant exhibited much lower proteolytic stability even as compared to the wild type FGF1 (Figure 24). It was concluded that the D28N mutation did not translate to significantly increasing the proteolytic stability of FGF1 when incorporated into the solubly expressed protein. Thus, further characterizations with the L131R mutant were continued.

[00347] We also wanted to determine whether the mutation at position 131 to arginine was unique for conferring proteolytic stability, or if the mutation away from leucine was significant. Thus, position 131 was alternatively mutated to either alanine (L131A) or lysine (L131K) to see if these single mutants maintained or lost their enhancement in proteolytic stability. Their degradation rates were evaluated in plasmin; it was found that the L131K maintained similar levels of degradation as compared to L131R, while L131A exhibited higher levels of degradation even as compared to the wild type FGF1 (Figure 25).

Thermal stability of wild type FGF1 and FGF1 L131R mutant

[00348] In order to determine whether the improvement in proteolytic stability of the FGF1 L131R mutant is attributable to an improvement in thermal stability, we measured the melting temperature of the wild type FGF1 and the FGF L131R mutant. We used the ThermoFluor assay with a hydrophobic dye to measure the unfolding of each protein as the temperature is gradually increased^{54,55}. It was found that while the L131R mutation leads to a slight increase in the melting temperature as compared to the wild type FGF1, the difference

is not statistically significant (Figure 26). Thus, it was concluded that the thermal stability does not contribute significantly to the increase in proteolytic stability for the FGF1 L131R mutant.

Stability of FGF1 L131R mutant in cell culture

5 **[00349]** The stability of the FGF1 L131R mutant was tested in culture with MDA-MB-231, a breast cancer cell line that expresses urokinase plasminogen activator (uPA) to activate plasminogen and convert it into plasmin. 500 ng of protein was incubated for various incubation times with MDA-MB-231 in culture. All of the protein for each condition was concentrated and loaded each condition into a separate well for analysis by Western blot. The
10 amount of protein left was quantified by measuring the band intensity and normalizing by 500 ng of protein that was not incubated in culture. It was found that the FGF1 L131R mutant exhibited increased stability in culture as compared to the wild type protein (Figure 27).

FGF1 L131R mutant is an FGFR antagonist

15 **[00350]** To characterize the ability of FGF1 L131R to modulate the FGF pathway, we evaluated its ability to modulate phosphorylation of ERK (MAPK), a key signaling molecule that is downstream of FGFR activation and is important for induction of cell proliferation^{56,57}. NIH3T3 cells, which express FGFRs, were incubated with wild type FGF1 alone, the FGF1 L131R mutant alone, or wild type FGF1 with various concentrations of the FGF1 L131R mutant. It was found that while the FGF1 L131R mutant is unable to induce ERK
20 phosphorylation, the mutant can effectively inhibit ERK phosphorylation by wild-type FGF1 (Figure 28). For 1 nM wild type FGF1, we generated a dose-response curve for the inhibition of ERK phosphorylation by the FGF1 L131R mutant and found that its IC₅₀ (1 nM) is equimolar to the concentration of wild type FGF1 (Figure 29).

Binding of FGF1 L131R mutant to NIH3T3 cells

25 **[00351]** The binding affinity of the FGF1 L131R mutant was characterized and compared to that of wild-type FGF1. NIH3T3 cells which express FGFRs were incubated with varying concentrations of FGF1 at 4°C to prevent incubation. The cells were labeled with a fluorescently tagged anti-His antibody to detect bound His-tagged FGF1. It was found that both FGF1 WT and the FGF1 L131R mutant exhibit a binding affinity of 10 nM for
30 NIH3T3 cells (Figure 30).

DISCUSSION

[00352] In this example, we solubly expressed and characterized the FGF mutants that were identified in the screen for proteolytic stability in plasmin from Example 2. Upon recombinant expression, it was found that FGF1 was expressed and purified easily. However, it was found that the yield of the expressions was fairly low at 1-3 mg/L of expression. This low protein yield may be due to the poor stability of FGF1.

[00353] The soluble FGF1 BS4M1 (D28N, L131R) mutant was successfully confirmed to exhibit increased proteolytic stability in plasmin as compared to wild-type FGF1. When the mutations D28N and L131R were combined with the mutations from the stabilized FGF1 PM2 mutant (Q40P, S47I, H93G) from *Zakrzewska et al.*¹⁴, it was found that new mutations further enhanced the proteolytic stability of FGF1 in plasmin. In addition, the BS4M1 mutant was found to be more stable in trypsin, a protease that cleaves after lysine and arginine in a manner similar to plasmin⁵⁸. This demonstrates the ability of the screen to increase the protein's proteolytic stability in the presence of other proteases that share primary specificity with the protease used for selection. For example, the BS4M1 mutant may also be more proteolytically stable in the presence of cathepsins, which are responsible for lysosomal degradation and share primary specificity with plasmin.

[00354] Through characterization of the FGF1 D28N and L131R single mutants, it was found that most of the increased proteolytic stability was attributable to L131R, while the D28N single mutant was even less proteolytically stable than the wild-type. The difference in the significance of the D28N mutation between the yeast-displayed FGF1 and the soluble *E. coli*-derived FGF1 may be attributable to glycosylation which only occurs in the yeast displayed protein. Mutation of the aspartic acid to asparagine leads to the introduction of an NGx glycosylation site for eukaryotes⁵⁹. Thus, FGF1 BS4M1 mutant that is expressed in yeast or mammalian cells may have additional proteolytic stability.

[00355] The L131R mutation is a counter-intuitive one, as plasmin has primary specificity for arginine. Indeed, no rational design strategy would involve introducing new potential cleavage sites to the protein. However, as discussed in Example 1, primary specificity is not the only determinant of whether protein cleavage occurs at a potential cleavage site; multiple amino acids around the site and the steric accessibility of the site to the protease also contribute greatly. To probe further into whether the mutation away from leucine or the mutation to arginine at position 131 is important for increasing proteolytic stability, FGF1 L131A and L131K single mutants were characterized. It was found that changing leucine 131 to an alanine, an amino acid commonly used for substitution of a

protease cleavage site by rational design, led to a decrease in proteolytic stability even as compared to the wild-type FGF1. However, mutation of leucine 131 to a lysine, the other amino acid that plasmin has primary specificity for, led to a retention in the increased proteolytic stability in plasmin. While it was considered whether cleavage of the FGF1 protein at position 131 by plasmin leads to an increased proteolytic stability of the resulting protein fragment, it was concluded that the screen could not have selected for such a mutation. Any cleavage within the yeast displayed FGF1 would have led to a loss of c-myc signal and selection away from this mutant. Because lysine and arginine are both positively charged amino acids, it may instead be possible that the addition of a positive charge to this position is important for increasing the proteolytic stability of FGF1. We found that there was no statistically significant difference in melting temperature between wild-type FGF1 and the FGF1 L131R mutant, suggesting that an increase in thermal stability does not explain the increase in proteolytic stability. Thus, further studies would be required to definitively find the mechanism of L131R for increasing proteolytic stability.

[00356] It was also found that the FGF1 L131R mutant appears to be more stable than wild-type FGF1 in cell culture with MDA-MB-231 breast cancer cells. These cells express urokinase plasminogen activator (uPA), which cleaves and activates plasminogen into plasmin. That result was significant for demonstrating that the FGF1 L131R mutant exhibits increased stability in a more biologically relevant context.

[00357] Finally, using the ERK phosphorylation assay in NIH3T3 cells, it was found that the L131R mutation turns FGF1 into an FGF pathway antagonist. This result is interesting while reasonable, given that the screen for increasing proteolytic stability only selects for mutants that bind to FGFR but does not select for whether the protein acts as an agonist or an antagonist. The binding affinity of the FGF1 L131R mutant to NIH3T3 cells is roughly equivalent to that of wild-type FGF1, which explains why the IC₅₀ of the FGF1 L131R mutant is roughly equimolar to the concentration of wild-type FGF1 used in the inhibition assay. The inhibition of ERK phosphorylation by the FGF1 L131R mutant is not complete, as the samples treated with the highest concentrations of the FGF L13R mutant in the presence of wild-type FGF1 show a low level of ERK phosphorylation that is above that of untreated cells. However, this phenomenon is also observed in the FGF1 R50E mutant, which is the only other FGF1 mutant that is reported to act an antagonist in the literature⁶⁰. It is reported that sustained, high levels of ERK phosphorylation for the induction of FGF pathway-associated cell proliferation and the activation of downstream effector proteins such

as cyclin D1^{57,61}. The FGF1 R50E mutant is defective in its binding to integrin $\alpha v \beta 3$, and it also shows incomplete inhibition of ERK phosphorylation. However, in a follow-up study by *Mori et al.*, they successfully show that their FGF1 R50E antagonist is able to inhibit FGF1-induced cell migration, HUVEC tube formation, angiogenesis in Matrigel plug assays, and the outgrowth of cells in aorta ring assays⁴¹. Thus, this example provides good evidence that the FGF1 L131R can similarly act as an FGF pathway antagonist in functional biological assays.

[00358] In conclusion, the results described in this Examples 2 and 3 show that we were able to successfully utilize our high-throughput screen for increasing the proteolytic stability of FGF1 in plasmin and identify key proteolytically stabilized candidates for FGF2. It was shown that the FGF1 mutants exhibit increased proteolytic stability in plasmin and trypsin, and increased stability in culture. It was demonstrated that the FGF1 L131R mutant acts as a potent FGF pathway antagonist that can be used to inhibit FGF1-induced ERK phosphorylation in NIH3T3 cells. The FGF1 mutants demonstrate their promise for development of a proteolytically stabilized therapeutic molecule for anti-angiogenesis therapy in the treatment of diseases such as cancer and unwanted neovascularization in the eye.

MATERIALS & METHODS

Recombinant FGF1 expression and purification

[00359] FGF1 was expressed using Rosetta (DE3) competent cells (Novagen). The gene was cloned from human FGF1 cDNA (Dharmacon) into the pBAD/His B vector (Invitrogen) with an N-terminal 6x His tag and an arabinose-inducible promoter. The restriction sites XhoI and HindIII were used for cloning. The pBAD FGF1-His plasmid was transformed into chemically competent Rosetta (DE3) cells, recovered in 1 mL LB at 37°C with shaking at 235 rpm, and plated on LB plates with ampicillin (Amp) selection. Colonies were inoculated into 5 mL LB Amp and grown at 37°C overnight. 1 mL of the overnight culture was used to inoculate a 100 mL LB Amp expression culture. Cells were grown at 37°C with shaking at 235 rpm for 2 to 2.5 hours. At an OD₆₀₀ of ~0.5, the cells were induced with 0.2% L-arabinose (Sigma Aldrich). The proteins were expressed and maintained in the cell cytoplasm. The expression culture was grown for 6 hours at 37°C before the cells were spun down and collected.

[00360] The cells were lysed in B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) with lysozyme, DNase I, and heparin sulfate for 30 minutes. The extraction mixture was spun down at 15,000 g for 10 minutes, and the supernatant was collected and filtered through a 0.22 µm filter. The supernatant containing the FGF1 was diluted in a 1:10 dilution with binding buffer for Ni-NTA affinity purification, as detailed in Section 2.5.6.1. The supernatant and binding buffer mixture was loaded onto the Ni-NTA column. The elution from Ni-NTA affinity purification was concentrated and buffer exchanged into PBS using the Amicon Ultra-4 Centrifugal Filter Unit with 10 kDa cutoff. Size exclusion chromatography with the Superdex 75 column was used to purify the final FGF1-His protein, as described in Section 2.5.6.1.

Cloning of FGF1 single mutants

[00361] Overlap extension PCR was used to mutate wild-type FGF1 into single amino acid mutants⁶². The codon mutations are as follows: D28N – GAT to AAT; L131R – CTA to CGA; L131A – CTA to GCA; L131K – CTA to AAA. The site-specific mutagenesis primers incorporated the codon mutations as well as 20 bp overhangs on each side that overlap with the wild-type FGF1 sequence.

Proteolytic stability assay

[00362] For each condition, 125 ng of protein was incubated in 20 µL of plasmin digest buffer (100 mM Tris-HCl, 0.01% BSA, pH 8.5) with varying concentrations of plasmin or for varying incubation times at 37°C. At the end of the appropriate incubation time for each sample, the protease digestion was stopped by storage of the sample at -20°C. After the completion of all incubations, samples were thawed on ice for analysis. Each 20 µL sample was mixed with 5 µL of NuPAGE LDS Sample Buffer and 2 µL of NuPAGE Sample Reducing Agent. The samples were heated to 95°C for 10 minutes prior to running SDS-PAGE gels. Gels were incubated with 20% ethanol for 10 minutes prior to blotting onto a nitrocellulose membrane using the Invitrogen iBlot Gel Transfer Device (Program 0, 7 minutes).

[00363] The Western blots were blocked with 5% nonfat dry milk (Bio-Rad) in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.1% Tween 20) for one hour. Primary staining was done with 1:1000 dilution of mouse anti-FGF1 (Sigma Aldrich, clone 2E12) in 5% milk in TBST for one hour. After washing three times in TBST for 15 minutes, secondary staining was done with 1:2500 dilution of goat anti-mouse HRP (ThermoFisher Scientific) for 2

hours. After washing three more times in TBST for 15 minutes, the blots were imaged by BioRad ChemiDoc XRS System in Chemi Hi Sensitivity mode. Band intensities were quantified using ImageJ and normalized band intensities were plotted using GraphPad Prism 6.

5 ThermoFluor assay for measuring melting temperature

[00364] 50 μ L of 1.2 mg/mL protein was loaded into a 96-well, thin-wall PCR plate (Bio-Rad). 0.5 μ L of SYPRO Orange (Molecular Probes) was added to the sample and mixed thoroughly. The plate was sealed with a plastic cover prior to plate analysis with BioRad CFX96 RT System C1000 Touch. The plate was cooled to 4°C for 5 minutes and then the
10 plate was heated slowly up to 100°C at a rate of 1°C per minute. Fluorescence changes were monitored and measured at each °C. The fluorescence over temperature was plotted on Microsoft Excel and the melting temperature was calculated by finding the temperature at which the fluorescence equals the average of the maximum and minimum fluorescence signals.

15 Cell culture stability assay

[00365] MDA-MB-231 cells were seeded on 6-well plates (Sigma Aldrich) at a density of 100,000 cells/well in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and grown at 37°C in 5% CO₂. After 24 hours, the media was aspirated and replaced with DMEM for serum starvation. After 24 hours, the DMEM
20 was aspirated. For each sample, 500 ng of protein in 1 mL of DMEM was added to each well and incubated at 37°C in 5% CO₂ for varying incubation times. At the end of each incubation, the supernatant was collected, filtered with 0.22 μ m filter, and frozen down at -20°C prior to analysis. After all incubations were complete, supernatants were thawed on ice. Each supernatant sample was concentrated down to 50 μ L volume using Amicon 3K MWCO
25 Ultra-0.5 mL Centrifugal Filters. 15 μ L of the concentrated sample was mixed with 5 μ L of NuPAGE LDS Sample Buffer and 2 μ L of NuPAGE Sample Reducing Agent. The samples were heated to 95°C for 10 minutes prior to running SDS-PAGE gels. Gels were incubated with 20% ethanol for 10 minutes prior to blotting onto a nitrocellulose membrane using the Invitrogen iBlot Gel Transfer Device (Program 0, 7 minutes).

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in TBST for one hour. After washing three times in TBST for 15 minutes, secondary staining was done with 1:2500 dilution of goat anti-mouse HRP (ThermoFisher Scientific) for 2 hours. After washing three more times in TBST for 15 minutes, the blots were imaged by BioRad ChemiDoc XRS System in Chemi Hi Sensitivity mode. Band intensities were
5 quantified using ImageJ and normalized band intensities were plotted using GraphPad Prism 6.

NIH3T3 ERK Phosphorylation Assay

[00367] MDA-MB-231 cells were seeded on 6-well plates (Sigma Aldrich) at a density of 100,000 cells/well in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10%
10 newborn calf serum (NBCS) (Gibco) and grown at 37°C in 5% CO₂. After 24 hours, the media was aspirated and replaced with DMEM for serum starvation. After 24 hours, the DMEM was aspirated. Cells were stimulated with wild-type FGF1 and/or varying concentrations of FGF1 L131R mutant for 15 to 18 hours at 37°C without any phosphatase inhibitors. After stimulation, cells were washed with ice-cold PBS and treated with 100 µl of
15 lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% Glycerol, 1% Nonidet P-40) with 1X phosphatase inhibitor cocktail 2 and 1X protease inhibitor cocktail 2 (Sigma) for 1 hour at 4°C. Lysates were frozen down at -80 °C prior to analysis. Lysates were thawed on ice and clarified by centrifugation. Protein concentrations were quantified with Pierce BCA Protein Assay. 2 µg of protein lysate for each sample was diluted to 14.6 µL with MilliQ H₂O. Each
20 diluted sample was mixed with 5.6 µL of NuPAGE LDS Sample Buffer and 2.25 µL of NuPAGE Sample Reducing Agent. The samples were heated to 95°C for 10 minutes prior to running SDS-PAGE gels. Gels were incubated with 20% ethanol for 10 minutes prior to blotting onto a nitrocellulose membrane using the Invitrogen iBlot Gel Transfer Device (Program 0, 7 minutes).

[00368] The Western blots were blocked with 5% nonfat dry milk (Bio-Rad) in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.1% Tween 20) for one hour. Primary staining was done with 1:1000 dilution of rabbit anti-phospho-ERK1/2 (Y202/Y204) antibody (Cell Signaling) or rabbit anti-ERK1/2 (Cell Signaling) in 5% milk in TBST for one hour. After
25 washing three times in TBST for 15 minutes, secondary staining was done with 1:2500 dilution of goat anti-rabbit HRP (Santa Cruz Biotechnology) for 2 hours. After washing three
30 more times in TBST for 15 minutes, the blots were imaged by BioRad ChemiDoc XRS System in Chemi Hi Sensitivity mode. Band intensities were quantified using ImageJ and plotted using GraphPad Prism 6.

NIH3T3 cell binding assay

[00369] NIH3T3 cells were incubated with varying concentrations of wild-type FGF1 or FGF1 BS4M1 mutant in binding buffer (20 mM Tris-HCl (pH 7.5) with 1 mM MgCl₂, 1 mM MnCl₂, 2 mM CaCl₂, 100 mM NaCl, and 0.1% BSA) for 3 hours at 4°C. Cells were incubated in sufficiently large volumes to avoid ligand depletion. After incubation with FGF, the cells were washed and incubated with 1:100 dilution of anti-His Hilyte Fluor 488 (Anaspec) on ice for 15 min. The cells were washed, pelleted, and resuspended in binding buffer immediately before analysis by flow cytometry using EMD Millipore Guava EasyCyte. Flow cytometry data were analyzed using FlowJo (v7.6.1). Binding curves were plotted and K_d values were obtained using GraphPad Prism 6.

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WHAT IS CLAIMED IS:

1. A variant of human fibroblast growth factor 1 (FGF1) comprising at least one member selected from the group consisting of an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof, wherein the resulting FGF1 variant exhibits increased proteolytic stability as compared to wild-type FGF1 of SEQ ID NO:1.
2. The variant according to claim 1, wherein said FGF1 variant comprises an amino acid substitution, an amino acid deletion, an amino acid addition and combinations thereof in the β -loop or near the C-terminus.
3. The variant according to claims 1 to 2, wherein said FGF1 variant is a fibroblast growth factor receptor (FGFR) antagonist.
4. The variant according to claims 1 to 3, wherein said FGF1 variant comprises at least one amino acid substitution at position 28, 40, 47, 93, or 131.
5. The variant according to claim 3, wherein said FGF1 variant comprises at least one amino acid substitution selected from the group consisting of D28N, Q40P, S47I, H93G, L131R, and L131K.
6. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitution L131R.
7. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitution L131K.
8. The variant according to claim 3, wherein said variant comprises amino acid substitutions D28N and L131R.

9. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitutions D28N and L131K.
10. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitutions Q40P, S47I, H93G, and L131R.
11. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitutions Q40P, S47I, H93G, and L131K.
12. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitutions D28N, Q40P, S47I, H93G, and L131R.
13. The variant according to claim 3, wherein said FGF1 variant comprises amino acid substitutions D28N, Q40P, S47I, H93G, and L131K.
14. The variant according to claim 3, wherein said FGF1 variant does not comprise the amino acid substitution L131A.
15. The variant according to claim 1, wherein said FGF1 variant is conjugated to a member selected from a detectable moiety, a water-soluble polymer, a water-insoluble polymer, a therapeutic moiety, a targeting moiety and a combination thereof.
16. The variant according to claim 14, wherein said FGF1 variant is conjugated to a detectable moiety selected from a radioisotope, a paramagnet, a fluorophore and combinations thereof.
17. The variant according to claim 15, wherein said FGF1 variant is a diagnostic imaging agent.
18. A pharmaceutical formulation comprising a FGF1 variant according to claim 1, wherein said variant is in combination with a pharmaceutically acceptable carrier.

- 19.** A method of inhibiting or preventing angiogenesis a subject in need thereof, comprising administering a variant according to claims **1** to **17** to the subject in need thereof, thereby preventing or inhibiting angiogenesis.
- 20.** The method of claim **19**, wherein the subject has cancer.
- 21.** The method of claim **19**, wherein the subject is treated to prevent neovascularization in the eye.
- 22.** A method of treating cancer in a subject in need of the treatment, the method comprising administering to the subject a therapeutically effective amount of a variant according to claim **1**, thereby treating the cancer.
- 23.** A method of reducing a process which is a member selected from tumor progression, angiogenesis, metastasis and combinations thereof in a subject, the method comprising administering to said subject an amount of said FGF1 variant according to claim **1** sufficient to reduce the process.
- 24.** The method according to claim **23**, wherein said cancer is a member selected from colorectal, oral, hepatocellular, renal, breast, lung, ovarian, stomach, brain, prostate, and combinations thereof.
- 25.** A nucleic acid encoding said FGF1 variant polypeptide of claims **1** to **17**.
- 26.** An isolated cell wherein said nucleic acid of claim **25** is expressed.
- 27.** A method of screening for proteolytically stable growth factor variants, said method comprising:
- i. expressing a library of growth factor variants in a yeast display system;

- ii. testing the yeast displayed growth factor variants from i) for proper folding by measuring binding activity of the yeast displayed growth factor variants to the relevant growth factor receptor;
 - iii. incubating the yeast displayed growth factor variants from ii) with at least one protease;
 - iv. determining the protease cleavage of the yeast displayed growth factor variants from iii) as compared to the protease cleavage of the wild-type growth factor; and
 - v. selecting the variants from iv) that exhibit reduced protease cleavage by and/or increased proteolytic stability to at least one protease as compared to the protease cleavage of the wild-type growth factor by the same protease, wherein said selected growth factor variants are proteolytically stable growth factor variants.
- 28.** The method of claim **27**, wherein the at least one protease is a protease capable of cleaving the wild-type growth factor.
- 29.** The method of claim **27**, wherein the at least one protease is capable of selectively cleaving the growth factor and which exhibits minimal and/or no non-specific cleavage of the yeast display proteins
- 30.** The method of claim **27**, wherein the at least one protease is selected from the group consisting of serum, trypsin, chymotrypsin, and plasmin.
- 31.** The method of claim **30**, wherein the at least one protease is serum.
- 32.** The method of claim **30**, wherein the at least one protease is trypsin.
- 33.** The method of claim **30**, wherein the at least one protease is chymotrypsin.
- 34.** The method of claim **30**, wherein the at least one protease is plasmin.

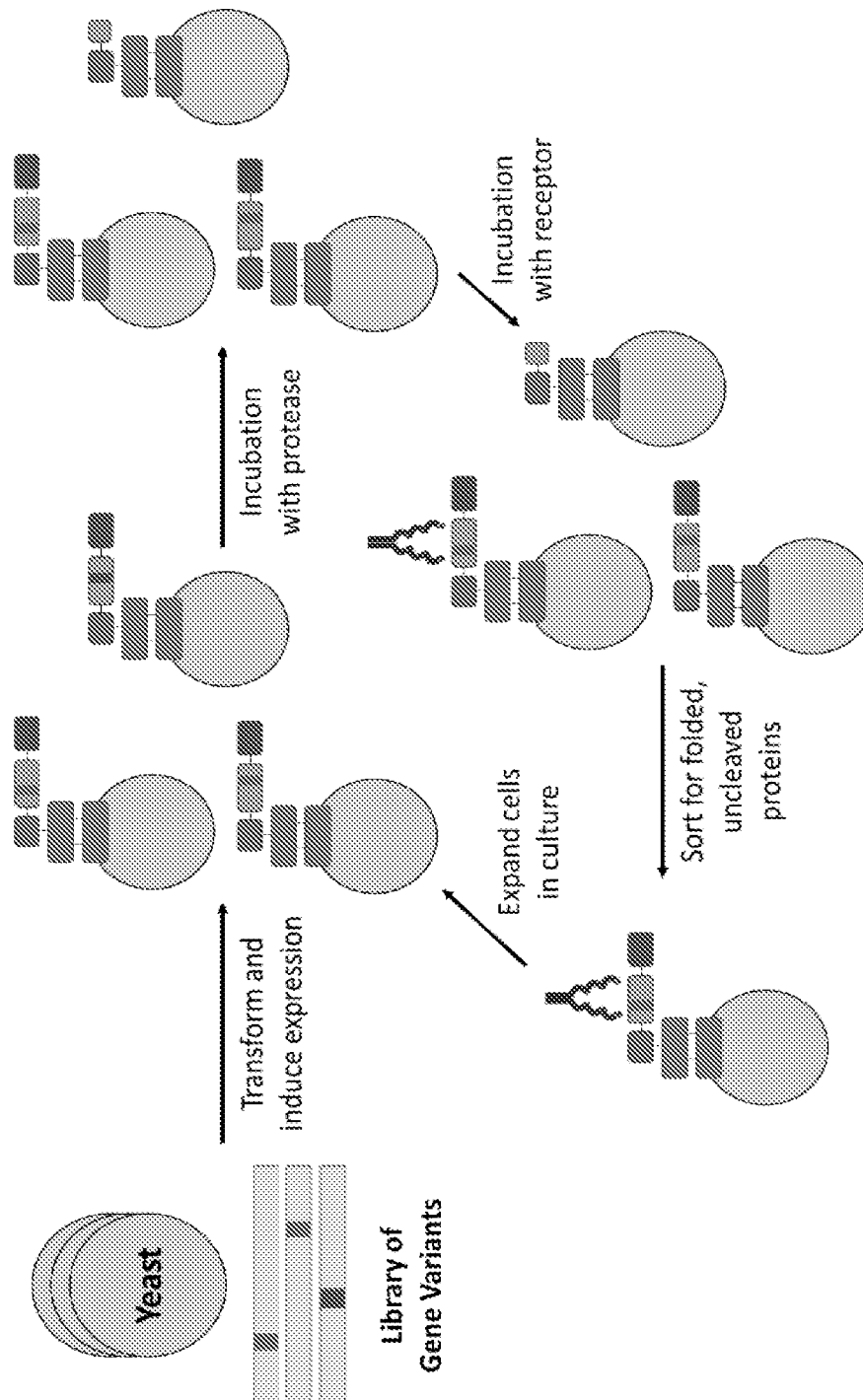
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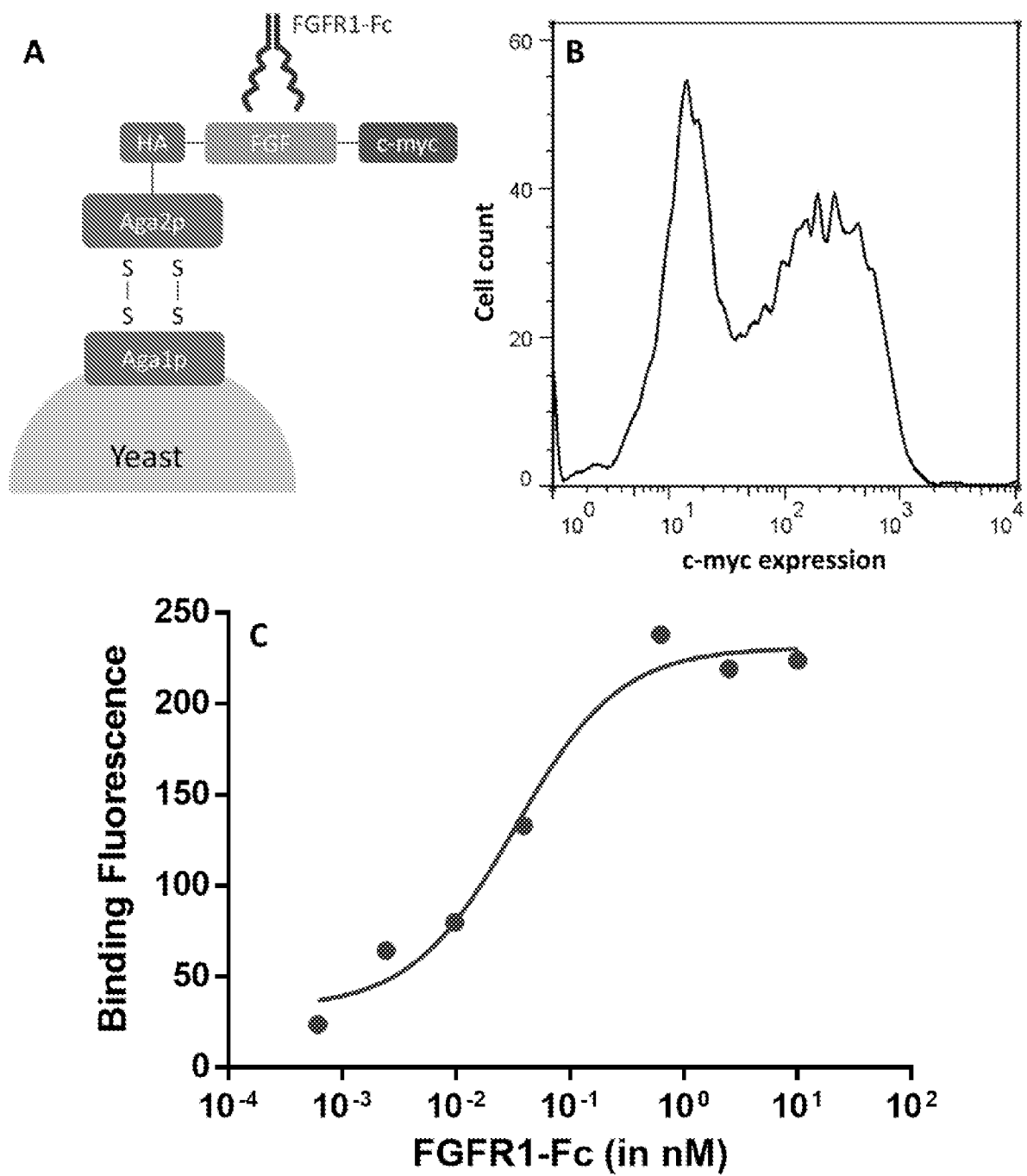
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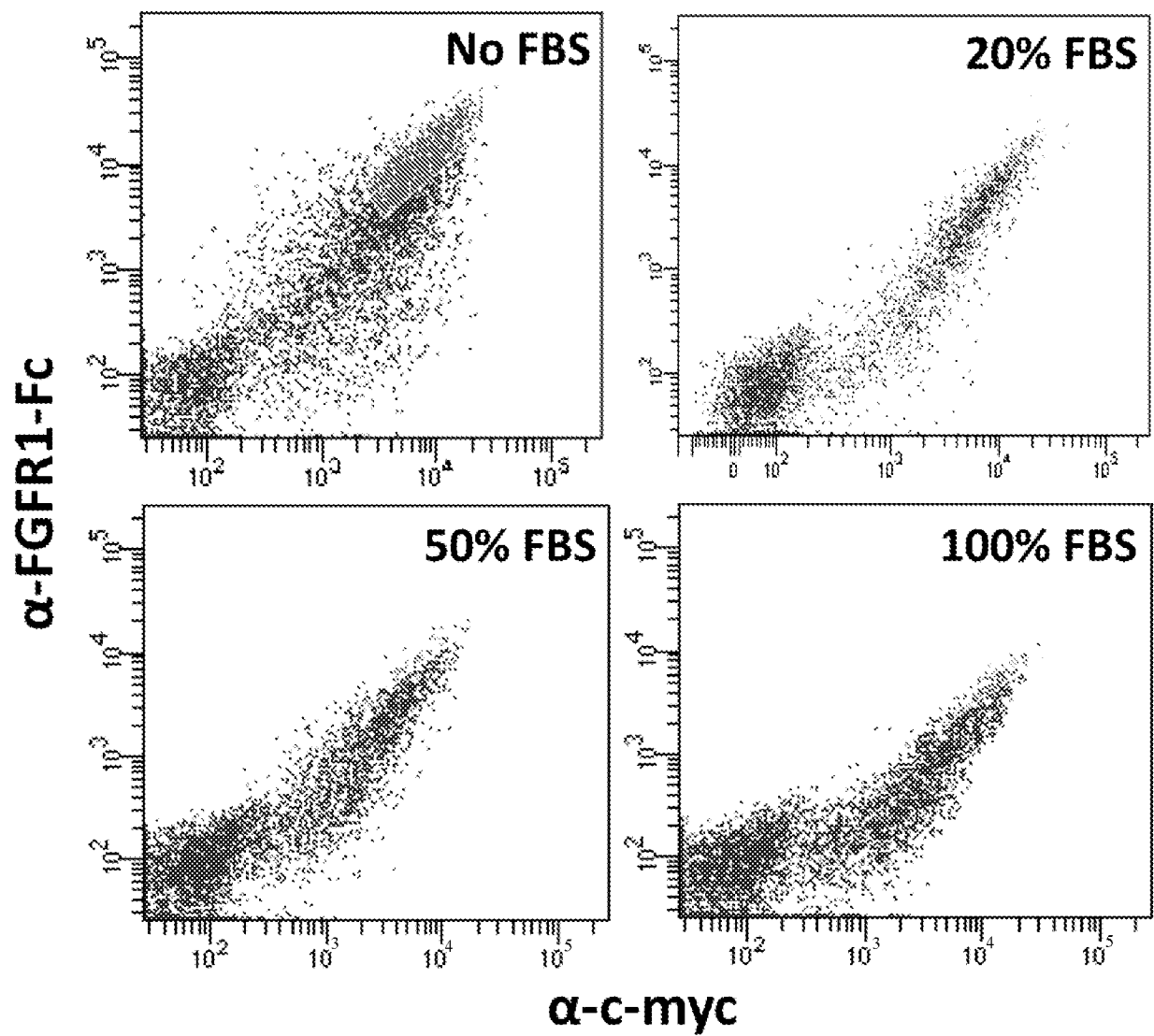
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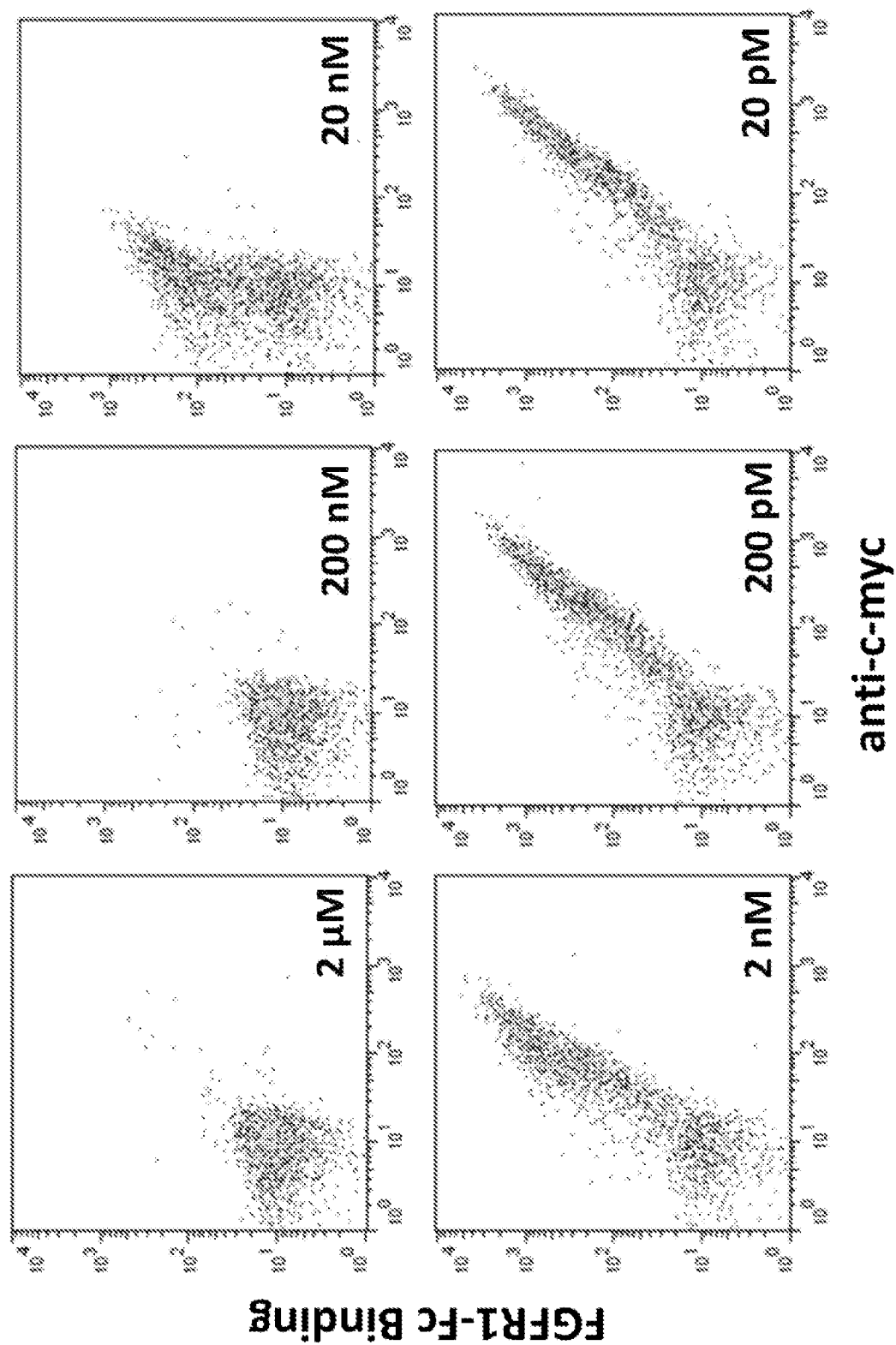
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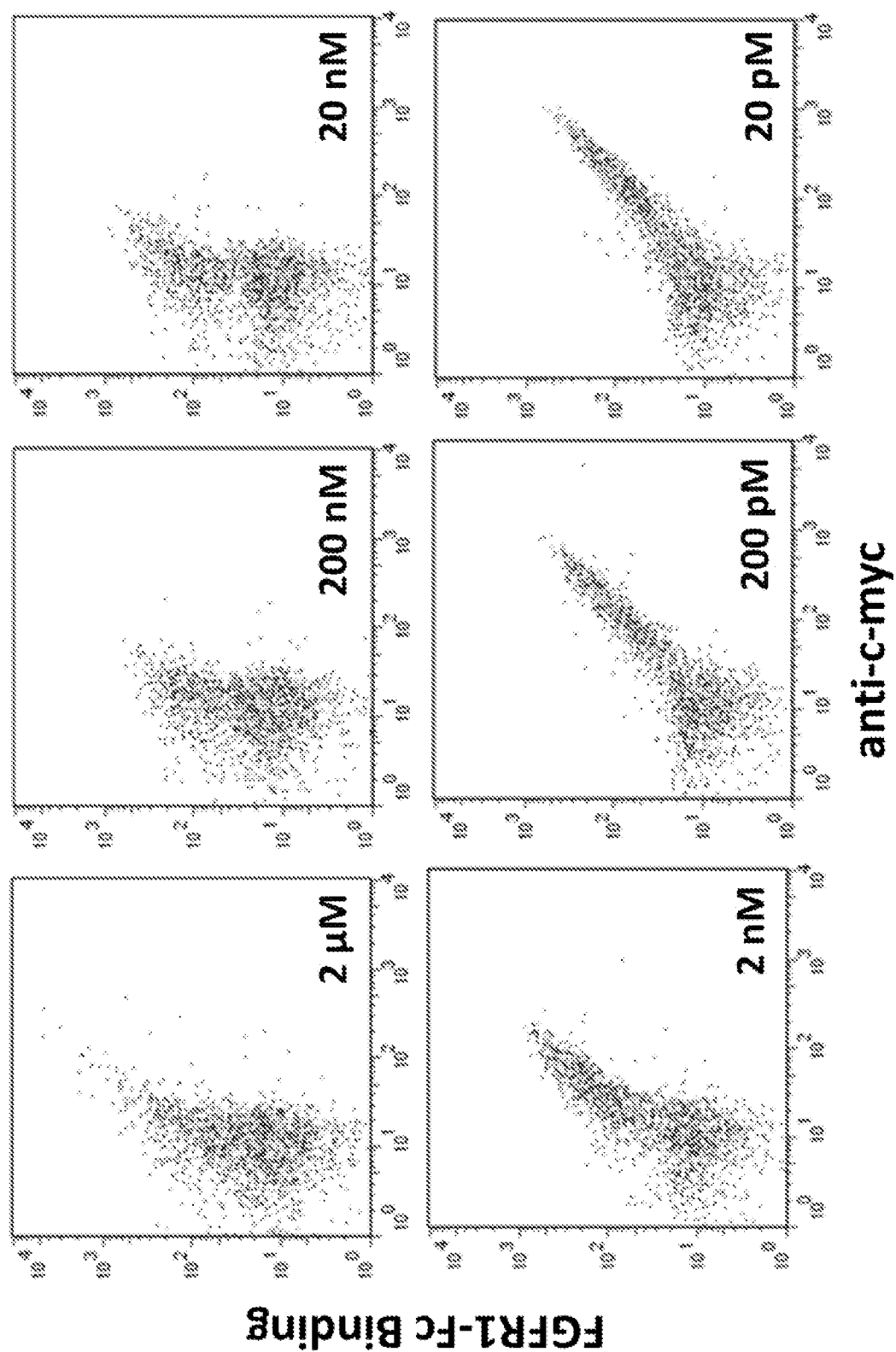
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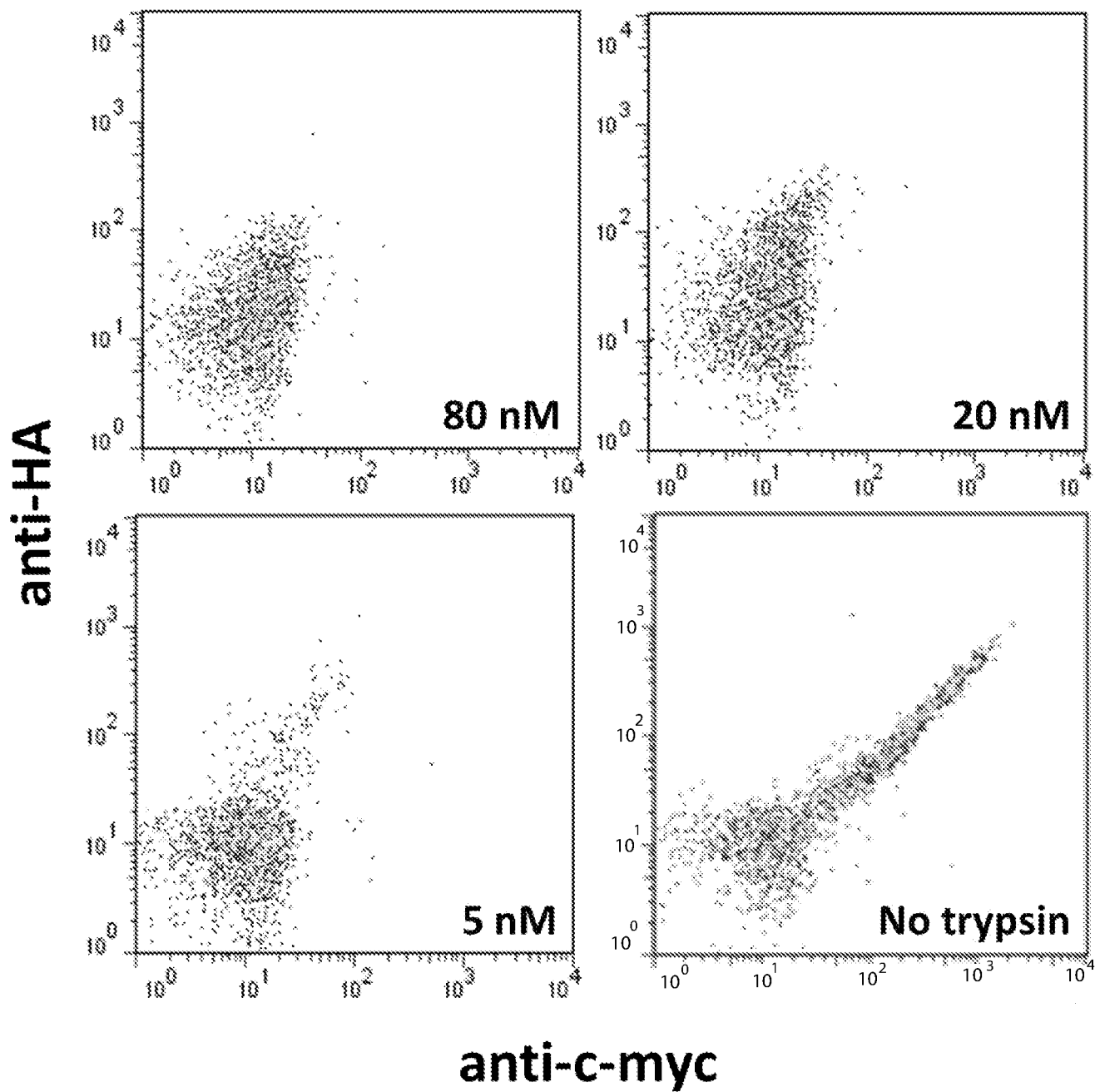
Figure 7A

