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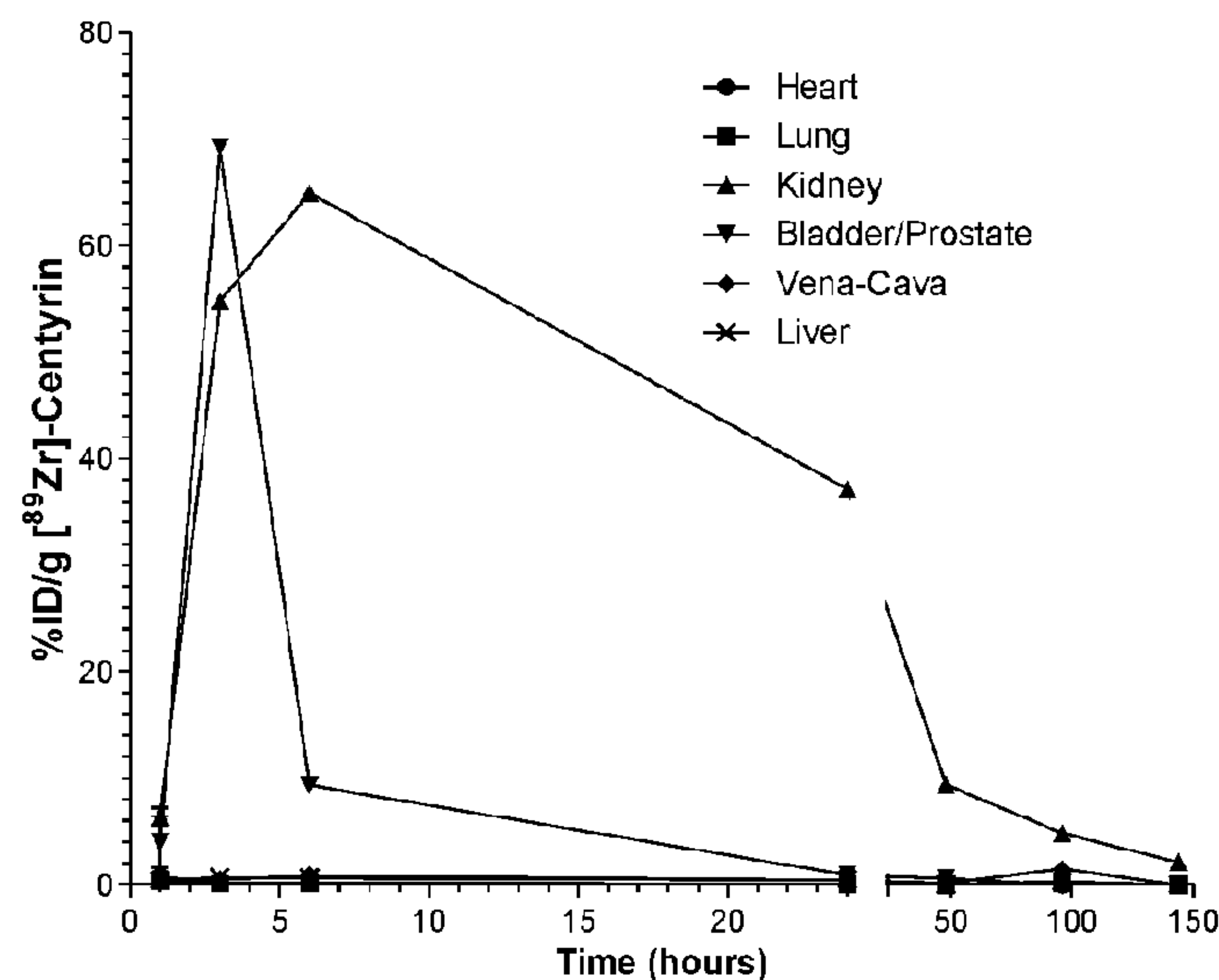
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(54) Title: PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA) BISPECIFIC BINDING AGENTS AND USES THEREOF

Fig. 1



(57) **Abrégé/Abstract:**

Provided herein are isolated CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragment thereof wherein a FN3 domain specifically binds human prostate specific membrane antigen (PSMA) and a second antigen-binding site immunospecifically binds CD3. Also described are fusion proteins and related polynucleotides capable of encoding the provided fusion proteins and, cells expressing the provided fusion proteins. In addition, methods of using the provided isolated CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragment thereof are described.



