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(54) Title: INSULIN-LIKE GROWTH FACTOR 2 (IGF2) BINDING AGENTS

(57) Abstract: This invention relates to mutant polypeptides comprising the IGF2 binding domain of the Insulin-like Growth Factor 2 Receptor (IGF2R) with residue P1597 is substituted for a different residue, for example H or K. Other residues which may be mutated include S1543, E1544, K1545, G1546, L1547, Q1569, S1602, G1603 and/or K1631. These IGF2 binding domains display dramatically increased binding affinity for IGF2 compared to both the wild-type and previously identified mutants and may be useful for example in cancer therapy.

Insulin-like Growth Factor 2 (IGF2) Binding Agents

Field

This invention relates to binding agents for use in the sequestration of Insulin-like Growth Factor 2 (IGF2), for example, in the treatment of cancer.

Background

Insulin-like Growth Factor 2 (IGF2) is a small mitogenic peptide hormone that functions principally during embryonic growth, where its activity is tightly regulated, but is also frequently deregulated in tumours, which predominantly express IGF2 rather than IGF1. Like IGF1, IGF2 exerts its mitogenic affect predominantly by signalling through the IGF1 receptor (IGF1R), but also unlike IGF1 through isoform A of the Insulin receptor, both leading to tyrosine kinase activation and stimulation of both the mitogen-activated protein (MAP) kinase and PKB/AKT signalling.

Many therapies targeting IGF1R have failed in the clinic. This failure may be due to redundancy, as IGF2 still functions through the Insulin receptor. Furthermore, inhibition of IGF1 signalling causes a feedback loop via the pituitary gland, which produces more growth hormone, which in turn tells the liver to produce more IGF1 [1]. Inhibition of IGF2 signalling does not cause such a feedback loop and preferential targeting of IGF2 over IGF1 may reduce negative feedback effects.

The genes *Igf2* and *Igf2r* are imprinted in mammals, and code for the ligand and cell growth promoter, insulin-like growth factor 2 (IGF2) and the mannose 6-phosphate/IGF2 receptor (M6P/IGF2R or IGF2R), respectively [2, 3]. As imprinted genes, both genes are mono-allelically expressed.

Unlike products of other mammalian imprinted genes, IGF2 and M6P/IGF2R are unusual because they specifically bind with high affinity[4-10]. IGF2 binding is specific for domain 11 of the 15 extra-cellular domains of IGF2R. Membrane bound IGF2R acts to negatively regulate free IGF2 levels by receptor internalisation and intra-cellular degradation of IGF2 [2].

Loss of function of *Igf2r* through disruption of the maternal allele results in *Igf2* dependent overgrowth and fatality[11-15]. Loss of function of IGF2R and gain of function of IGF2 through somatic mutation and increased expression, respectively, are also frequently observed in human cancer[16-20]. Conversely, titration of IGF2 via gain of function of *Igf2r*, for example by bi-allelic expression, leads to an *Igf2* dependent growth reduction [21]. The effects of *Igf2r* on *Igf2* dependent growth are thus modified by two fold changes in allelic dosage, suggesting also that the normal capacity of IGF2R to reduce IGF2 bioavailability depends on the affinity of domain 11 for IGF2.

Wild-type IGF2R domain 11 is selective for IGF2 over IGF1 because of a key specific interaction with threonine 19 of IGF2. The IGF2 binding site within domain 11 of human IGF2R consists of a hydrophobic pocket centred on the CD loop, surrounded by polar and charged residues in the AB, FG and HI loops that complement surface charge on IGF2[3]. The exception to this is an otherwise unfavourable charge-charge interaction between E1544 on the AB loop and D23 on IGF2 [6, 22, 23]. Domain 13 interacts with the AB loop of domain 11, breaking this interaction in the full length receptor and thus contributes to the stability of the complex by decreasing the 'off-rate' (k_{off}) of the IGF2 interaction compared to domain 11 alone [22]. Mutations in this structurally sensitive AB loop have, however, resulted in isolated domain 11 analogues with increased affinity for IGF2 (e.g. domain $11^{E1544K, K1545S, L1547V}$ or clone AB3, K_D = 15 nM)[3, 23]$^{, 57}$. The higher affinity domain 11^{AB3} AB loop mutant also led to the solution structure of a stable 24.2 kDa complex (IGF2: domain 11^{AB3})[3]. Domain 11^{AB3} retains a relatively fixed conformation of the CD loop upon complex formation, and the mutated AB loop moves to accommodate IGF2 helix 1 and the packing of IGF2 residues T16 and F19. The FG loop also repositions between helices 2 and 3 of IGF2 to accommodate burial of IGF2 residue L53 in the domain 11^{AB3} binding site. All three of these IGF2 residues are critical for IGF2R binding [22, 24]. Both conformational changes also allow the formation of complementary

hydrophobic surfaces and support a range of H-bonding and salt bridging interactions with amino acids in the other loops[3].

Importantly, IGF2 binding to domain 11 co-evolved with the evolution of mammals, as in primitive mammals (monotremes) IGF2 binds with ten-fold lower affinity (K_D = 250-400 μM vs domain 11^{WT} K_D = 40-60 nM) [3-5, 10]. The structural evolution of domain 11 suggests that the IGF2 binding site has fully evolved in mammals[3].

Summary

The present inventors have unexpectedly identified combinations of mutations in the IGF2 binding domain of the Insulin-like Growth Factor 2 Receptor (IGF2R) which dramatically increase the binding affinity for IGF2 compared to both the wild-type and previously identified mutants.

One aspect of the invention provides a mutant IGF2 binding domain comprising the amino acid sequence of residues 1511 to 1650 of human IGF2R with 30 or fewer, 20 or fewer, 15 or fewer or 10 or fewer of said residues mutated, wherein residue P1597 of said amino acid sequence is substituted for a different residue.

Another aspect of the invention provides a mutant IGF2 binding domain comprising an amino acid sequence which has at least 80% sequence identity, at least 90% sequence identity or at least 95% sequence identity with residues 1511 to 1650 of human IGF2R, wherein residue P1597 of said amino acid sequence is substituted for a different residue.

Another aspect of the invention provides a mutant IGF2 binding domain consisting of the amino acid sequence of residues 1511 to 1650 of human IGF2R with residue P1597 and at least five residues selected from S1543, E1544, K1545, G1546, L1547, Q1569, S1602, G1603 and K1631 substituted for different residues.

Another aspect of the invention provides a mutant IGF2 binding domain consisting of the amino acid sequence of residues 1511 to 1650 of human IGF2R with one of the following sets of substitutions;

1) S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597H and S1602H
2) S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597K and S1602H
3) S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597H and G1603K
4) S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597K and G1603K
5) E1544K, K1545S, L1547V, Q1569R, P1597H, and S1602H
6) E1544K, K1545S, L1547V, Q1569R, P1597K, and S1602H
7) E1544K, K1545S, L1547V, Q1569R, P1597H, S1602H, and G1603K
8) E1544K, K1545S, L1547V, Q1569R, P1597K, S1602H, and G1603K.

Other aspects of the invention provide polypeptides comprising mutant IGF2 binding domains described herein, nucleic acids encoding mutant IGF2 binding domains described herein and methods and uses of such domains, polypeptides and nucleic acids in therapy, for example in the treatment of cancer.

Brief Description of Figures

Figure 1 shows a heat map of $IGF2^{1-67}$ binding to single point mutants in loops of domain 11^{AB5} (left) and domain 11^{AB3} (right) relative to their respective AB loop background. Top panel, effect on the k_{off}, Bottom panel, effect on the K_D. The scale represents the $\log_2$ of the domain $11^{reference}$/ domain 11^{mutant} parameter ratio, where domain $11^{reference}$ is domain 11^{AB5} (left panel) or domain 11^{AB3} (right panel). In this colour code, black indicates increase of the affinity (decrease in the K_D value) or a decrease in the k_{off}, and light grey decrease in affinity (increase in the K_D value) or increase in the off-rate.

Figure 2 shows the effect of combinations of loop mutants on the (fold decrease in 'off rate') of the interaction with IGF2 compared to domain 11^{AB3}. Boxes show the change in the binding free energy contribution, $\Delta\Delta G°$, for the indicated mutants. The loop/s that contains the mutation/s is indicated in each box. The $\Delta\Delta G°$ values were calculated as $\Delta\Delta G° = \Delta G°$domain $11^{mutant} - \Delta G°$domain $11^{AB3} = RT\ln(K_D$ domain $11^{mutant}/K_D$ domain

11^{AB3}).Additive and non-additive contributions of single point mutations are shown when calculated with a box solid line and dashed line, respectively. Domain 11^{AB5} Q1569R P1597H S1602H, K_D 650 pM (on rate 5.90 x10^6 $M^{-1}s^{-1}$ and off rate 0.0038 s^{-1}); domain 11^{AB3} Q1569R P1597H S1602H G1603K, K_D 620 pM (k_{on}1.75 x10^7 $M^{-1}s^{-1}$ and k_{off} 0.0108 s^{-1}). Raw data are shown in Tables 1, 2 and 3.

Figure 3 shows the effect of combinations of loop mutants on the binding free energy ($\Delta G°$) of the interaction with IGF2 compared to domain 11^{AB3}. Boxes show the change in the binding free energy contribution, $\Delta\Delta G°$, for the indicated mutants. The loop/s that contains the mutation/s is indicated in each box. The $\Delta\Delta G°$ values were calculated as $\Delta\Delta G°=\Delta G°$domain 11^{mutant} – $\Delta G°$domain 11^{AB3} = $RT\ln(K_D$ domain $11^{mutant}/K_D$ domain $11^{AB3})$. Additive and non-additive contributions of single point mutations are shown when calculated with a box solid line and dashed line, respectively. Domain 11^{AB5} Q1569R P1597H S1602H, K_D 650 pM (on rate 5.90 x10^6 $M^{-1}s^{-1}$ and off rate 0.0038 s^{-1}); domain 11^{AB3} Q1569R P1597H S1602H G1603K, K_D 620 pM (k_{on}1.75 x10^7 $M^{-1}s^{-1}$ and k_{off} 0.0108 s^{-1}). Raw data are shown in Tables 1, 2 and 3.

Figure 4 shows the thermodynamic profile of the IGF2 interaction with domain 11 variants shown. Data were obtained by fitting the temperature dependence of the dissociation constant according to the van't Hoff equation.

Figure 5 shows a comparison of k_{off} between domain 11^{AB3} and domain 11^{AB5} with additional mutations.

Figure 6 shows that a human IgG1 Fc-domain 11^{AB5} acts as an IGF2 super-antagonist. Surface plasmon resonance sensorgrams of the interaction between Fc-domain 11^{AB5} (a), Fc-domain $11^{AB5-RHH}$ (b), and Fc-domain 11^{I1572A} (c) with IGF1 (dashed), $IGF2^{1-67}$, $IGF2^{1-104}$ and $IGF2^{1-156}$. Recombinant IGF1 and the different IGF2 forms were injected at concentrations ranging from 64 nM to 0.25 nM over Fc-domain 11^{AB5} immobilised on a CM5 surface by antibody capture. The data were fitted to a 1:1 interaction.

Figure 7 shows an Fc-domain 11 pull-down assay of different recombinant IGF2 forms. Recombinant mature IGF2, proIGF2$^{1-104}$ and proIGF2$^{1-156}$ were incubated with Fc-domain 11^{AB5} or with the low binder Fc-domain 11^{I1572A} as a control. Complexes were pulled down using protein A agarose and IGF2 was detected using anti-IGF2 antibody.

Figure 8 shows an Fc-domain 11 pull-down assay as in Fig 7 of the different IGF2 forms produced by tumour cell lines. Supernatants of the HCC cell lines Hep3B and Huh7, and of the NIH 3T3 control cell line expressing pro-IGF2^{R104A} were incubated with Fc-domain 11^{AB5} or with the low binding affinity Fc-domain 11^{I1572A} as a control.

Figure 9 shows the effect of Fc-domain 11^{AB5} in an IGF2-induced hypoglycaemia mouse model. (A) Left: Premixing human IGF2 with Fc-domain 11^{AB5} prevents IGF2-induced hypoglycaemia. Following normalisation of blood glucose levels either 1 mg kg^{-1} IGF2$^{1-67}$ alone (circles, n=4), or 1 mg kg^{-1} IGF2$^{1-67}$ premixed with Fc-domain 11^{AB5} (1:1) (squares, n=3) were injected and blood glucose levels monitored. 2 way ANOVA with Bonferonni post-test p=0.0204. Middle: Preloading Fc-domain 11^{AB5} in vivo protects against IGF2-induced hypoglycaemia. Either Fc-domain 11^{AB5} (squares, n=4) or PBS (circles, n=3) were injected immediately after induction of anaesthesia, after an additional 30 min 1 mg kg^{-1} IGF2$^{1-67}$ was administered and blood glucose levels monitored. 2 way ANOVA with Bonferonni post-test p=0.0358. Right: Fc-domain 11^{AB5} can reverse IGF2-induced hypoglycaemia *in vivo*. 1 mg kg^{-1} IGF2$^{1-67}$ was injected immediately after induction of anaesthesia, after an additional 30 min either Fc-domain 11^{AB5} (squares, n=3) or PBS (circles, n=3) were administered and blood glucose levels monitored. (B) Fc-domain 11$^{AB5-RHH}$ (IGF2-TRAP) abrogates an IGF2$^{1-67}$ -induced hypoglycaemia in a mouse model to a greater extent than Fc-domain 11^{AB5}. As in (A) Left above, mice were anaesthetized (time= -30 min) and blood glucose levels were allowed to stabilize for 30 mins (expressed relative to this blood glucose level). Subsequently (time= 0 min), they received 1 mg kg^{-1} IGF2$^{1-67}$ alone (circles n=4), or premixed with Fc-domain 11^{AB5} (squares) or with Fc-domain 11$^{AB5-RHH}$ (triangles) at a molar ratio of 1:1 (n=3) (p=0.0133) or 1:0.5 (n=3) (p=0.0023), respectively.

When using a molar ratio of 1:0.23 Fc-domain $11^{AB5-RHH}$ (triangles and dotted line), is a more efficient IGF2 antagonist than Fc-domain 11^{AB5} (squares and dotted line) (n=3; p=0.0026). Statistics 2 way ANOVA with Bonferonni post test.

Figure 10 shows the effect of Fc-domain 11^{AB5} on cell viability, proliferation and apoptosis on the human hepatocellular carcinoma cell lines Hep 3B2.1-7 and Huh-7D12. Cell viability: Fc-domain 11^{AB5}, black closed squares and solid line; Fc-domain 11^{I1572A}, grey squares; OSI-906, open squares and dashed line. Proliferation: untreated, black; 0.5 uM Fc-domain 11^{AB5}, dashed line; 1.5uM Fc-domain 11^{AB5}, dotted line; 1 uM OSI-906, grey line; 2 uM U0126, light grey line.

Figure 11 shows Fc-domain $11^{AB5-RHH}$ (IGF2-TRAP) on cell signaling and viability of SKNMC-$IGF2^{1-67}$ (A) IGF2-TRAP decreases the cell viability of IGF2-autocrine SKNMC-$IGF2^{1-67}$ cell line compared to luciferase only SKNMC control. (B) Western blot of Fc-domain IGF2-TRAP effect on IGF2 dependent phosphorylation of IGF1R and IR-A, and of AKTS473, in SKNMC-$IGF2^{1-67}$.

Figure 12 shows Fc-domain $11^{AB5-RHH}$ (IGF2-TRAP) inhibition of IGF2 signalling in vivo.
(A) IGF2-TRAP reduces IGF2 dependent xenograft growth (SKNMC-$IGF2^{1-67}$). $5x10^6$ per injection site in CD-1 nude mice, single infused concentration of IGF2-TRAP (40 mg kg-1 per week) (green, n=10) or PBS control (blue, n=7, n=2 injection error, n=1 unexplained death). (p=0.002, Wilcoxon test across all time points). (B) IGF2-TRAP resulted in reduced levels of serum IGF2 independent of IGF1, Growth Hormone (GH) or IGFBP levels (Day 28, control PBS n= 10 out of 10, IGF2-TRAP n=6 out of 7).

Figure 13 shows IGF2-TRAP synergistic screening validation dose response curves for PI3 kinase inhibitors (PF-04691502 and Pictilisib) in the presence (light grey line) and absence (dark grey line) of the IGF2-TRAP in Ewing sarcoma cell line (SKNMC). IC50 values are shown. Leftward shift indicates synergism ($P<0.0001$ when comparing the IC50 of drug alone vs.

drug + IGF2-TRAP using the F-test). Asterisks indicate the concentrations at which there are synergistic interactions (Q>1.15).

Figure 14 IGF2-TRAP modifies the molecular distribution of IGF2 in human serum. Normal (left) and NICTH (right) sera were fractionated in a gel filtration column at neutral pH, alongside a molecular weight calibration marker, before and after incubation and depletion with IGF2-TRAP-loaded protein G beads. Elution fractions were analysed by western blot.

Detailed Description

This invention relates to mutant IGF2 binding domains in which residue P1597 and optionally one or more other residues are mutated. Proline has an important structural function in proteins, so the beneficial effect of mutation at P1597 was unexpected.

The mutant IGF2 binding domains have increased affinity for IGF2 compared to the wild-type IGF2 binding domain (residues 1511 to 1650) of human IGF2R, for example 20 fold or greater, 30 fold or greater, 50 fold or greater, 75 fold or greater or 100 fold or more greater affinity. In some embodiments, a mutant IGF2 binding domain may have 50 to 75 fold greater affinity for IGF2 than the wild-type human IGF2 binding domain.

A mutant IGF2 binding domain described herein may bind to IGF2 with a lower dissociation constant (K_D) than the wild-type IGF2 binding domain of IGF2R (K_D of 40-60nM). For example, a mutant IGF binding domain may bind IGF2 with a Kd of 10nM or less, 5nM or less or 1nM or less. In some embodiments, a mutant IGF2 binding domain may bind IGF2 with a Kd of 0.1 nM to 1 nM, preferably 0.5 nM to 1 nM.

Preferably, the specificity of the mutant IGF2 binding domain is similar to that of the wild-type IGF2 binding domain (residues 1511 to 1650) of human IGF2R, i.e. it binds to IGF2 and shows no binding or substantially no binding to IGF1.

Wild-type human IGF2R has the amino acid sequence of SEQ ID NO: 1 and database entry NP_000867.1 GI: 4504611 and is encoded by the nucleotide sequence of SEQ ID NO: 2 and database entry (NM_000876.1 GI: 4504610). The IGF2 binding domain is located at residues 1511 to 1650 of the full length human IGF2R sequence (SEQ ID NO: 1). The amino acid sequence of residues 1511 to 1650 of human IGF2R is shown in SEQ ID NO: 3.

Residues are numbered herein with reference to the full length human IGF2R sequence of SEQ ID NO: 1 unless otherwise stated. A residue identified by its position in the human IGF2R sequence may easily be identified in a truncated or variant IGF2R sequence, such as the IGF2 binding domain sequence shown in SEQ ID NO: 3, or variants thereof, using standard sequence analysis tools.

A mutant IGF2 binding domain described herein may consist of the amino acid sequence of residues 1511 to 1650 of wild-type human IGF2R with 30 or fewer amino acid residues mutated or otherwise altered, preferably 20 or fewer, 15 or fewer, 10 or fewer, or 8 or fewer. The mutated amino acid residues in the mutant IGF2 binding domain may include substitution at residue P1597 and optionally further substitutions at one or more of residues S1543, E1544, K1545, G1546, L1547, Q1569, S1602, G1603 and K1631 as described herein.

Unless otherwise stated, an amino acid residue in the mutant IGF2 binding domain may be mutated by insertion, deletion or substitution of one or more amino acids relative to the wild-type IGF2R amino acid sequence. Such alterations may be caused by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the encoding nucleic acid.

In some embodiments, one or both of residues F1567 and I1572 are not mutated in the mutant IGF2 binding domain.

Preferably, the mutant IGF-II domain retains the β-barrel structure of the wild-type domain.

In some preferred embodiments, the IGF2 binding domain may consist of the amino acid sequence of residues 1511 to 1650 of human IGF2R with residue P1597 substituted for a different residue and one, two, three, four, five, six, seven, eight or all nine residues selected from S1543, E1544, K1545, G1546, L1547, Q1569, S1602, G1603 and K1631 substituted for different residues.

The mutant IGF2 binding domain may share at least 80% sequence identity with the wild-type amino acid sequence of residues 1511 to 1650 of human IGF2R, at least 85%, at least 90%, at least 95% or at least 98%. The sequence may share at least 80% sequence similarity with the wild-type sequence, at least 85% similarity, at least 90% similarity, at least 95% similarity or at least 99% similarity.

Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine.

Sequence similarity and identity are commonly defined with reference to the algorithm GAP (Wisconsin GCG package, Accelerys Inc, San Diego USA). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST or TBLASTN (which use the method of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 405-410), NBLAST and XBLAST (Altschul et al., 1991, *Nucleic Acids Res.*, 25:3389-3402), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* 85: 2444-2448), the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol. 147*: 195-197), Gapped BLAST, BLAST-2, WU-BLAST-2 (Altschul et al., 1996, *Methods in Enzymology*, 266:460-480), ALIGN, ALIGN-2 (Genentech, CA USA), Megalign (DNASTAR), and the Bestfit program (Wisconsin Sequence

Analysis Package, Genetics Computer Group, WI USA 53711), generally employing default parameters.

Sequence comparisons are preferably made over the full-length of the relevant sequence described herein.

In the mutant IGF2 binding domain, residue P1597 may be substituted for an uncharged polar residue, such as S, N or Q, an aliphatic residue such as L, or a basic residue, such as K, R or H. Preferably, residue P1597 is substituted for K or H.

In addition to substitution at residue P1597, residues Q1569, S1602 and/or G1603 of said amino acid sequence may also be substituted for a different residue.

For example, Q1569 of said amino acid sequence may be substituted for a basic residue, such as K, R or H, most preferably R. S1602 of said amino acid sequence may be substituted for N or Q or a basic residue, such as K, R or H, most preferably H. G1603 of said amino acid sequence may be substituted for a basic residue, such as K, R or H, most preferably K.

In addition to substitution at P1597 and optionally Q1569, S1602 and/or G1603, the mutant IGF2 binding domains described herein may further comprise one, two, three or more substitutions in the AB loop at residues 1542 to 1547 of the wild type IGF2R amino acid sequence.

In some embodiments, the AB loop of the mutant IGF2 binding domain may contain no more than eight substituted amino acid residues in total.

S1543 of the wild-type IGF2R amino acid sequence may be substituted for a different residue, for example an aliphatic residue, such as G, A, V, L or I, most preferably A.

E1544 of the wild type IGF2R amino acid sequence may be substituted for an aliphatic residue such as A, V, L or I, a basic residue such as K, R

or H, a sulphur containing residue such as C or M, or a hydroxyl residue, such as S or T. More preferably, E1544 may be substituted for a polar residue, such as S, or a basic residue, such as K, R or H. For example, the mutant IGF2 binding domain may include a K, R, H or S residue at position 1544 (position 34 in SEQ ID NO: 3). In some embodiments, E1544 is substituted for R or K, preferably K. In other embodiments, E1544 is not mutated.

K1545 of the wild type IGF2R amino acid sequence may be substituted for a different residue, most preferably G or S.

G1546 of the wild type IGF2R amino acid sequence may be substituted for a different residue, most preferably a hydrophobic residue, such as W.

L1547 of the wild type IGF2R amino acid sequence may be substituted for a different residue, most preferably an aliphatic residue, such as G or V.

In some embodiments, the mutant IGF2 binding domain may comprise the substitutions S1543A, E1544K, K1545G, G1546W and L1547G i.e. the mutant IGF2 binding domain may comprise the sequence YAKGWG at residues 1542 to 1547 (i.e. the AB loop region).

In other embodiments, the mutant IGF2 binding domain may comprise the substitutions E1544K, K1545S, and L1547V i.e. the mutant IGF2 binding domain described herein may further comprise the sequence YSKSGV at residues 1542 to 1547 (i.e. the AB loop region).

In preferred embodiments, a mutant IGF2 binding domain may comprise substitutions at 6 or more, 7 or more or 8 or more positions selected from S1543, E1544, K1545, G1546, L1547, Q1569, P1597, S1602 and G1603 of the wild-type IGF2R sequence.

In a first group of preferred embodiments, a mutant IGF2 binding domain may comprise substitutions at S1543, E1544, K1545, G1546, L1547, Q1569, P1597 and S1602 of the wild-type IGF2R sequence. For example, a mutant IGF2

binding domain may comprise the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R P1597H and S1602H or the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597K and S1602H. Suitable mutant IGF2 binding domains may comprise the amino acid sequence of residues 1511 to 1650 of human IGF2R with 30 or fewer of said residues mutated; or an amino acid sequence which has at least 80% sequence identity with residues 1511 to 1650 of human IGF2R, as described above.

In some embodiments, the mutant IGF2 binding domain may consist of the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597H and S1602H or the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597K and S1602H.

In a second group of preferred embodiments, a mutant IGF2 binding domain may comprise substitutions at S1543, E1544, K1545, G1546, L1547, Q1569, P1597 and G1603. For example, a mutant domain may comprise the substitutions S1543A, E1544K, K1545G, G1546W, L154G, Q1569R, P1597H and G1603K or the substitutions S1543A, E1544K, K1545G, G1546W, L154G, Q1569R, P1597K and G1603K. Suitable mutant IGF2 binding domains may comprise the amino acid sequence of residues 1511 to 1650 of human IGF2R with 30 or fewer of said residues mutated; or an amino acid sequence which has at least 80% sequence identity with residues 1511 to 1650 of human IGF2R, as described above.

In some embodiments, the mutant IGF2 binding domain may consist of the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions S1543A, E1544K, K1545G, G1546W, L154G, Q1569R, P1597H and G1603K or the substitutions S1543A, E1544K, K1545G, G1546W, L154G, Q1569R, P1597K and G1603K.

In a third group of preferred embodiments, a mutant IGF2 binding domain may comprise substitutions at E1544, K1545, L1547, Q1569, P1597 and S1602 of the wild-type IGF2R sequence. For example, a mutant domain may comprise

the substitutions E1544K, K1545S, L1547V, Q1569R, P1597H and S1602H or the substitutions E1544K, K1545S, L1547V, Q1569R, P1597K and S1602H. Suitable mutant IGF2 binding domains may comprise the amino acid sequence of residues 1511 to 1650 of human IGF2R with 30 or fewer of said residues mutated; or an amino acid sequence which has at least 80% sequence identity with residues 1511 to 1650 of human IGF2R, as described above.