Figure 7B

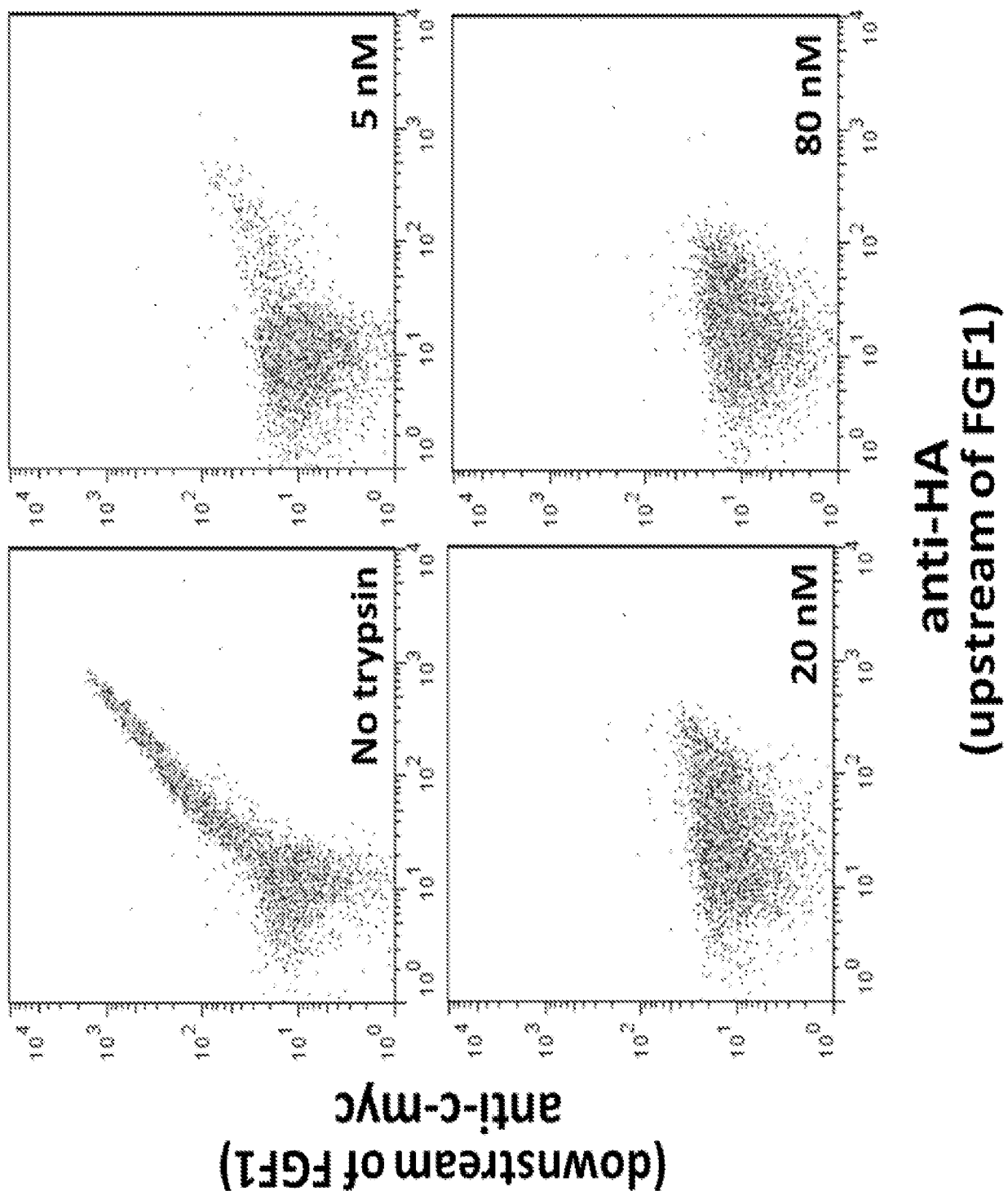


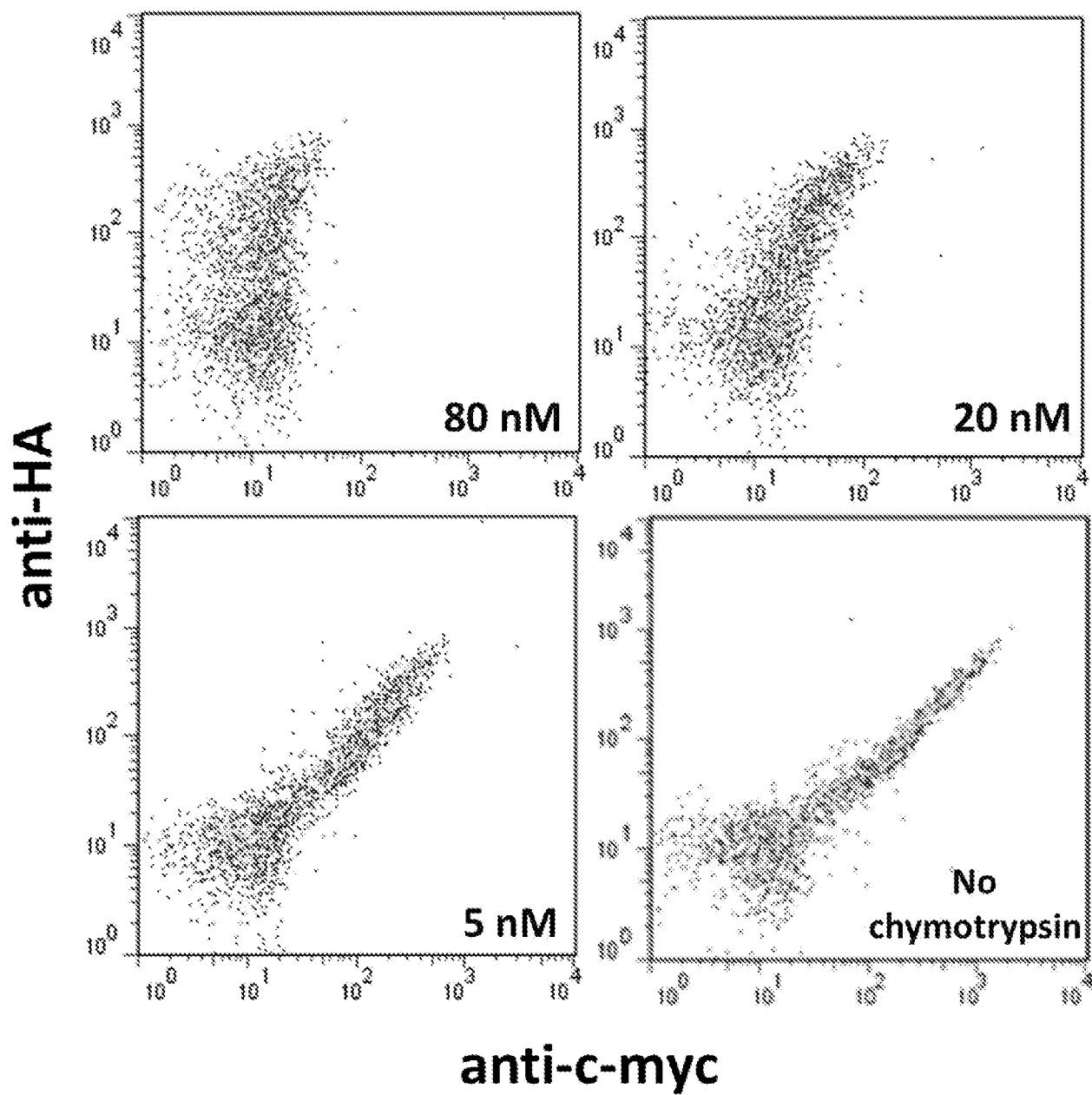
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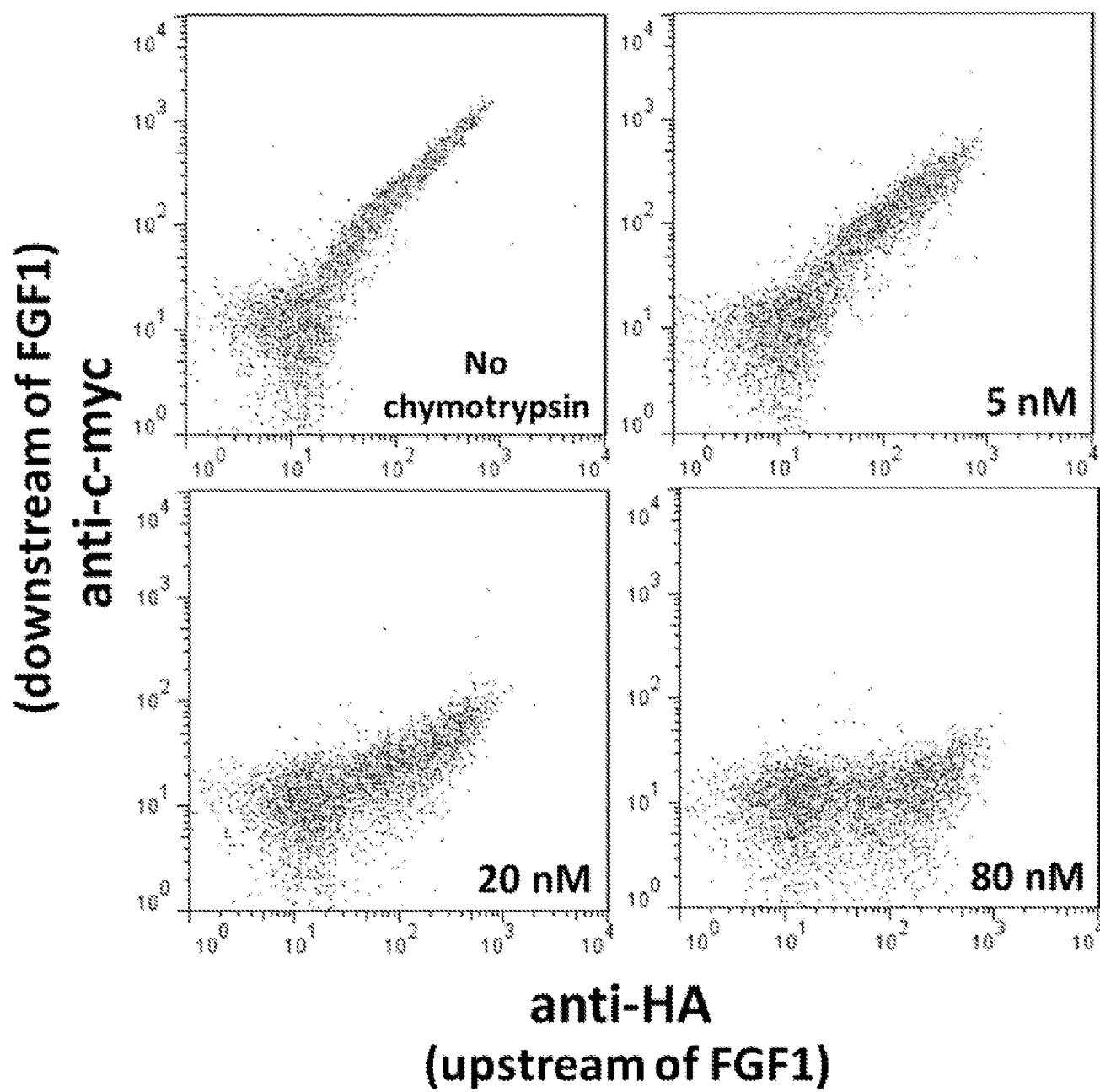
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Figure 9