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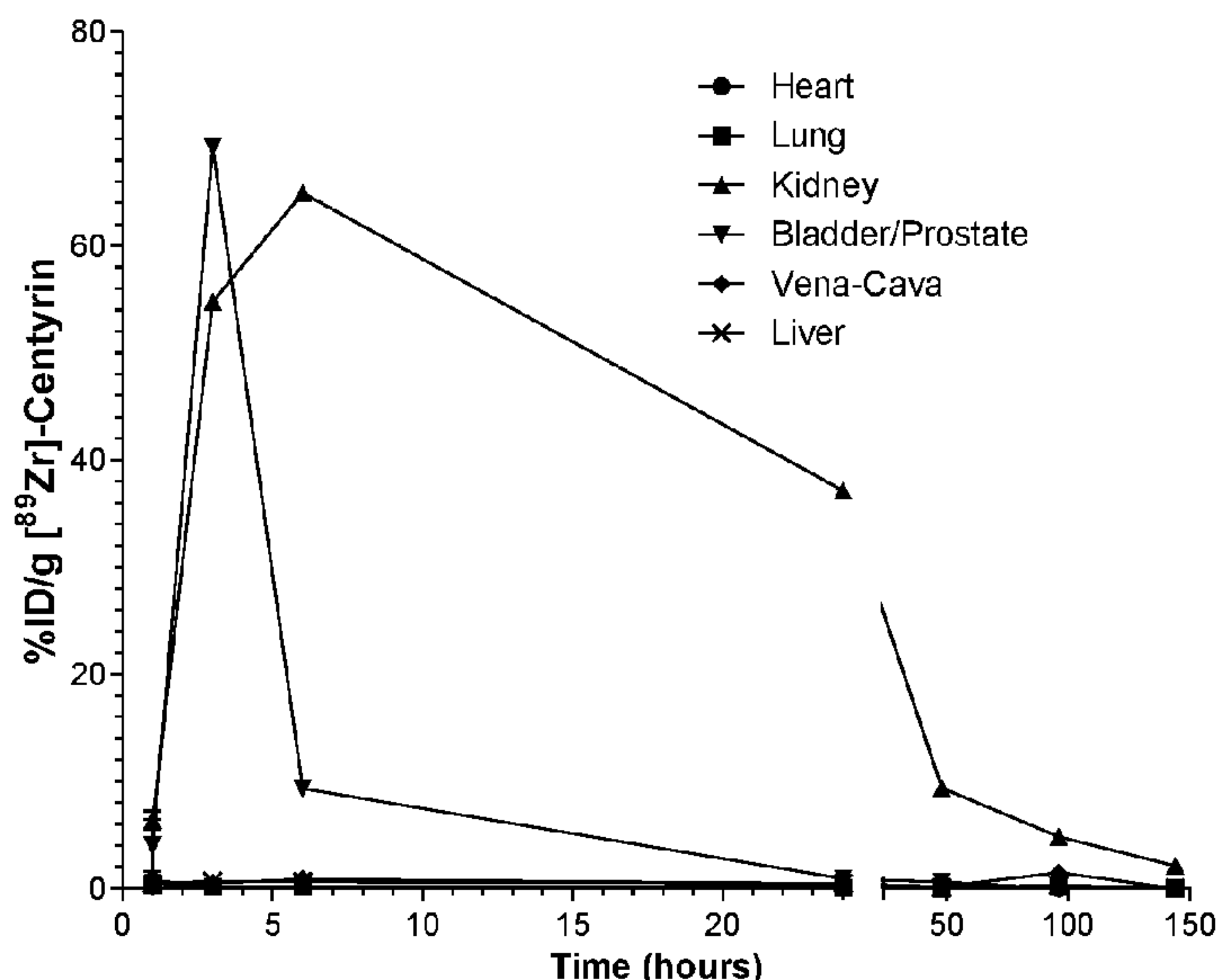
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Fig. 1



(57) Abstract: Provided herein are isolated CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragment thereof wherein a FN3 domain specifically binds human prostate specific membrane antigen (PSMA) and a second antigen-binding site immunospecifically binds CD3. Also described are fusion proteins and related polynucleotides capable of encoding the provided fusion proteins and, cells expressing the provided fusion proteins. In addition, methods of using the provided isolated CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragment thereof are described.