In some embodiments, the mutant IGF2 binding domain may consist of the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions E1544K, K1545S, L1547V, Q1569R, P1597H, and S1602H or the substitutions E1544K, K1545S, L1547V, Q1569R, P1597K and S1602H.

In a fourth group of preferred embodiments, a mutant IGF2 binding domain may comprise substitutions at E1544, K1545, L1547, Q1569, P1597, S1602 and G1603 of the wild-type IGF2R sequence. For example, a mutant domain may comprise the substitutions E1544K, K1545S, L1547V, Q1569R, P1597H, S1602H and G1603K or the substitutions E1544K, K1545S, L1547V, Q1569R, P1597K, S1602H and G1603K. Suitable mutant IGF2 binding domains may comprise the amino acid sequence of residues 1511 to 1650 of human IGF2R with 30 or fewer of said residues mutated; or an amino acid sequence which has at least 80% sequence identity with residues 1511 to 1650 of human IGF2R, as described above.

In some embodiments, the mutant IGF2 binding domain may consist of the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions E1544K, K1545S, L1547V, Q1569R, P1597H, S1602H and G1603K or the substitutions E1544K, K1545S, L1547V, Q1569R, P1597K, S1602H and G1603K.

A mutant IGF2 binding domain as described herein may comprise one or more non-natural amino acids, modified amino acids or d-amino acids. The use of such amino acids is well-known to those of skill in the art.

A mutant IGF2 domain as described above may be comprised within a polypeptide.

The polypeptide may comprise multiple IGF2 binding domains, including, for example, one or more mutant IGF2 binding domains as described herein. For example, a polypeptide may comprise two, three, four or more IGF2 binding domains. The presence of multiple domains may increase the ability of the polypeptide to bind to IGF2. The domains may be identical (i.e. copies) or may be non-identical (i.e. they may differ at one or more amino acid residues).

The mutant IGF2 binding domains may be directly connected without linkers or may be linked by amino acid sequences from human IGF2R, synthetic amino acid sequences, synthetic organic molecules or polypeptides that multimerise or assemble into polymeric structures. In some embodiments, the IGF2 binding domains may be linked via biotin-streptavidin tags.

The polypeptide may further comprise one or more amino acid sequences additional to the one or more mutant IGF2 binding domains. For example, the IGF2 binding polypeptide may comprise one or more additional domains.

Additional domains may include domains of human IGF2R, such as domain 13 (residues 1800 to 1991 of the IGF2R sequence), and domain 12 (residues 1651 to 1799 of the IGF2R sequence) or domains from other polypeptides (i.e. heterologous domains) which improve the stability, pharmacokinetic, targeting, affinity, purification and production properties of the polypeptide, such as an immunoglobulin Fc domain, which confers improved stability/pharmacokinetic parameters in biological fluid.

A mutant IGF2 binding domain may be linked to an immunoglobulin, such as IgG1 or IgG2, or part of an immunoglobulin, such as an Fc domain. Suitable immunoglobulins include human immunoglobulins.

In some embodiments, the polypeptide may comprise an affinity tag, which may, for example, be useful for purification. An affinity tag is a heterologous peptide sequence which forms one member of a specific binding pair. Polypeptides containing the tag may be purified by the

binding of the other member of the specific binding pair to the polypeptide, for example in an affinity column. For example, the tag sequence may form an epitope which is bound by an antibody molecule.

Suitable affinity tags include for example, glutathione-S-transferase, (GST), maltose binding domain (MBD), MRGS(H)$_6$, DYKDDDDK (FLAG™), T7-, S-(KETAAAKFERQHMDS), poly-Arg (R_{5-6}), poly-His (H_{2-10}), poly-Cys (C_4) poly-Phe(F_{11}) poly-Asp(D_{5-16}), Strept-tag II (WSHPQFEK), c-myc (EQKLISEEDL), Influenza-HA tag [25], Glu-Glu-Phe tag [26], SPY-TAG (AHIVMVDAYKPTK; [52]) Tag.100 (Qiagen; 12 aa tag derived from mammalian MAP kinase 2), Cruz tag 09™ (MKAEFRRQESDR, Santa Cruz Biotechnology Inc.) and Cruz tag 22™ (MRDALDRLDRLA, Santa Cruz Biotechnology Inc.). Known tag sequences are reviewed in Terpe et al (2003) [53].

In preferred embodiments, a FLAG™ or poly-His tag such as $(H)_6$ or MRGS$(H)_6$ may be used.

The affinity tag sequence may be removed after purification, for example, using site-specific proteases.

In some embodiments, the polypeptide may be coupled to an appropriate signal leader peptide to direct secretion from cell into the culture medium. A range of suitable signal leader peptides are known in the art. The signal leader peptide may be heterologous to the IGF binding domain i.e. it may be a non-IGF2R signal sequence. For example, an α-factor secretion signal may be employed. Preferably, the signal peptide is removed by post-translational processing after expression of the polypeptide.

Preferably, a polypeptide comprising or consisting of one or more IGF2 binding domains is soluble. A soluble polypeptide does not naturally associate with membranes after expression and does not form aggregates in aqueous solution under physiological conditions. A soluble polypeptide may, for example, lack a transmembrane domain.

In some embodiments, the polypeptide may be immobilised. For example, the polypeptide may be covalently or non-covalently attached to an insoluble support. The support may be in particulate or solid form and may include a plate, a test tube, beads, a ball, a filter or a membrane. A polypeptide may, for example, be fixed to an insoluble support that is suitable for use in affinity chromatography. Methods for fixing polypeptides to insoluble supports are known to those skilled in the art. A polypeptide may be immobilised, for example, to isolate IGF2 from a sample.

Mutant IGF2 binding domains and polypeptides may be generated wholly or partly by chemical synthesis. For example, the domains and polypeptides may be synthesised using liquid or solid-phase synthesis methods; in solution; or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof. Chemical synthesis of polypeptides is well-known in the art (J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984); M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); J. H. Jones, The Chemical Synthesis of Peptides. Oxford University Press, Oxford 1991; in Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California; G. A. Grant, (Ed.) Synthetic Peptides, A User's Guide. W. H. Freeman & Co., New York 1992, E. Atherton and R.C. Sheppard, Solid Phase Peptide Synthesis, A Practical Approach. IRL Press 198 9 and in G. B. Fields, (Ed.) Solid Phase Peptide Synthesis (Methods in Enzymology Vol. 289). Academic Press, New York and London 1997).

In some embodiments, a polypeptide comprising or consisting of one or more IGF2 binding domains may be labelled with a detectable or functional label.

Detectable labels may include radionuclides, such as iodine-131, yttrium-90, indium-111 and technicium-99, which may be attached to polypeptides of the invention using conventional chemistry known in the art. A polypeptide labelled with a radioactive isotope may be used to selectively deliver radiation to a specific target, such as a tumour. This may be useful in imaging the tumour or in delivering a cytotoxic dose of radiation, as described below.

Other detectable labels may include enzyme labels such as horseradish peroxidase, chemical moieties such as biotin which may be detected via binding to a specific cognate detectable moiety, e.g. labelled avidin, fluorochromes such as fluorescein, rhodamine, phycoerythrin and Texas Red and near infrared fluorophores, including cyanine dye derivatives such as Cy7 (Amersham Pharmacia) and Alexa750 (Molecular probes).

In some embodiments, a polypeptide comprising or consisting of one or more IGF2 binding domains may be linked to a reactive moiety for the covalent attachment of additional molecules. Suitable reactive moieties include photoaffinity groups, such as cyclopropenones, e.g. for click chemistry reactions.

Mutant IGF2 binding domains and polypeptides may be generated wholly or partly by recombinant techniques. For example, a nucleic acid encoding a mutant IGF2 binding domain or polypeptide may be expressed in a host cell and the expressed polypeptide isolated and/or purified from the cell culture.

Another aspect of the invention provides a nucleic acid encoding a polypeptide comprising or consisting of one or more mutant IGF2 binding domains as described herein, and optionally one or more additional domains, as described above.

Nucleic acid encoding a polypeptide may be comprised in a vector. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments,

polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Preferably, the vector contains appropriate regulatory sequences to drive the expression of the nucleic acid in mammalian cells. A vector may also comprise sequences, such as origins of replication and selectable markers, which allow for its selection and replication in bacterial hosts such as *E. coli*.

Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 3rd edition, Russell et al., 2001, Cold Spring Harbour Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds. John Wiley & Sons, 1992.

A nucleic acid or vector as described herein may be introduced into a host cell.

A range of host cells suitable for the production of recombinant polypeptides are known in the art. Suitable host cells may include prokaryotic cells, in particular bacteria such as *E. coli*, and eukaryotic cells, including mammalian cells such as CHO and CHO-derived cell lines (Lec cells), HeLa, COS, and HEK293 cells, amphibian cells such as Xenopus oocytes, insect cells such as *Trichoplusia ni*, Sf9 and Sf21 and yeast cells, such as *Pichia pastoris*.

Techniques for the introduction of nucleic acid into cells are well established in the art and any suitable technique may be employed, in accordance with the particular circumstances. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. adenovirus, AAV, lentivirus or vaccinia. For bacterial cells, suitable techniques may include calcium

chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduced nucleic acid may be on an extra-chromosomal vector within the cell or the nucleic acid may be integrated into the genome of the host cell. Integration may be promoted by inclusion of sequences within the nucleic acid or vector which promote recombination with the genome, in accordance with standard techniques.

The introduction may be followed by expression of the nucleic acid to produce the encoded polypeptide comprising or consisting of one or more mutant IGF2 binding domains. In some embodiments, host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) may be cultured *in vitro* under conditions for expression of the nucleic acid, so that the encoded IGF2 binding polypeptide is produced. When an inducible promoter is used, expression may require the activation of the inducible promoter.

The expressed polypeptide comprising or consisting of one or more mutant IGF2 binding domains may be isolated and/or purified, after production. This may be achieved using any convenient method known in the art. Techniques for the purification of recombinant polypeptides are well known in the art and include, for example HPLC, FPLC or affinity chromatography. In some embodiments, purification may be performed using an affinity tag on the polypeptide as described above.

Polypeptides comprising or consisting of one or more mutant IGF2 binding domains which are produced as described may be investigated further, for example the pharmacological properties and/or activity may be determined. Methods and means of protein analysis are well-known in the art.

In some embodiments, a nucleic acid or vector as described herein may be introduced into a host cell that is suitable for administration and expression of the mutant IGF2 binding domain or polypeptide in an individual.

Another aspect of the invention provides a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described herein for use in a method of treatment of the human or animal body by therapy. For example, the polypeptide comprising a mutant IGF2 binding domain, nucleic acid or host cell as described herein may be used in a method of treatment of cancer.

Another aspect of the invention provides the use of a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described herein in the manufacture of a medicament for use in the treatment of cancer.

Cancers which may be treated as described herein include cancers characterised by up-regulation of IGF2 or the down-regulation of IGF2R, for example colorectal cancers such as intestinal adenoma, cervical cancers such as cervical carcinoma, lung cancers such as lung carcinoma, kidney cancers such as Wilms' tumour, muscle cancers such as rhabdomyosarcoma, bone cancers such as Ewing's sarcoma, endocrine cancers such as phaeochromocytoma, liver cancers such as hepatocellular carcinoma, brain tumours such as glioblastoma, breast cancers such as inflammatory breast cancers, upper gastrointestinal cancers such as pancreatic cancer, haematological cancers such as myeloma, soft tissue sarcomas, such as haemangiopericytoma, and cancers that either result in tumour related hypoglycaemia (Non-Islet cell tumour associated hypoglycaemia, or NICTH, or IGF2 syndrome, due to high circulating supply of big-IGF2 isoforms) related to increased circulating levels of IGF2 and/or express the IGF2 gene at high levels.

Another aspect of the invention provides a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or

a host cell as described herein for use in a method of treatment of a non-cancer disorder characterised by up-regulation of IGF2 or the down-regulation of IGF2R, for example a disorder associated with proliferative vascular growth, such as diabetic retinopathy, a growth disorder associated with aberrant expression of IGF2, such as Beckwith-Wiedemann syndrome, or a bone-related metabolic disorder, such as hepatitis C-associated osteosclerosis (HCAO).

Another aspect of the invention provides the use of a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described herein in the manufacture of a medicament for use in the treatment of a disorder associated with proliferative vascular growth such diabetic retinopathy, a growth disorder associated with aberrant expression of IGF2, such as Beckwith-Wiedemann syndrome, or a bone-related metabolic disorder, such as hepatitis C-associated osteosclerosis (HCAO).

An individual with cancer or another condition who is suitable for treatment with a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described herein may display increased IGF2 protein expression, increased IGF2 mRNA expression, the presence of pro-forms of IGF2 and/or low blood glucose.

An individual suitable for treatment as described above may be a mammal, such as a rodent (e.g. a guinea pig, a hamster, a rat, a mouse), murine (e.g. a mouse), canine (e.g. a dog), feline (e.g. a cat), equine (e.g. a horse), a primate, simian (e.g. a monkey or ape), a monkey (e.g. marmoset, baboon), an ape (e.g. gorilla, chimpanzee, orang-utan, gibbon), or a human.

In some preferred embodiments, the individual is a human. In other preferred embodiments, non-human mammals, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (*e.g.* murine, primate, porcine, canine, or leporid) may be employed.

In some embodiments, the individual may have minimal residual disease (MRD) after an initial cancer treatment.

An individual with cancer may display at least one identifiable sign, symptom, or laboratory finding that is sufficient to make a diagnosis of cancer in accordance with clinical standards known in the art. Examples of such clinical standards can be found in textbooks of medicine such as Harrison's Principles of Internal Medicine, 15th Ed., Fauci AS et al., eds., McGraw-Hill, New York, 2001. In some instances, a diagnosis of a cancer in an individual may include identification of a particular cell type (e.g. a cancer cell) in a sample of a body fluid or tissue obtained from the individual. In some embodiments, the individual may have been previously identified or diagnosed with cancer or a method of the invention may comprise identifying or diagnosing cancer in the individual for example by determining the presence of an identifiable sign, symptom, or laboratory finding indicative of cancer in the individual.

Mutant IGF2 binding domains and polypeptides as described herein may also be useful as cancer-targeting agents to deliver other anti-cancer molecules to tumours, radiolabels to detect and treat tumours, and sensitising agents that sensitise tumours to other cancer therapies, including chemotherapy and radiotherapy.

Whilst a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described herein may be administered alone, it is preferable to present it as a pharmaceutical composition (e.g. formulation) which comprises the polypeptide, nucleic acid or cell, together with one or more pharmaceutically acceptable carriers, adjuvants, excipients, diluents, fillers, buffers, stabilisers, preservatives, lubricants, or other materials well known to those skilled in the art and, optionally, other therapeutic or prophylactic agents. Such materials should be non-toxic and should not interfere with the efficacy of the active compound. The precise nature of the carrier or other material will depend on the route of administration, which may be

by bolus, infusion, injection or any other suitable route, as discussed below. Suitable materials will be sterile and pyrogen-free, with a suitable isotonicity and stability. Examples include sterile saline (e.g. 0.9% NaCl), water, dextrose, glycerol, ethanol or the like or combinations thereof. The composition may further contain auxiliary substances such as wetting agents, emulsifying agents, pH buffering agents or the like.

Methods of the invention may therefore comprise the step of formulating a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described herein with a pharmaceutically acceptable carrier, adjuvant or excipient.

The term "pharmaceutically acceptable" as used herein pertains to compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgement, suitable for use in contact with the tissues of a subject (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation. The precise nature of the carrier or other material will depend on the route of administration, which may be oral or by injection, e.g. cutaneous, subcutaneous, or intravenous or intraocular, for example intraorbital.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the

art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Suitable carriers, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990.

The pharmaceutical compositions and formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the mutant IGF2 binding domain, polypeptide, nucleic acid or host cell with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active compound with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations may be in the form of liquids, solutions, suspensions, emulsions, elixirs, syrups, tablets, lozenges, granules, powders, capsules, cachets, pills, ampoules, suppositories, pessaries, ointments, gels, pastes, creams, sprays, mists, foams, lotions, oils, boluses, electuaries, or aerosols.

Whether it is a mutant IGF2 binding domain, polypeptide, nucleic acid or host cell according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the circumstances of the individual to be treated.

The mutant IGF2 binding domain or polypeptide or pharmaceutical composition comprising the mutant IGF2 binding domain or polypeptide may be administered to a subject by any convenient route of administration, whether systemically/peripherally or at the site of desired action, including but not limited to, oral (e.g. by ingestion); and parenteral, for example, by injection, including subcutaneous, intradermal, intramuscular, intravenous, intraarterial, intracardiac, intrathecal, intraspinal, intracapsular, subcapsular, intraorbital, intraperitoneal, intratracheal, subcuticular, intraarticular, subarachnoid, and intrasternal, preferably subcutaneous or intraorbital; by implant of a depot, for example, subcutaneously or intramuscularly. Usually administration will be by the oral route, although other routes such as intraperitoneal, subcutaneous, transdermal, intravenous, nasal, intramuscular or other convenient routes are not excluded.

The pharmaceutical compositions comprising the active compounds may be formulated in a dosage unit formulation that is appropriate for the intended route of administration.

Treatment may be any treatment or therapy, whether of a human or an animal (e.g. in veterinary applications), in which some desired therapeutic effect is achieved, for example, the inhibition or delay of the onset or progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress, inhibition of metastasis, amelioration of the condition, cure or remission (whether partial or total) of the condition, preventing, delaying, abating or arresting one or more symptoms and/or signs of the condition or prolonging survival of a subject or individual beyond that expected in the absence of treatment.

Cancer growth generally refers to any one of a number of indices that indicate change within the cancer to a more developed form. Thus, indices for measuring an inhibition of cancer growth include a decrease in cancer cell survival, a decrease in tumor volume or morphology (for example, as determined using computed tomographic (CT), sonography, or other imaging method), delayed tumor growth, or destruction of tumor vasculature. Administration of a mutant IGF2 binding domain as described herein in an individual with cancer, as described herein may improve the capacity of the individual to resist cancer growth, in particular growth of a cancer already present the subject and/or decrease the propensity for cancer growth in the individual.

Treatment as described herein may include prophylactic treatment (i.e. prophylaxis) i.e. the individual being treated may not have or may not be diagnosed as having a cancer at the time of treatment. For example, an individual susceptible to or at risk of the occurrence or re-occurrence of cancer may be treated as described herein. Such treatment may prevent or delay the occurrence or re-occurrence of cancer in the individual or reduce its symptoms or severity after occurrence or re-occurrence. In some embodiments, the individual may have been previously identified as having increased susceptibility or risk of cancer compared to the general population or a method may comprise identifying an individual who has increased susceptibility or risk of cancer. Prophylactic or preventative treatment may be preferred in some embodiments.

A mutant IGF2 binding domain may be administered as described herein in therapeutically-effective amounts.

The term “therapeutically-effective amount" as used herein, pertains to that amount of an active compound, or a combination, material, composition or dosage form comprising an active compound, which is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio.

The appropriate dosage of an active compound may vary from individual to individual. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the administration. The selected dosage level will depend on a variety of factors including, but not limited to, the route of administration, the time of administration, the rate of excretion of the active compound, other drugs, compounds, and/or materials used in combination, and the age, sex, weight, condition, general health, and prior medical history of the individual. The amount of active compounds and route of administration will ultimately be at the discretion of the physician, although generally the dosage will be to achieve therapeutic plasma concentrations of the active compound without causing substantial harmful or deleterious side-effects.

In general, a suitable dose of the active compound is in the range of about 100 µg to about 400 mg per kilogram body weight of the subject per day, preferably 200 µg to about 200 mg per kilogram body weight of the subject per day, for example 5-10 mg/kg/day. Where the active compound is a salt, an ester, prodrug, or the like, the amount administered is calculated on the basis of the parent compound and so the actual weight to be used is increased proportionately.

Administration in vivo can be effected in one dose, continuously or intermittently (e.g., in divided doses at appropriate intervals).

Methods of determining the most effective means and dosage of administration are well known in the art and will vary with the formulation used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the physician.

Multiple doses of the mutant IGF2 binding domain may be administered, for example 2, 3, 4, 5 or more than 5 doses may be administered. The administration of the mutant IGF2 binding domain may continue for

sustained periods of time. For example treatment with the mutant IGF2 binding domain may be continued for at least 1 week, at least 2 weeks, at least 3 weeks, at least 1 month or at least 2 months. Treatment with the mutant IGF2 binding domain may be continued for as long as is necessary to reduce cancer symptoms or achieve complete remission.

The mutant IGF2 binding domain may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the individual circumstances. For example, a mutant IGF2 binding domain as described herein may be administered in combination with one or more additional active compounds.

For example, a mutant IGF2 binding domain as described herein may be administered in combination with a phosphatidylinositol 3-kinase (PI3 kinase) inhibitor.

Suitable PI3 kinase inhibitors are well-known in the art and include F-04691502 ([2-amino-8-[trans-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxypyridin-3-yl)-4-methylpyrido- [2,3-d]pyrimidin-7(8H)-one), Pictilisib (GDC-0941; 4-[2-(1*H*-indazol-4-yl)-6-[(4-methylsulfonylpiperazin-1-yl)methyl]thieno[3,2-*d*]pyrimidin-4-yl]morpholine), perifosine (1,1-Dimethylpiperidinium-4-yl octadecyl phosphate), idelalsib (5-Fluoro-3-phenyl-2-[(1*S*)-1-(7*H*-purin-6-ylamino)propyl]-4(3*H*)-quinazolinone), PX866 ((1E,4S,4aR,5R,6aS,9aR)-5-(acetyloxy)-1-[(di-2-propen-1-ylamino)methylene]-4,4a,5,6,6a,8,9,9a-octahydro-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-cyclopenta[5,6]naphtho[1,2-c]pyran-2,7,10(1H)-trione), duvelisib (S)-3-(1-((9H-purin-6-yl)amino)ethyl)-8-chloro-2-phenylisoquinolin-1(2H)-one), copanlisib (2-Amino-*N*-[7-methoxy-8-(3-morpholin-4-ylpropoxy)-2,3-dihydroimidazo[1,2-c]quinazolin-5-yl]pyrimidine-5-carboxamide), pilaralisib (N-(3-{[(3-{[2-chloro-5-(methoxy)phenyl]amino}quinoxalin-2-yl)amino]sulfonyl}phenyl)-2-methylalaninamide); XL-765 (N-[2-[(3,5-Dimethoxyphenyl)amino]quinoxalin-3-yl]-4-[(4-methyl-3-methoxyphenyl)carbonyl]aminophenylsulfonamide) and BEZ-235 (2-methyl-2-(4-(3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-1-yl)phenyl)propanenitrile).

Preferred PI3 kinase inhibitors include F-04691502 and pictilisib.

Other aspects of the invention provide a method of treating cancer or a non-cancer disorder characterised by up-regulation of IGF2 or the down-regulation of IGF2R comprising; administering a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described above to an individual in need thereof in combination with a PI3 kinase inhibitor.

Another aspect of the invention provides a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described above for use in a method of treating cancer or a non-cancer disorder characterised by up-regulation of IGF2 or the down-regulation of IGF2R, wherein the method comprises administering the polypeptide, nucleic acid or host cell in combination with a PI3 kinase inhibitor.

Another aspect of the invention provides a PI3 kinase inhibitor for use in a method of treating cancer or a non-cancer disorder characterised by up-regulation of IGF2 or the down-regulation of IGF2R, wherein the method comprises administering the PI3 kinase inhibitor with a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described above.

Another aspect of the invention provides a polypeptide comprising or consisting of one or more mutant IGF2 binding domains, a nucleic acid, or a host cell as described above and a PI3 kinase inhibitor for use in a method of treating cancer or a non-cancer disorder characterised by up-regulation of IGF2 or the down-regulation of IGF2R.

A mutant IGF2 binding domain as described herein may be useful in binding IGF2 in *in vitro* assays. For example, an assay may comprise contacting a mutant IGF2 binding domain as described herein with a sample that comprises IGF2 or is to be tested for the presence of IGF2, such that

IGF2 in the sample binds to the mutant IGF2 binding domain to form a complex. The complex may be washed following binding of the IGF2 and the mutant IGF2 binding domain. The IGF2 may be separated from the mutant IGF2 binding domain following washing and subjected to further analysis. The amount of IGF2 may be determined, for example by mass spectrometry or immunostaining, before or after release from the complex.

The mutant IGF2 binding domain may be immobilised, for example on a bead or column.

In vitro binding assays using a mutant IGF2 binding domain may be useful in improving the assessment of the level of IGF2 in an individual, for example in IGF2 quantification prior to patient selection for treatment, the diagnosis of NICTH and the detection of exogenous administration of IGF2, such as in doping screens for athletes.

Another aspect of the invention provides the use of a mutant IGF2 binding domain to bind IGF2 *in vitro*.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

Other aspects and embodiments of the invention provide the aspects and embodiments described above with the term "comprising" replaced by the term "consisting of" and the aspects and embodiments described above with the term "comprising" replaced by the term "consisting essentially of".

It is to be understood that the application discloses all combinations of any of the above aspects and embodiments described above with each other, unless the context demands otherwise. Similarly, the application discloses all combinations of the preferred and/or optional features either singly or together with any of the other aspects, unless the context demands otherwise.

Modifications of the above embodiments, further embodiments and modifications thereof will be apparent to the skilled person on reading this disclosure, and as such these are within the scope of the present invention.

All documents and sequence database entries mentioned in this specification are incorporated herein by reference in their entirety for all purposes.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described above and the tables described below.

Table 1 shows the affinity kinetics of site directed mutants following structural predictions (domain 11^{AB3}: IGF2). Surface plasmon resonance (SPR) binding analysis of recombinant M6P/IGF2R domain 11 mutants interaction with IGF2. *K*on indicates association (on) rate; *k*off, dissociation (off) rate; *RU*, response units. Binding to IGF2 assessed with BIAcore T200 using purified biotinylated IGF2 and IGF1 on a streptavidin biosensor chip. Recombinant proteins were expressed either in *E. coli* and/or *P. pastoris*. All the mutants in the FG and CD loops are on the domain 11^{AB5} background for the AB loop unless otherwise stated. All experiments were carried out at 25°C in HBS-EP buffer (pH 7.4), were repeated 2-5 times on two independent BIAcore chips. nd = not determined. Data for *K*D expressed as mean of each experiment, and means of *k*on and *k*off are shown.

Table 2 shows HI Loop mutagenesis library directed screen and domain 11^{AB3} random mutant characterisation.