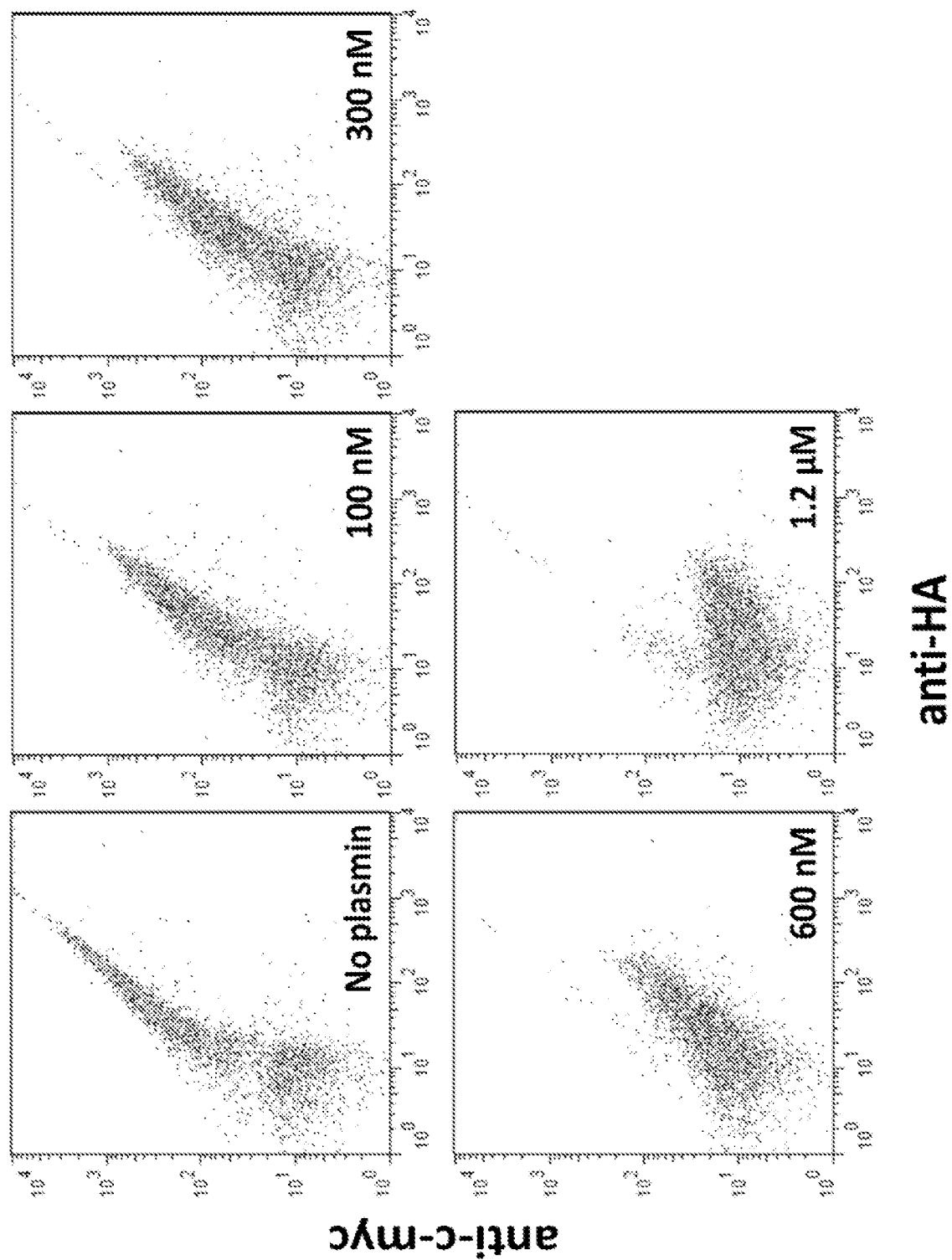


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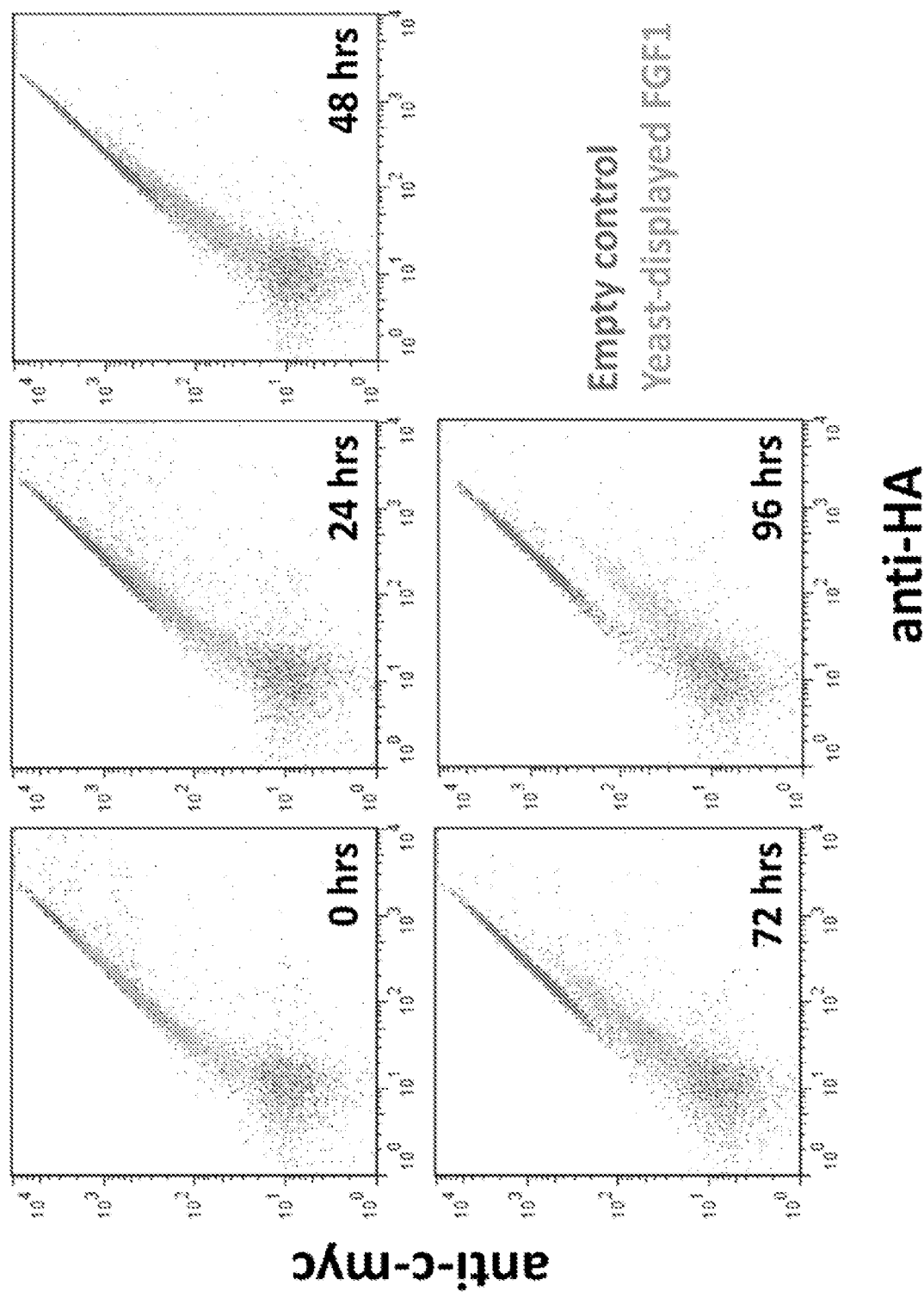
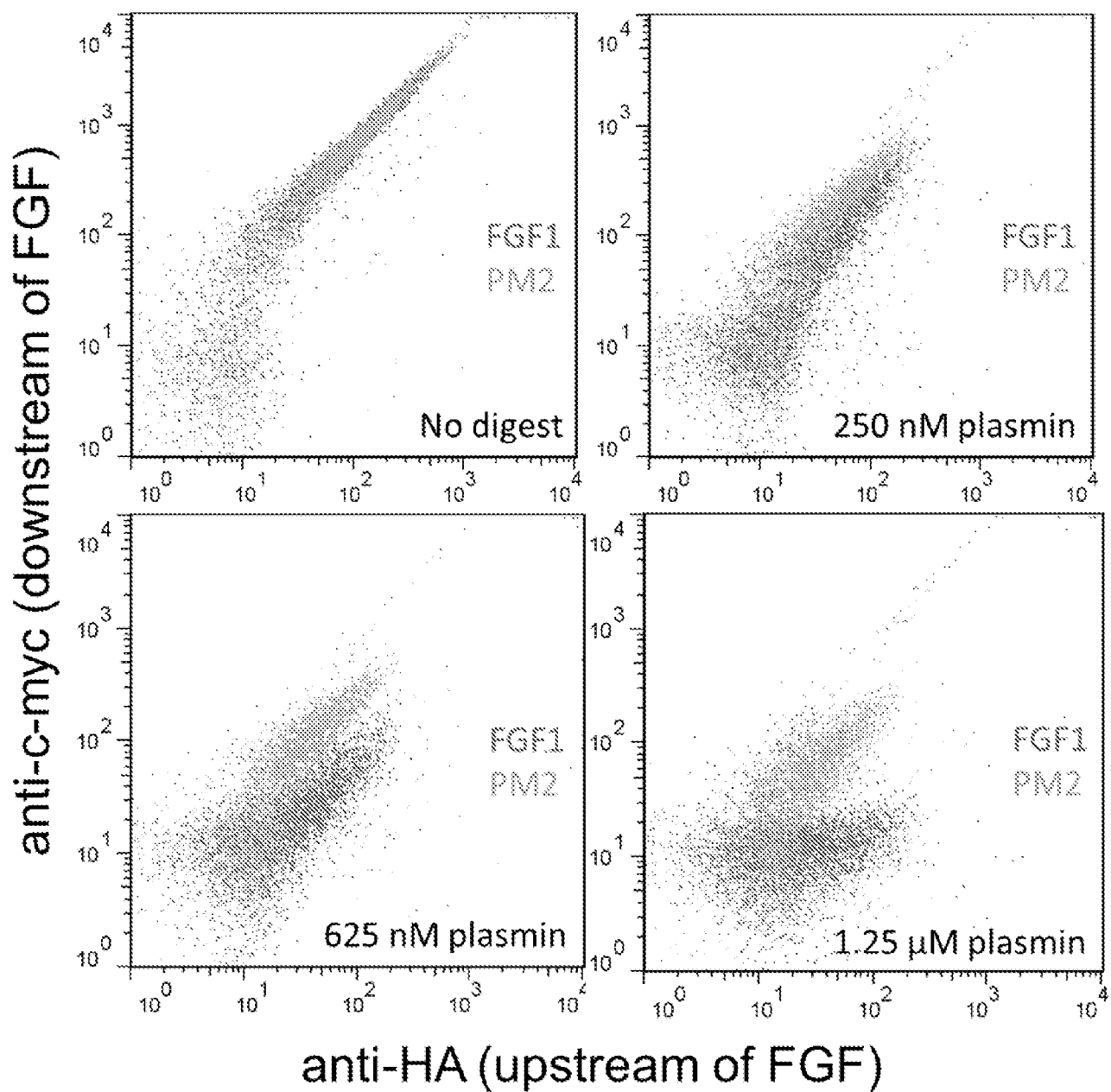