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PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA) BISPECIFIC BINDING AGENTS
AND USES THEREOF

SEQUENCE LISTING

[0000] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 3, 2016, is named JBI5066WOPCT_SL.txt and is 195,436 bytes in size.

TECHNICAL FIELD

[0001] The disclosure provided herein relates to multispecific agents that specifically bind human prostate specific membrane antigen (PSMA) and immunospecifically bind cluster determinant 3 (CD3), and methods of producing and using the described agents.

BACKGROUND

[0002] Prostate specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II or N-acetylated alpha-linked acidic dipeptidase 1, is a dimeric type 2 transmembrane glycoprotein. PSMA cleaves several substrates, including folate and N-acetyl-L-aspartyl-L-glutamate, and is expressed in a number of tissues with highest expression in prostate, and to a lesser extent in the small intestine, central and peripheral nervous system, kidney and lung. PSMA is constitutively internalized through clathrin coated pits.

[0003] PSMA is a prostate-cancer related cell membrane antigen frequently overexpressed in prostatic intraepithelial neoplasia (PIN), a condition in which some prostate cells have begun to look and behave abnormally; primary and metastatic prostate cancers; and the neovasculature of other solid tumors (e.g. breast, lung, bladder, kidney). PSMA expression correlates with disease progression and Gleason score. PSMA expression is increased in metastatic disease, hormone refractory cases, and higher-grade lesions, and it is further upregulated in androgen-insensitive tumors

[0004] Prostate cancer is the leading cause of cancer among males, and the 2nd leading cause of cancer-induced death. Globally, there are approximately 1,100,000 new cases and 300,000 mortalities every year, translating to about 4% of all cancer deaths. It is estimated that 1 in every 6 men will be diagnosed with the disease. In the U.S., more than 90% of prostate

cancers are found in local or regional stages. At these early stages, the 5-year survival rate is close to 100%. When the cancer has metastasized, however, the 5-year survival rate is reduced to about 28%.

[0005] Current treatments for prostate cancer include surgery, radiation, hormone and antibody-drug conjugate (ADC) therapies. However, tumor cells often become androgen insensitive, and, when this occurs, limited treatment options remain. Typically, the cancer vaccine sipuleucel-T, a radiopharmaceutical agent (such as radium-223 chloride), secondary hormone therapies (such as abiraterone or enzalutamide), and/or chemotherapies (docetaxel and cabazitaxel) are added to the hormonal therapy in sequence.

[0006] While each of these treatments can delay growth of the cancer for several months and palliate symptoms produced by the disease, the disease ultimately becomes resistant to them.

[0007] Therefore, there remains a need for additional and improved therapeutics to treat prostate cancer and other cancers overexpressing PSMA.

SUMMARY

[0008] Described herein are isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA (“CD3 x PSMA multispecific molecules”) or multispecific antigen-binding fragments thereof. In one embodiment, an isolated multispecific molecule or multispecific antigen-binding fragment thereof that binds specifically to PSMA is provided. In one embodiment, the PSMA that is bound comprises the amino acid sequence of SEQ ID NO: 144.