Table 3 shows Affinity kinetics of combined CD, FG and HI, loop mutants with AB loop backgrounds domain 11^{AB3} and domain 11^{AB5}. Measurements were carried out at 25oC in HBS-EP buffer (pH 7.4) (nd= not determined, np= no protein). Data for *K*D expressed as mean of each experiment, and means of *k*on and *k*off are shown.

Table 4 shows pH dependent affinity kinetics of combined CD, FG and HI, loop mutants with AB loop backgrounds domain 11^{AB3} and domain 11^{AB5} (pH 6.5). Measurements were carried out at 25oC in MES-EP buffer, 20 mM MES pH 6.5, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20). Data for *K*D expressed as mean of each experiment, and means of *k*on and *k*off are shown.

Table 5 shows the affinity of IGF1 and the different forms of IGF2 ($IGF2^{1-67}$, $IGF2^{1-104}$ and $IGF2^{1-156}$) for the Fc domain 11^{AB5} and Fc-domain 11^{I1572A} fusion proteins. SPR analysis of the interaction of recombinant IGF1 and IGF2 forms with Fc-domain 11 proteins immobilised by antibody capture (CM5 chip containing monoclonal mouse anti-human IgG antibody immobilised by amine coupling). Measurements were carried out at 25oC in HBS-EP buffer (pH 7.4).

Experiments

Materials and Methods

Construction of plasmids for yeast surface display of IGF2R domain 11 and loop-specific mutagenesis

The yeast surface display vector, pPICFlagDomain 11-Agα1, was generated by ligating IGF2R domain 11 (residues 1508-1654) into the *Eco*RI and *Avr*II restriction sites of pPIC9K preceded by the Flag peptide and followed by the C-terminal region of the *Saccharomyces cerevisiae AGα1* gene. The resulting protein is a N-terminal Flag tagged fusion of domain 11 and the C-terminal domain (residues 311-650) of α-agglutinin, which targets the fusion protein to the cell surface through the secretory pathway, where it is attached by a GPI anchor [27]. The pLITMUS-Domain 11 vector was also generated by ligation of the Domain 11 insert into the

*Eco*RI and *Avr*II restriction sites in the multipurpose cloning pLITMUS28i vector.

Construction of loop-specific mutant libraries

Loop-specific mutant libraries were generated by either whole plasmid PCR (using Kappa HiFi HotStart DNA polymerase, Kappa Biosystems, or Phusion Polymerase, Finnzymes), using tail-to-tail mutagenic primers pairs (Supplementary Table 1) and the pPICFlagDomain11-Agα1 vector or the pLITMUS-Domain 11 vector as template for the AB loop and the HI loop libraries, respectively. The PCR product was treated with *Dpn*I in order to remove the template, self-ligated and then transformed into XL10-Gold® ultra-competent *E. coli* cells (Stratagene) to generate the *E. coli* libraries. The presence of random amino acid mutations in the desired positions was confirmed by sequencing of 50 different clones of each library. In the case of the HI loop library, a midiprep of the *E. coli* pLITMUS-Domain 11^{HI} library (containing 8.3×10^6 clones and therefore covering the number of possible mutants by more than 10-fold) was digested with *Eco*RI and *Avr*II and the excised HI loop-mutated domain 11 was then ligated into the pPICFlag-Agα1 and transformed into the ultra-competent *E. coli* cells to generate the *E. coli* pPICFlagDomain 11^{HI}-Agα library (containing 1.32 x 10^6 mutants). pPICFlagDomain 11^{AB}-Agα1 and pPICFlagDomain 11^{HI}-Agα1 were linearised within the HIS4 gene by *Sal*I digestion. Targeted integration of the expression constructs into the *HIS4* locus of the *P. pastoris* genome was achieved by electroporation of the *Sal*I linearised vector into the histidine auxothrophic *P. pastoris* strain GS115 as described by [28]. For selection, *P. pastoris* libraries were grown in histidine deficient minimal medium MD (1.34% yeast nitrogen base (YNB), 4×10^{-5} % biotin, 2% dextrose).

Induction of Surface Display

For surface display expression, *P. pastoris* yeast libraries selected on histidine-deficient medium for 72 hr were kept at 4ºC for a further 24 hr. The libraries were passaged on MD and an excess of at least 10-fold of the library size of freshly grown cells was grown at 30ºC in BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base (YNB), 4 x 10-5%

biotin, 1% (v/v) glycerol, 100 mM potassium phosphate, pH 6.0) for 24-48 hr. Surface display of the mutated domain 11 was induced by transfer to BMMY (1% yeast extract, 2% peptone, 1.34% YNB, 4 × 10-5% biotin, 0.5% (v/v) methanol, 100 mM potassium phosphate, pH 6.0). For maintained induction, cultures were supplemented with methanol to a concentration of 1% (v/v) every 24 hr for three days. Yeast cultures of control proteins (domain 11^{WT}, domain 11^{AB3} and domain 11^{I1572A}) were induced in the same manner.

FACS screening validation

Freshly induced yeast cells displaying domain 11^{WT}, domain 11^{AB3} and domain 11^{I1572A} were pelleted and washed with PBSA (PBS+0.5% (v/v) BSA). Aliquots of 5 × 10^5 cells were incubated in PBSA for 1 hr at room temperature with anti-Flag M2 antibody (1:1000 dilution, Sigma) and increasing concentrations of biotinylated IGF2 ranging from 15 pM to 1.5 µM in a total volume such that a 10-fold molar excess of IGF2 over the domain 11 displayed on the surface was maintained (assuming a maximum of $5x10^4$ molecules per cell) [29]. The cells were then pelleted, washed 3 times with cold PBSA and labelled with Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen) and Alexa Fluor® 647 streptavidin (Invitrogen) at 20 µg mL^{-1} in a volume of 50 µL for 30 min at 4ºC. Negative controls with secondary only and a positive control with anti-Flag+Alexa Fluor 488 only were also included. The cells were pelleted, washed 3 times with cold PBSA and re-suspended in 1 mL of PBSA and run in CyAn ADP Cytometer (Dako). The yeast population was gated on forward- and side-scatter channels to remove debris and the percentage of double positive cells was measured for each IGF2 concentration.

FACS library screening

For the yeast loop libraries sorting, a 10-fold excess of the library population diversity was labelled in a similar manner, alongside the yeast cells displaying domain 11^{AB3}. The cells were pelleted, washed with PBSA and incubated first with the Anti-Flag M2 antibody (0.76 µg/10^6 cells, Sigma) for 1 hr at room temperature followed by 3x PBSA washes. The subsequent incubation with 5 nM IGF2, maintaining a 10-fold molar

excess of IGF2 over domain 11, was also carried out for 1hr at room temperature. After 3x PBSA washes, the yeast cells were incubated with Alexa Fluor 488 goat anti-mouse IgG1 (0.02 µg/10^6 cells, Invitrogen) and Alexa Fluor® 647 streptavidin (2 µg/10^6 cells, Invitrogen) for 30 min-1 hr at 4ºC. Negative and positive controls (1 × 10^6 cells) were similarly labelled and used, together with the yeast cells displaying domain 11^{AB3} (also labelled with 5 nM IGF2), to gate the library yeast cells that should display higher affinity binders on the surface [29].

Biacore 3000 screen

FACS gated cells were grown for 48 hr in YPD (1% yeast extract, 2% peptone, 2% dextrose) containing 100U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 100 µg mL^{-1} kanamycin and 0.5 mg mL^{-1} Geneticin. Genomic DNA was extracted using the YeaStar Genomic DNA kit (Zymoresearch). Mutated domain 11 was amplified by PCR from the genomic DNA using primers that incorporated *Eco*RI and *Avr*II restriction sites into the pPIC-HIS vector in frame with the α-factor secretion signal to generate pPIC-Domain 11 construct of the mutants [23]. The vector was then linearised with *Sal*I and integrated into the HIS4 locus of the *P. pastoris* genome by electroporation [28]. Yeast cells incorporating domain 11 into their genome were selected at 30ºC on MD plates. Single yeast colonies were inoculated into 96 well plates containing 500 µl of BMGY and grown at 30ºC for 24 hr (covered with a sterile breathable seal). Each plate contained a set of controls, including non-transformed cells and cells secreting domain 11^{WT}, domain 11^{AB3} and domain 11^{I1572A} mutants. The medium was replaced with BMMY and induction was sustained by supplementing with methanol to a final concentration of 1% (v/v) every 24 hr for two days.

100 µl of the supernatants, containing the secreted domain 11 mutants, were transferred to a 96 well plate, diluted with 100 µL of HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20) and screened for IGF2 binding ability by SPR on a Biacore 3000. Biotinylated IGF2 (GroPep, Aus) was immobilized on the flow cells of the sensor chip SA by affinity capture to streptavidin after pre-conditioning the sensor surface with three 1 min injections of 1 M NaCl, 50 mM NaOH.

Kinetic experiments were performed at 25ºC in HBS-EP at a flow-rate of 40 µL min^{-1} and consisted of a 2 min injection of diluted supernatant followed by a 2 min dissociation phase in HBS-EP running buffer, after which the binding surface was regenerated with a 2 min injection of 2M $MgCl_2$. The resulting sensorgrams were visually analysed using the standard BiaEvaluation software version 4.0.1 for Biacore 3000. Additionally, the dissociation phase was fitted to a first order exponential decay curve ($Y=Y_0 \cdot e^{-Kx}$) from which the 'off rate' (K) could be calculated.

Protein expression and purification of IGF2R domain 11 mutants for SPR studies.

For expression, yeast colonies selected on histidine-deficient plates were grown at 30ºC in 10 mL of BMGY for 24 hr and induced by being transferred to 50 mL BMMY. For maintained induction, cultures were supplemented with methanol to a final concentration of 1% (v/v) every 24 hr for two days. Supernatants were then subjected to SDS-PAGE and western blot using anti-His_6 mouse monoclonal antibody conjugated to peroxidase (Roche Diagnostics, USA). Domain 11 secreting cultures were scaled up to 500 mL BMMY and grown for further 72 hr and supplemented with 1% methanol every 24 hr. Supernatants were cleared by centrifugation (6000 rpm for 20 min at 4ºC in a Beckman-Coulter Avanti-J2 refrigerated centrifuge), diluted 5-fold in 20 mM sodium phosphate pH 8.0 buffer and His-purified using a Ni-NTA superflow column (Qiagen) on an Äkta FPLC system (GE Life Sciences, UK) washed with 20 mM imidazole and eluted with 250 mM imidazole. The fractions containing the protein were pooled and, when required, diluted in 20 mM MES pH 6.0 and purified using a Resource S column on an Äkta FPLC system (GE Life Sciences, UK). Purified proteins were dialyzed and concentrated using centrifugal filter units of 5 kDa cut-off (Millipore). Protein concentration was determined spectrophotometrically (Nanodrop) using the theoretical extinction coefficient calculated by ProtParam and protein size and homogeneity was verified using 12% SDS-PAGE.

Structure-informed loop-specific site directed mutagenesis

The domain 11^{AB3}:IGF2 structure (PDB: 2L29) was used to identify residues in the binding loops that could interact with IGF2. These were manually mutated in Pymol v1.5 to determine whether or not they might stabilise the complex. In addition the Robetta server was used to perform interface alanine scanning mutagenesis on the Domain 11^{AB3} complex to identify residues important for complex formation. A number of possible amino acids were introduced at each site to assess the residue's role in binding. M6P/IGF2R domain 11 sub-cloned from the pPICHis vector into pET26a (Novagen, Merck Chemicals Ltd, Nottingham, UK) was used as a template for site directed mutagenesis. The template was amplified in a thermocycler (PeqStar) using mutagenic primers and KOD DNA polymerase (Novagen) to introduce the mutations. Following amplification the parental DNA was digested with DpnI and transformed into *E. coli* 5α cells (NEB) for sequencing. The mutant proteins were expressed and refolded using existing protocols in *E. coli* BL21 (DE3) before the binding kinetics were analysed by SPR[3].

Protein expression and purification of domain 11 mutants for NMR studies.
For NMR studies, domain 11^{AB5} was sub-cloned from the pPICHis vector into pET26a (Novagen, Merck Chemicals Ltd, Nottingham, UK) for expression in *E. coli* BL21 (DE3). The proteins were refolded using existing protocols and purified by gel filtration [3]. Uniformly ^{13}C, ^{15}N-labelled proteins were expressed in *E. coli* BL21 (DE3) grown in minimal media containing $^{15}NH_4Cl$ and ^{13}C glucose as the sole sources of carbon and nitrogen.

Expression and purification of domain 11^{WT}, domain 11^{AB5} and IGF2-domain 11^{AB5} complexes for NMR
His_6-tagged domain 11^{WT} and domain 11^{AB5} (residues 1508-1654) cloned into pET26 (Novagen, Merck Chemicals Ltd, Nottingham, UK) were transformed into *E. coli* BL21(DE3) CodonPlus™ competent cells, purified as inclusion bodies and refolded according to well-established protocols [3]. Proteins were isotopically labelled using 1 g L^{-1} ^{15}N-ammonium chloride and/or 2 g L^{-1} $^{13}C_6$-glucose (99%, Cambridge Isotope Laboratories) in M9 minimal media. Lyophilised unlabelled IGF2 obtained from Novozymes Biopharma, AU, was added to NMR samples of ^{15}N-single (dynamics studies)

and ^{15}N, ^{13}C-double labelled domain 11^{AB5} mutant (structural studies) in a 1:1 ratio at pH 4, which provided optimum stability for the complex. Structural calculations of the domain 11^{AB5} both free and complexed with IGF2 were as described for the domain 11^{AB3}:IGF2 complex [3]. Validation was performed using the iCing online server (version r76). The ensembles of NMR structures and associated NMR chemical shifts have been deposited with the protein database and BioMagResBank with the following accession codes: Free domain 11^{AB5} 2M6T and rcsb103281, IGF2 bound domain 11^{AB5}, 2M68 and rcsb103260.

NMR dynamics studies

NOE, ^{15}N-T_1 and ^{15}N-T_2 NMR relaxation data were acquired at two fields (600 and 900 MHz) at 25°C on a Varian VNMRS or INOVA spectrometer respectively, both fitted with cryogenically cooled probe heads for domain 11^{WT} and domain 11^{AB5}. T_1 delays of 0.01, 0.02*, 0.03, 0.05, 0.07, 0.10, 0.15, 0.30, 0.40, 0.6, 0.8, 1.0, 1.5, 2.0** and 2.5** s (*600 MHz only, **900 MHz only) and T_2 delays of 0.1–2.1 s with intervals of 0.2 s were gathered using BioPack pulse sequences provided by VnmrJ 2.2. T_1 and T_2 experiments were run in duplicate. Automatic spectral compression (ASCOM) [30] was applied to increase signal: noise without increasing the length of the experiments. NOE ratios were calculated between peak heights in HSQC spectra recorded both with and without NOE proton saturation. At the beginning of each transient a 5 s ultra-low power pulse was employed for those spectra without NOE and a 3 s proton saturation pulse with a 2 s delay (total 5 s) for those experiments with NOE. Data collected was processed (NMRPipe, [31]) and assigned (Analysis 2.1.5 [32]) before being exported for curve fitting and model free analysis (relax 1.3.9 [33, 34]. Relaxation rates were calculated by an eleven parameter grid search, followed by simplex minimisation curve fitting to a two-parameter (I_0, R_x) exponential decay. Monte Carlo error analysis used the standard deviation of simulated curves back calculated from fitted parameters across replicated spectra or from the Sparky 3.113 (Goddard T. D.). RMSD calculation of noise in a peak-free region of non-replicated spectra. Relaxation datasets recorded at 600 and 900 MHz were validated using self-consistency tests within relax. Extended model free

analysis [35-37] was performed within relax 1.3.9 which involved AIC model-free model selection [38] and elimination of failed models followed by Monte Carlo simulations [39]. After initial calculation of local τ_m values, isotropic (sphere) and anisotropic (prolate/oblate spheroids, ellipsoid) diffusion models were optimised followed by AIC selection of the best model.

Surface plasmon resonance (SPR) and binding analysis

Final kinetic analysis of mutants by SPR was conducted using a Biacore T200 biosensor at 25ºC in either HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20) or MES-EP (20 mM MES pH 6.5, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20) at a flow-rate of 40 µL min^{-1}. For analysis of the domain 11:IGF2 interaction, approximately 20-50 RU of biotinylated IGF2 (GroPep, Aus) or IGF1 were immobilized on each flow cell of a CM5 chip by affinity capture to streptavidin and recombinant domain 11 at concentrations ranging from 64 nM to 0.125 nM was injected. For the analysis of the Fc-domain 11^{AB5} interaction, approximately 50 RU of a biotinylated IGF2:biotinylated ubiquitin mixture (1:8 proportion) was immobilized on the CM5-streptavidin chip. This interaction was also analysed by immobilizing the Fc-domain 11^{AB5} and Fc-domain $11^{AB5\text{-}RHH}$ (or Fc-domain 11^{I1572A} as control) by Human Antibody Capture (monoclonal mouse anti-human IgG antibody immobilised on a CM5 chip by amine coupling, GE Healthcare) with and without human IgG1 Fc fragment (Bethyl Laboratories, UK) in the control flow cell. Increasing concentrations of $IGF2^{1-67}$, $proIGF2^{1-104}$, $proIGF2^{1-156}$ and IGF1 ranging from 0.125 to 32 nM were injected. T200 BiaEvaluation software (GE Healthcare) was used to fit the resulting curves according to a 1:1 binding model. Steady state affinities were determined using the kinetic data, taking the response average at 5 seconds before injection end and for 5 seconds average. Thermodynamic data was performed across the temperature range of 10°C to 30°C, van't Hoff and Erying plots generated and ΔH, $-T\Delta S$ and ΔG values determined as previously described[23].

Fc-fusion protein expression and purification

Fc-domain 11^{AB5} fusion protein was cloned as previously described[40], produced by transient transfection of HEK293T cells with pHLSec-AB5-hIgG1 and utilising Corning Cellstack10's to scale-up cell culture. Purified Fc-domain 11^{AB5} was analysed for the presence of aggregates by chemical crosslinking with bis[sulfosuccinimidyl]suberate (BS3). 100-200 ng of concentrated Fc-domain 11^{AB5} was diluted into PBS and incubated in the presence or absence of 0.5 mM BS3 for 30 min at room temperature. The reaction was quenched by the addition of SDS-PAGE sample buffer and samples were analysed by SDS-PAGE and western blotting using an anti-IGF2 antibody (AF-292, R&D). For bulk production in Chinese Hamster Ovary cells GS knockout (CHOK1SV GS-KO cells, Lonza Biologics, PLC), three IgG2 Fc-fusion constructs were first transiently expressed, Fc-domain $7\text{-}13^{T926\text{-}P1189}$, Fc-domain $11^{AB5\text{-}RHH}$, and a tandem construct of domain $11^{AB5\text{-}RHH}$. Prior evaluation of *in silico* antigenicity utilised Epibase™ HLA class II allotypes against amino acid sequences of Fc constructs, including linkers, showed that domain 11^{AB5} was less antigenic and so was selected for bulk production. Bulk cultures from stable selected CHOK1SV GS-KO resulted in protein A affinity purified yields in the range of 27 to 187 mg/L. SDS-Page and size exclusion HPLC confirmed a single Fc product with endotoxin levels < 0.74 EU/mg. For Fc-domain 11^{AB5}:IGF2 co-precipitation, an aliquot of 400 µL of media of cells treated with Fc-domain 11^{AB5} or Fc-domain 11^{I1572A} or a previously incubated mixture of recombinant IGF2 and Fc-domain 11^{AB5} was incubated overnight at 4 ºC with Protein A/G Agarose (Immuno-precipitation, Protein A, Roche). Agarose beads were washed according to the manufacturer´s instructions, resuspended in 2 × sample buffer, boiled and the supernatant used for western blot analysis as described below.

Fc-domain 11^{AB5}:IGF2 co-precipitation

An aliquot of 400 µL of media of cells treated with Fc-domain 11^{AB5} or Fc-domain 11^{I1572A} or a previously incubated mixture of proIGF2 and Fc-domain 11^{AB5} was incubated overnight at 4ºC with Protein A/G Agarose (Immunoprecipitation kit (Protein A), Roche). Agarose beads were washed according to the manufacturer´s instructions, resuspended in 2 × sample

buffer, boiled and the supernatant used for western blot analysis as described previously.

IGF2 induced hypoglycaemia

All animal experiments were approved by the animal use ethical committee of Oxford University and fully complied with UK Home Office guidelines (PPL 30/2695). Wild-type C57BL/6J and 129S2 mice were used throughout. Mice were anaesthetised using a rising concentration of isoflurane in oxygenated air and maintained using 2% isoflurane with 2 L min^{-1} oxygen. Blood glucose measurements were taken using an Advantage diabetic glucose meter (Roche Diagnostics, Burgess Hill, UK). In order to test the direct inhibition of IGF2-induced hypoglycaemia by Fc-domain 11^{AB5}, blood glucose levels were allowed to normalise for 30 min prior to taking a baseline blood glucose reading, then either 1 mg kg^{-1} IGF2 only (R&D systems), or 1 mg kg^{-1} IGF2 premixed with 11.7 mg kg^{-1} Fc-domain 11^{AB5}, Fc-domain 11^{RHH}, or Fc-domain 11^{I1572A} (1:1 molar ratio) was injected. Blood glucose measurements were taken every 10 min for the following hour. For the Fc-domain 11^{AB5} mouse preloading experiment, 11.7 mg kg^{-1} Fc-domain 11^{AB5} or PBS, as negative control, was injected immediately after induction of anaesthesia, 30 mins later a baseline blood glucose reading was taken and 1 mg kg^{-1} IGF2 (1:1 molar ratio) was injected, readings were then taken every 10 min for the following hour. For the mouse IGF2 preloading experiment, 1 mg kg^{-1} IGF2 was injected immediately after induction of anaesthesia, 30 min later a baseline blood glucose reading was taken and either 11.7 mg kg^{-1} Fc-domain 11^{AB5} (1:1 molar ratio) or PBS was injected, readings were then taken every 10 min for the following hour.

In vitro cell viability

The human hepatocellular carcinoma cell lines Hep 3B2.1-7 and Huh-7D12 were obtained from the ATCC (HB-8065) and the ECACC (Catalogue number 1042712), respectively. Hep 3B2.1-7 was grown in EMEM (Lonza) supplemented with 10% (v/v) fetal bovine serum, 100 U mL-1 penicillin, 100 µg mL-1 streptomycin, and 2mM Lglutamine Huh-7D12 was grown in DMEM (Sigma) supplemented with 10% fetal bovine serum, 100 U mL-1 penicillin, 100 µg mL-1 streptomycin, and 2mM L-glutamine. Cells were plated in 96-

well plates at a density of 1-2 x10^4 cells/well in serum free medium. After 24 hr, Fc-domain 11^{AB5} (orange), Fc-domain 11^{I1572A} as negative control (grey) or OSI-906 (purple) were added to each well to different final concentrations and the cells were incubated for further 48-72 hr. Cell viability was assayed at 48 and 72 hr after Fc-domain 11^{AB5} treatment using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). The half maximal inhibitory concentration (IC50) was calculated using graph prism.

Cell proliferation assay

EdU incorporation was used to measure cell proliferation (Invitrogen, C10338). Briefly, cells were plated at a density of 2x105 cells/well in 6-well plates and cultured in serum free media. After 24 hr of serum starvation, PBS (black) and 1.5 µM Fc-domain 11^{I1572} (grey) as negative controls, 0.5 and 1.5 µM Fc-domain 11^{AB5} (orange), 1 µM OSI-906 (purple) and 0.1 and 0.5 µM doxocycline (green) as positive controls were added to the different wells and cells were further cultured. After 48 hr, 10 µM EdU was added for 3 hr and cells were then washed and trypsinised, followed by fixation in 4% PFA and permeabilisation in TBS/5% Triton. The Alexa 555 dye was then attached to the EdU molecules by click chemistry following the manufacturer's instructions and the % of EdU incorporation under different conditions was then analysed by flow cytometry.

IGF2 dependent human cancer cell xenografts

IGF2 sensitive autocrine cell line SKNMC-$IGF2^{67}$ was generated from the Ewing sarcoma cell line SKNMC by retroviral infection with constructs containing IRES-luciferase. Autocrine growth and signalling function of the SKNMC line, including inhibition by IGF2-TRAP, was confirmed in culture using an MTS reagent (Cell Titer 96 Aqueous One Solution cell proliferation assay, Promega). For xenografts, approximately 5x10^6 SKNMC-$IGF2^{67}$ cells harvested during the growth phase of sub-confluent cell cultures were then mixed with matrigel (50:50 v/v, 100µl/ mouse) and injected (sub-cutaneous)into CD-1® (Crl:NU-1 Foxn1nu) female mice, 24 hours after establishing ALZET® mini-pump placement delivering a single concentration 40 mg kg-1 per week either IGF2-TRAP or PBS vehicle

control. Tumour growth was monitored with both caliper measurements and bioluminescence. The latter was performed with a Bruker Xtreme 2D optical system 15 minutes (10 second exposure) following luciferin injection (100 µl per 20g body weight). Tumours were excised at 28 days or when size was > 1000 mm3. Fixation in formalin, embedding in paraffin and Hematoxylin and Eosin (H&E) staining was done using standard protocols. Apoptotic area was quantified using automated image analysis of brightfield images in ImageJ, applying H AEC color deconvolution filter and automated thresholding. Serum was also collected at the time of culling.

IGF2-TRAP Synthetic Lethality Screen

For synthetic lethality screens (Target Discovery Institute, University of Oxford), either 2,500 or 5,000 SKNMC-IGF2104 -IRES-luc cells were plated in medium containing 0.5% charcoal stripped FBS in 384 well plates and the IGF2-TRAP was added 24h later to half of the plates. Using automated dispensing 307 oncology drugs that have been tested in man (TDI expanded Oncology Drug library) were added to cells 3hrs later 10 µM, 1 µM, 100 nM and 10 nM, in the presence and absence of 250 nM IGF2-TRAP. After 48hr of incubation, resazurin was added to a final concentration of 10 µg/mL and the fluorescence (Ex: 560 nm, Em: 590 nm) recorded at 2hr. Normalisation was performed with untreated cells and cells treated with the fixed concentration of IGF2-TRAP alone. Pearson's product-moment correlation coefficient (r) was calculated to determine replicate correlation. For hit selection, we used a bespoke 'interaction score' metric (a variation on standard Δz-score analysis) followed by hit selection using the robust non-parametric Rank Product method.