Figure 11

3.7 FIGURES

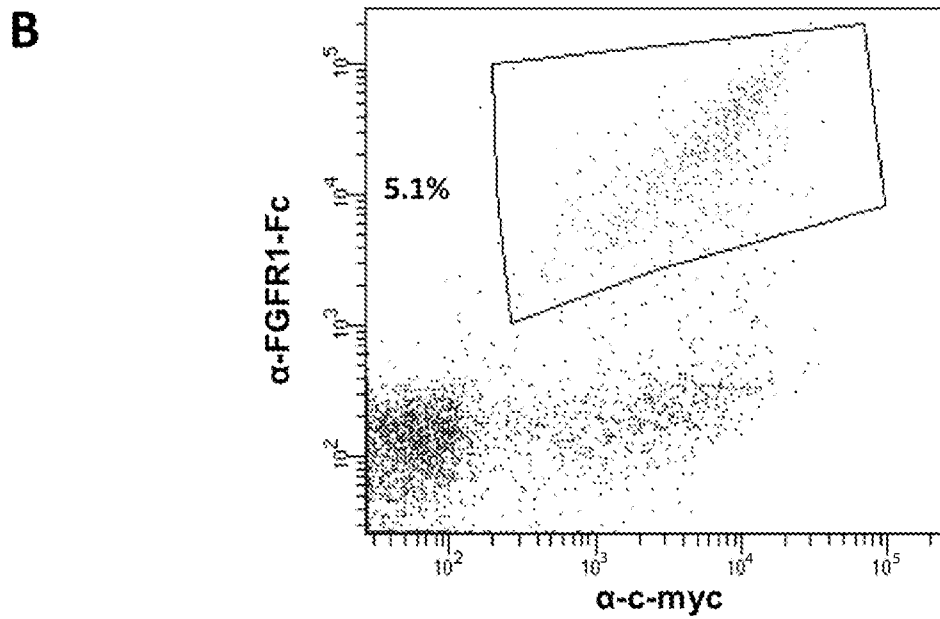
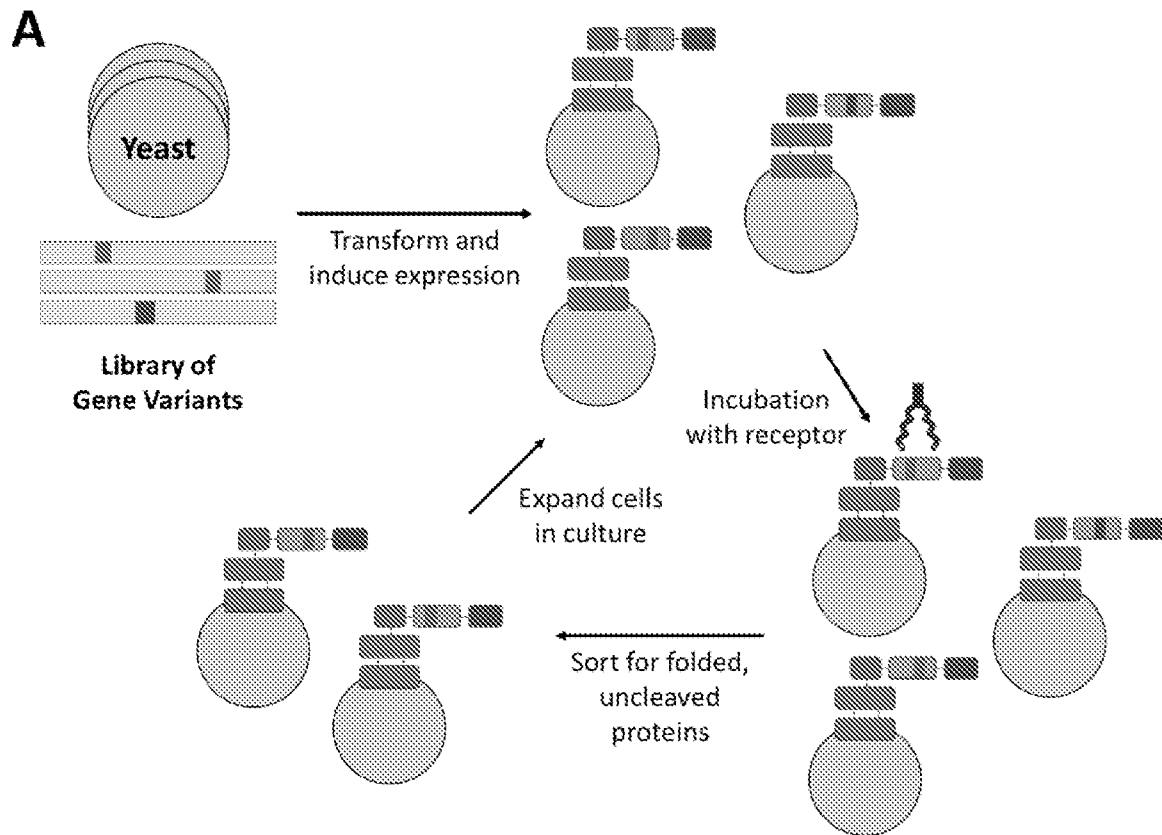
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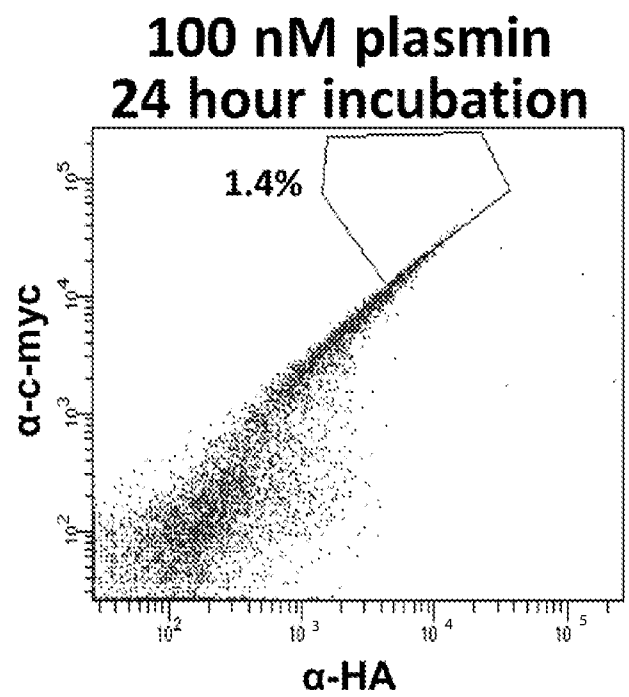
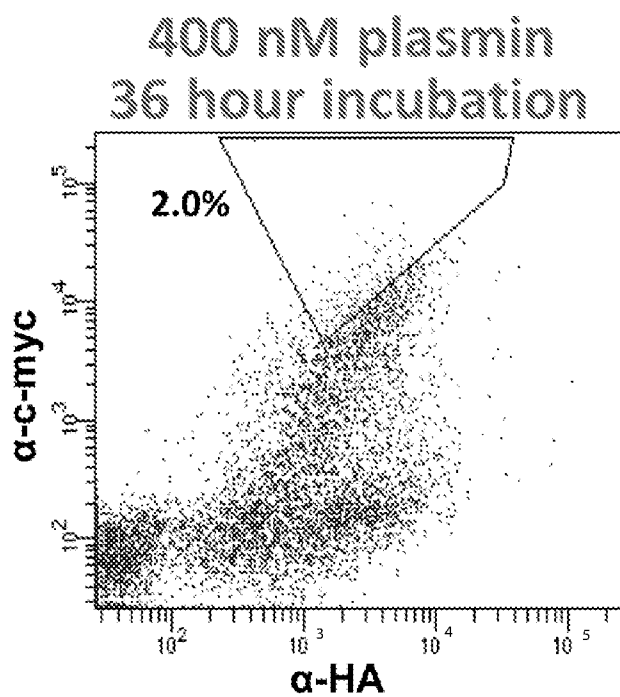
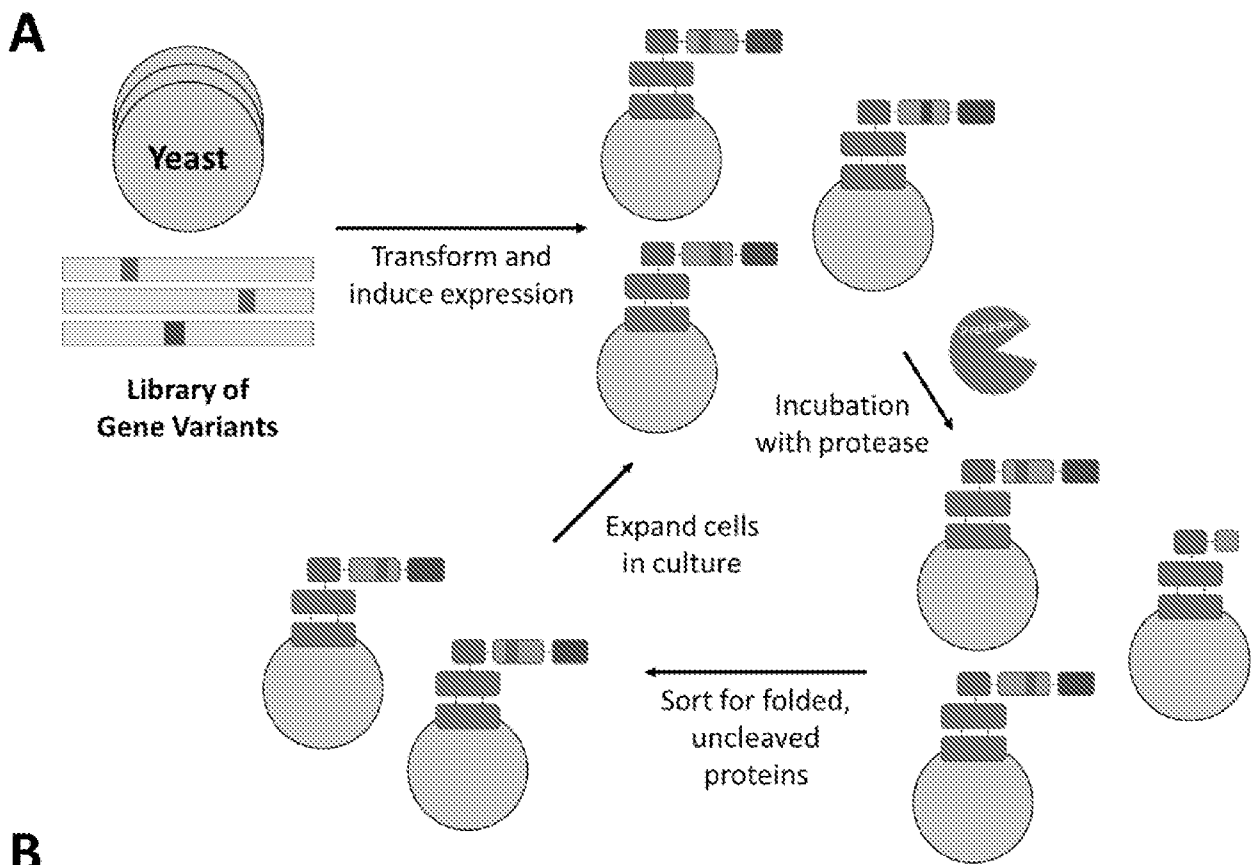
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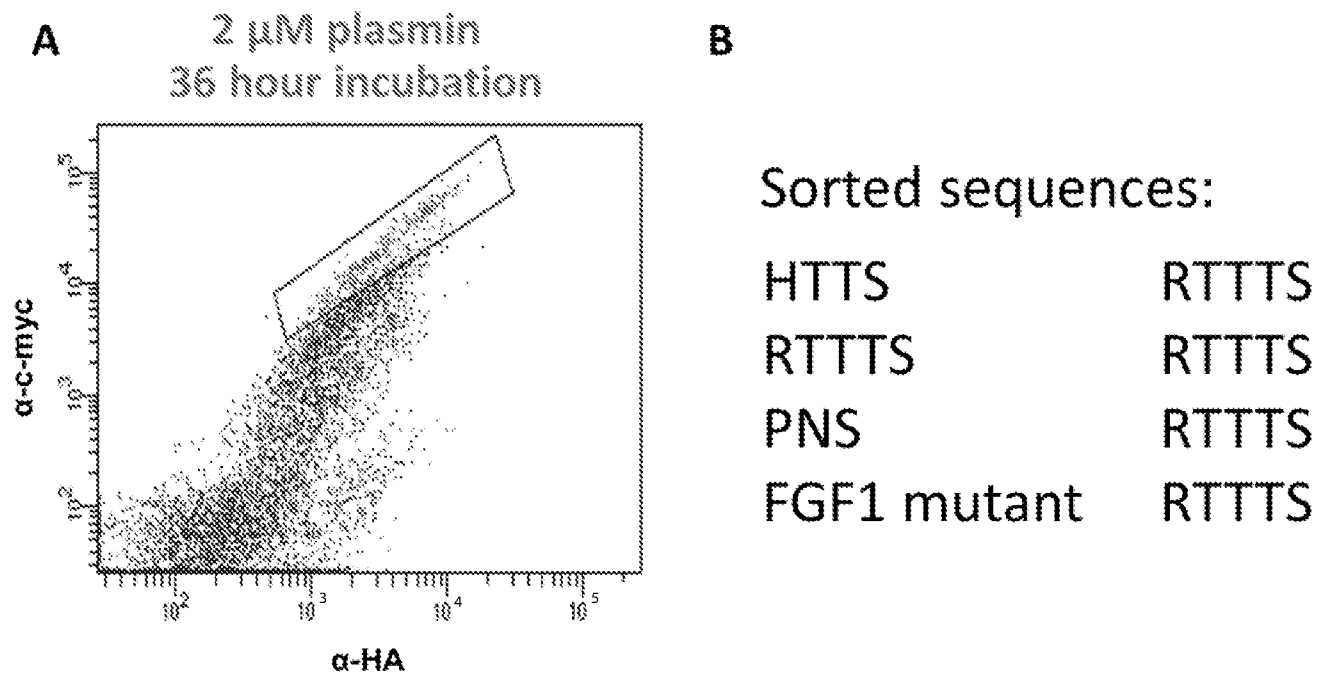
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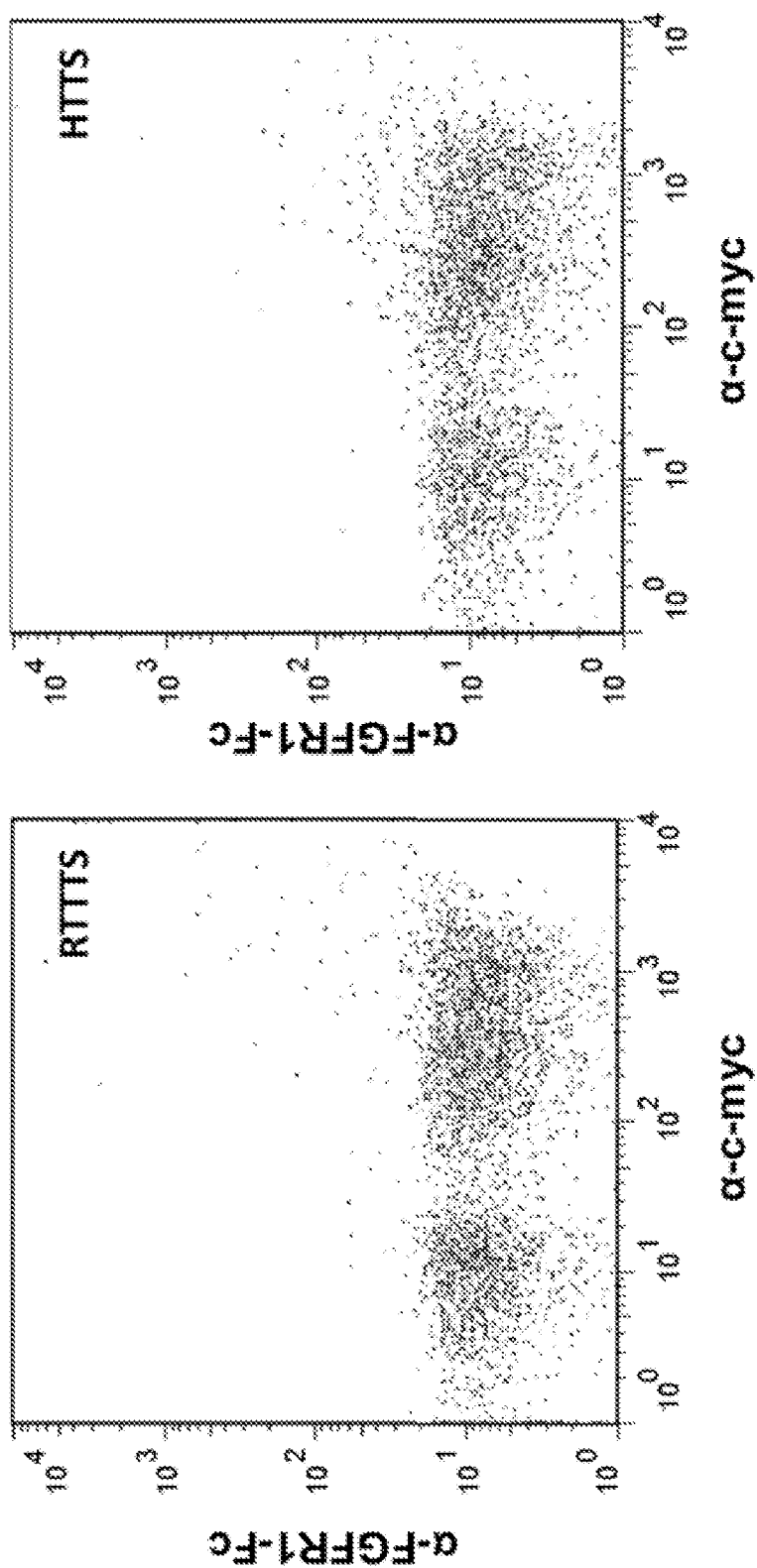
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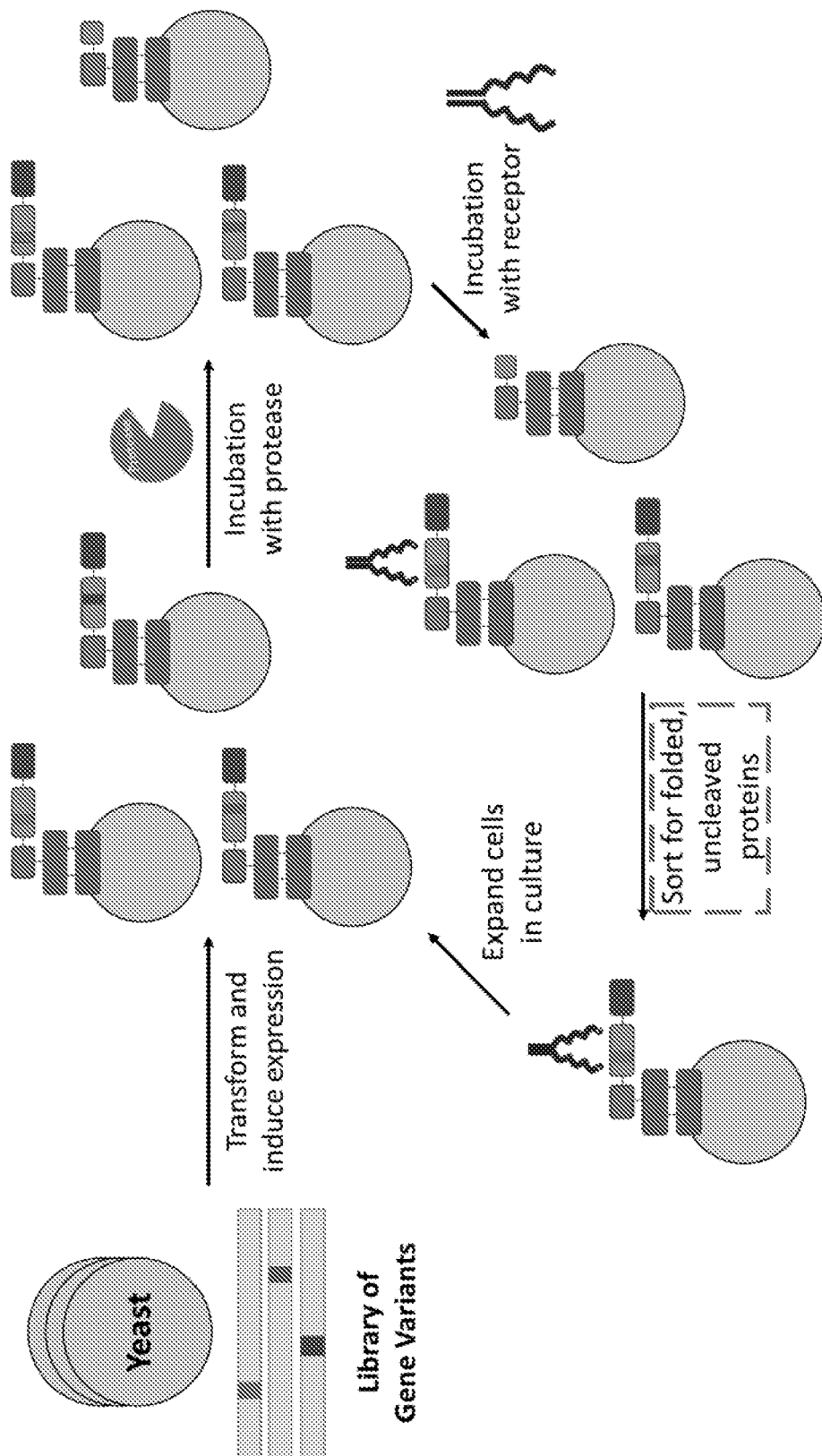
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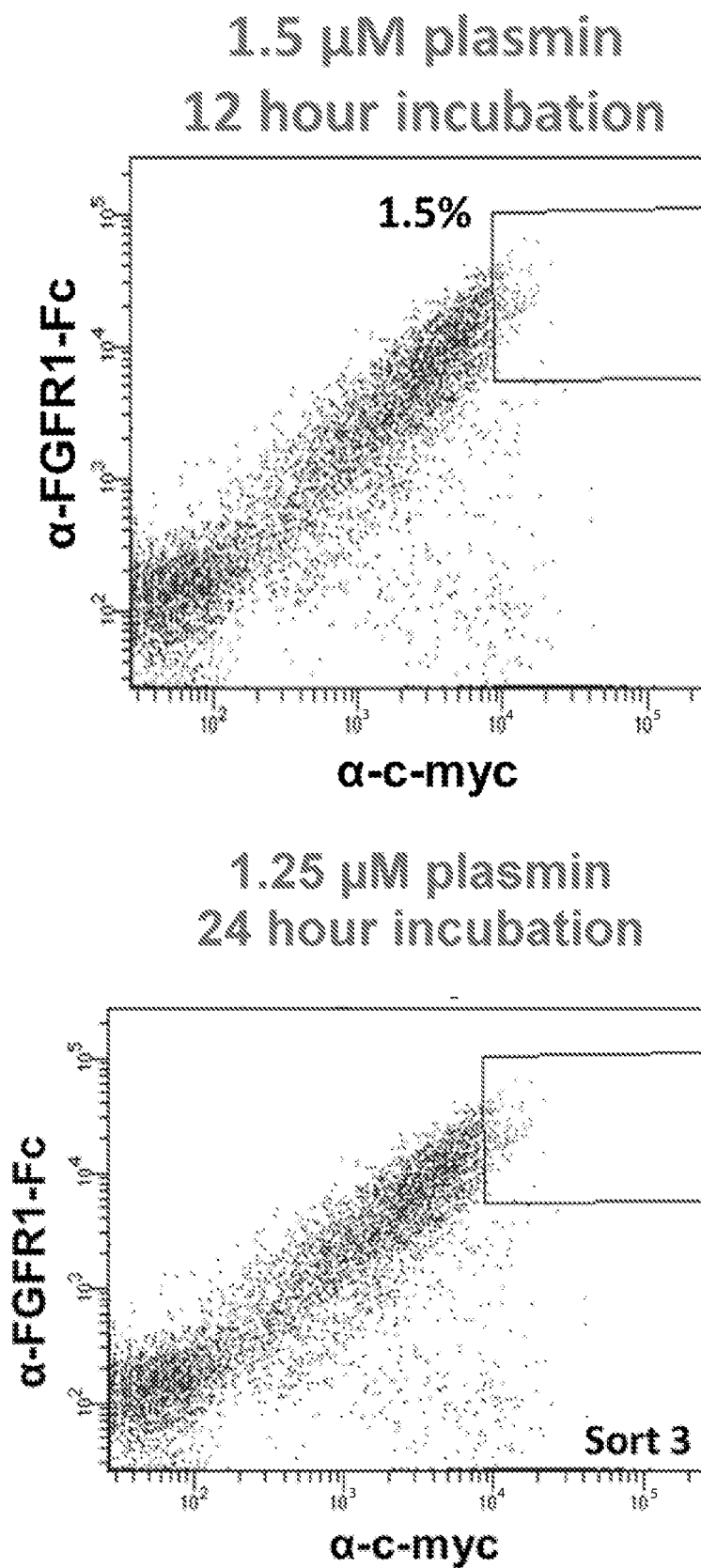
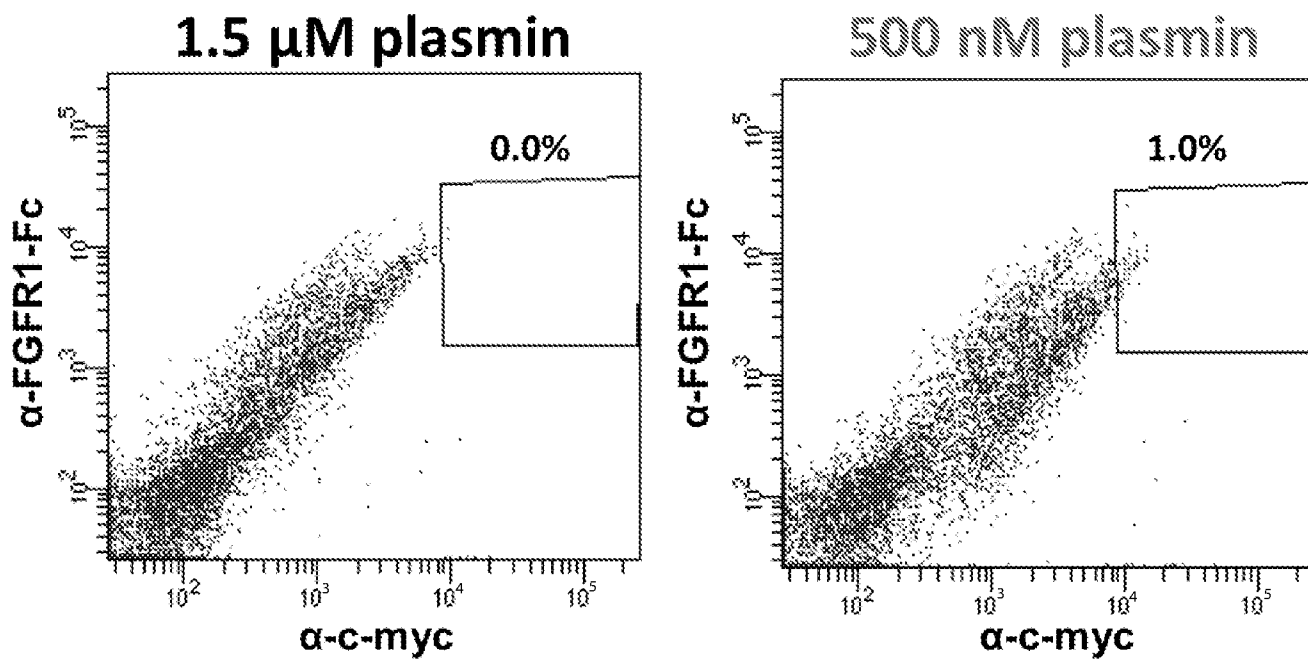
Figure 17