FN3 Domain

[0009] In some embodiments, the PSMA-specific domain of the multispecific antigen-binding molecule or multispecific antigen-binding fragment thereof binds human PSMA. In some embodiments, the PSMA-specific domain of the multispecific or multispecific antigen-binding fragment thereof cross reacts with *Macaca fascicularis* PSMA or with *Pan troglodytes* PSMA. In preferred embodiments, the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragment thereof is a CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof. In some embodiments, an isolated antigen-binding molecule or multispecific antigen-binding fragment thereof comprises: a) a FN3 domain; b) a light chain (LC); and c) a heavy chain (HC), wherein the FN3 domain forms a first

antigen-binding site that specifically binds human prostate specific membrane antigen (PSMA), and the HC and the LC pair to form a second antigen-binding site that immunospecifically binds CD3, or a PSMA-binding FN3 domain x CD3 bispecific antigen-binding fragment thereof is provided. In another embodiment, an isolated cell expressing the CD3 x PSMA-multispecific antigen-binding molecule or bispecific antigen-binding fragment thereof is provided. In some embodiments, the FN3 domain (or “PSMA-specific arm”) of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof is derived from a Tencon sequence of SEQ ID NO: 1 or Tencon27 of SEQ ID NO: 4, the SEQ ID NO: 1 or SEQ ID NO: 4 optionally having substitutions at residue positions 11, 14, 17, 37, 46, 73, and/or 86; or the FN3 domain is isolated from a library comprising the sequence of SEQ ID NOs: 2, 3, 5, 6, 7, or 8 described herein. Examples of FN3 domains having these sequences are listed in Table 1).

[0010] Table 1.

Clone		
Clone ID	SEQ ID NO:	Sequence
P229CR5P819_H1 1	40	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSSNPLSAI FTT
P258AR6P1071_G 03	35	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1070_A 05	36	LPAPKNLVVSRVTEDSARLSWTIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_F 04	37	LPAPKNLVVSRVTEDSARLSWVIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1070_F 09	38	LPAPKNLVVSRVTEDSARLSWTIDEQRDWFESFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_D 02	39	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFESFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT

P234CR9_H01	46	LPAPKNLVVSRVTEDSARLSWEWWVIPGDFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVVNSGQWNDTSNPLSAI FTT
P234CR9_A7	45	LPAPKNLVVSRVTEDSARLSWGEQFTIFDSFLIQ YQESEKVGGEAIVLTVPGSERSYDLTGLKPGTEYT VSIYGASGYEWFHAFGSSNPLSAI FTT
P233FR9_H10	41	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYP VYIAGVKGGQWSFPLSAI FTT
P233FR9P1001_D 9	44	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFPIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYW VYIAGVKGGQWSFPLSAI FTT
P233FR9P1001_B 5-5	42	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFTIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYP VYIAGVKGGQWSFPLSAI FTT
P233FR9P1001_H 3-1	43	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFPIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYH VYIAGVKGGQWSFPLSAI FTT

[0011] In another embodiment, the FN3 domain of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof may be selected from the sequences listed in Table2.

[0012] Table 2.

Clone ID	SEQ ID NO:	Sequence
P258AR6P10 71_D02_v1	75	LPAPKNLVVSRVTEDSARLSWAADEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v2	76	LPAPKNLVVSRVTEDSARLSWAIAEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v3	77	LPAPKNLVVSRVTEDSARLSWAIDAQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v4	78	LPAPKNLVVSRVTEDSARLSWAIDEARDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P10	79	LPAPKNLVVSRVTEDSARLSWAIDEQADWFE

71_D02_v5		SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v6	80	LPAPKNLVVSRVTEDSARLSWAIDEQRAWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v7	81	LPAPKNLVVSRVTEDSARLSWAIDEQRDAFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v8	82	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v9	83	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYAVYRSNPLSAI FTT
P258AR6P10 71_D02_v10	84	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHAYRSNPLSAI FTT
P258AR6P10 71_D02_v11	85	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVARSNPLSAI FTT
P258AR6P10 71_D02_v12	86	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYASNPLSAI FTT
P258AR6P10 71_D02_v13	87	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFA SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v14	88	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v15	89	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFD SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v16	90	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSSNPLSAI FTT
P258AR6P10 71_D02_v17	91	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFD SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSNPLSAI FTT
P258AR6P10 71_D02_v18	92	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSSNPLSAI FTT
P258AR6P10 71_D02_v19	93	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFD SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIIYGVYHVYRSSNPLSAI FTT
P233FR9_H1 0_v1	94	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDGGEAIVLTVPGSCRSYDLTGLK PGTEYRVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v2	95	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDGGEAIVLTVPGSCRSYDLTGLK

		PGTEYKVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v3	96	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYEVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v4	97	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v5	98	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYDVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v6	99	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYAVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v7	100	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYGVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v8	101	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYVVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v9	102	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYLVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v10	103	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYIVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v11	104	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYFVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v12	105	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYWVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v13	106	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYNVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v14	107	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYQVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v15	108	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYSVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v16	109	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYTVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v17	110	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYYVYIAGVKGGQWSFPLSAI FTT
P233FR9_H1 0_v18	111	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIA ~~Y~~ WEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAI FTT

P233FR9_H1 0_v19	112	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIS Y WEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT
P233FR9_H1 0_v20	113	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDTDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT
P233FR9_H1 0_v21	114	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWSDSDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT
P233FR9_H1 0_v22	115	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY YEW DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ W SFPLSAIFTT
P233FR9_H1 0_v23	116	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY FEW DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ W SFPLSAIFTT
P233FR9_H1 0_v24	117	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY LEW DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ W SFPLSAIFTT
P233FR9_H1 0_v25	118	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY WEY DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ W SFPLSAIFTT
P233FR9_H1 0_v26	119	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY WEF DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ W SFPLSAIFTT
P233FR9_H1 0_v27	120	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY WEL DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ W SFPLSAIFTT
P233FR9_H1 0_v28	121	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY WEW DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ Y SFPLSAIFTT
P233FR9_H1 0_v29	122	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY WEW DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ F SFPLSAIFTT
P233FR9_H1 0_v30	123	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGY WEW DDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ L SFPLSAIFTT
P233FR9P10 01-H3-1_v1	124	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF RIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v2	125	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF KIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v3	126	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF EIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10	127	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF

01-H3-1_v4		HIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v5	128	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE DIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v6	129	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v7	130	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE GIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v8	131	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE VIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v9	132	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE LIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v10	133	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE IIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v11	134	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE FIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v12	135	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE WIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v13	136	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE NIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v14	137	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE QIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v15	138	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE SIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v16	139	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE TIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P10 01-H3-1_v17	140	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE YIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT

[0013] In another embodiment, the isolated FN3 domain of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof that specifically binds human PSMA of SEQ ID NO: 144 comprises an amino acid sequence that is

89% identical to the amino acid sequence of SEQ ID NO: 41, or that has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 substitutions when compared to the amino acid sequence of SEQ ID NO: 41.

[0014] In another embodiment, the FN3 domain of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof that specifically binds human PSMA of SEQ ID NO: 144 comprises the amino acid sequence of SEQ ID NO: 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139 or 140.

Fusion Proteins

[0015] Another embodiment of the invention is a fusion protein comprising: a heavy chain; a FN3 domain; and a linker. The heavy chain Fc region can be IgG4 PAA. Another embodiment of the invention is a fusion protein comprising: a Fc region; a FN3 domain; and a linker. The linker and FN3 domain may be attached to the amino-terminus or the the carboxyl-terminus of the Fc region. The linker can comprise the amino acid sequence of SEQ ID NO: 175 (GGGGSGGGGS). Another embodiment of the invention is an isolated polynucleotide encoding a fusion protein comprising: a heavy chain; an FN3 domain; and a linker. The heavy chain Fc region can be IgG4 PAA. Another embodiment of the invention is an isolated polynucleotide encoding a fusion protein comprising: an Fc region; an FN3 domain; and a linker. The linker and FN3 domain may be attached to the amino-terminus or the the carboxyl-terminus of the Fc region. The linker can comprise the amino acid sequence of SEQ ID NO: 175 (GGGGSGGGGS). Another embodiment of the invention is a vector comprising the polynucleotide of the invention. Another embodiment of the invention is a host cell comprising the vector of the invention.

CD3-Binding Arm

[0016] In some embodiments, the CD3-binding arm (or “CD3-specific arm”) of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof is derived from the mouse monoclonal antibody SP34, a mouse IgG3/lambda isotype. (K.R. Abhinandan and A. C. Martin, 2008. Mol. Immunol. 45, 3832-3839). In some embodiments, the CD3-binding arm of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof comprises one VH domain and one VL domain

selected from Table 3. Table 3 provides a summary of examples of some the heavy chains and light chains of the CD3-specific antibodies and antigen-binding fragments.