The interaction score was calculated where v was the viability readout for the drug condition, $\bar{x}$ was the sample mean of the negative controls and s was the sample standard deviation of the negative controls, z was calculated as $z_{treatment}=(v-\bar{x})/s$. $z_{(no\_IT)}$ was calculated from PBS control plates, comparing 'drug d' to same- plate negative controls 'cells + DMSO'. z_{IT} was calculated from treated plates, comparing 'drug d + IGF2-TRAP (IT)' to same-plate negative controls 'cells + IT + DMSO'. The magnitude of these z-scores reflects the degree of inhibition of cell

viability by 'drug d ± IT' relative to negative controls, also taking into account well-to-well variation. The interaction score is defined as z_(no_LT)-z_IT. Thus, a more negative interaction score represents a greater synergistic lethal effect: the inhibitory effect of the drug is potentiated by IT. Interaction scores were calculated for each replicate r_(d,1)/r_(d,2)/r_(d,3) independently. The rank product was calculated in each data set (16 data sets: cell density x drug library concentration x rezasurin incubation time) each replicate for each drug was ranked independently, with 1st rank assigned to the most negative interaction score. Using these ranks, a Rank Product RP(d) for drug d in each data set was calculated, where r_(d,i) is the rank of the ith replicate for drug d: $RP(d)=\sqrt[3]{((r_{(d,1)})(r_{(d,2)})(r_{(d,3)}))}$

In a permutation test, the RP for each drug is compared to a simulated data set giving an estimated p-value of false positive. These p values, in the context of our experiment, informed us about effect size, direction and reliability, and allowed us to control the false discovery rate in contrast to many other hit selection protocols43. Hits were selected where $p<0.05$.

For synthetic lethality hit validation, fresh stocks of the four drugs selected as hits were obtained (pictilisib, SYNkinase and PF-04691502, Cayman Chemical). The interaction of the selected drugs and IGF2-TRAP were evaluated by Q-value calculated by the formula Q=FD+T/FD+(1-FD)FT, where FD+T represents the fraction affected by treatment with the drug plus IGF2-TRAP compared with the untreated control group, FD represents the fraction affected by the drug alone, and FT represents the fraction affected by the IGF2-TRAP alone. A value of $Q>1.15$ indicates a synergistic effect, $Q<0.85$ indicates an antagonistic effect, and Q between 0.85 and 1.15 indicates an additive effect [54].

Cell assays, protein extraction and Immunoblotting

For analysis of IGF2 secretion by the different cell lines, cells were plated in 6-well plates in serum-free medium at a concentration of approximately 106 cells per well. The medium was collected after 48 hr,

spun at 4000g for 20 min and the supernatant filtered through a 0.22 µm filter. For signaling analysis, cells were plated in 6-well plates at a concentration of approximately 3-6x105 cells per well in serum-free medium. After 24 hr, Fc-domain $11^{AB5/AB5-RHH}$ (or PBS and Fc-domain 11^{I1572A} as negative controls) were added to each well to the corresponding concentration and the cells were incubated for further 48-72 hr. Cells were washed with PBS and lysed on ice in RIPA buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (Halt cocktail, Pierce). Lysates were cleared by centrifugation at 4°C. Protein levels were quantified using the CB-X assay (G-Biosciences) as per manufacturer's instructions. SDS-PAGE gel electrophoresis and protein transfer of either 20 µg of protein or 5 µL of serum were done following standard protocols. Antibodies used were anti hIGF2 (R&D Systems AF-292, 2 µg mL-1), anti pAktS473 (Cell Signaling 4060, 1:1000), anti β-actin (AbCam 8227, 1:20000), anti pIGF1RY1135/1136/pIRY1150/1151 (Cell Signaling 3024, 1:1000), CaptureSelect Biotin Anti-hIgG-Fc (Life Technologies, 1:1000), anti mIGF1 (R&D Systems AF-791, 1 µg mL-1), anti mIGFBP2 (Millipore 06-107, 1/1000 dilution), anti mIGFBP3 (R&D Systems MAB775, 1 µg mL-1), anti mIGFBP5 (R&D Systems AF578, 0.1 µg mL-1), anti mIGFBP6 (R&D Systems AF776, 0.1 µg mL-1), anti mGH (R&D Systems BAF1566, 0.5 µg mL-1), anti hIGFBP3 (Santa Cruz Biotechnology Sc-9028, 1 µg mL-1), anti hALS (Novus Biologicals NBP1-89118, 1 µg mL-1). Secondary antibodies were from Dako, used at 1:2000, except Streptavidin HRP at 1:4000 (Thermo Scientific Pierce). Densitometry was performed using ImageJ (NIH) and was normalized to actin.

Results

M6P/IGF2R domain 11 has been previously expressed using *P. pastoris* to generate stable soluble protein suitable for surface plasmon resonance (SPR) and isothermal calorimetry (ITC)[23]. A yeast surface display system was adapted using the Agα1 system in *P. pastoris*[41]. By expressing an N-terminal flag-tagged domain 11, we measured the cell surface expression of the domain with anti-flag antibody and Alexa 488 labelled secondary antibody. Following incubation of live yeast

expressing domain 11 with biotinylated human $IGF2^{1-67}$, IGF2 binding was quantified with Alexa647 labelled streptavidin. When incubated with a range of IGF2 concentrations, the system was sensitive enough to discriminate different affinities for domain 11^{WT}, domain 11^{AB3} and a non-binding mutant domain 11^{I1572A}. Yeast induced to express mutant domain 11s underwent flow sorting to select high affinity IGF2 binders to a low concentration of IGF2 (5 nM). Gates were set based on the binding of IGF2 to domain 11^{WT} and domain 11^{I1572A} (reduced binding mutant), prior to setting thresholds using domain 11^{E1544K} or domain 11^{AB3} (higher IGF2 affinity) [23]. Selected live yeast were cultured, mutant domain 11s sub-cloned in a soluble protein expression vector, cells plated and single selected clones screened in a 96 well format using a Biacore 3000 and k_{off} ($y=Y_0.e^{-k_{off}X}$) determination. Yeast expressing soluble mutant his_6-domain 11s with slow k_{off} were expanded and purified prior to real time kinetic quantification using a sensitive Biacore T200.

Screens of the AB loop (^{1542}YSKKGL^{1547}) library including the E1544K mutation led to the identification of a mutant (AB5) with ten-fold higher IGF2 affinity compared to domain 11^{WT} (K_D 5.07 nM vs 46-64 nM, Table 1). Sequencing of clone AB5 identified the presence of five AB loop mutations (^{1542}YAKGWG^{1547}) compared to the three mutations of domain 11^{AB3} (^{1542}YSKSGV^{1547}) relative to wild-type domain 11 (^{1542}YSEKGL^{1547}). Of the mutants characterized, domain 11^{AB5} was the highest IGF2 affinity AB loop mutant that retained IGF2 specificity with respect to IGF1.

To determine how the mutations incorporated into domain 11^{AB5} altered the IGF2 binding site, high-resolution NMR structures of domain 11^{AB5}, both in the free form and in complex with IGF2 were then solved. ^{15}N-relaxation data for domain 11^{WT} and domain 11^{AB5} was also recorded at two field strengths (600 and 900 MHz). Domain 11^{AB5} showed the characteristic flattened β-barrel domain 11 fold and was well defined over this core secondary structure (RMSD = 0.55 Å). All the hydrophobic residues in the binding site, including foundation residues, are conserved between domain 11^{WT}, ^{E1544K}, ^{AB3} and ^{AB5}[3]. The hydrophobic residues (Y1542, F1567, I1572, Y1606, L1629 and the hydrophobic portion of K1631) and the foundation

residues (V1574, L1626 and L1636) create a complementary binding surface for IGF2 and mutation of these residues reduces or abrogates IGF2 binding [23]. Despite these observations, the AB loop adopts a dramatically different structure in domain 11^{AB5}. W1546, which substitutes for a G1546, has the aromatic side-chain rotated to pack into and extend the hydrophobic patch. The bulky tryptophan side chain causes a re-orientation of adjacent residues within the binding site, forcing the displacement of Y1542 and F1567 on the AB and CD loops, respectively, and projecting the remainder of the AB loop between Y1542 and W1546 back away from the IGF2 binding region. In both the domain 11^{WT} and domain 11^{AB3} structures, this portion of AB is more closely associated with the hydrophobic patch, with the small G1546 side chain allowing facile rearrangement and conformational flexibility in both the AB loop and the neighbouring Y1542 and F1567. Globally, when compared to domain 11^{WT}, accommodation of the tryptophan residue in domain 11^{AB5} displaces the β-A strand and causes a displacement of the GH-loop (at the opposite end of the protein) and the C-terminal α-helical turn.

Upon complex formation, the AB loop was observed to shift in domain 11^{AB3} to accommodate IGF2, whereas there is almost no rearrangement of the AB loop in domain 11^{AB5} as this superimposes closely with the bound form. The re-organisation in domain 11^{AB5}, while allowing the original hydrophobic residues to contact IGF2, would also favour the interaction of W1546 with F19, the most important residue for the docking of IGF2 to domain 11[3, 22]. The bulky side-chain of W1546 causes a reorientation of residues within the binding site, forcing the displacement of Y1542 and F1567 on the AB and CD loops, respectively while still allowing them to contact F19 of IGF2. Increased interaction via aromatic ring-stacking between Y1542, W1546 and F1567 also causes an overall increase in rigidity in domain 11^{AB5} in comparison to the more flexible domain 11^{WT}, shown by an increase in S^2 and a decrease in R_{ex} in the AB, BC and CD loops of domain 11^{AB5}. The loss of flexibility stabilises the binding site of domain 11, providing a stable platform for the docking of IGF2. In addition to the five hydrophobic binding site residues of wild-type domain 11, there are also charged and polar residues that interact with IGF2. These are found

among the more flexible regions of all four binding loops. The surface of IGF2 is negatively charged overall, and residue E1544 in human domain 11^{WT} is the only acidic residue in the binding site. Mutation from a negative to a positive charge at this position forms a salt bridge with D23 and therefore plays a major part in increasing binding affinity to IGF2. In domain 11^{AB3} the additional K1545S mutation may have also contributed to increased binding affinity since a hydrogen bond could be formed between Q18 of IGF2 and the hydroxyl group of the serine. In domain 11^{AB5}, however, K1545G would be incapable of forming a hydrogen bond with Q18, implicating structural importance of the G1546W mutation for the gain in affinity.

We next looked at structural data for residues in the binding loops that could interact with IGF2, and mutated these in Pymol to predict whether or not they might stabilise the complex. In addition the Robetta server was used to perform interface alanine scanning mutagenesis on the AB3 complex to identify residues important for complex formation. These additional mutations and SPR affinity are shown in Table 1 and Figure 1. Introduction of W1546F into the AB loop reduced the k_{off} by two fold, yet retained the similar overall K_D (4.35 nM) as a result of a reduced k_{on}. Mutations of FG loop residues P1599, S1600, and L1604 failed to enhance affinity, but K1601R, S1602H, S1602Q and G1603K all exhibited up to 2-fold improvement. Interestingly, S1602H, S1602N and G1603K all reduced k_{off}, suggesting that AB loop and FG loop mutants may also function in combination. Introduction of structurally predicted site directed mutations in the HI loop slightly improved affinity, although a number of single point mutations could be introduced that appeared to slow the k_{off}, such as Q1632A (K_D= 3.76 nM) and K1631W (K_D =3.23 nM).

The affinity of IGF2 for domain 11^{AB5} was at least two fold higher than our highest previously detected mutant, and we had already detected functionally additive single point mutations of the FG and HI loops that enhanced domain 11^{AB5} affinity. We then systematically determined the potential for the HI loop to specifically stabilise the IGF2 binding interaction by introducing a library of mutants for four residues of the HI loop (^{1630}DKQT^{1633}) into domain 11^{WT}, rather than domain 11^{AB5}, leading to

a predicted $1x10^5$ fully representative clones of every possible amino acid combination. By validating flow sorting gates with domain 11^{AB3}, we sub-cloned 2.2 x 10^5 flow sorted mutants with high binding to IGF2 into a soluble expression system. We purified soluble proteins and screened over >1000 clones (Table 3). One mutant amongst several false positives was identified (^{1630}HFQS^{1633}) with slower k_{off} than domain 11^{WT} (Table 2). The majority of false positives also indicated that introducing a more specific kinetic screen with the T200 platform using purified recombinant protein was required.

In parallel we screened a domain 11^{AB3} cultures and obtained clones from a control culture with slow k_{off} to IGF2 binding. Expressed proteins confirmed a number of mutants that bound IGF2 with apparent high affinity (Table 3). In particular, a four-fold reduction in k_{off} was detected with the FG loop mutant, P1597H that was also associated with a ten-fold reduction in affinity compared to domain 11^{AB3} (K_D = 1.23 nM vs 15.3 nM, respectively). Similarly, a two-fold reduction in k_{off} was detected with P1597K that was also associated with a six-fold reduction in affinity compared to domain 11^{AB3} (K_D = 2.78 nM vs 15.3 nM, respectively). Moreover, we unexpectedly detected a mutant in the hydrophobic CD loop (Q1569R) that had enhanced affinity (K_D= 2.30 nM). This was a notable observation because of the known importance of hydrophobicity and shape complementarity of the CD loop component during evolution of the binding site [3], even though we knew that Q1569A had little impact on IGF2 affinity [23]. As a result of the combined structural predictions and directed evolution approaches described, we had evolved domain 11 with respect to higher IGF2 affinity *in vitro* with several novel and independent AB, CD, FG and HI loop mutants. Moreover, these specific mutations in CD, FG and HI loops, on either domain 11^{AB5} or domain 11^{AB3} AB loop backgrounds, modified the k_{off} and overall affinity (Fig. 1 and Tables 1 and 3).

We next sought to determine whether individual loop mutations, identified on different AB loop mutagenic backgrounds, could be combined to either generate additive, subtractive or synergistic binding affinities and specificity for IGF2. In particular, CD loop (Q1596R), FG loop (P1597H/K,

S1602H, G1603K) and HI (K1631W, ^{1630}HFQS^{1633}) loop mutations appeared to each enhance affinity, with retained specificity for IGF2, and were selected as potential candidates to combine. Successive additions of the majority of mutations resulting in combinations of CD and FG mutations, additively improved the binding kinetics on either a domain 11^{AB3} or domain 11^{AB5} background (Fig. 2, 3 and Table 3).

The thermodynamic parameters for the domain 11–IGF2 interaction were obtained from the temperature dependence of the dissociation constant according to the van't Hoff equation for domain 11^{WT}, AB loop mutants (domain 11^{AB3} and domain 11^{AB5}), double loop mutants (domain 11$^{AB5\ Q1569R}$ (AB+CD loops) and domain 11$^{AB5\ P1597H\ S1602H}$ (AB+FG loops) and the triple loop mutant domain 11$^{AB5\ Q1569R\ P1597H\ S1602H}$ [23, 42]. The interaction of IGF2 with domain 11^{WT} appeared driven by enthalpy ($\Delta H<0$), with a relatively unfavourable entropic contribution ($-T\Delta S>0$) (Fig. 4). Mutations in the AB loop appeared to have different effects. Domain 11^{AB5} retained the same thermodynamic profile as domain 11^{WT}, whereas in domain 11^{AB3}, the entropic contribution appeared more favourable as we had previously shown for the E1544K AB loop mutant[23]. Addition of mutations in domain 11^{AB5} CD or FG loops introduced more favourable changes in the entropic contribution and k_{off} similar to domain 11^{AB3} (Fig. 4, 5). The CD loop mutation in domain 11$^{AB5\ Q1569R}$ was the only mutant tested with both improvement in both enthalpy and entropy terms compared to domain 11^{WT}. Moreover, introduction of mutations in FG loop appeared to generate a clearer contribution of entropy to the improved affinity, as both energetic terms contribute favourably to the free energy of binding of domain 11$^{AB5\ P1597H\ S1602H}$ and of the triple loop mutant domain 11$^{AB5\ Q1569R\ P1597H\ S1602H}$.

In the combined mutants, additive rather than synergistic effects were detected (Fig. 3). It was possible that combining loop mutants in a single domain might also disrupt of protein folding, attributed to epistatic interactions that modify the successful accommodation of additional mutations into stable folded protein. For example, addition of HI loop K1631W and ^{1630}HFQS^{1633} to the combined loop mutants, all failed to result in stable expressed protein, implicating a probable effect on protein folding on both domain 11^{AB3} and domain 11^{AB5} backgrounds.

Introduction of multiple mutations (9 to 11) into domain 11^{AB5} appeared less tolerated in terms of protein yields, than similar mutations in domain 11^{AB3}, potentially implicating the structural configuration adopted by the effect of W1546 in domain 11^{AB5} on protein folding of other loop mutants (Table 3). Four higher affinity IGF2 super-antagonists were identified. For mutants on a domain 11^{AB5} background, the high affinity mutants appeared relatively independent of pH (domain $11^{AB5\ (1542YAKGWG1547)\ Q1569R\ P1597H\ S1602H}$ K_D= 0.65 nM pH 7.4/ = 0.71 nM pH 6.5, domain $11^{AB5\ (1542YAKGWG1547)\ Q1569R\ P1597H\ G1603K}$ K_D= 0.87 nM pH 7.4/ = 0.69 nM pH 6.5). For domain 11^{AB3}, lower pH (Table 4) enhanced affinity of both mutants due to slower k_{off} (domain $11^{AB3(1542YSKSGV1547)\ Q1569R\ P1597H\ S1602H}$ K_D= 0.74 nM pH 7.4/ = 0.40 nM pH 6.5, domain $11^{AB3(1542YSKSGV1547)\ Q1569R\ P1597H\ S1602H\ G1603K}$ K_D= 0.78 nM pH 7.4/ = 0.43 nM pH 6.5), such that there a 100 fold improved overall affinity of mutant domain 11 with retained IGF2 specificity compared to domain 11^{WT}.

We next determined if the initial optimised AB loop mutant (domain 11^{AB5}) could be translated into the basis of an improved IGF2 super-antagonist ligand trap. An IgG1 Fc-fusion with domain 11^{E1544K} functioned as an IGF2 signalling antagonist in cell lines based on signalling and cell proliferation responses[40]. We expressed and purified a human IgG1 Fc-fusion domain 11^{AB5}. Fc-domain 11^{AB5} binding kinetics revealed an affinity and specificity for $IGF2^{1-67}$ close to that of the single soluble domain 11^{AB5}, either when immobilised on a Biacore chip using an anti-human IgG antibody (3.89 nM, Table 5, Fig. 6) or when immobilising a low response units of IGF2 mixed with a neutral binder (ubiquitin). IGF2 in serum, and commonly in tumours, occurs as a range of differentially glycosylated pro-IGF2 isoforms attributed to incomplete processing by pro-protein convertases (PC4) [43]. We determined the binding kinetics of recombinant pro-IGF2 isoforms to Fc-domain 11^{AB5} and specificity relative to IGF1. Recombinant mature ($IGF2^{1-67}$ [7.5 kDa] and pro-IGF2 ($pro\text{-}IGF2^{1-104}$ [11 kDa] and $pro\text{-}IGF2^{1-156}$ [17 kDa]) binding kinetics were performed using Fc-domain 11^{AB5} and Fc-domain 11^{I1572A} control immobilised with antibody capture. We detected higher relative affinity binding of the pathological variants of IGF2, $pro\text{-}IGF2^{1-104}$ and $pro\text{-}IGF2^{1-156}$ isoforms, to Fc-domain 11^{AB5} and Fc-domain $11^{AB5\text{-}RHH}$, and surprisingly binding of Fc-domain 11^{I1572A} to both $pro\text{-}IGF2^{1-156}$ and to a lesser extent $pro\text{-}IGF2^{1-104}$ (Fig. 6, Table 5). Binding of

Fc-domain 11 constructs was independent of the Fc-fragment, as the same affinity to pro-IGF2 isoforms was obtained when immobilised IgG1 human Fc was used in the control flow cell. Solution pull-downs confirmed the specific capture of recombinant $IGF2^{1-67}$, pro-$IGF2^{1-104}$ and pro-$IGF2^{1-156}$ (multiple forms) by Fc-domain 11^{AB5} (Fig. 7). In order to further test the functional effects, we selected human hepatocellular carcinoma cell lines (HuH7, Hep3B) that expressed IGF2, a colorectal cancer (HT29/219) cell line that did not express high levels of IGF2, but was dependent on IGF2 in culture and a NIH 3T3 cell line expressing human pro-$IGF2^{R104A}$ following retroviral integration of a human IGF2 construct with a mutation (R104A), known to impair proteolytic cleavage of the E-domain [43]. We validated IGF2 signalling and growth dependency of cell lines by addition of dual IGF1 receptor/ insulin receptor kinase inhibitor OSI-906. Immuno-precipitation with domain 11^{AB5} identified both mature IGF2 and pro-IGF2 isoforms in spent serum free culture supernatants, confirming that Fc-domain 11^{AB5} bound human IGF2 isoforms generated by tumour cell lines (Fig. 8).

To test the inhibitory effects of Fc-domain 11^{AB5} *in vivo*, we added recombinant protein in serum free conditions, to Hep3B and Huh7 (IC_{50}, Hep3B= 35.5 µM, Huh7= 1.2 µM and HT29/219= 1.7 µM). Proliferation was inhibited relative to OSI-06, in Hep3B, but apoptosis was promoted in Huh7 (Fig. 10). As expected, the inhibitory concentration IC_{50} for Fc-domain 11^{AB5} in the context of autocrine IGF2 in the media varied between cell lines, and was at least 100 times higher than the apparent K_D for the binding interaction to IGF1 receptor (10-15nM). There was no effect of an Fc-domain 11^{I1572A} mutant control that lacked binding to IGF2.

We next reproduced the metabolic effects of exogenous addition of $IGF2^{1-67}$ to induce hypoglycaemia in mice, similar to the cancer syndrome of non-islet tumour associated hypoglycaemia (NICTH). Pre-loading, co-injection or post-loading of Fc-domain 11^{AB5} resulted in significant abrogation of the hypoglycaemic effects, an effect not detected with the Fc-domain 11^{I1572A} non-IGF2 binding control (Fig. 9A). Whereas the co-injection of $IGF2^{1-67}$ and Fc-domain 11^{AB5} or Fc-domain $11^{AB5-RHH}$ in a 1 : 1 or 1:0.5 molar ratio, respectively, resulted in abrogation of the $IGF2^{1-67}$ induced

hypoglycaemia; when the IGF2 to Fc-domain 11 ratio was reduced, the advantages of a higher affinity mutant were evident. A molar ratio of 1 : 0.23 significantly counteracted IGF2-induced hypoglycaemia in the case of domain $11^{AB5-RHH}$, but not with Fc-domain 11^{AB5} (Fig. 9B). These data demonstrate that Fc-domain 11^{AB5} and Fc-domain $11^{AB5-RHH}$ function as IGF2 antagonists *in vivo*.

An alternative autocrine-$IGF2^{1-67}$ tumour model was developed using a Ewing sarcoma cell line (SKNMC) selected for IGF2 dependency. Growth of a retroviral transformed SKNMC cells expressing constitutive $IGF2^{1-67}$ and a luciferase reporter was inhibited by Fc-domain $11^{AB5-RHH}$ in culture (Fig. 11). SKNMC-$IGF2^{1-67}$ xenografts are predicted to be dependent on autocrine-$IGF2^{1-67}$ supply as adult mice do not express IGF2. IgG2 Fc-domain $11^{AB5-RHH}$ purified from CHO cells was infused using osmotic mini-pumps prior to SKNMC-$IGF2^{1-67}$ cell line injection. Reduced xenograft growth rates in mice was observed with Fc-domain $11^{AB5-RHH}$ (Fig. 12A), with induced regions of tumour cell death that resulted in reduced bioluminescence signal to volume ratio. Circulating $IGF2^{1-67}$ was readily detected in serum following 28 days of SKNMC-IGF^{267} tumour growth, but was less evident in treated Fc-domain $11^{AB5-RHH}$ mice, and without alteration in total serum IGF1, Growth Hormone or IGFBP levels (Fig. 12B).

A number of candidate additive and synergistic lethality interactions were observed in SKNMC-$IGF2^{104}$ treated with Fc-domain $11^{AB5-RHH}$ and an extended oncology compound library (222 agents plus 77 additional agents from the DTP Approved Oncology Drug Set contains oncology specific compounds that have been tested in man.). The high throughput screen compared the effects of the oncology drugs in the presence and absence of a fixed concentration of the Fc-domain $11^{AB5-RHH}$ (IGF2-TRAP) that resulted in partial reduction in cell viability (10-20%) at the assay end point of 72hrs. Evaluation of the metrics of raw data (Pearson's r scores) indicated good replicate correlation, with average r $\geq$0.92 for all plates with an average inhibitory effect of the IGF2-TRAP alone of □10%. Drugs showing $p<0.05$ in the rank product method applied to their interaction score were selected. A number of hits were identified, including two independent PI3-kinase inhibitors, F-04691502 ([2-amino-8-[trans-4-(2-

hydroxyethoxy)cyclohexyl]-6-(6-methoxypyridin-3-yl)-4-methylpyrido- [2,3-d]pyrimidin-7(8H)-one) (p=0.011) and Pictilisib (GDC-0941; 4-[2-(1*H*-indazol-4-yl)-6-[(4-methylsulfonylpiperazin-1-yl)methyl]thieno[3,2-*d*]pyrimidin-4-yl]morpholine) (p=0.017) . These were further validated by comparison of the dose response curve both in the presence and absence of Fc-domain $11^{AB5-RHH}$ (IGF2-TRAP) (Fig. 13). The IC50 for PF-04691502 shifted from 0.42 µM to 92 nM in the presence of the Fc-domain $11^{AB5-RHH}$ (IGF2-TRAP), and from 1.1 µM to 0.14 µM in the case of pictilisib, both of which are significant based on an F-test. Such ten-fold differences following combination with Fc-domain $11^{AB5-RHH}$ (IGF2-TRAP) provide indication that antagonising IGF2 signalling can reveal clinically meaningful synergistic activity, for example, with PI3 kinase inhibitors.