Figure 18

36 hour incubation

3.75 μ M plasmin
36 hour incubation

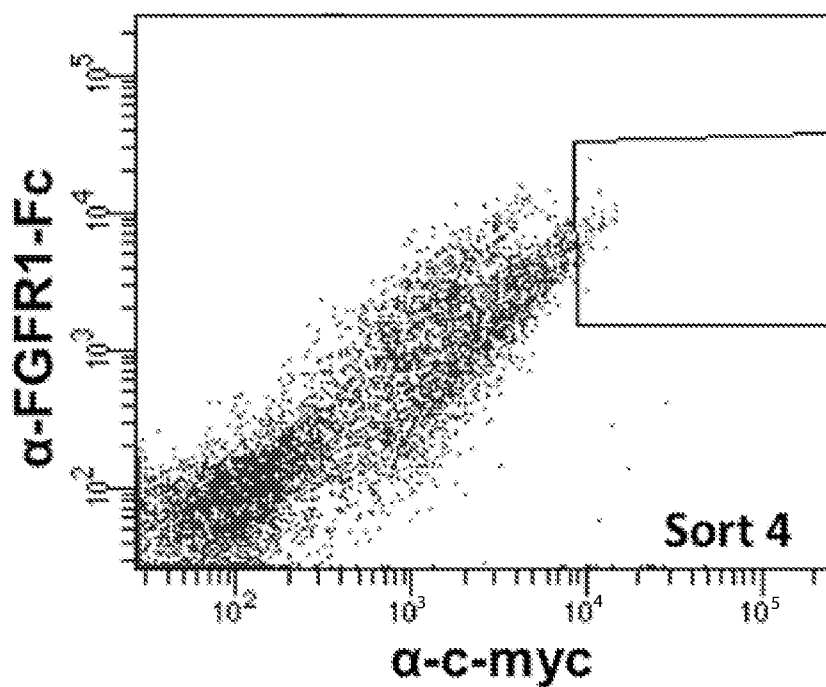


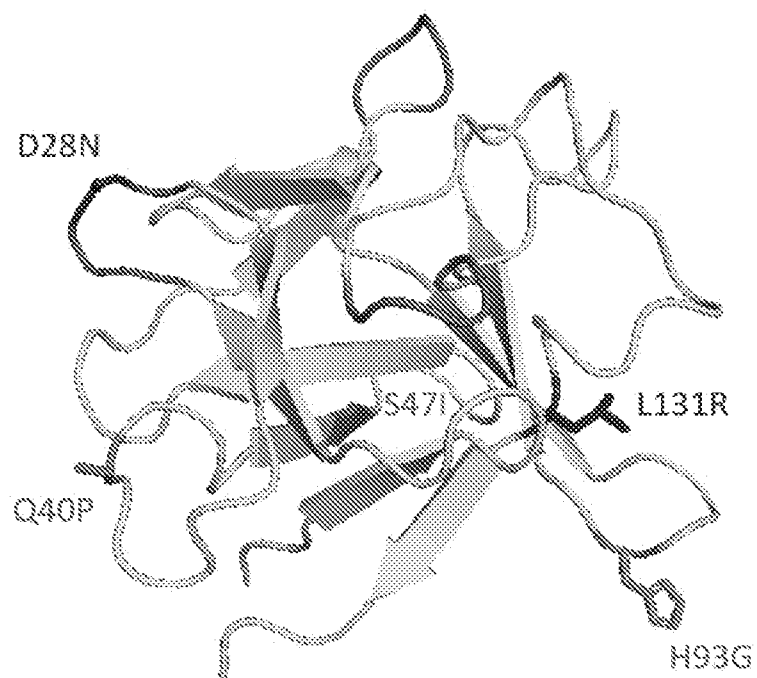
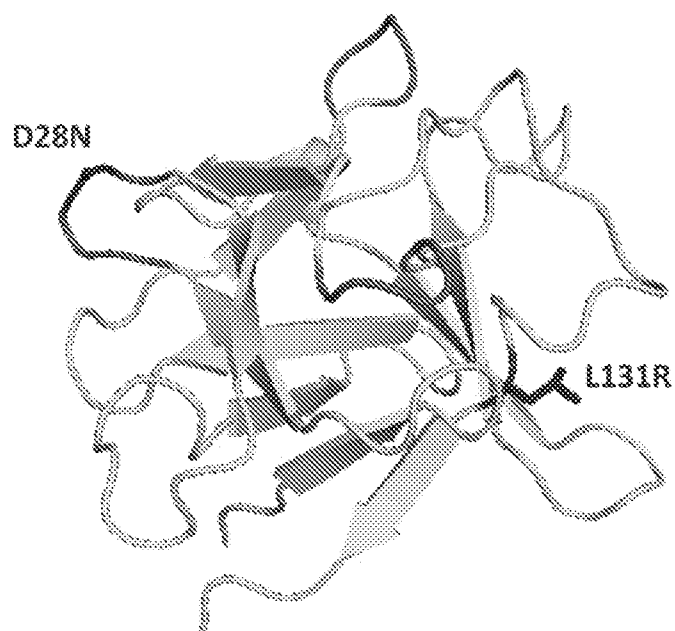
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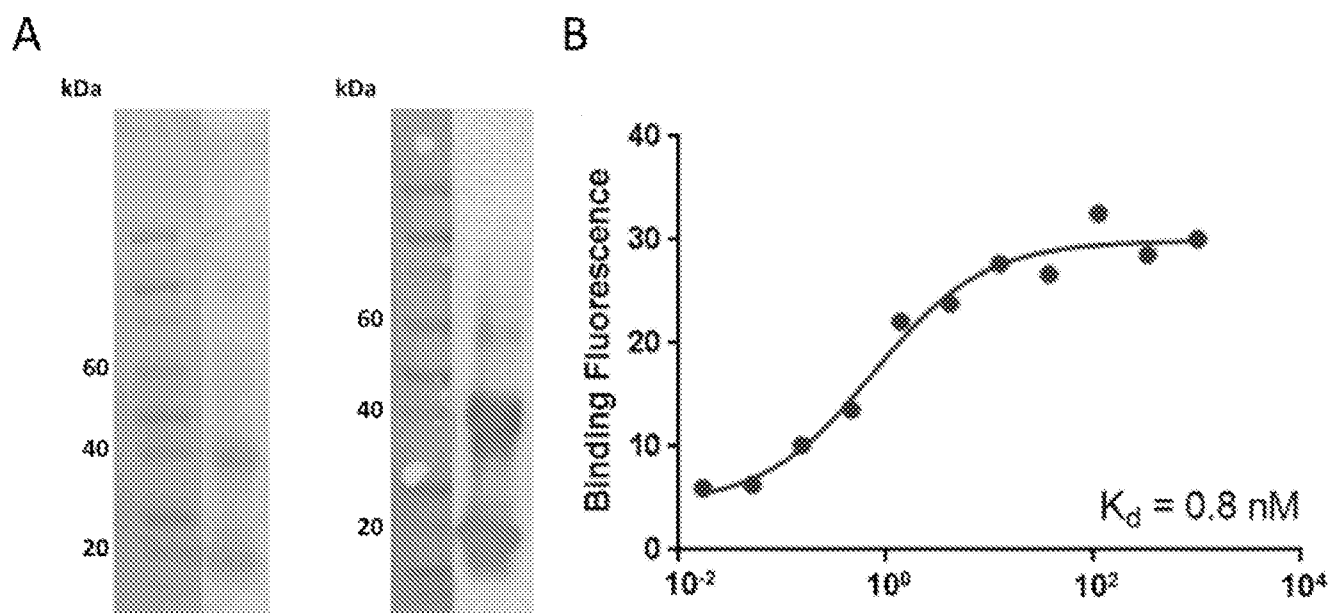
Figure 20