[0017] Table 3. The VH and VL sequences of the antibodies are shown below:

mAb	HC	VH Amino Acid sequence	SEQ ID NO.	LC	VL Amino Acid sequence	SEQ ID NO.
CD3B1 43	CD3H 141	EVQLVESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYAASVKGRFTISR DSKNSLYLQMNSLKT EDTAVYYCARHGNFG NSYVSWFAYWGQGT LTVSS	163	CD3L 63	QAVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167
CD3B1 44	CD3H 141	EVQLVESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYAASVKGRFTISR DSKNSLYLQMNSLKT EDTAVYYCARHGNFG NSYVSWFAYWGQGT LTVSS	163	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 46	CD3H 141	EVQLVESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYAASVKGRFTISR DSKNSLYLQMNSLKT EDTAVYYCARHGNFG NSYVSWFAYWGQGT LTVSS	163	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	169
CD3B1 47	CD3H 142	EVQLLESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCAKHGNFG NSYVSWFAYWGQGT LTVSS	164	CD3L 63	QAVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167

CD3B1 48	CD3H 142	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCAKHGNGF NSYVSWFAYWGQGT LTVSS	164	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 50	CD3H 142	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCAKHGNGF NSYVSWFAYWGQGT LTVSS	164	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	169
CD3B1 51	CD3H 143	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCVKHGNGF NSYVSWFAYWGQGT LTVSS	165	CD3L 63	QAVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167
CD3B1 52	CD3H 143	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCVKHGNGF NSYVSWFAYWGQGT LTVSS	165	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 54	CD3H 143	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCVKHGNGF	165	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW	169

		NSYVSWFAYWGQGT LVTVSS			VFGGGTKLTVL	
CD3B1 55	CD3H 144	EVQLVESGGGLVQPG GSLKLSCAASGFTFN TYAMNWVRQASGKGL EWVGRIRSKYNGYAT YYAASVKGRFTISR D DSKNTAYLQMNSLKT EDTAVYYCTRHGNF G NSYVSWFAYWGQGT LVTVSS	166	CD3L 63	QAVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167
CD3B1 56	CD3H 144	EVQLVESGGGLVQPG GSLKLSCAASGFTFN TYAMNWVRQASGKGL EWVGRIRSKYNGYAT YYAASVKGRFTISR D DSKNTAYLQMNSLKT EDTAVYYCTRHGNF G NSYVSWFAYWGQGT LVTVSS	166	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 58	CD3H 144	EVQLVESGGGLVQPG GSLKLSCAASGFTFN TYAMNWVRQASGKGL EWVGRIRSKYNGYAT YYAASVKGRFTISR D DSKNTAYLQMNSLKT EDTAVYYCTRHGNF G NSYVSWFAYWGQGT LVTVSS	166	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	169

[0018] In some embodiments, the CD3-specific antibodies and antigen-binding fragments comprise a heavy chain from Table 4 and a light chain from Table 4. Table 4 provides a summary of the matrix of the heavy chains and light chains of the CD3-specific antibodies and antigen-binding fragments.

Table 4. The antibodies created by combining the heavy and light chains.

Heavy chain	Light chain		
	CD3L63 (LV746/W59G)	CD3L64 (LV1-51)	CD3L66 (LV743/W59G)
CD3H141	CD3B143	CD3B144	CD3B146
CD3H142 (HV3-23 + S49A)	CD3B147	CD3B148	CDB150

CD3H143 (HV3-23 + S49A, A99V)	CD3B151	CD3B152	CD3B154
CD3H144 (VH3-73 with G49)	CD3B155	CD3B156	CD3B158

[0019] The IgG class is divided in four isotypes: IgG1, IgG2, IgG3 and IgG4 in humans. They share more than 95% homology in the amino acid sequences of the Fc regions but show major differences in the amino acid composition and structure of the hinge region. The Fc region mediates effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, the Fc region of an antibody binds to Fc receptors (FcγRs) on the surface of immune effector cells such as natural killers and macrophages, leading to the phagocytosis or lysis of the targeted cells. In CDC, the antibodies kill the targeted cells by triggering the complement cascade at the cell surface.