Changes in the molecular distribution of circulating IGF2 upon IGF2-TRAP treatment were analysed by neutral size fractionation of human serum (Fig. 14). In adult human serum, western blots of the fractions reveal that IGF2 is mainly the mature lower molecular weight isoform, and is predominantly present in a ternary complex with IGFBP3 and the acid labile subunit (ALS) (~150 kDa), and to a lesser extent in a binary complex with IGFBP3 (~40 kDa), with free IGF2 below the limit of detection. In this sample, IGF2-TRAP depleted IGF2 in the binary complex, and also partially decreased the amount IGF2 in the ternary complex (lower left panel, Fig. 14). Pull-downs confirmed that the depleted IGF2 specifically bound IGF2-TRAP. In a second experiment, serum from a patient with Non-Islet-Cell Tumour-associated Hypoglycaemia (NICTH) secondary to a GIST tumour was utilised. Western blotting of the fractions showed that IGF2 was present with additional larger molecular weight isoforms, predominantly in a binary complex. IGF2-TRAP depleted both lower and higher molecular weight isoforms of IGF2, and pull-downs confirmed specific binding to both isoforms (lower right panel, Fig. 14). These data suggest that IGF2-TRAP can target larger molecular weight pro-IGF2 isoforms associated with human cancer and NICTH (IGF2 syndrome).

The above experiments unexpectedly show that domain 11 of human IGF2R, despite having evolved over 150 million years, could be further evolved

to an even higher affinity. In order to ensure correct folding of the β-barrel of the expressed protein, we adopted the yeast surface display technology and mutation strategies focused on the prior knowledge of specific IGF2 interacting residues in the binding site loops. We screened a library of binding loop mutants in two stages. Firstly, flow sorting with prior binding controls to select for high IGF2 affinity mutants. The advantages were the selection on the cell surface of correctly folded protein combined with live cell selection. Secondly, the expression of soluble protein derived from selected high affinity clones individually, so that selection was via a high throughput SPR based on defined criteria for selection on the k_{off} of the interaction. The mutagenesis of domain 11 was focused on a number of interacting residues, informed by the high-resolution structure of these sites within the binding loops of domain 11:IGF2 [3].

Our starting premise was that mammals (human) had evolved an optimal domain 11 binding site, such that further evolutionary selection for higher affinity would likely to have been exhausted. Our data suggest that single mutations in loops CD, HI and FG may have resulted in relatively small (<2 fold) incremental gains in affinity, supporting a premise that the binding site has fully evolved in human. In the experimental situation described here-in, our evidence also suggests the contrary, as we have been able identify forms of domain 11 with up to 100 fold increase in affinity without compromising specificity for IGF2.

Comparison of the high solution NMR structures of the domain 11^{WT}, ^{AB3} and ^{AB5} reveals that structural perturbations within the binding site are localized to the AB loop[3]. Although the G1546W mutation in domain 11 ^{AB5} introduces a bulky side-chain into the AB loop and binding site, residues in the neighbouring CD and FG loops are only minimally perturbed. The observation that affinity for IGF2 can be dramatically increased whilst preserving the shape complementarity to T16 of IGF2 may be a critical factor underpinning the success of this method whilst retaining specificity for IGF2 over IGF1. Longer range effects transmitted via strand β-A to the C-terminal helix may reflect a

structural coupling between the conformation of the AB loop and inter-domain conformational changes induced in the full length receptor, perhaps triggered by IGF2 binding.

The thermodynamic pay-offs between entropy (predominantly solvent exclusion) and enthalpy (charge attraction) of the interaction between IGF2 and domain 11 showed some consistent differences between domain 11 AB loop mutants, including the effects of CD and FG loop mutations. Previously we showed that the reduced k_{off} associated with an AB loop E1544K was via improvement of the entropic barrier of the interaction compared to wild type domain 11 [23]. Mutation of the AB loop in the domain 11^{AB3} mutant would be consistent with rigidification and improved solvent exclusion as with domain 11^{E1544K}. This effect was only unmasked in domain 11^{AB5} with respect to thermodynamic terms, after the introduction of additional mutations in the CD and FG loops. Thus the overall improvement in Gibbs free energy of the interaction (ΔG°) appeared to require combinations of mutations that reduced the entropic barrier (improved solvent exclusion).

The increased bioavailability of IGF2, and downstream activation of the IGF1 receptor pathway, accounts for the clinical phenotypes in over-growth syndromes such as Beckwith-Weidemann syndrome, and more common somatic events in human cancer (GCID: GC11M002113). Systemic excess of pro-IGF2 ($IGF2^{87}$, $IGF2^{104}$) in NICTH, mediated via activation of the insulin receptor, is associated with large bulk tumours such as in sarcoma and hepatocellular carcinoma [44, 45] that express *IGF2*. There is no current specific treatment for NICTH. Local IGF2 supply appears to regulate early angiogenic stages of tumour progression and the later stages of transformation to carcinoma [46-48]. Increased IGF2 supply through *IGF2* loss of imprinting, genomic amplification and decreased ligand clearance occurs frequently in common cancers, e.g. colon [16, 17, 20]. The clinical development of single agent/ single receptor IGF1R inhibitors and anti-IGF1R humanised monoclonal antibodies have been limited, mainly because of incomplete IGF2 pathway inhibition and frequent bypass of IGF1R via IGF2 activation of isoform A of the insulin receptor (IR-A)

[49]. In addition, frequent activation of a central feedback loop in the brain following IGF1 targeting also results in paradoxical increased systemic IGF1 supply [50]. Targeting either bioavailable IGF ligands or by dual IGF1R/ IR-A receptor kinase inhibitors may circumvent many of the limitations of just targeting IGF1R alone [49, 50]. Few agents have been developed that solely target IGF2 without cross-reacting with IGF1, e.g. an antibody or soluble domain 11 [40, 51]. The specific high affinity IGF2 super-antagonists generated here may be adapted and tested in appropriate models, including human tumour models, in order to translate these findings to treatment in man.

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Loop	Mutant Domain 11	Kinetics				Steady state	
		K_D (nM)	k_{on} (x10^6 M^{-1}s^{-1})	k_{off} (s^{-1})	Chi^2 (RU^2)	K_D (nM)	Chi^2 (RU^2)
AB	WT	46±1.8	5.0	0.23	0.33	64±1.0	0.24
	AB3	15.3±2.0	2.76	0.044	0.41	15.5±3.3	0.77
	AB5	**5.07±0.87**	**4.21**	**0.0208**	**0.87**	**6.44±1.06**	**0.14**
	W1546F	4.35±0.49	4.5	0.0180	0.767	5.00±0.93	0.129
FG	P1599A*	>70	Nd	>0.1	-	>70	-
	P1599V*	>100	Nd	>0.1	-	>100	-
	P1599G*	>200	Nd	>0.2	-	>200	-
	S1600A	89.10±5.1	2.39	0.2126	nd	84.65±15.20	0.04
	S1600V	>200	Nd	>0.2	nd	>200	-
	S1600L	>200	Nd	>0.2	nd	>200	-
	S1600Y	>200	Nd	>0.2	nd	>200	-
	S1600N	24.44±8.70	3.95	0.0910	0.15	29.67±9.54	0.01
	K1601A	6.80±0.65	3.26	0.0220	0.51	8.51±0.39	0.04
	K1601R	4.75±0.08	5.28	0.0251	0.37	5.78±0.18	0.07
	S1602A	16.48±3.83	1.57	0.0250	0.63	19.92±3.77	0.17
	S1602H	3.19±1.00	3.61	0.0112	1.24	5.44±1.41	0.96
	S1602N	4.75±0.52	2.38	0.0112	0.56	7.93±2.29	0.46
	S1602Q	3.61±1.19	5.88	0.0191	0.57	5.11±0.73	0.13
	G1603A	8.69±1.48	2.14	0.0183	0.45	12.09±0.69	0.16
	G1603K	2.73±0.95	4.66	0.0121	1.18	4.54±0.91	1.18
	L1604A	48.34±8.4	0.79	0.0397	0.26	59.96±19.8	0.01
HI	K1631A	>200	-	-	-	>200	-
	K1631R	12.29±2.81	7.18	0.0867	0.23	13.51±2.65	0.08
	K1631E	>100	-	>0.1	-	>100	-
	K1631W	2.94±0.37	1.18	0.0034	1.47	12.16±2.35	3.16
	Q1632A	3.76±0.54	4.50	0.0167	0.57	4.84±0.22	0.12
	Q1632K	11.07±2.10	1.96	0.0212	0.34	12.84±0.63	0.05
	Q1632E	39.83±7.10	0.52	0.0202	2.11	52.41±4.29	0.06

Q1632L	5.75 ±1.05	3.18	0.0175	1.17	9.34±1.69	2.00
T1633A	26.41±9.49	0.80	0.0196	0.21	53.55±10.0	0.19

Table 1

Mutant Domain 11	Kinetics				Steady state	
	K_D (nM)	k_{on} ($x10^6 M^{-1}s^{-1}$)	k_{off} (s^{-1})	Chi^2 (RU^2)	K_D (nM)	Chi^2 (RU^2)
WT	46±1.8	5.0	0.23	0.33	64±1.0	0.24
AB3	15.3±2.0	2.76	0.044	0.41	15.5±3.3	0.77
AB3 + 62B (FG-P1597H)	1.23±0.32	10.6	**0.013**	1.62	1.76±0.47	1.43
AB3 + 73E (CD-Q1569R)	2.30±0.27	13.6	**0.031**	1.54	3.15±0.41	0.25
WT + 23D (HI-HFQS)*	176	0.25	**0.065**	0.56	220.5	0.45
WT + 26C (HI-DMQT)*	130	1.89	0.24	0.17	127	0.19
WT + 27G (HI-NMAL)*	195	2.0	0.25	0.19	194	0.13
WT+ 312G (HI-VKLM)*	119	1.07	0.15	0.27	135	0.45
AB5 + 51F (HI-NRQS)*	13.5	7.4	0.10	0.31	6.94	0.26

*Protein quality poor based on absorption spectra

Table 2

Domain 11 Mutant	*Kinetics*			*Steady State*	Number of mutations
	K_D (nM)	k_{on} ($x10^6 M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)	
AB3	15.3±2.0	2.76	0.044	15.5±3.3	3
AB3 Q1569R	2.30±0.27	13.6	0.031	3.15±0.41	4
AB3 P1597H	1.23±0.32	10.6	0.013	1.76±0.47	4
AB3 P1597K	2.78±0.61	10.5	0.0287	3.59±0.77	4
AB3 Q1569R P1597H	0.90±0.29	18.9	0.0153	1.73±0.4	5
AB3 Q1569R P1597K	1.34±0.47	20.0	0.0241	2.27±1.04	5
AB3 P1597H K1631W	17.55±1.88	19.7	0.3478	18.67±2.23	5
AB3 P1597H HI^{23D}	nd	nd	0.012	nd	8
AB3 P1597K S1602H	3.73	2.74	0.0102	5.332	5
AB3 Q1569R P1597H S1602H	0.74±0.22	14.1	0.010	1.53±0.46	6
AB3 Q1569R P1597K S1602H	0.79±0.25	20.1	0.016	1.39±0.48	6
AB3 Q1569R P1597H K1631W	np	np	np	np	6
AB3 Q1569R P1597K K1631W	np	np	np	np	6
AB3 Q1569R P1597H S1602H G1603K	0.62±0.03	17.5	0.0108	1.31±0.16	7
AB3 Q1569R P1597K S1602H G1603K	0.78±0.22	20.8	0.0171	1.62±0.41	7
AB3 Q1569R P1597H S1602H K1631W	np	np	np	np	7
AB5	5.07±0.87	4.21	0.0208	6.44±1.06	5
AB5 Q1569R	2.24±0.44	8.04	0.0175	3.22±0.55	6
AB5 P1597H	1.90±0.60	3.68	0.0070	2.79±0.52	6
AB5 P1597A	9.07±1.08	1.90	0.0174	12.44±0.69	6
AB5 P1597K	1.94±0.39	9.54	0.0120	2.21±0.14	6
AB5 P1597L	3.86±1.68	4.18	0.0150	4.85±1.82	6
AB5 P1597N	4.13±1.74	4.72	0.0180	5.36±2.24	6
AB5 P1597Q	3.83±2.42	6.53	0.0203	4.51±2.66	6
AB5 P1597S	4.19±1.80	5.00	0.0193	4.90±1.88	6
AB5 HI^{23D}	np	np	0.012	np	9
AB5 Q1569R P1597H	1.95±0.60	3.2	0.0058	5.40±0.98	7
AB5 Q1569R P1597K	0.95	9.59	0.0091	1.69	7

AB5 P1597H S1602H	1.17±0.2	3.50	0.0046	2.93±0.47	7
AB5 P1597K S1602H	4.62	2.09	0.0096	7.85	7
AB5 P1597H HI^{2-3D}	np	np	np	np	10
AB5 Q1569R P1597H S1602H	0.65±0.11	5.90	0.0038	2.0±0.50	8
AB5 Q1569R P1597K S1602H	0.92±0.13	7.87	0.0073	2.11±0.55	8
AB5 Q1569R P1597H G1603K	0.87±0.09	6.67	0.0058	2.01±0.30	8
AB5 Q1569R P1597H Q1632L	np	np	np	np	8
AB5 Q1569R P1597H K1631W	1.02±0.06	11.20	0.0115	1.95±0.47	8
AB5 Q1569R P1597K K1631W	np	np	np	np	8
AB5 Q1569R P1597H HI2-3D	np	np	np	np	11
AB5 Q1569R P1597H S1602H G1603K	np	np	0.005	np	9
AB5 Q1569R P1597K S1602H G1603K	np	np	0.008	np	9
AB5 Q1569R P1597H S1602H K1631W	np	np	np	np	9
AB5 Q1569R P1597K S1602H K1631W	np	np	np	np	9
AB5 Q1569R P1597K S1602H Q1632L	np	np	np	np	9
AB5 Q1569R P1597H S1602H G1603K K1631W	np	np	np	np	10
AB5 Q1569R P1597K S1602H G1603K K1631W	np	np	np	np	10

Table 3

Mutant	*Kinetics*		
	K_D (nM)	k_{on} (x10^6 $M^{-1}s^{-1}$)	k_{off} (s^{-1})
WT	35.06±10.45	2.68	0.1441
AB3	11.85±0.20	3.43	0.0381
AB3 Q1569R P1597H	0.47±0.06	19.9	0.0091
AB3 Q1569R P1597H S1602H	0.40±0.07	16.1	0.0064
AB3 Q1569R P1597H S1602H G1603K	0.43±0.03	24.3	0.0087
AB5	4.38±0.78	4.17	0.0180
AB5 Q1569R P1597H	1.46±0.29	2.37	0.0034
AB5 Q1569R P1597H S1602H	0.71±0.10	4.41	0.0030
AB5 P1597H S1602H	0.62±0.34	5.32	0.0029
AB5 Q1569R P1597H G1603K	0.69±0.06	7.54	0.0051

Table 4

IGF form	Kinetics		
	K_D (nM)	k_{on} ($x10^6 M^{-1}s^{-1}$)	k_{off} (s^{-1})
Interaction with Fc-domain 11^{AB5}			
$IGF2^{1-67}$	3.89	6.47	0.025
$IGF2^{1-104}$	0.54	12.87	0.0069
$IGF2^{1-156}$	0.21*	4.19*	0.0009
IGF1	–	–	–
Interaction with Fc-domain $11^{AB5-RHH}$			
$IGF2^{1-67}$	0.54	2.60	0.0014
$IGF2^{1-104}$	0.39	1.29	0.0005
$IGF2^{1-156}$	1.13*	0.23*	0.0003
IGF1	–	–	–
Interaction with Fc-domain 11^{I1572A}			
$IGF2^{1-67}$	–	–	–
$IGF2^{1-104}$	4.41	0.52	0.002
$IGF2^{1-156}$	2.71*	0.29*	0.0008
IGF1	–	–	–

* Note, kinetic analysis may be inaccurate because concentration of the recombinant $IGF2^{1-156}$ (Gropep). This consists of a mixture of three isoforms, the full-length protein and truncated peptides (151 and 141 residues long).

Table 5

Sequences

```
   1 mgaaagrsph lgpaparrpq rsllllqlll lvaapgstqa qaapfpelcs ytweavdtkn
  61 nvlykinicg svdivqcgps savcmhdlkt rtyhsvgdsv lrsatrslle fnttvscdqq
 121 gtnhrvqssi aflcgktlgt pefvtatecv hyfewrttaa ckkdifkank evpcyvfdee
 181 lrkhdlnpli klsgaylvdd sdpdtslfin vcrdidtlrd pgsqlracpp gtaaclvrgh
 241 qafdvgqprd glklvrkdrl vlsyvreeag kldfcdghsp avtitfvcps erregtipkl
 301 taksncryei ewiteyachr dylesktcsl sgeqqdvsid ltplaqsggs syisdgkeyl
 361 fylnvcgete iqfcnkkqaa vcqvkksdts qvkaagryhn qtlrysdgdl tliyfggdec
 421 ssgfqrmsvi nfecnktagn dgkgtpvftg evdctyfftw dteyacvkek edllcgatdg
 481 kkrydlsalv rhaepeqnwe avdgsqtete kkhffinich rvlqegkarg cpedaavcav
 541 dkngsknlgk fisspmkekg niqlsysdgd dcghgkkikt nitlvckpgd lesapvlrts
 601 geggcfyefe wrtaaacvls ktegenctvf dsqagfsfdl spltkkngay kvetkkydfy
 661 invcgpvsvs pcqpdsgacq vaksdektwn lglsnaklsy ydgmiqlnyr ggtpynnerh
 721 tpratlitfl cdrdagvgfp eyqeednsty nfrwytsyac peeplecvvt dpstleqydl
 781 sslakseggl ggnwyamdns gehvtwrkyy invcrplnpv pgcnryasac qmkyekdqgs
 841 ftevvsisnl gmaktgpvve dsgsllleyv ngsacttsdg rqttyttrih lvcsrgrlns
 901 hpifslnwec vvsflwntea acpiqtttdt dqacsirdpn sgfvfnlnpl nssqgynvsg
 961 igkifmfnvc gtmpvcgtil gkpasgceae tqteelknwk parpvgieks lqlstegfit
1021 ltykgplsak gtadafivrf vcnddvysgp lkflhqdids gqgirntyfe fetalacvps
1081 pvdcqvtdla gneydltgls tvrkpwtavd tsvdgrkrtf ylsvcnplpy ipgcqgsavg
1141 sclvsegnsw nlgvvqmspq aaangslsim yvngdkcgnq rfstritfec aqisgspafq
1201 lqdgceyvfi wrtveacpvv rvegdncevk dprhgnlydl kplglndtiv sageytyyfr
1261 vcgklssdvc ptsdkskvvs scqekrepqg fhkvaglltq kltyengllk mnftggdtch
1321 kvyqrstaif fycdrgtqrp vflketsdcs ylfewrtqya cppfdltecs fkdgagnsfd
1381 lsslsrysdn weaitgtgdp ehylinvcks lapqagtepc ppeaaacllg gskpvnlgrv
1441 rdgpqwrdgi ivlkyvdgdl cpdgirkkst tirftcsesq vnsrpmfisa vedceytfaw
1501 ptatacpmks nehddcqvtn pstghlfdls slsgragfta aysekglvym sicgenencp
1561 pgvgacfgqt risvgkankr lryvdqvlql vykdgspcps ksglsyksvi sfvcrpeagp
1621 tnrpmlisld kqtctlffsw htplaceqat ecsvrngssi vdlsplihrt ggyeaydese
1681 ddasdtnpdf yinicqplnp mhavpcpaga avckvpidgp pidigrvagp pilnpianei
1741 ylnfesstpc ladkhfnyts liafhckrgv smgtpkllrt secdfvfewe tpvvcpdevr
1801 mdgctltdeq llysfnlssl ststfkvtrd srtysvgvct favgpeqggc kdggvcllsg
1861 tkgasfgrlq smkldyrhqd eavvlsyvng drcppetddg vpcvfpfifn gksyeeciie
1921 sraklwcstt adydrdhewg fcrhsnsyrt ssiifkcded edigrpqvfs evrgcdvtfe
1981 wktkvvcppk kleckfvqkh ktydlrllss ltgswslvhn gvsyyinlcq kiykgplgcs
2041 erasicrrtt tgdvqvlglv htqklgvigd kvvvtyskgy pcggnktass vieltctktv
2101 grpafkrfdi dsctyyfswd sraacavkpq evqmvngtit npingksfsl gdiyfklfra
2161 sgdmrtngdn ylyeiqlssi tssrnpacsg anicqvkpnd qhfsrkvgts dktkyylqdg
2221 dldvvfasss kcgkdktksv sstiffhcdp lvedgipefs hetadcqylf swytsavcpl
2281 gvgfdsenpg ddgqmhkgls ersqavgavl slllvaltcc llalllykke rretvisklt
2341 tccrrssnvs ykyskvnkee etdenetewl meeiqlpppr qgkegqengh ittksvkals
2401 slhgddqdse devltipevk vhsgrgagae sshpvrnaqs nalqereddr vglvrgekar
2461 kgksssaqqk tvsstklvsf hddsdedllh i
```

Domain 11 (1511 - 1650) is underlined

SEQ ID NO: 1 Human IGF2R

```
   1 cgagcccagt cgagccgcgc tcacctcggg ctcccgctcc gtctccacct ccgcctttgc
  61 cctggcggcg cgaccccgtc ccggcgcggc ccccagcagt cgcgcgccgt tagcctcgcg
 121 cccgccgcgc agtccgggcc cggcgcgatg ggggccgccg ccggccggag ccccccacctg
 181 gggcccgcgc ccgcccgccg cccgcagcgc tctctgctcc tgctgcagct gctgctgctc
 241 gtcgctgccc cggggtccac gcaggcccag gccgccccgt tccccgagct gtgcagttat
 301 acatgggaag ctgttgatac caaaaataat gtactttata aaatcaacat ctgtggaagt
 361 gtggatattg tccagtgcgg gccatcaagt gctgtttgta tgcacgactt gaagacacgc
 421 acttatcatt cagtgggtga ctctgttttg agaagtgcaa ccagatctct cctggaattc
 481 aacacaacag tgagctgtga ccagcaaggc acaaatcaca gagtccagag cagcattgcc
 541 ttcctgtgtg ggaaaaccct gggaactcct gaatttgtaa ctgcaacaga atgtgtgcac
 601 tactttgagt ggaggaccac tgcagcctgc aagaaagaca tatttaaagc aaataaggag
 661 gtgccatgct atgtgtttga tgaagagttg aggaagcatg atctcaatcc tctgatcaag
 721 cttagtggtg cctacttggt ggatgactcc gatccggaca cttctctatt catcaatgtt
 781 tgtagagaca tagacacact acgagaccca ggttcacagc tgcgggcctg tccccccggc
 841 actgccgcct gcctggtaag aggacaccag gcgtttgatg ttggccagcc ccgggacgga
 901 ctgaagctgg tgcgcaagga caggcttgtc ctgagttacg tgagggaaga ggcaggaaag
 961 ctagactttt gtgatggtca cagccctgcg gtgactatta catttgtttg cccgtcggag
1021 cggagagagg gcaccattcc caaactcaca gctaaatcca actgccgcta tgaaattgag
1081 tggattactg agtatgcctg ccacagagat tacctggaaa gtaaaacttg ttctctgagc
1141 ggcgagcagc aggatgtctc catagacctc acaccacttg cccagagcgg aggttcatcc
1201 tatatttcag atggaaaaga atatttgttt tatttgaatg tctgtggaga aactgaaata
1261 cagttctgta ataaaaaaca agctgcagtt tgccaagtga aaaagagcga tacctctcaa
1321 gtcaaagcag caggaagata ccacaatcag accctccgat attcggatgg agacctcacc
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1441 tttgagtgca ataaaaccgc aggtaacgat gggaaaggaa ctcctgtatt cacaggggag
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SEQ ID NO: 2 Human IGF2R coding sequence