Figure 21

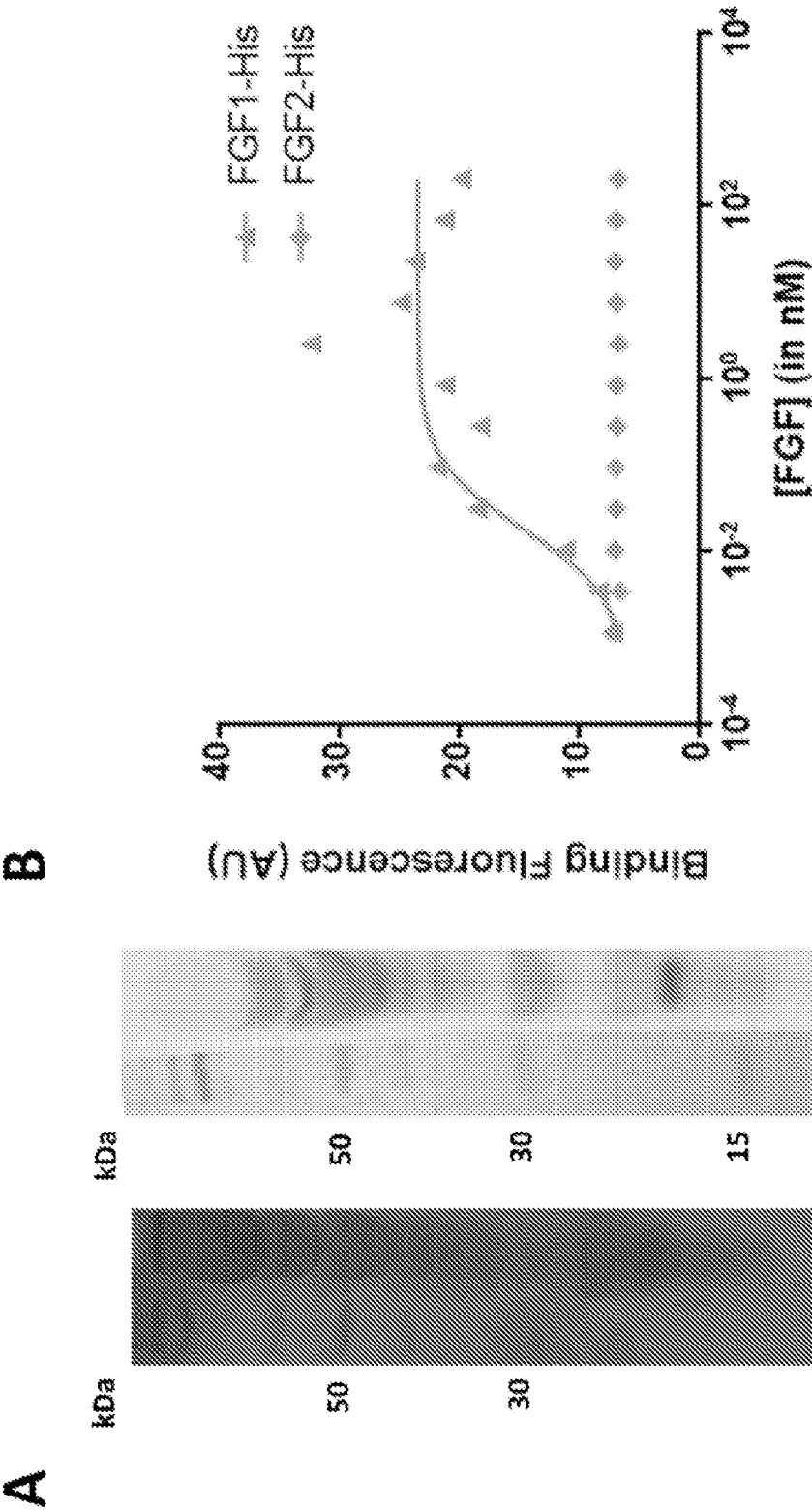


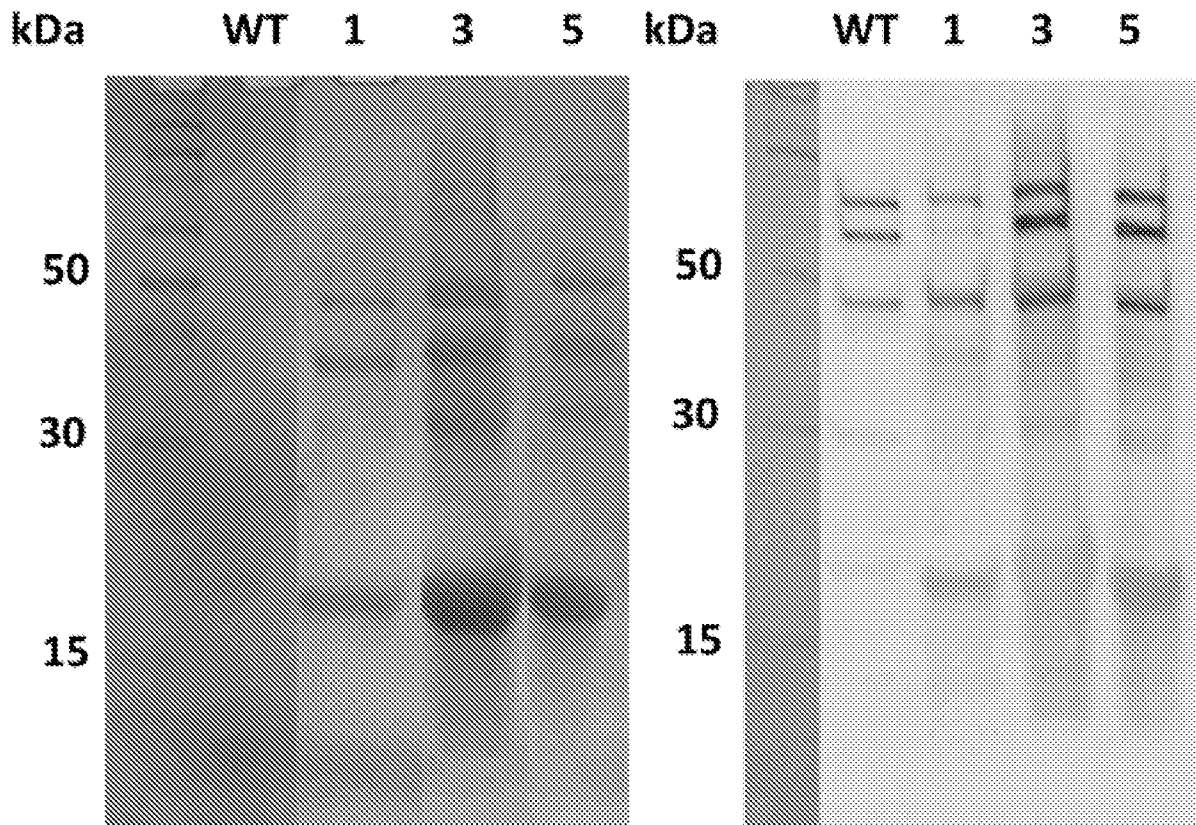
Figure 22

Figure 23

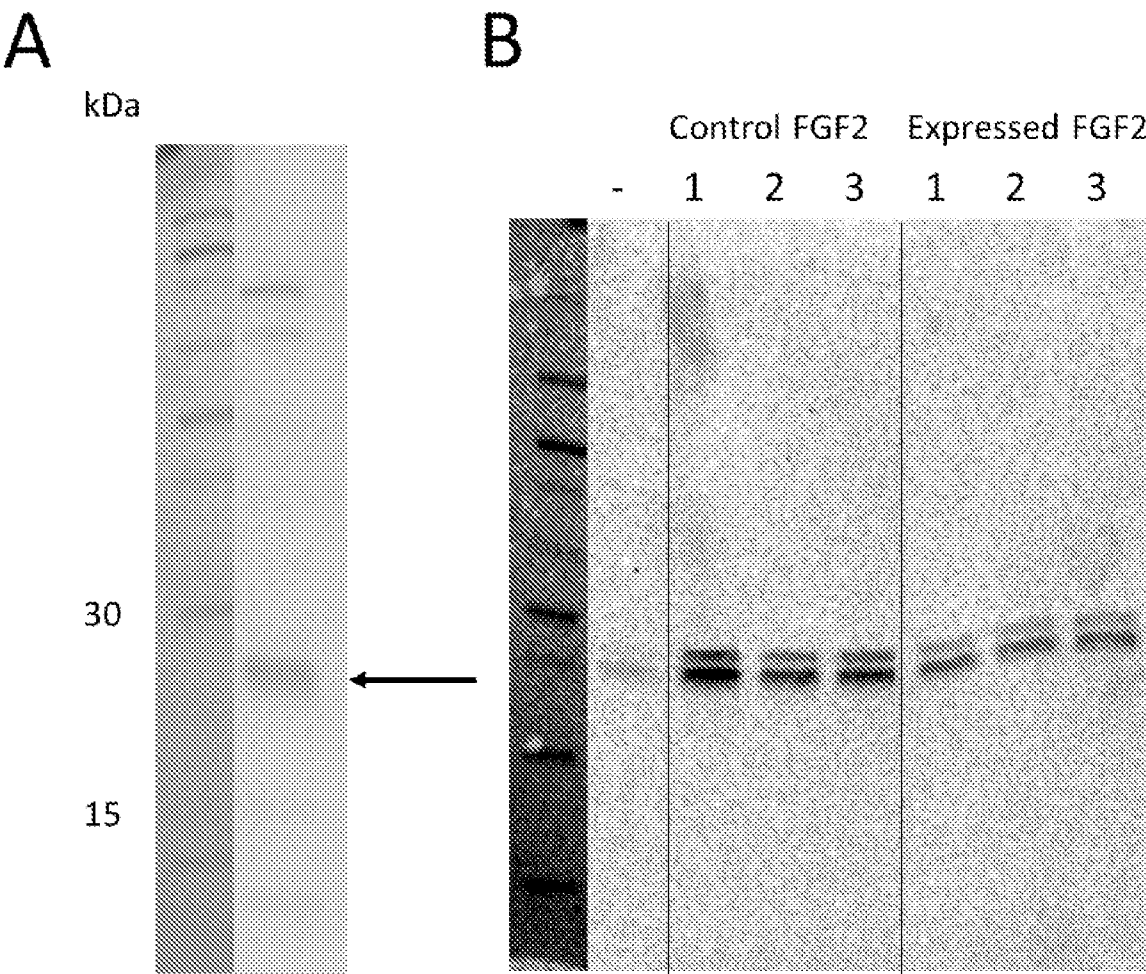


Figure 24

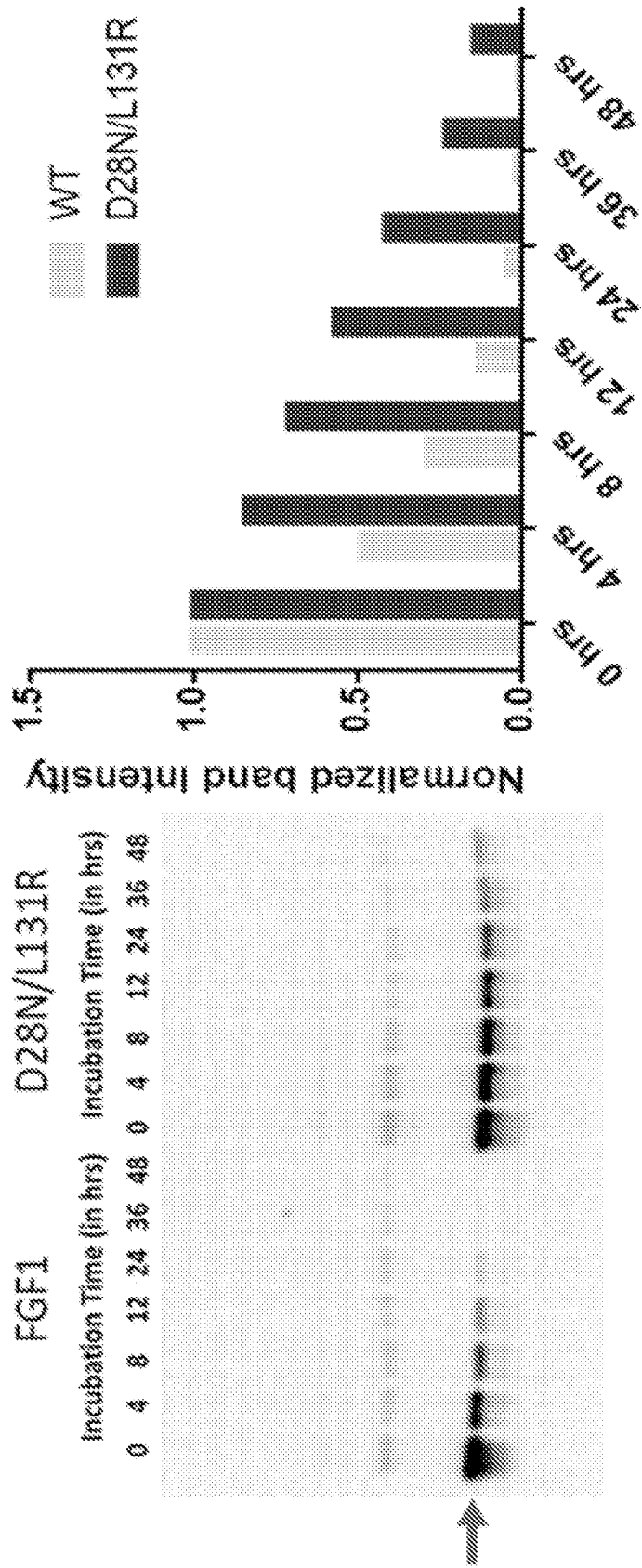


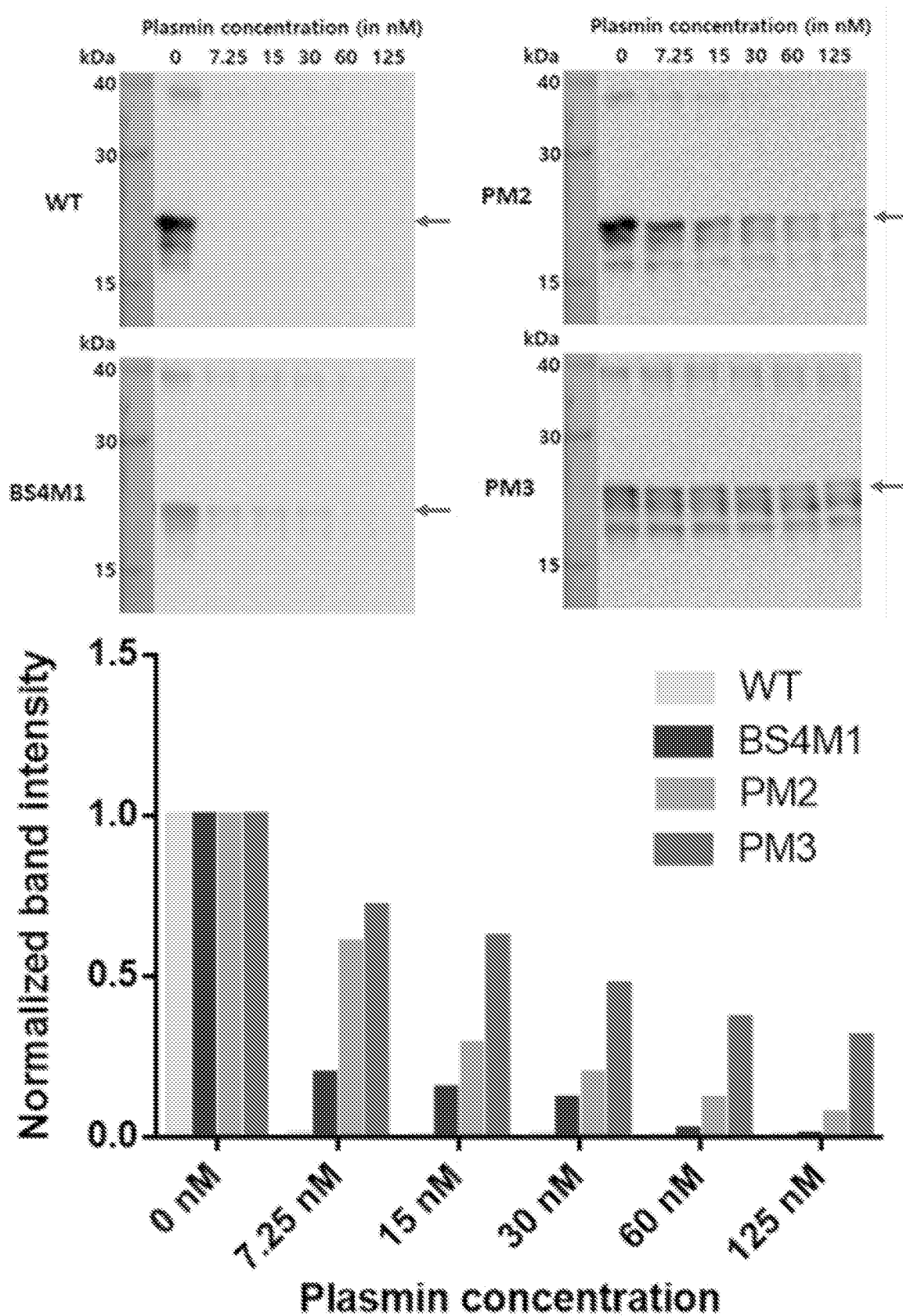
Figure 25

Figure 26

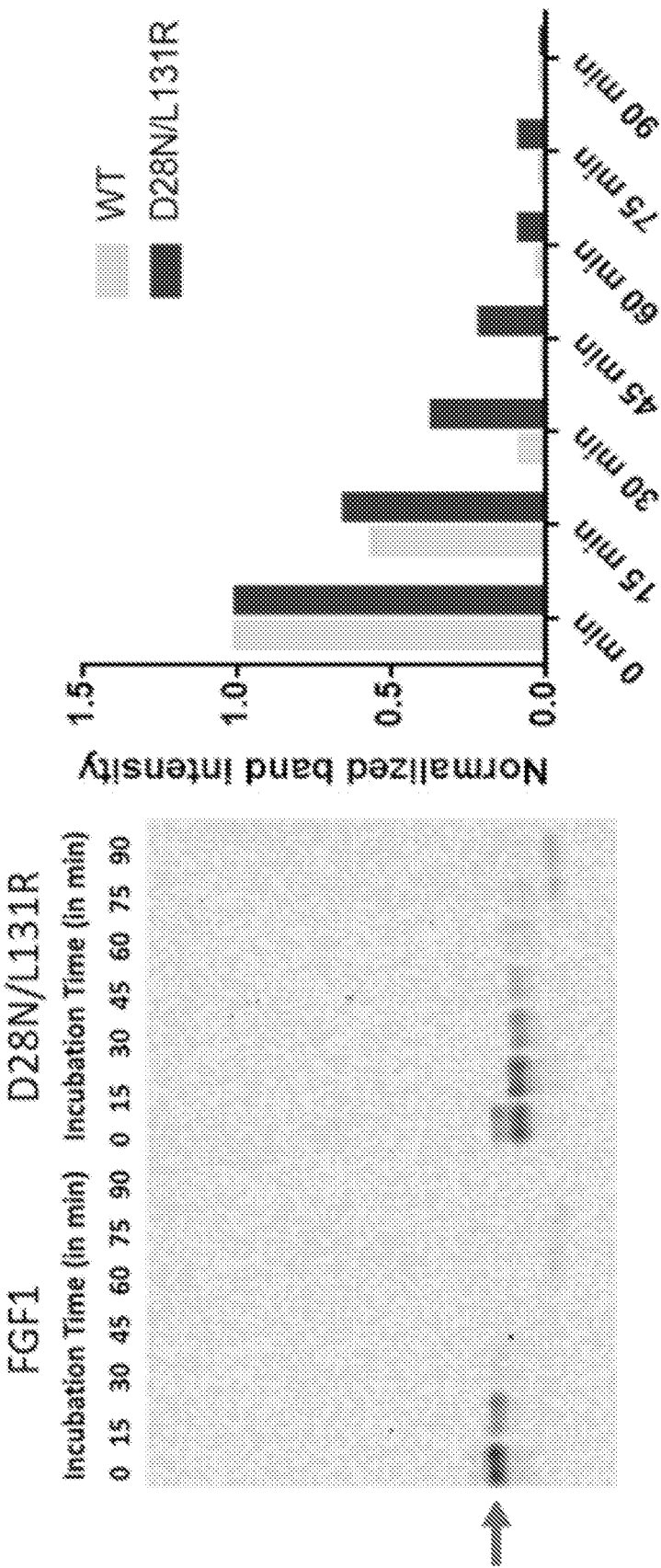


Figure 27

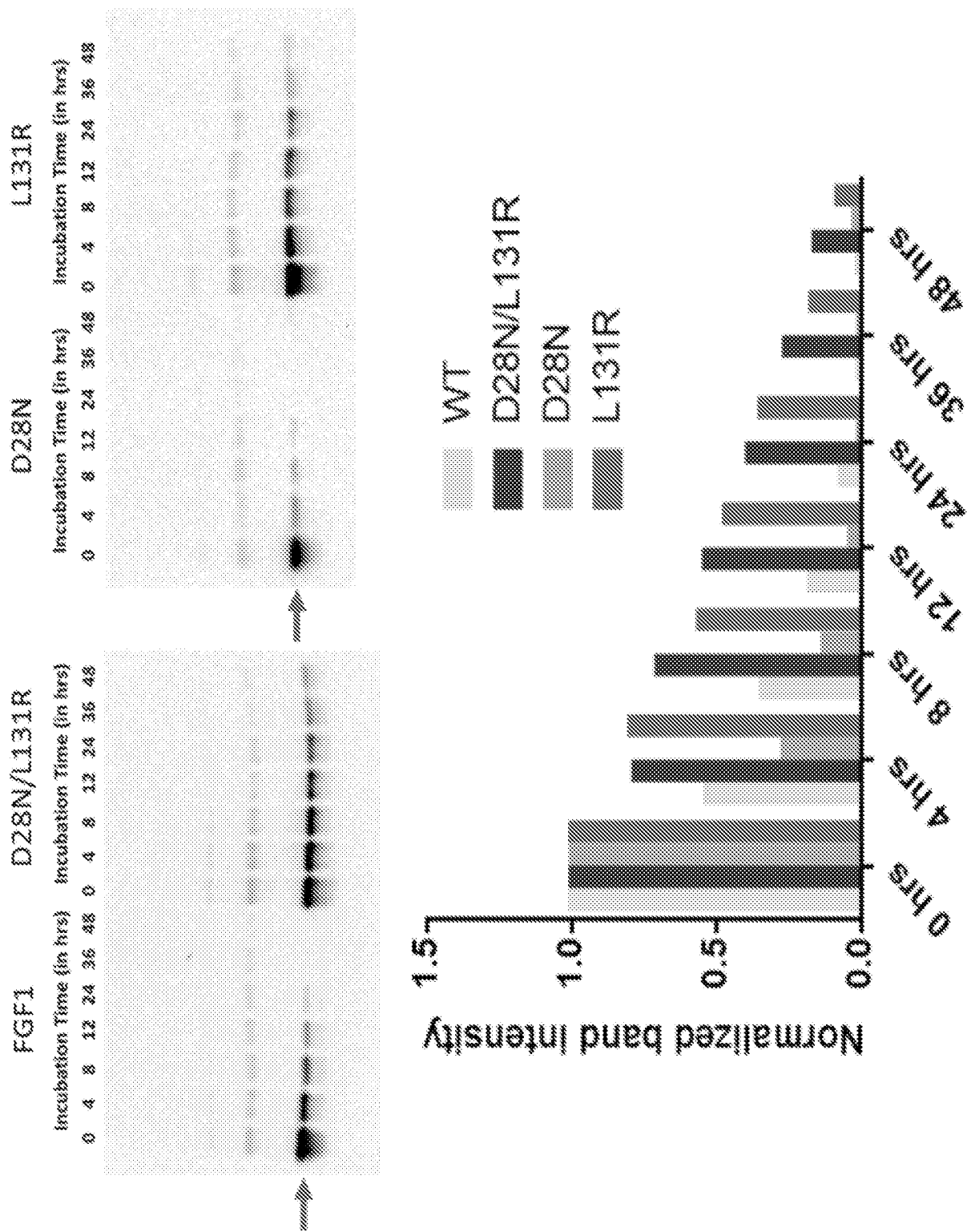


Figure 28

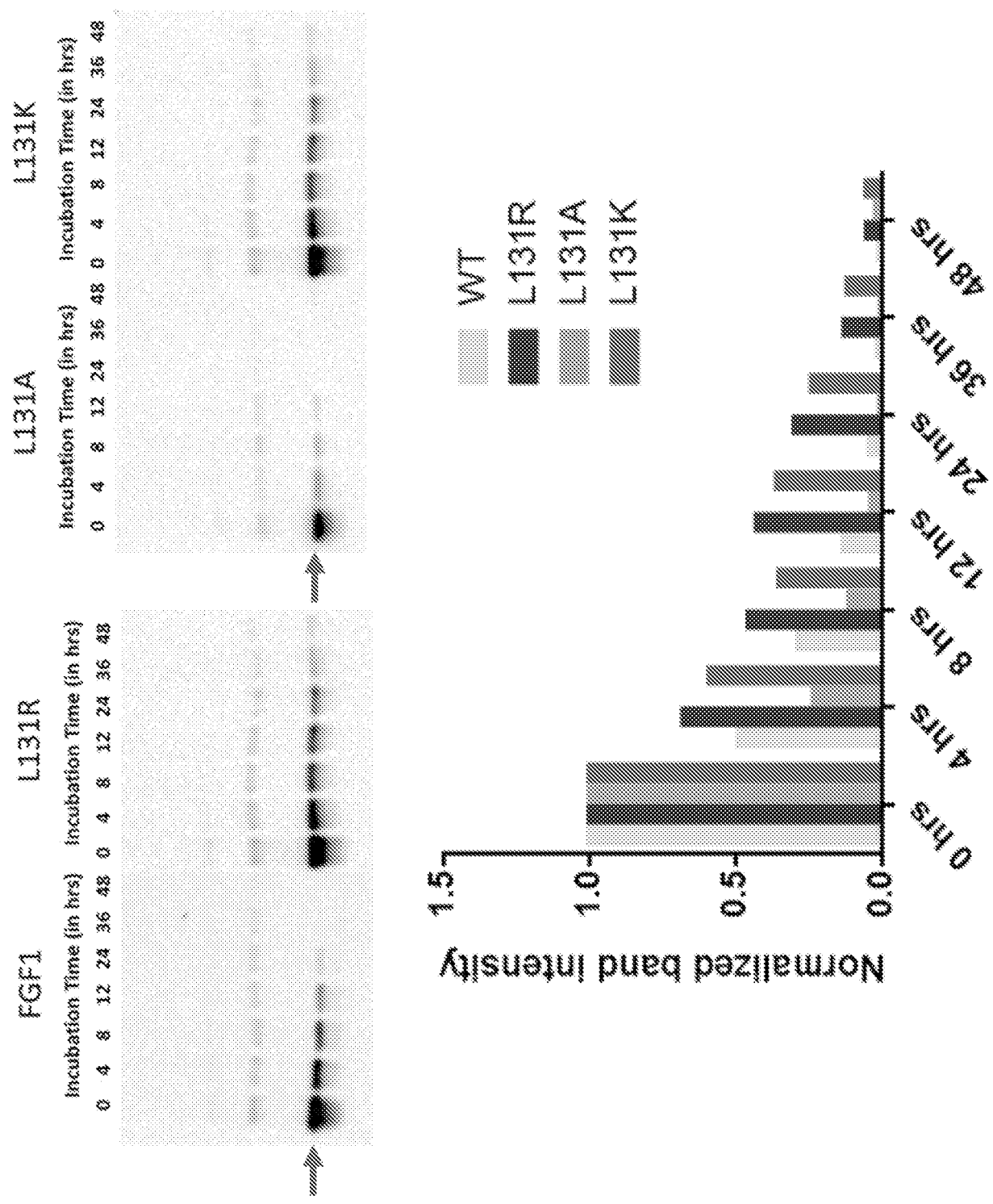


Figure 29

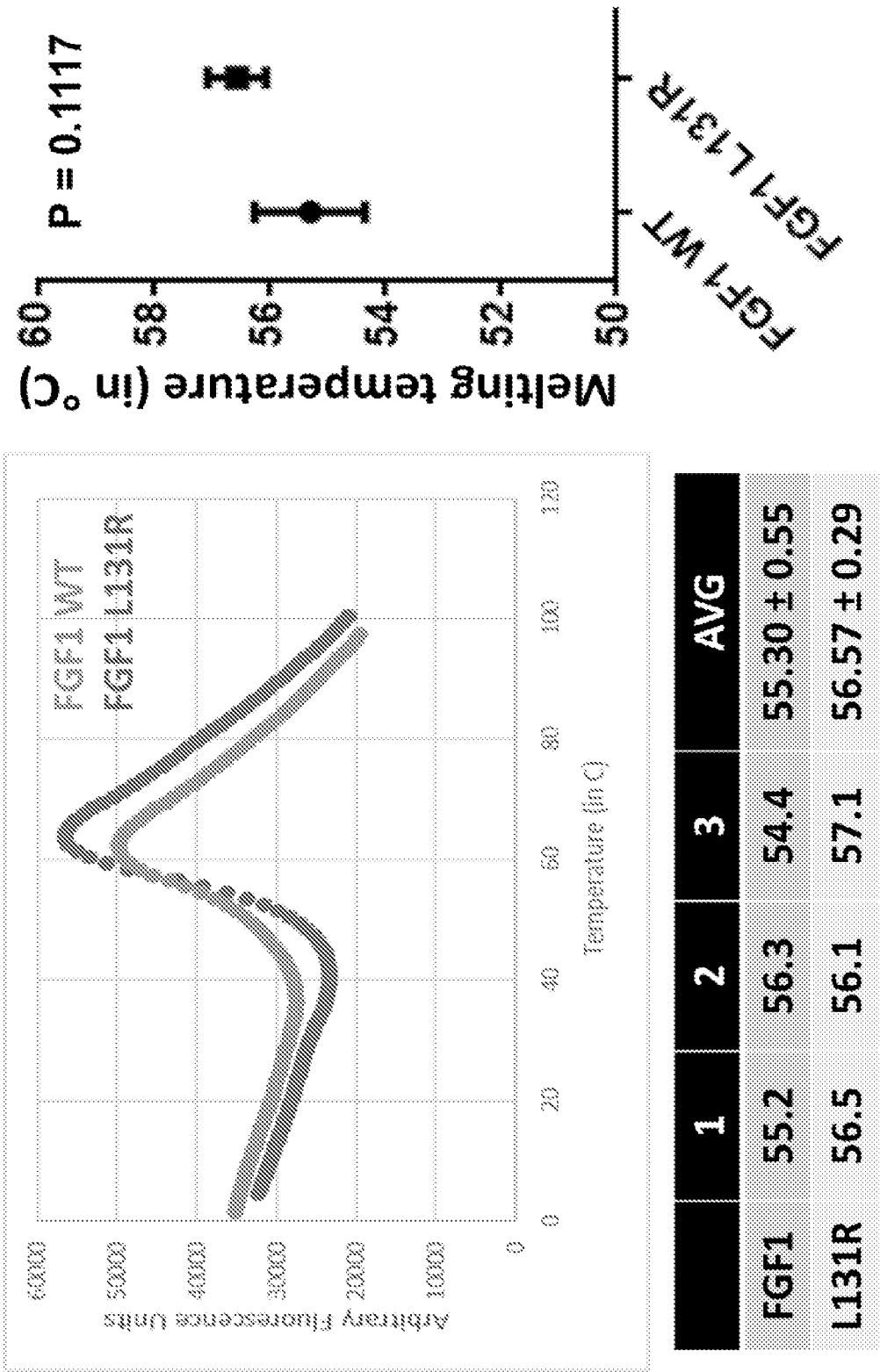


Figure 30

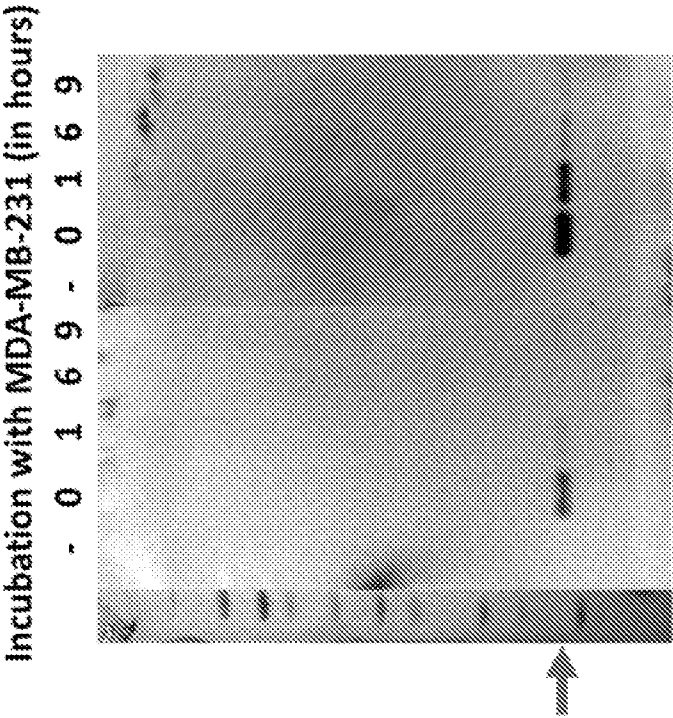
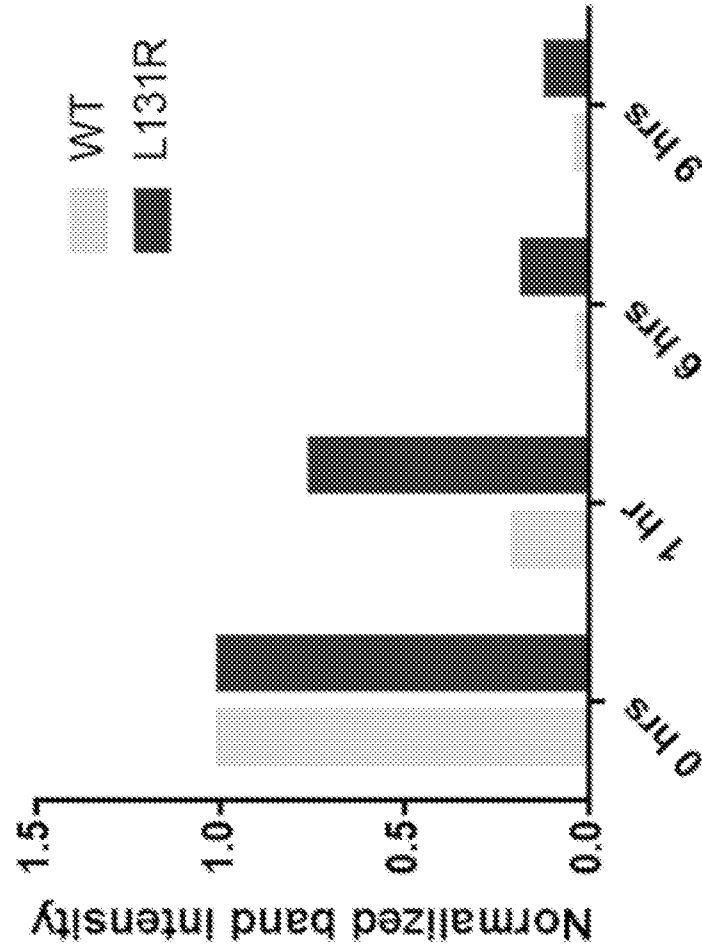
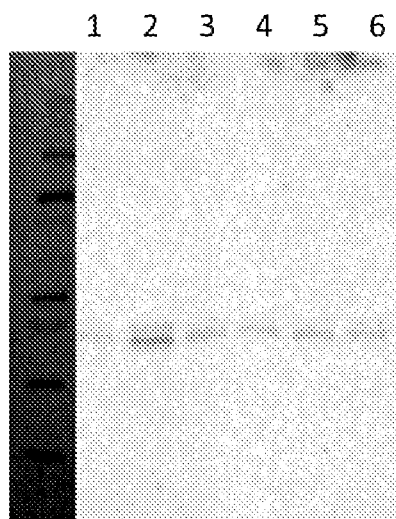
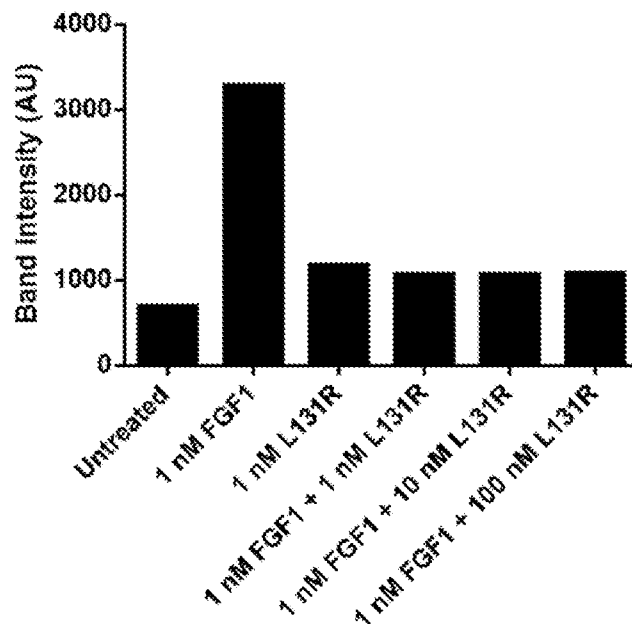


Figure 31

NIH3T3 ERK Phosphorylation

15 hr pulse, α -phosphoERK

Sample #:

1 = Untreated

2 = 1 nM FGF1

3 = 1 nM L131R

4 = 1 nM FGF1 + 1 nM L131R

5 = 1 nM FGF1 + 10 nM L131R

6 = 1 nM FGF1 + 100 nM L131R

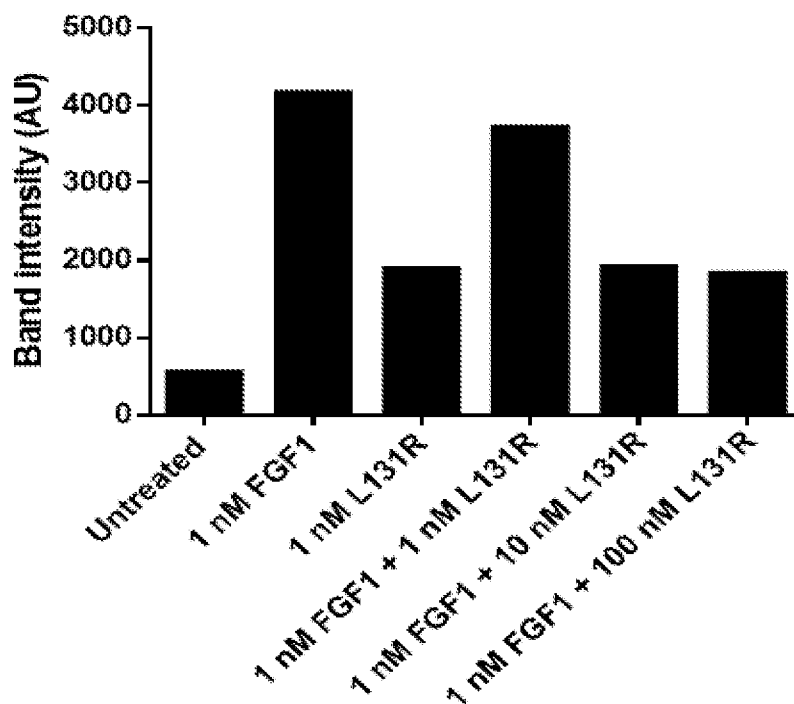
10 hour pulse

Figure 32

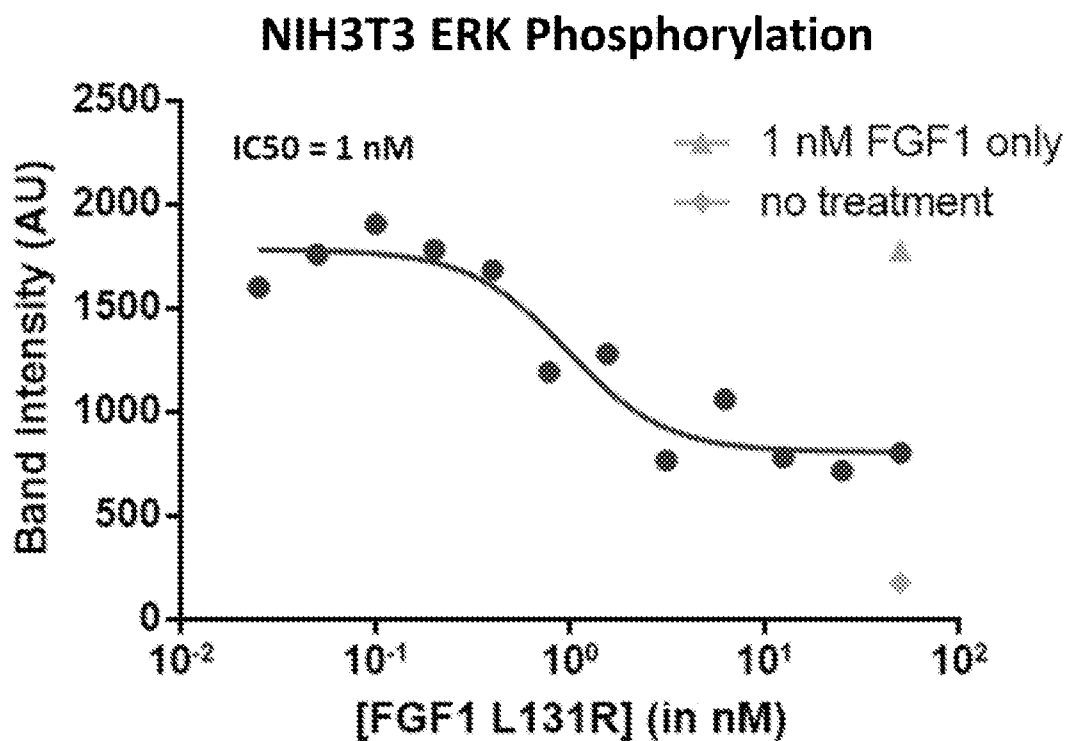
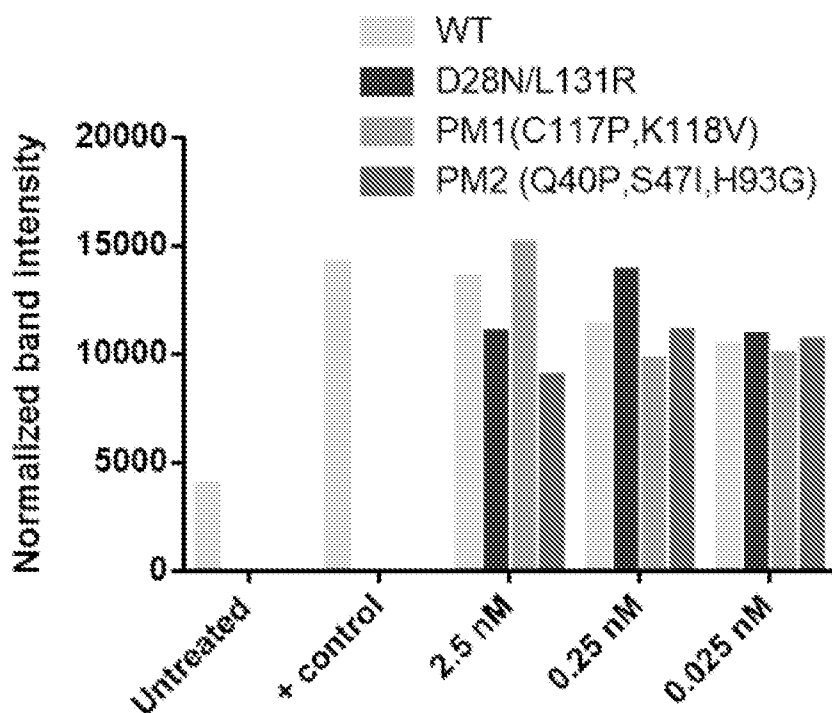
**NIH3T3 ERK phosphorylation (15 min)**

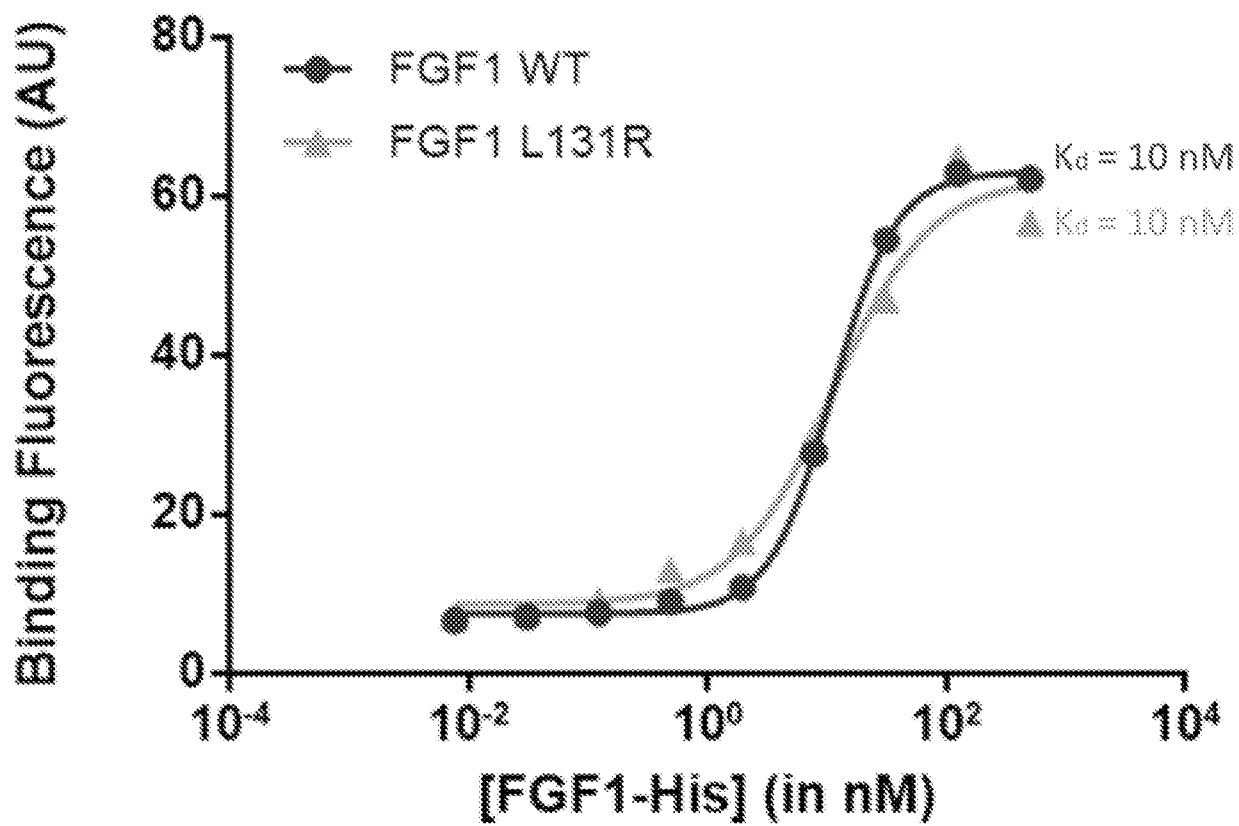
Figure 33

Figure 34A

CH1																							
EU Index	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138		
IgG1	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	G		
IgG2	A	S	T	K	G	P	S	V	F	P	L	A	P	C	S	R	S	T	S	E	S		
IgG3	A	S	T	K	G	P	S	V	F	P	L	A	P	C	S	R	S	T	S	G	G		
IgG4	A	S	T	K	G	P	S	V	F	P	L	A	P	C	S	R	S	T	S	E	S		
EU Index	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159		
IgG1	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N		
IgG2	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N		
IgG3	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N		
IgG4	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N		
EU Index	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180		
IgG1	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	Q	S	S	G	L	Y		
IgG2	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	Q	S	S	G	L	Y		
IgG3	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	Q	S	S	G	L	Y		
IgG4	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	Q	S	S	G	L	Y		
EU Index	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201		
IgG1	S	L	S	S	V	V	T	V	P	S	S	S	L	G	T	Q	T	Y	I	C	N		
IgG2	S	L	S	S	V	V	T	V	P	S	S	N	F	G	T	Q	T	Y	T	C	N		
IgG3	S	L	S	S	V	V	T	V	P	S	S	S	L	G	T	Q	T	Y	T	C	N		
IgG4	S	L	S	S	V	V	T	V	P	S	S	S	L	G	T	K	T	Y	T	C	N		
EU Index	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220				
IgG1	V	N	H	K	P	S	N	T	K	V	D	K	K	V	E	P	K	S	C				
IgG2	V	D	H	K	P	S	N	T	K	V	D	K	T	V	E	R	K	C	C				
IgG3	V	N	H	K	P	S	N	T	K	V	D	K	R	V	E	L	K	T	P				
IgG4	V	D	H	K	P	S	N	T	K	V	D	K	R	V	E	S	K	Y	G				
Hinge										Fc >													
EU Index	221				222	223	224	225	226	227	228												
IgG1	D				K	T	H	T	C	P	P												
IgG2					V		E		C	P	P												
IgG3	L	G	D		T	T	H	T	C	P	R	C	P	E	P	K	S	C	D	T	P	P	
IgG4							P	P	C	P	S												
EU Index																							
IgG1																							
IgG2																							
IgG3	P	C	P	R	C	P	E	P	K	S	C	D	T	P	P	P	C	P	R	C	P		
IgG4																							
										Fc >													
EU Index											229	230	231	232	233	234	235	236					