```
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121 kqtctlffsw htplaceqat
```

SEQ ID NO: 3 Human IGF2R domain 11

Claims:

1. A mutant IGF2 binding domain comprising the amino acid sequence of residues 1511 to 1650 of human IGF2R with 30 or fewer of said residues mutated,

wherein residue P1597 of said amino acid sequence are substituted for different residues.

2. A mutant IGF2 binding domain comprising an amino acid sequence which has at least 80% sequence identity, at least 90% sequence identity or at least 95% sequence identity with residues 1511 to 1650 of human IGF2R,

wherein residue P1597 of said amino acid sequence are substituted for different residues.

3. A mutant IGF2 binding domain according to claim 1 or claim 2 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with residue P1597 and at least five residues selected from S1543, E1544, K1545, G1546, L1547, Q1569, S1602, G1603 and K1631 are substituted for different residues.

4. A mutant IGF2 binding domain according to any one of the preceding claims that binds IGF2 with a Kd of 10nM or less.

5. A mutant IGF binding domain according to any one of the preceding claims which shows no binding or substantially no binding to IGF1.

6. A mutant IGF binding domain according to any one of the preceding claims wherein P1597 is substituted H or K.

7. A mutant IGF binding domain according to any one of the preceding claims wherein Q1569 of said amino acid sequence is substituted for a different residue.

8. A mutant IGF binding domain according to claim 7 wherein Q1569 of said amino acid sequence is substituted for R.

9. A mutant IGF binding domain according to any one of the preceding claims wherein S1602 of said amino acid sequence is substituted for a different residue.

10. A mutant IGF binding domain according to claim 9 wherein S1602 of said amino acid sequence is substituted for H.

11. A mutant IGF binding domain according to any one of the preceding claims wherein G1603 of said amino acid sequence is substituted for a different residue.

12. A mutant IGF binding domain according to claim 11 wherein G1603 is substituted for K.

13. A mutant IGF binding domain according to any one of the preceding claims wherein E1544 of said amino acid sequence is substituted for a different residue.

14. A mutant IGF binding domain according to claim 11 wherein E1544 is substituted for K.

15. A mutant IGF binding domain according to any one of the preceding claims wherein K1545 of said amino acid sequence is substituted for a different residue.

16. A mutant IGF binding domain according to any one of the preceding claims wherein L1547 of said amino acid sequence is substituted for a different residue.

17. A mutant IGF binding domain according to any one of the preceding claims wherein S1543 of said amino acid sequence is substituted for a different residue.

18. A mutant IGF binding domain according to any one of the preceding claims wherein G1546 of said amino acid sequence is substituted for a different residue.

19. A mutant IGF binding domain according to any one of the preceding claims wherein S1543 is substituted for A.

20. A mutant IGF binding domain according to any one of the preceding claims wherein K1545 is substituted for G.

21. A mutant IGF binding domain according to any one of the preceding claims wherein G1546 is substituted for W

22. A mutant IGF binding domain according to any one of the preceding claims wherein L1547 is substituted for G

23. A mutant IGF binding domain according to any one of the preceding claims wherein the sequence of residues 1542-1547 is YAKGWG

24. A mutant IGF binding domain according to any one claims 1 to 16 wherein K1545 is substituted for S.

25. A mutant IGF binding domain according to any one claims 1 to 16 and 24 wherein L1547 is substituted for V.

26. A mutant IGF binding domain according to any one claims 1 to 16, 24 and 25 wherein the sequence of residues 1542-1547 is YSKSGV.

27. A mutant IGF binding domain according to any one of the preceding claims comprising substitutions at 6 or more positions selected from S1543, E1544, K1545, G1546, L1547, Q1569, P1597, S1602 and G1603.

28. A mutant IGF2 binding domain according to any one of the preceding claims which comprises the amino acid sequence of residues 1511 to 1650

of human IGF2R with substitutions at S1543, E1544, K1545, G1546, L1547, Q1569, P1597 and S1602

29. A mutant IGF2 binding domain according to claim 28 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597H and S1602H.

30. A mutant IGF2 binding domain according to claim 28 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597K and S1602H.

31. A mutant IGF2 binding domain according to any one of claims 1 to 27 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with substitutions at S1543, E1544, K1545, G1546, L1547, Q1569, P1597 and G1603.

32. A mutant IGF2 binding domain according to claim 31 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597H and G1603K.

33. A mutant IGF2 binding domain according to claim 31 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions S1543A, E1544K, K1545G, G1546W, L1547G, Q1569R, P1597K and G1603K.

34. A mutant IGF2 binding domain according to any one of claims 1 to 27 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with substitutions at E1544, K1545, L1547, Q1569, P1597, and S1602.

35. A mutant IGF2 binding domain according to claim 34 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions E1544K, K1545S, L1547V, Q1569R, P1597H, and S1602H.

36. A mutant IGF2 binding domain according to claim 34 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions E1544K, K1545S, L1547V, Q1569R, P1597K, and S1602H.

37. A mutant IGF2 binding domain according to any one of claims 1 to 27 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with substitutions at E1544, K1545, L1547, Q1569, P1597, S1602 and G1603.

38. A mutant IGF2 binding domain according to claim 37 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions E1544K, K1545S, L1547V, Q1569R, P1597H, S1602H and G1603K.

39. A mutant IGF2 binding domain according to claim 37 which comprises the amino acid sequence of residues 1511 to 1650 of human IGF2R with the substitutions E1544K, K1545S, L1547V, Q1569R, P1597K, S1602H and G1603K.

40. A polypeptide comprising an IGF binding domain according to any one of claims 1 to 39.

41. A polypeptide according to claim 40 comprising two or more mutant IGF binding domains according to any one of claims 1 to 39.

42. A polypeptide according to claim 40 or claim 41 comprising domain 13 of human IGF2R.

43. A polypeptide according to any one of claims 40 to 42 comprising an immunoglobulin Fc domain.

44. A polypeptide according to any one of claims 40 to 43 comprising an affinity tag.

45. A nucleic acid encoding an IGF2 binding domain according to any one of claims 1 to 39 or a polypeptide according to any one of claims 40 to 44.

46. A vector comprising a nucleic acid according to claim 45.

47. A host cell comprising a vector according to claim 46.

48. A pharmaceutical composition comprising a mutant IGF2 binding domain according to any one of claims 1 to 39, a polypeptide according to any one of claims 40 to 44 a nucleic acid according to claim 45, a vector according to claim 46 or a host cell according to claim 47.

49. A method of producing a pharmaceutical composition comprising; admixing a mutant IGF2 binding domain according to any one of claims 1 to 39, a polypeptide according to any one of claims 40 to 44, a nucleic acid according to claim 45, a vector according to claim 46 or a host cell according to claim 47 with a pharmaceutically acceptable excipient.

50. An mutant IGF2 binding domain according to any one of claims 1 to 39, a polypeptide according to any one of claims 40 to 44, a nucleic acid according to claim 45, a vector according to claim 46 or a host cell according to claim 47 for use in a method of treatment of the human or animal body by therapy.

51. A composition comprising a mutant IGF2 binding domain according to any one of claims 1 to 39, a polypeptide according to any one of claims 40 to 44, a nucleic acid according to claim 45, a vector according to claim 46 or a host cell according to claim 47 for use in a method of treatment of cancer.

52. Use of a mutant IGF2 binding domain according to any one of claims 1 to 39, a polypeptide according to any one of claims 40 to 44, a nucleic acid according to claim 45, a vector according to claim 46 or a host cell

according to claim 47 in the manufacture of a medicament for use in the treatment of cancer.

53. A method of treating cancer in an individual comprising; administering a mutant IGF2 binding domain according to any one of claims 1 to 39, a polypeptide according to any one of claims 40 to 44, a nucleic acid according to claim 45, a vector according to claim 46 or a host cell according to claim 47 to the individual.

54. A composition, use or method according to any one of claims 51 to 53 wherein the cancer is associated with reduced IGF2R activity or function or increased IGF2 activity or function.

55. A composition, use or method according to any one of claims 51 to 54 wherein the cancer expresses IGF2.

56. A composition, use or method according to any one of claims 51 to 55 wherein the cancer is characterised by non-islet tumour associated hypoglycaemia (NICTH).

57. A composition, use or method according to any one of claims 51 to 56 wherein the mutant IGF2 binding domain, polypeptide, nucleic acid, vector, or host cell is administered in combination with a PI3 kinase inhibitor.

58. A composition, use or method according to claim 57 wherein the PI3 kinase inhibitor is pictilisib or F-04691502.

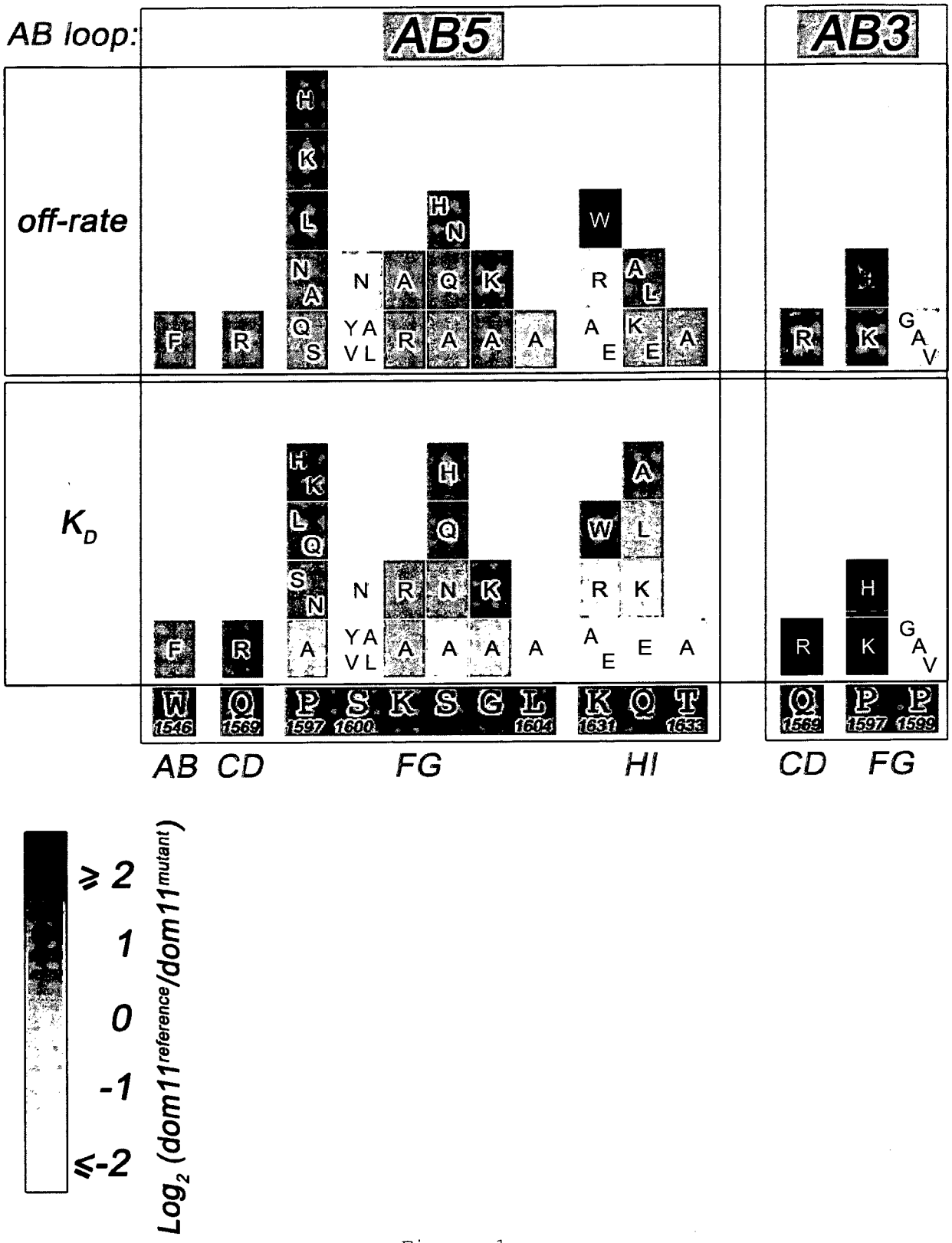
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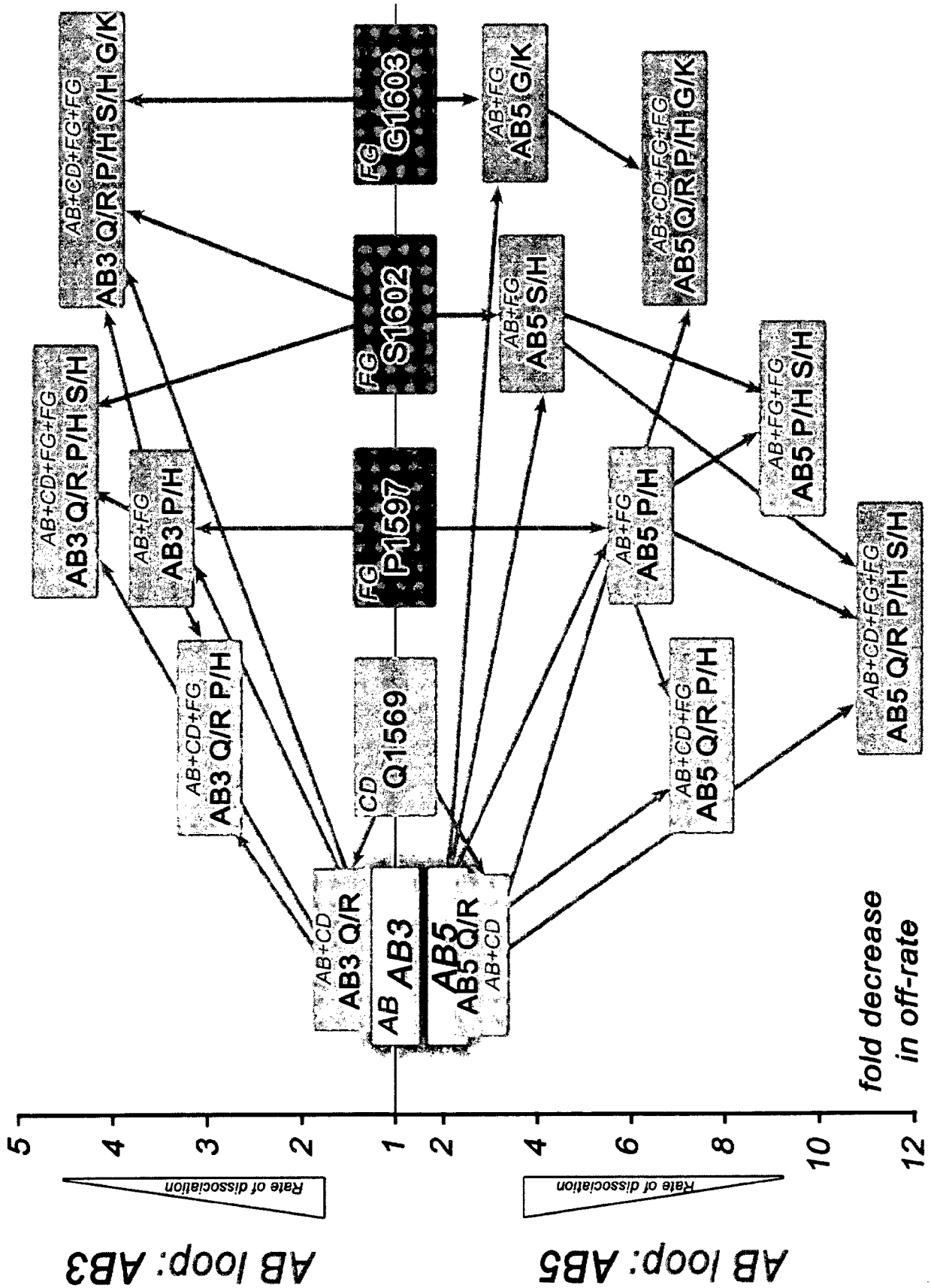
Figure 1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AB+CD+FG+FG AB5Q/R P/H G/K

Figure 2

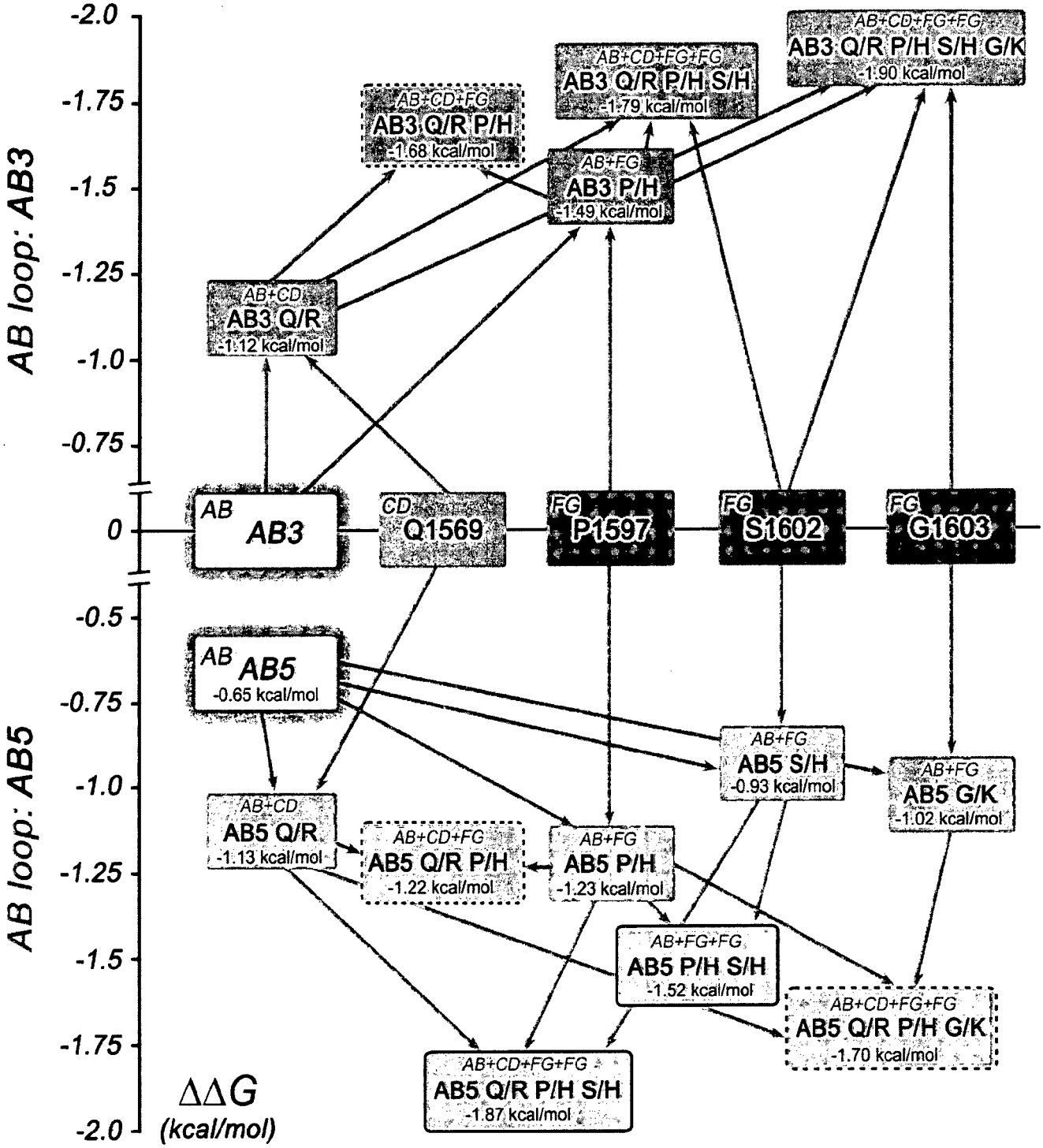

AB loop: AB3
AB loop: AB5
-2.0
-1.75
-1.5
-1.25
-1.0
-0.75
0
-0.5
-0.75
-1.0
-1.25
-1.5
-1.75
-2.0
ΔΔG
(kcal/mol)
AB AB3
CD Q1569
FG P1597
FG S1602
FG G1603
AB+CD AB3 Q/R -1.12 kcal/mol
AB+CD+FG AB3 Q/R P/H -1.68 kcal/mol
AB+FG AB3 P/H -1.49 kcal/mol
AB+CD+FG+FG AB3 Q/R P/H S/H -1.79 kcal/mol
AB+CD+FG+FG AB3 Q/R P/H S/H G/K -1.90 kcal/mol
AB AB5 -0.65 kcal/mol
AB+CD AB5 Q/R -1.13 kcal/mol
AB+CD+FG AB5 Q/R P/H -1.22 kcal/mol
AB+FG AB5 P/H -1.23 kcal/mol
AB+FG AB5 S/H -0.93 kcal/mol
AB+FG AB5 G/K -1.02 kcal/mol
AB+FG+FG AB5 P/H S/H -1.52 kcal/mol