IgG1											C	P	A	P	E	L	L	G					
IgG2											C	P	A	P	P	V	A						
IgG3	E	P	K	S	C	D	T	P	P	P	C	P	R	C	P	A	P	E	L	L	G		
IgG4											C	P	A	P	E	F	L	G					

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Figure 34B

CH2																					
EU Index	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257
IgG1	G	P	S	V	F	L	F	P	P	K	P	K	D	T	L	N	I	S	R	T	P
IgG2	G	P	S	V	F	L	F	P	P	K	P	K	D	T	L	N	I	S	R	T	P
IgG3	G	P	S	V	F	L	F	P	P	K	P	K	D	T	L	N	I	S	R	T	P
IgG4	G	P	S	V	F	L	F	P	P	K	P	K	D	T	L	N	I	S	R	T	P
EU Index	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278
IgG1	E	V	T	C	V	V	V	D	V	S	H	E	D	P	E	V	K	F	N	W	Y
IgG2	E	V	T	C	V	V	V	D	V	S	H	E	D	P	E	V	S	F	N	W	Y
IgG3	E	V	T	C	V	V	V	D	V	S	H	E	D	P	E	V	S	F	N	W	Y
IgG4	E	V	T	C	V	V	V	D	V	S	H	E	D	P	E	V	S	F	N	W	Y
EU Index	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299
IgG1	V	D	G	V	E	V	H	N	A	K	T	K	P	R	E	E	G	Y	N	S	T
IgG2	V	D	G	V	E	V	H	N	A	K	T	K	P	R	E	E	G	F	N	S	T
IgG3	V	D	G	V	E	V	H	N	A	K	T	K	P	R	E	E	G	Y	N	S	T
IgG4	V	D	G	V	E	V	H	N	A	K	T	K	P	R	E	E	G	F	N	S	T
EU Index	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320
IgG1	Y	R	V	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K
IgG2	F	G	V	V	S	V	L	T	V	V	H	Q	D	W	L	N	G	K	E	Y	K
IgG3	F	R	V	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K
IgG4	Y	R	V	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K
EU Index	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	
IgG1	C	K	V	S	N	K	A	L	P	A	P	I	E	K	T	I	S	K	A	K	
IgG2	C	K	V	S	N	K	L	P	A	P	I	E	K	T	I	S	K	T	K		
IgG3	C	K	V	S	N	K	A	L	P	A	P	I	E	K	T	I	S	K	T	K	
IgG4	C	K	V	S	N	K	L	P	A	P	I	E	K	T	I	S	K	A	K		
CH3																					
EU Index	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361
IgG1	G	G	P	R	E	P	Q	V	Y	T	L	P	P	S	R	D	E	L	T	K	N
IgG2	G	G	P	R	E	P	Q	V	Y	T	L	P	P	S	R	E	E	N	T	K	N
IgG3	G	G	P	R	E	P	Q	V	Y	T	L	P	P	S	R	E	E	N	T	K	N
IgG4	G	G	P	R	E	P	Q	V	Y	T	L	P	P	S	G	E	E	N	T	K	N
EU Index	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382
IgG1	G	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E
IgG2	G	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E
IgG3	G	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E
IgG4	G	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E
EU Index	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403
IgG1	S	N	G	G	P	E	N	N	Y	K	T	T	P	P	V	L	D	S	O	G	S
IgG2	S	N	G	G	P	E	N	N	Y	K	T	T	P	P	N	L	D	S	O	G	S
IgG3	S	G	G	G	P	E	N	N	Y	N	T	T	P	P	N	L	D	S	O	G	S
IgG4	S	N	G	G	P	E	N	N	Y	K	T	T	P	P	V	L	D	S	O	G	S
EU Index	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424
IgG1	F	F	L	Y	S	K	L	T	V	D	K	S	R	W	Q	Q	G	N	V	F	S
IgG2	F	F	L	Y	S	K	L	T	V	D	K	S	R	W	Q	Q	G	N	V	F	S
IgG3	F	F	L	Y	S	K	L	T	V	D	K	S	R	W	Q	Q	G	N	V	F	S
IgG4	F	F	L	Y	S	L	T	V	D	K	S	R	W	Q	E	G	N	V	F	S	
EU Index	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445
IgG1	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P
IgG2	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P
IgG3	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P
IgG4	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	L
EU Index	446	447																			
IgG1	G	K																			
IgG2	G	K																			
IgG3	G	K																			
IgG4	G	K																			