AB+CD+FG+FG AB5 Q/R P/H G/K -1.70 kcal/mol
AB+CD+FG+FG AB5 Q/R P/H S/H -1.87 kcal/mol

Figure 3

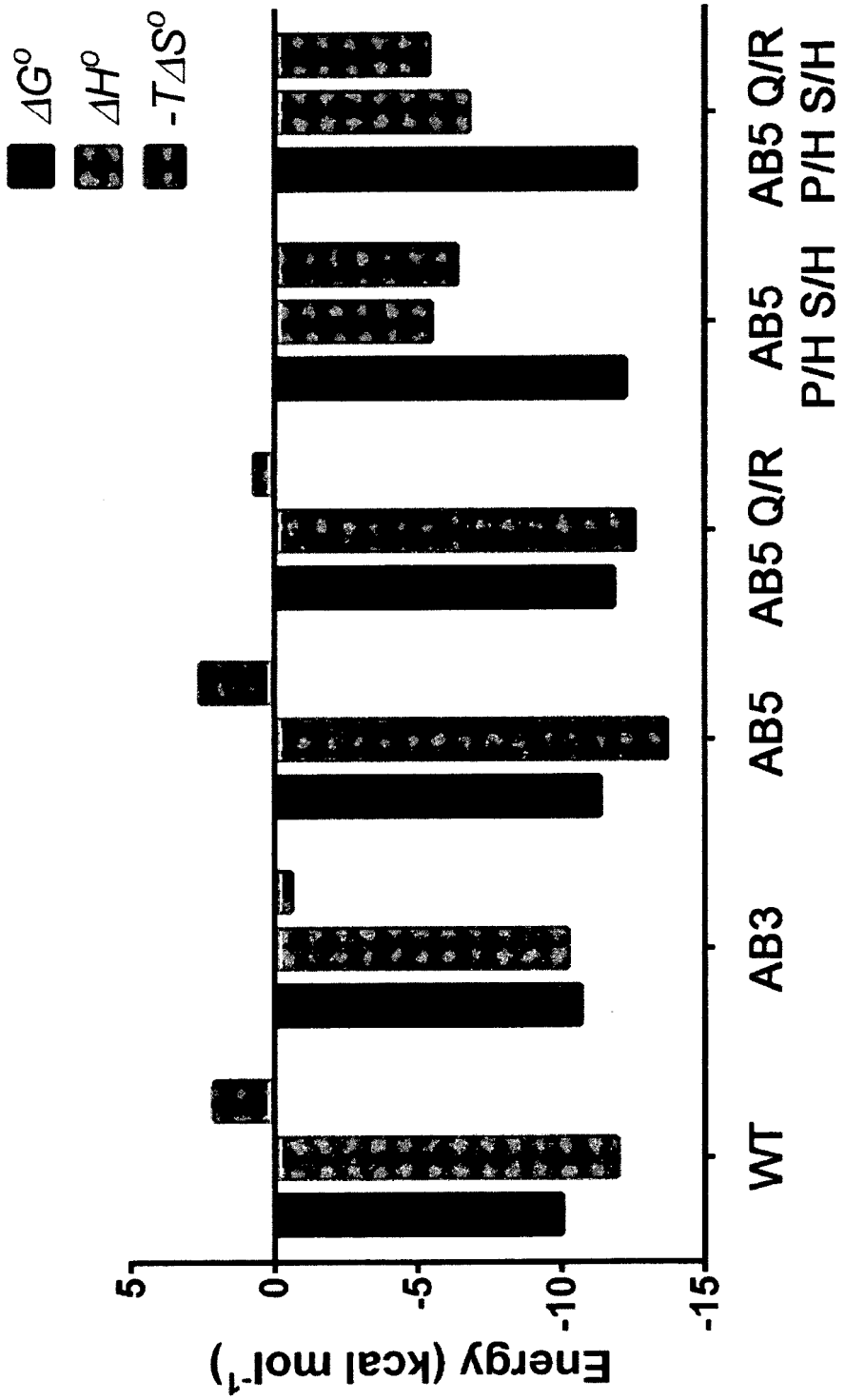

ΔG°
ΔH°
-TΔS°
Energy (kcal mol-1)
5
0
-5
-10
-15
WT
AB3
AB5
AB5 Q/R
AB5 P/H S/H
AB5 Q/R P/H S/H

Figure 4

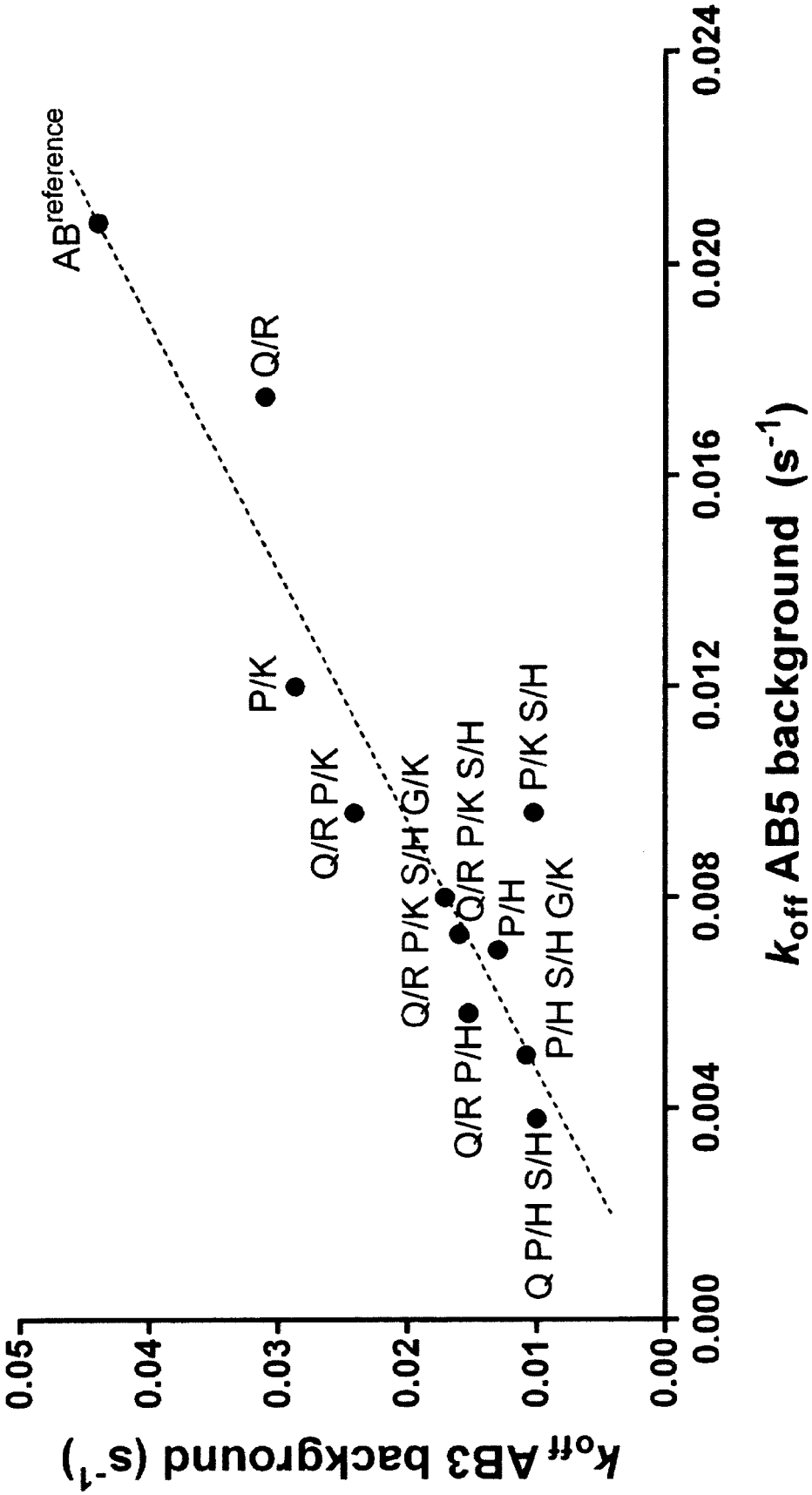

AB reference
Q/R
P/K
Q/R P/K
Q/R P/K S/H G/K
Q/R P/K S/H
P/H
P/K S/H
Q/R P/H
P/H S/H G/K
Q P/H S/H
0.05
0.04
0.03
0.02
0.01
0.00
0.000
0.004
0.008
0.012
0.016
0.020
0.024
k_{off} AB3 background (s^{-1})
k_{off} AB5 background (s^{-1})

Figure 5

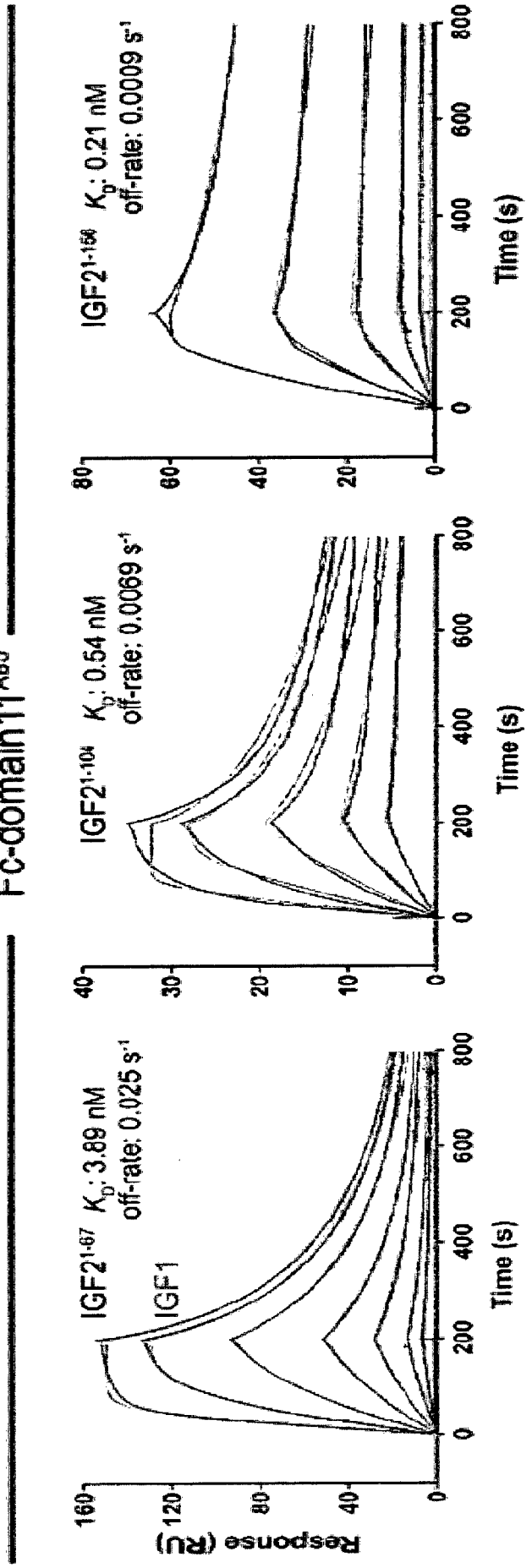

Fc-domain11AB5
IGF2^1-67 K_D: 3.89 nM
off-rate: 0.025 s^-1
IGF1
IGF2^1-104 K_D: 0.54 nM
off-rate: 0.0069 s^-1
IGF2^1-156 K_D: 0.21 nM
off-rate: 0.0009 s^-1
Response (RU)
Time (s)

Figure 6A

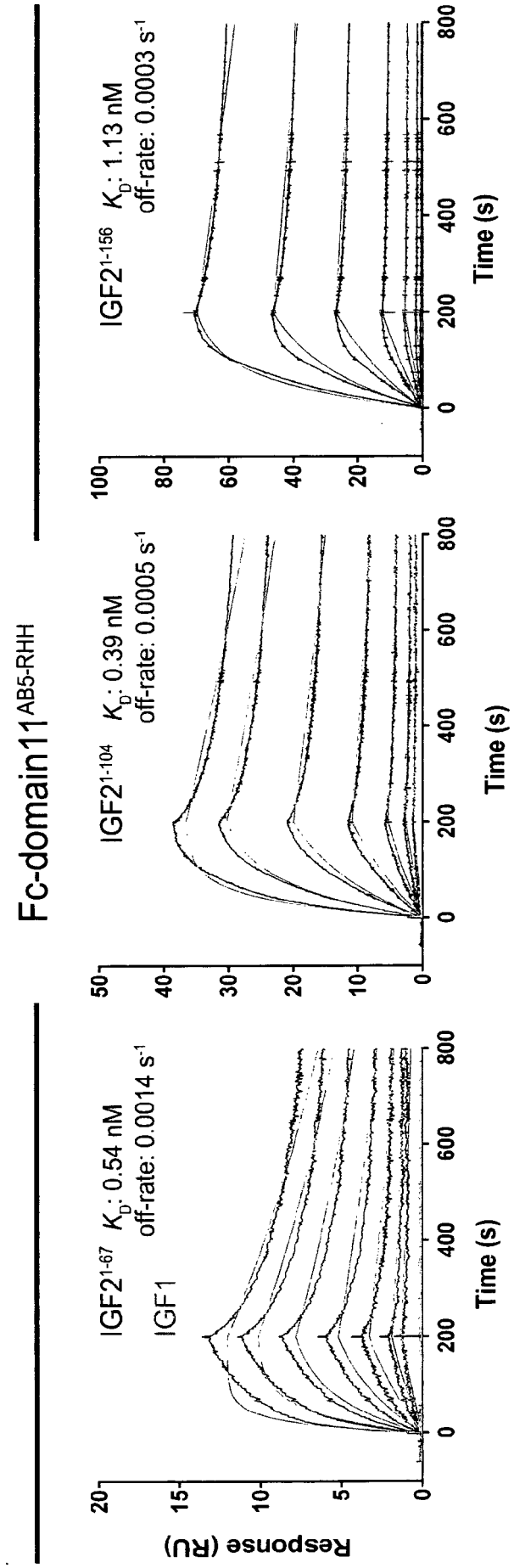

Fc-domain11AB5-RHH
IGF2^1-67 K_D: 0.54 nM
off-rate: 0.0014 s^-1
IGF1
IGF2^1-104 K_D: 0.39 nM
off-rate: 0.0005 s^-1
IGF2^1-156 K_D: 1.13 nM
off-rate: 0.0003 s^-1
Response (RU)
Time (s)

Figure 6B

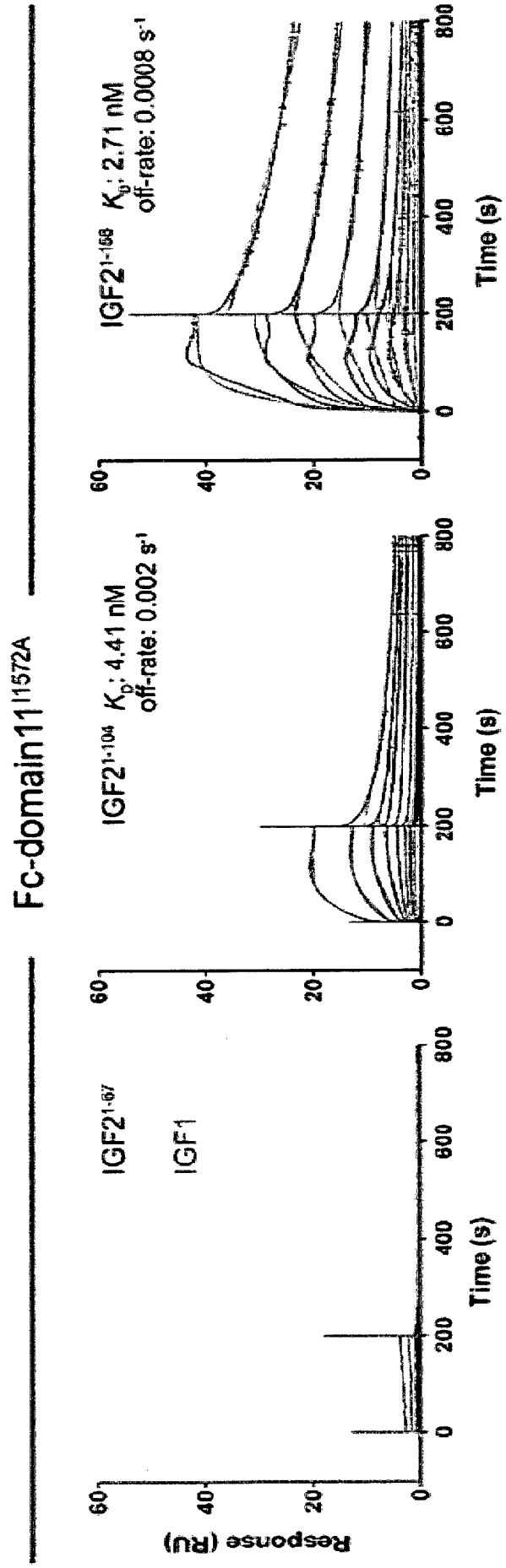

Fc-domain11I1572A
IGF2$^{1-168}$ K_D: 2.71 nM
off-rate: 0.0008 s^{-1}
IGF2$^{1-104}$ K_D: 4.41 nM
off-rate: 0.002 s^{-1}
IGF2$^{1-67}$
IGF1
Response (RU)
Time (s)
0
20
40
60
200
400
600
800

Figure 6C

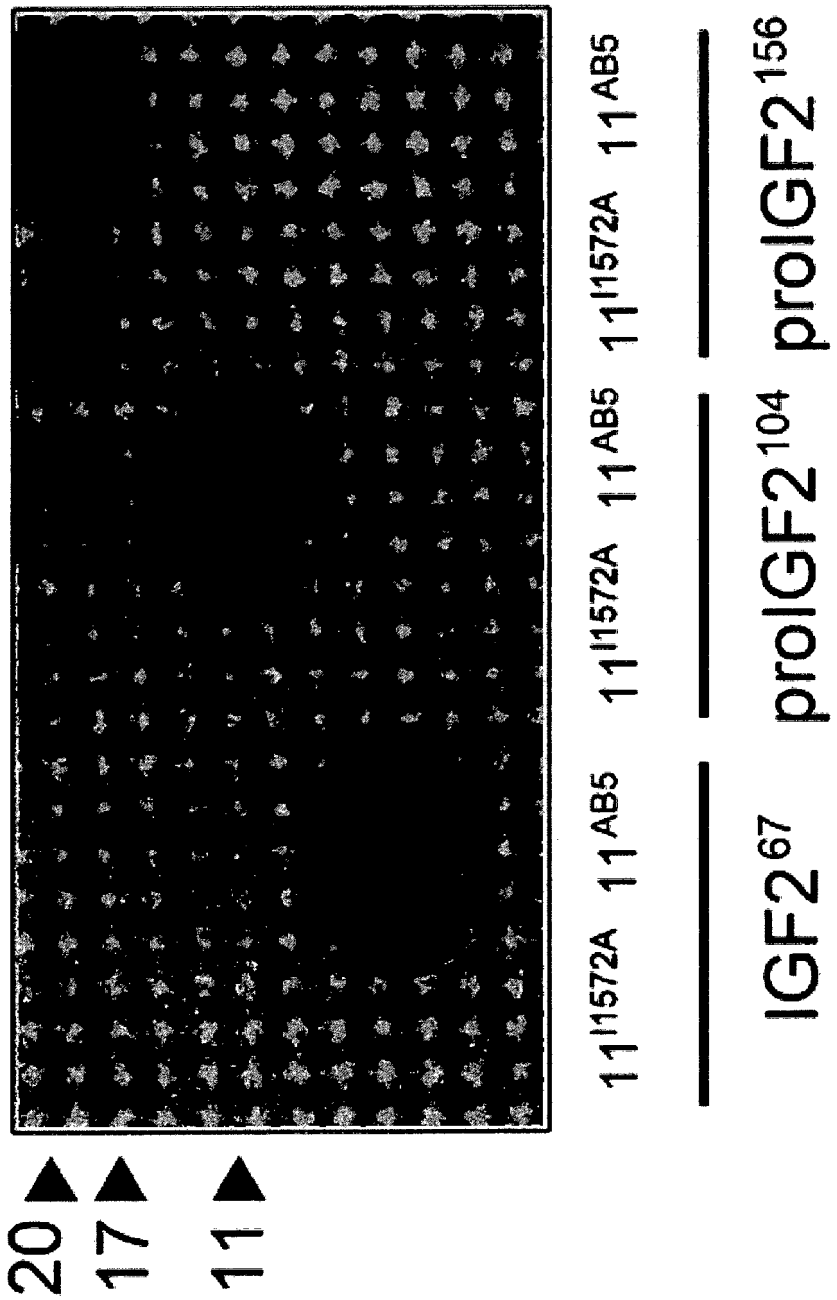

20
17
11
11I1572A 11AB5
IGF2 67
11I1572A 11AB5
proIGF2 104
11I1572A 11AB5
proIGF2 156

Figure 7

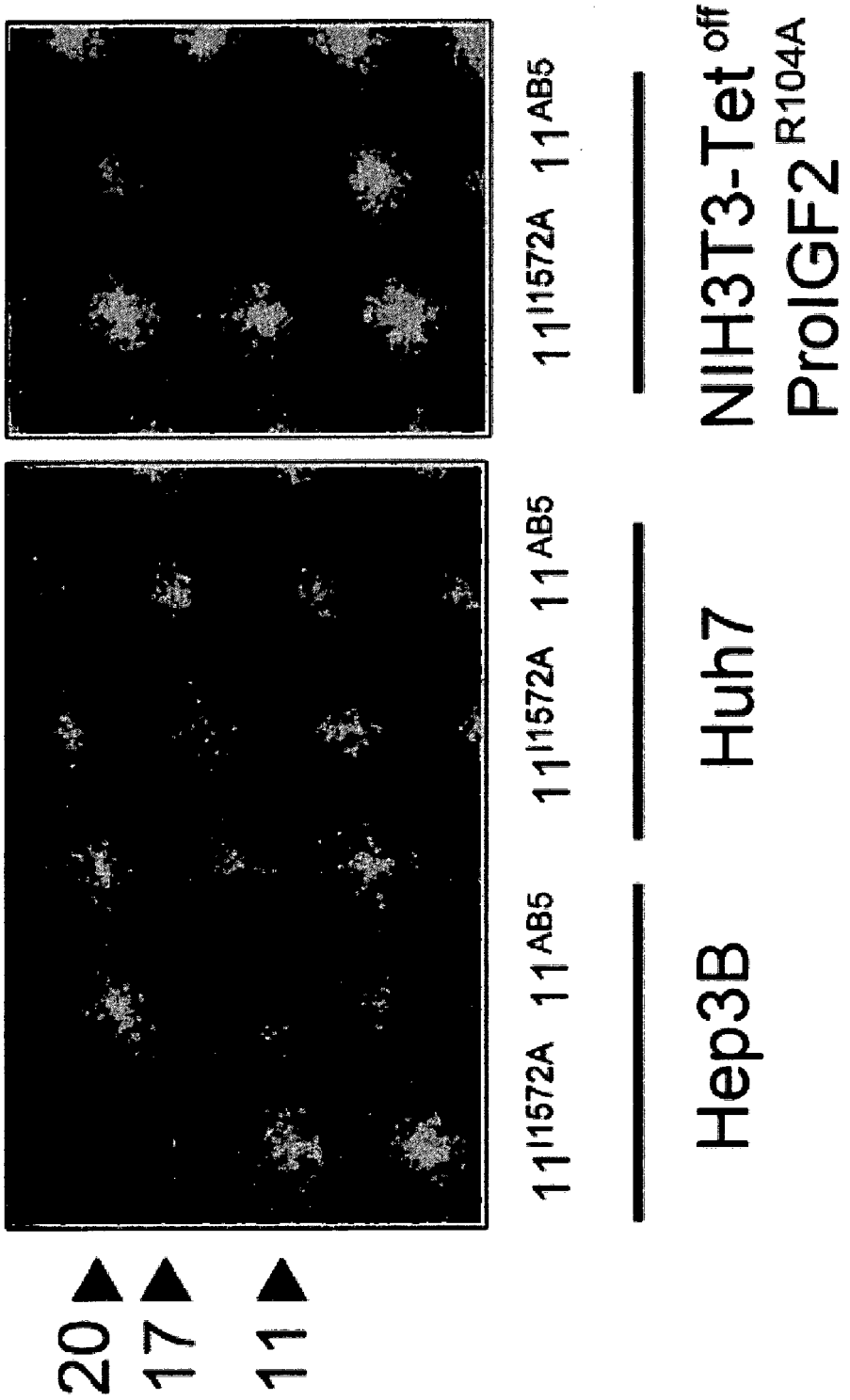

20
17
11
11I1572A 11AB5
11I1572A 11AB5
11I1572A 11AB5
Hep3B
Huh7
NIH3T3-Tet off
ProIGF2 R104A

Figure 8

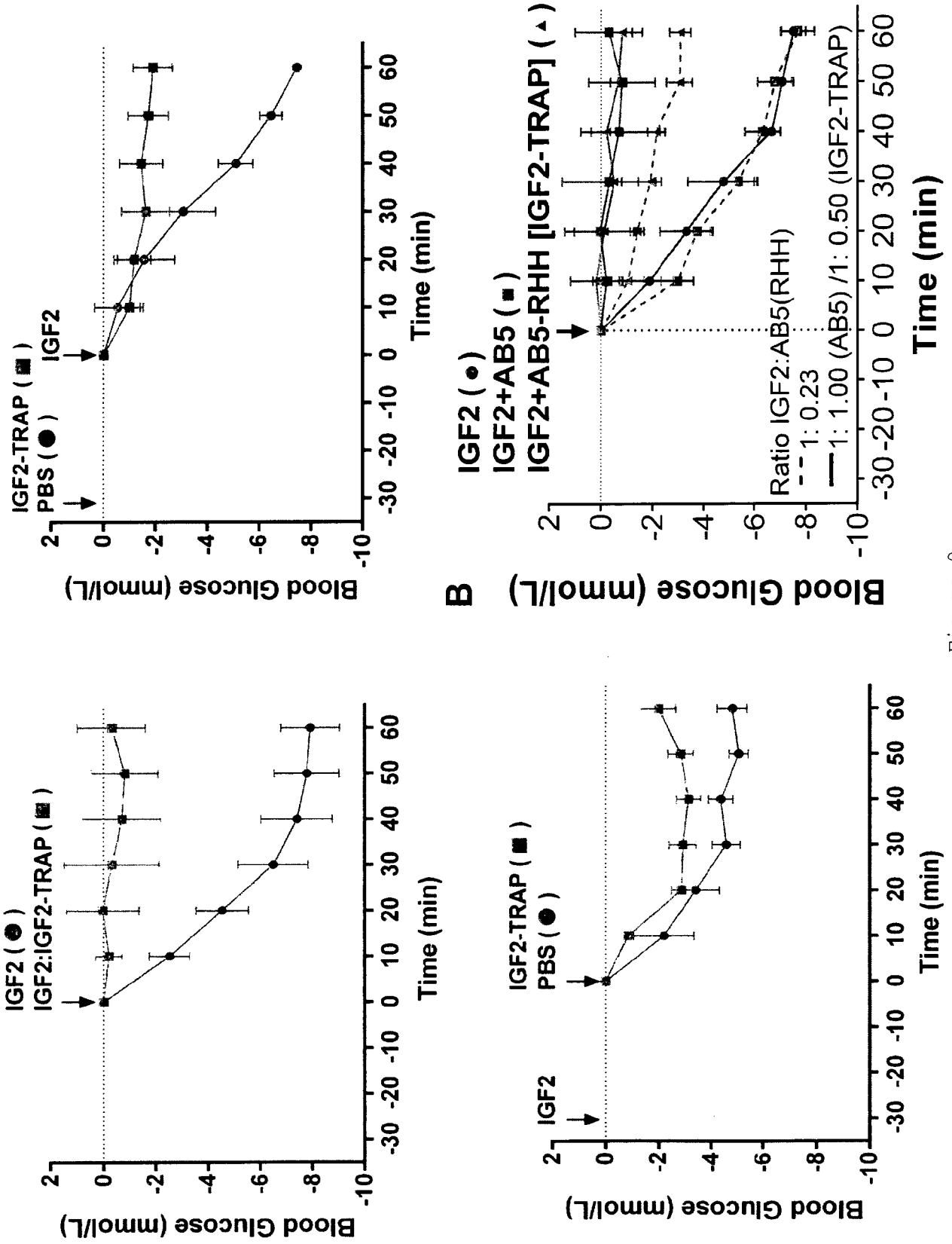

A
IGF2 (●)
IGF2:IGF2-TRAP (■)
Blood Glucose (mmol/L)
Time (min)
IGF2-TRAP (■)
PBS (●)
IGF2
B
IGF2 (●)
IGF2+AB5 (■)
IGF2+AB5-RHH [IGF2-TRAP] (▲)
Ratio IGF2:AB5(RHH)
1: 0.23
1: 1.00 (AB5) /1: 0.50 (IGF2-TRAP)

Figure 9

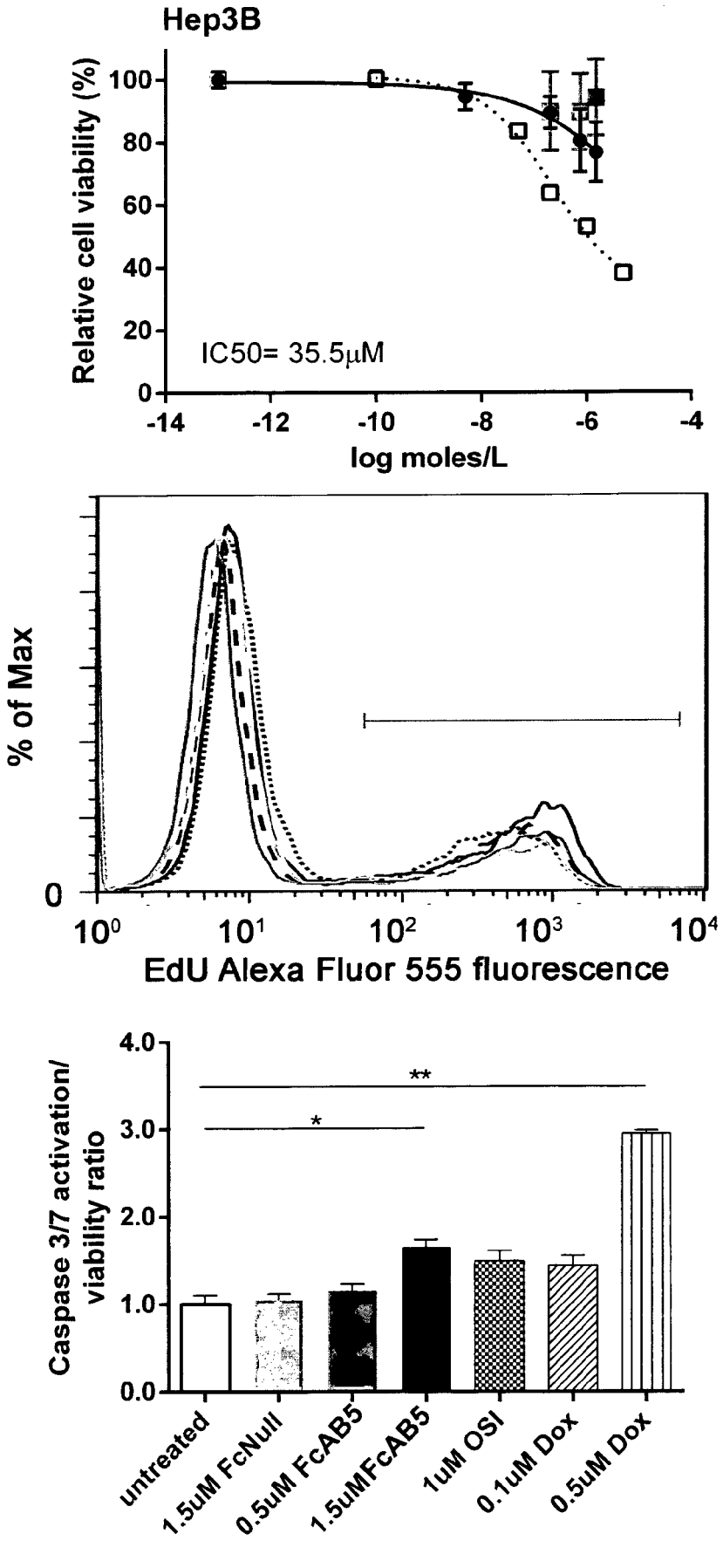
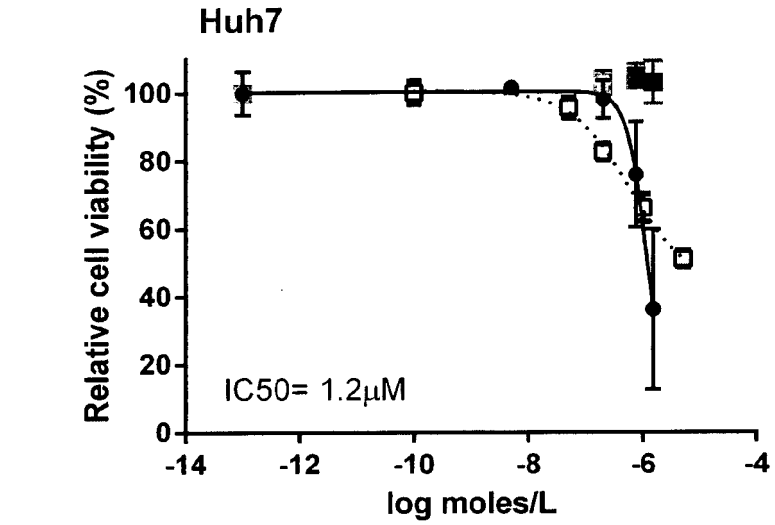
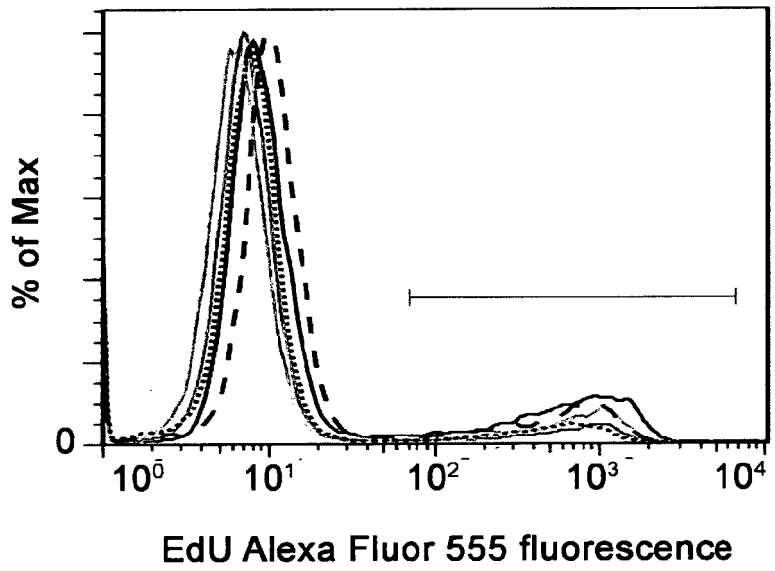

Hep3B
Relative cell viability (%)
100
80
60
40
20
0
IC50= 35.5μM
-14
-12
-10
-8
-6
-4
log moles/L
% of Max
0
10⁰
10¹
10²
10³
10⁴
EdU Alexa Fluor 555 fluorescence
Caspase 3/7 activation/ viability ratio
4.0
3.0
2.0
1.0
0.0
**
*
untreated
1.5uM FcNull
0.5uM FcAB5
1.5uMFcAB5
1uM OSI
0.1uM Dox
0.5uM Dox

Figure 10A


Huh7
Relative cell viability (%)
100
80
60
40
20
0
IC50= 1.2μM
-14
-12
-10
-8
-6
-4
log moles/L


% of Max
0
10^0
10^1
10^2
10^3
10^4
EdU Alexa Fluor 555 fluorescence

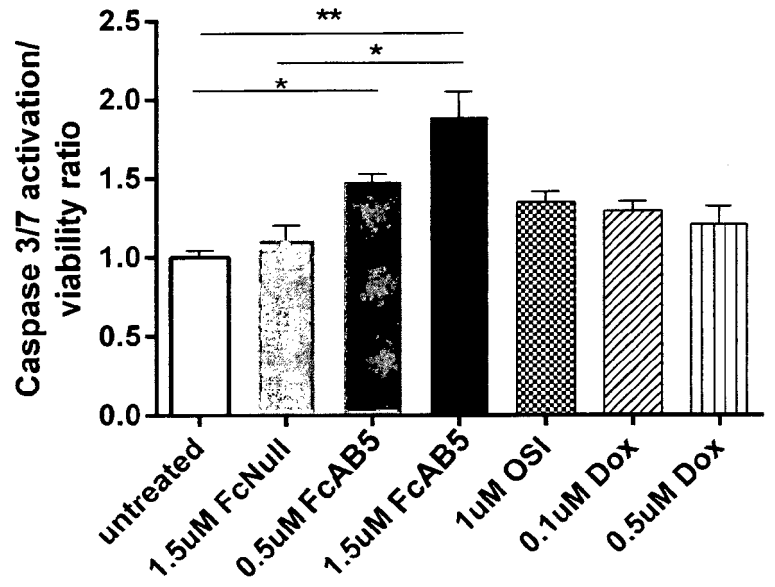

Caspase 3/7 activation/ viability ratio
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
untreated
1.5uM FcNull
0.5uM FcAB5
1.5uM FcAB5
1uM OSI
0.1uM Dox
0.5uM Dox

Figure 10B

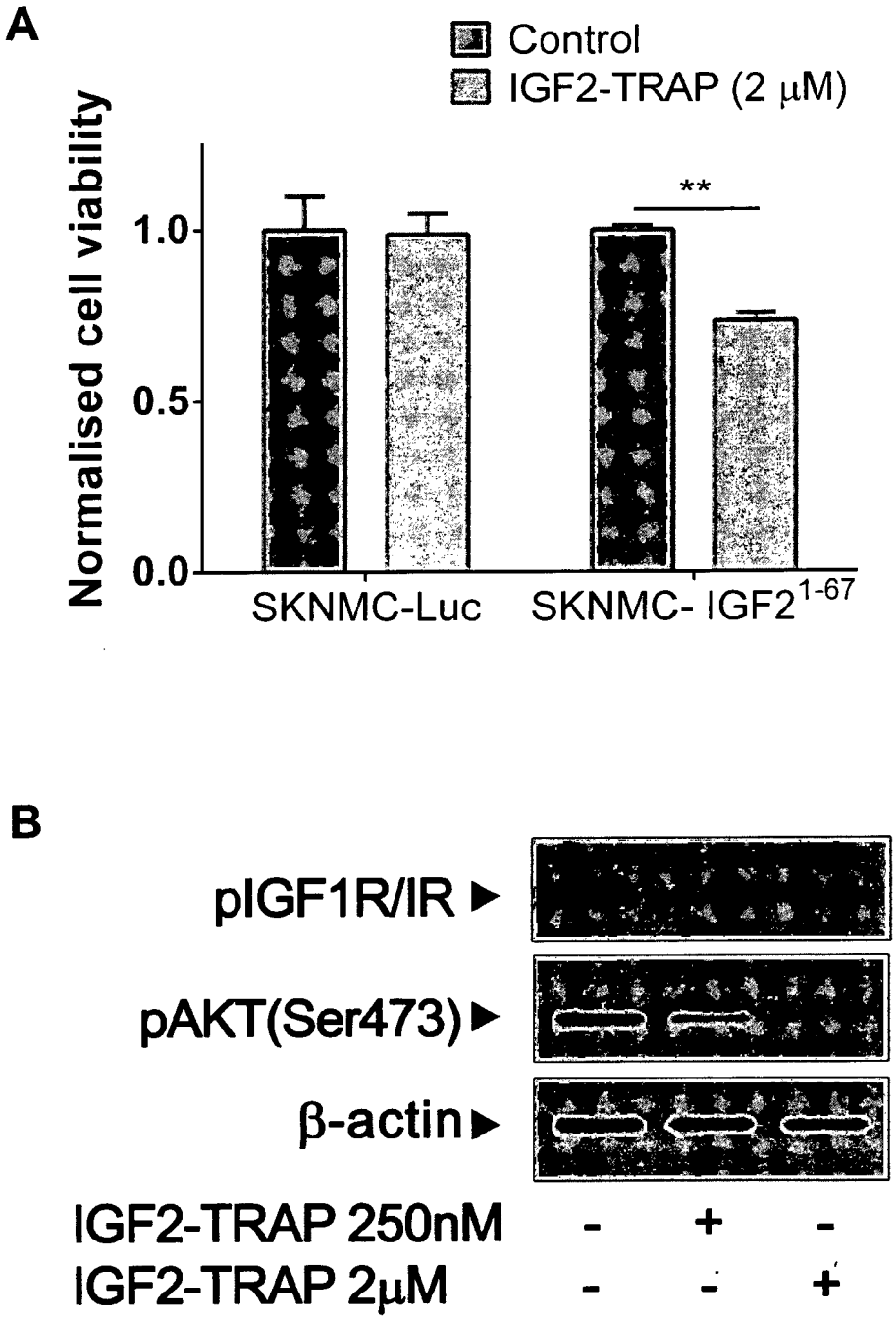

A
Control
IGF2-TRAP (2 μM)
Normalised cell viability
1.0
0.5
0.0
**
SKNMC-Luc
SKNMC- IGF2 1-67
B
pIGF1R/IR ▶
pAKT(Ser473) ▶
β-actin ▶
IGF2-TRAP 250nM - + -
IGF2-TRAP 2μM - - +

Figure 11

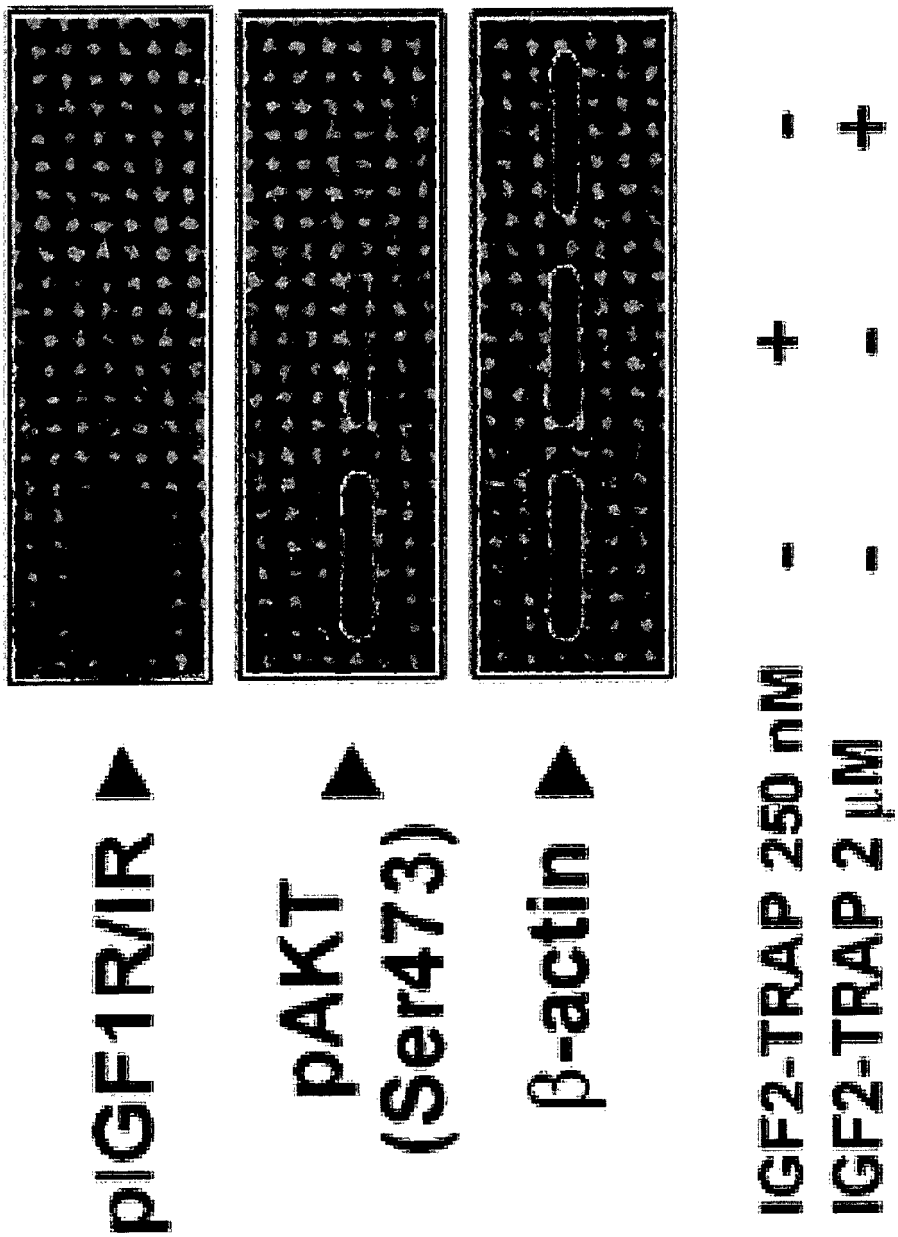


Figure 12A

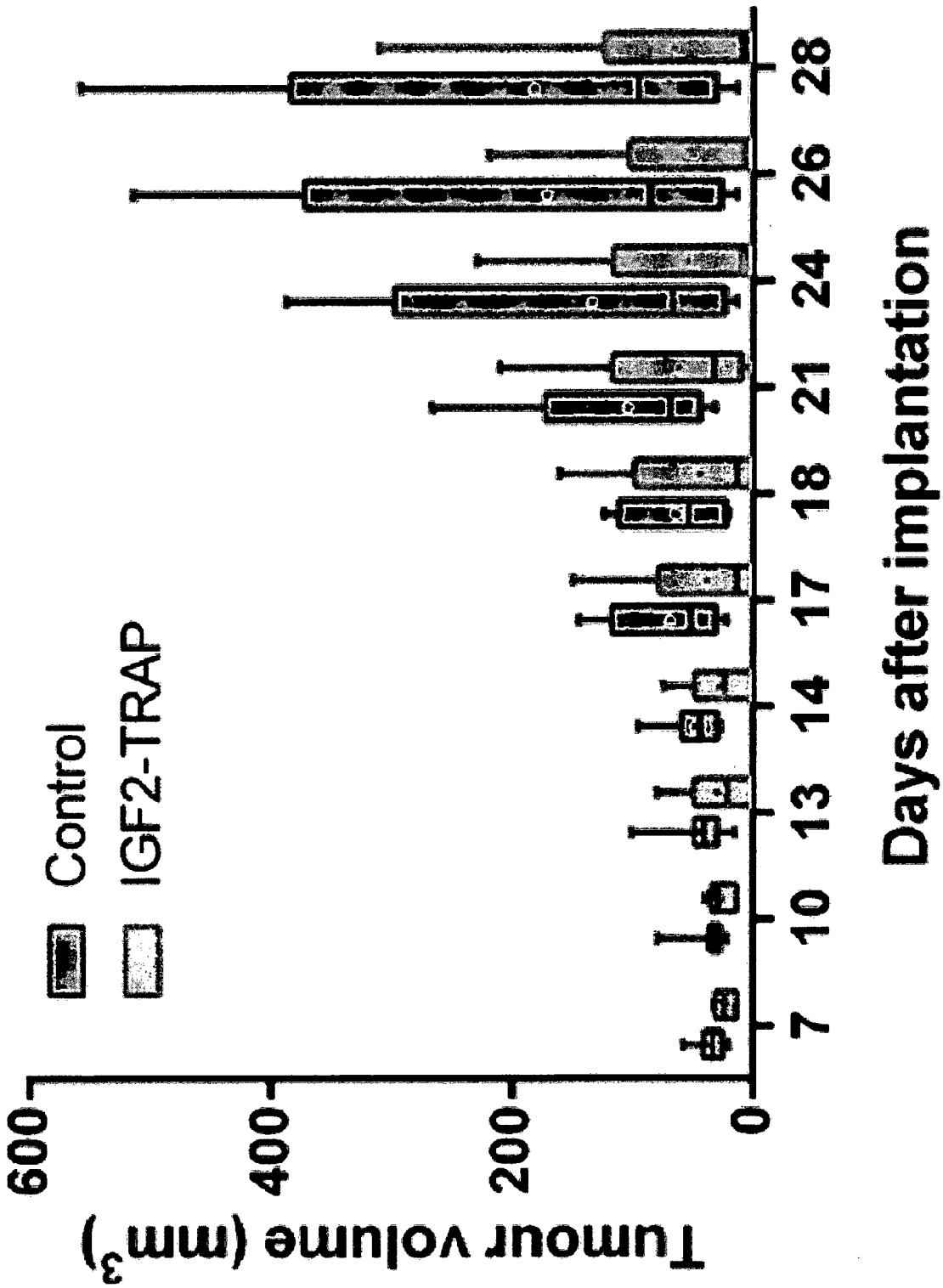

Tumour volume (mm³)
600
400
200
0
Control
IGF2-TRAP
7
10
13
14
17
18
21
24
26
28
Days after implantation

Figure 12B

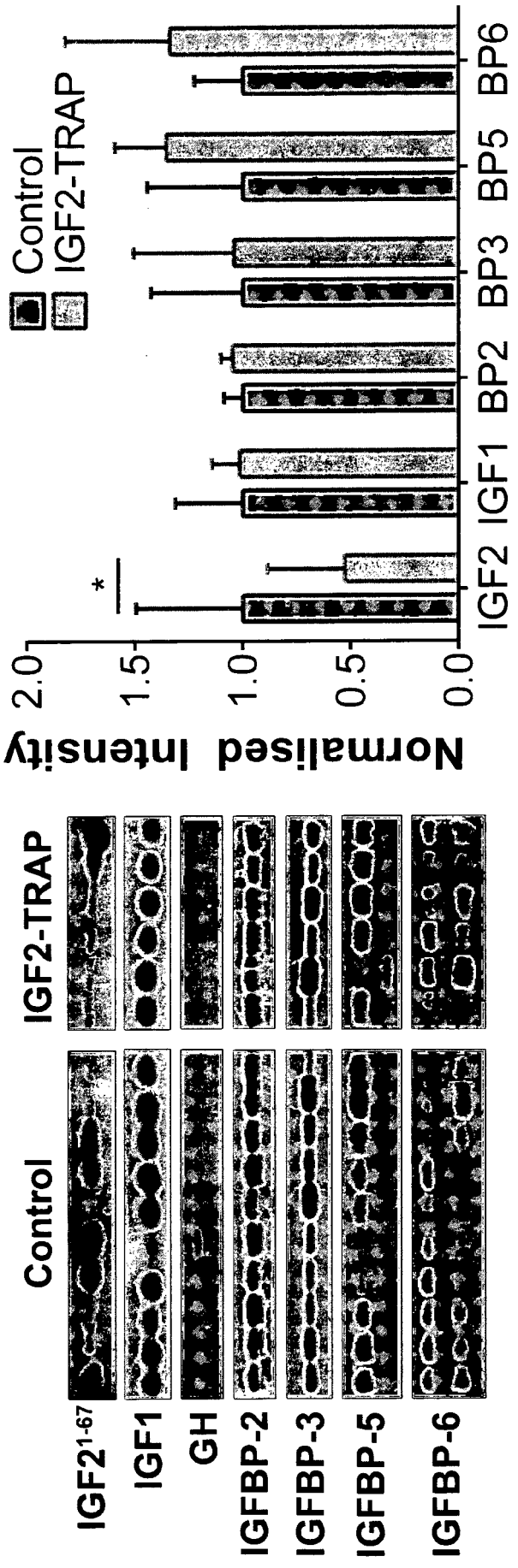

Control
IGF2-TRAP
IGF2 IGF1 BP2 BP3 BP5 BP6
Normalised Intensity
2.0
1.5
1.0
0.5
0.0
*
Control
IGF2-TRAP
IGF2^1-67
IGF1
GH
IGFBP-2
IGFBP-3
IGFBP-5
IGFBP-6

Figure 12C

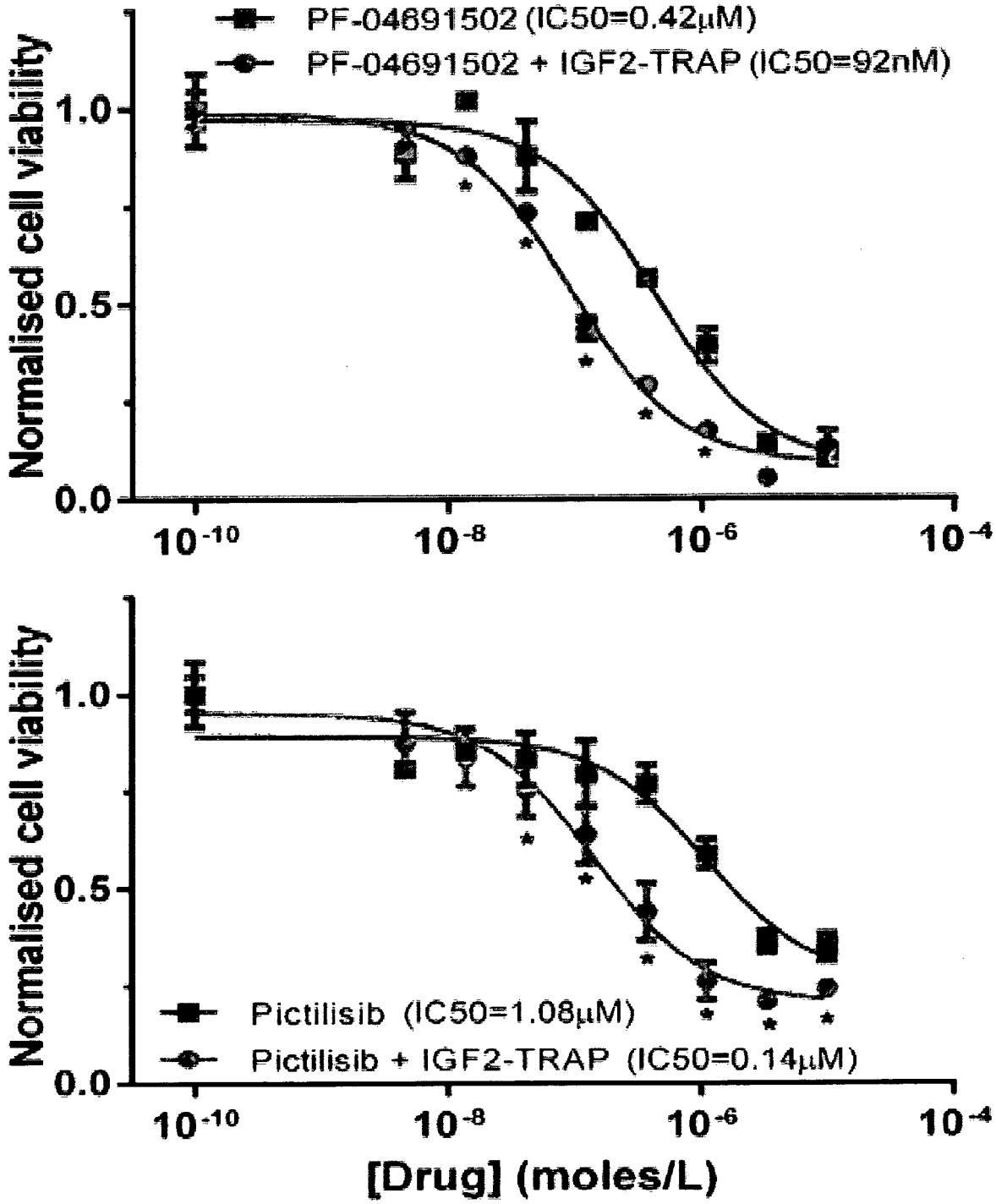

PF-04691502 (IC50=0.42μM)
PF-04691502 + IGF2-TRAP (IC50=92nM)
Normalised cell viability
1.0
0.5
0.0
10-10
10-8
10-6
10-4
Pictilisib (IC50=1.08μM)
Pictilisib + IGF2-TRAP (IC50=0.14μM)
[Drug] (moles/L)

Figure 13

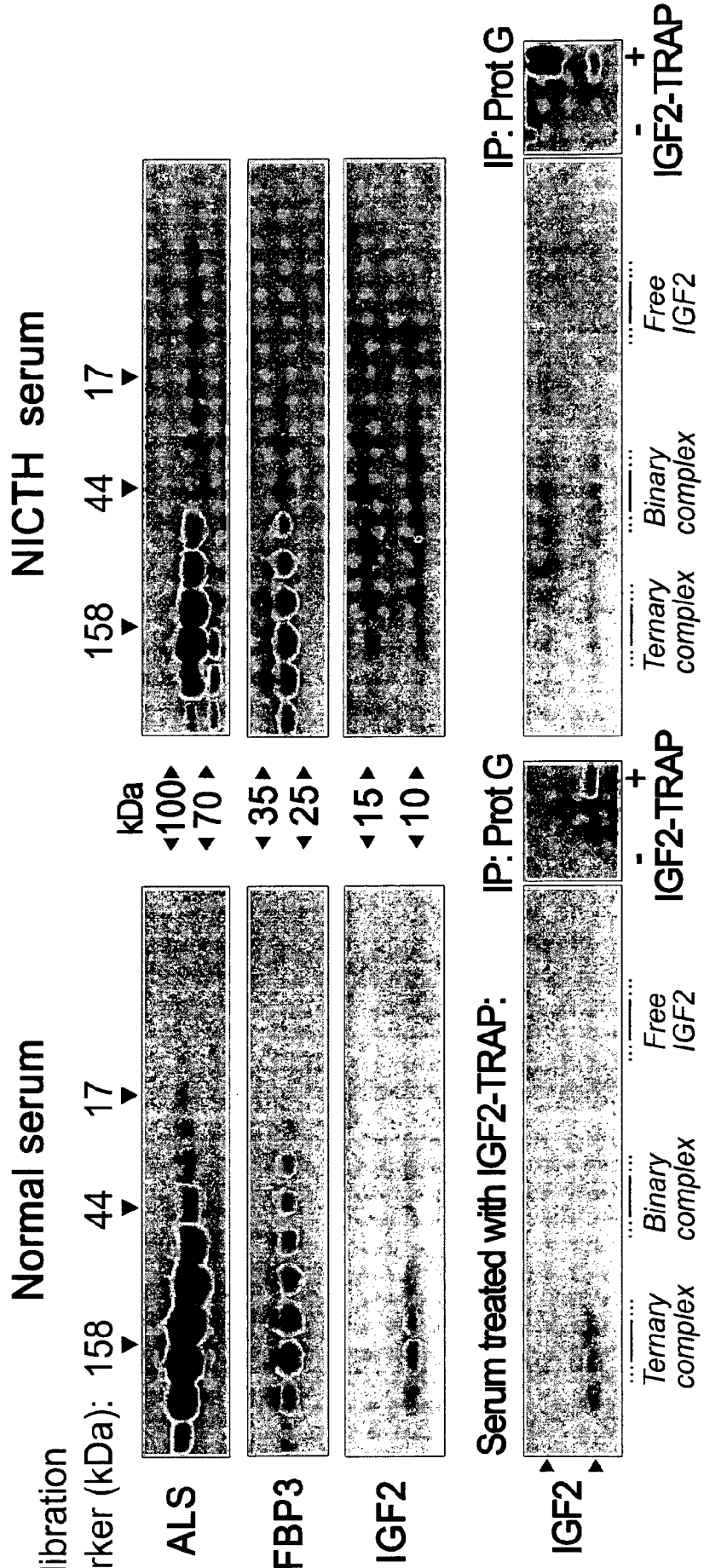

Normal serum
NICTH serum
Calibration marker (kDa):
158
44
17
kDa
100
70
35
25
15
10
ALS
IGFBP3
IGF2
Serum treated with IGF2-TRAP:
IP: Prot G
IGF2-TRAP
Ternary complex
Binary complex
Free IGF2

Figure 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/068904

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435 A61K38/17 C12N15/63
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2007/020402 A1 (CANCER REC TECH LTD [GB]; HASSAN ANDREW BASSIM [GB]; ZACCHEO OLIVER [G) 22 February 2007 (2007-02-22) whole document esp. Figure 2 -----	1-58
X	WO 2013/013017 A2 (ALNYLAM PHARMACEUTICALS INC [US]; ROSSOMANDO ANTHONY [US]; BETTENCOURT) 24 January 2013 (2013-01-24) whole document esp. seq id no 409 -----	1,40, 45-53
X	, 16 November 2011 (2011-11-16), XP002762422, Retrieved from the Internet: URL:http://www.uniprot.org/uniparc/UPI0001C5F204 [retrieved on 2016-09-26] the whole document -----	1,40

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 2016	05/12/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brück, Marianne

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOSCHOS S.J. . MANTZOROS C.S.: "The Role of the IGF System in Cancer: From Basic to Clinical Studies and Clinical Applications", ONCOLOGY, vol. 63, 7 November 2002 (2002-11-07), pages 317-332, XP009191817, whole document esp. pages 317,327 -----	1-58

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/068904

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007020402 A1	22-02-2007	AT 483730 T	15-10-2010
		EP 1945663 A1	23-07-2008
		ES 2353674 T3	04-03-2011
		US 2009304651 A1	10-12-2009
		US 2013071366 A1	21-03-2013
		WO 2007020402 A1	22-02-2007
WO 2013013017 A2	24-01-2013	NONE	