[0020] For many applications of therapeutic antibodies, Fc-mediated effector functions are not part of the mechanism of action. These Fc-mediated effector functions can be detrimental and potentially pose a safety risk by causing off-mechanism toxicity. Modifying effector functions can be achieved by engineering the Fc regions to reduce their binding to FcγRs or the complement factors. The binding of IgG to the activating (FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb) and inhibitory (FcγRIIb) FcγRs or the first component of complement (C1q) depends on residues located in the hinge region and the CH2 domain. In some cases, mutations have been introduced in IgG1, IgG2 and IgG4 to reduce or silence Fc functionalities.

[0021] In one embodiment, the antibody comprises an Fc region with one or more of the following properties: (a) reduced effector function when compared to the parent Fc; (b) reduced affinity to FcγRI, FcγRIIa, FcγRIIb, FcγRIIIb and/or FcγRIIIa, (c) reduced affinity to FcγRI (d) reduced affinity to FcγRIIa (e) reduced affinity to FcγRIIb, (f) reduced affinity to FcγRIIIb or (g) reduced affinity to FcγRIIIa.

[0022] In some embodiments, the CD3-specific antibody or antigen-binding fragment from which the CD3-specific arm of the multispecific antibody is derived is IgG, or a derivative thereof. In some embodiments, the CD3-specific antibody or antigen-binding fragment from which the CD3-specific arm of the multispecific antibody is derived is IgG1, or a derivative thereof. In some embodiments, for example, the Fc region of the CD3-specific IgG1 antibody from which the CD3-binding arm is derived comprises L234A, L235A, and F405L substitutions in its Fc region. In some embodiments, the CD3-specific antibody or antigen-binding fragment

from which the CD3-specific arm of the multispecific antibody is derived is IgG4, or a derivative thereof. In some embodiments, for example, the Fc region of the CD3-specific IgG4 antibody from which the CD3-binding arm is derived comprises S228P, L234A, L235A, F405L, and R409K substitutions in its Fc region. In some embodiments, for example, the Fc region of the CD3-specific IgG4 antibody from which the CD3-binding arm is derived comprises S228P, L234A, L235A, and F405L substitutions in its Fc region. In some embodiments, the CD3-specific antibody or antigen-binding fragment from which the CD3-specific arm of the multispecific antibody is derived is IgG-AA Fc. In some embodiments, the CD3-specific antibody or antigen-binding fragment from which the CD3-specific arm of the multispecific antibody is derived is IgG-AA Fc-L234A, L235A, and F405L (where L234A, L235A, and F405L are mutations). In some embodiments, the CD3-specific antibody or antigen-binding fragment from which the CD3-specific arm of the multispecific antibody is derived binds CD3 ϵ on primary human T cells and/or primary cynomolgus T cells. In some embodiments, the CD3-specific antibody or antigen-binding fragment from which the CD3-specific arm of the multispecific antibody is derived activates primary human CD4⁺ T cells and/or primary cynomolgus CD4⁺ T cells.

[0023] Further provided herein, are pharmaceutical compositions comprising the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof and a pharmaceutically acceptable carrier.

[0024] In some embodiments, the CD3 x PSMA-bispecific antigen-binding molecule comprises the amino acid sequence of SEQ ID NO: 171, the light chain comprises the amino acid sequence of SEQ ID NO: 170, and the FN3 domain comprises the amino acid sequence of SEQ ID NO: 172.

[0025] In some embodiments, the CD3 x PSMA-bispecific antigen-binding molecule comprises the amino acid sequence of SEQ ID NO: 171, the light chain comprises the amino acid sequence of SEQ ID NO: 170, and the FN3 domain comprises the amino acid sequence of SEQ ID NO: 173.

[0026] In some embodiments, the CD3 x PSMA-bispecific antigen-binding molecule comprises the amino acid sequence of SEQ ID NO: 174, the light chain comprises the amino acid sequence of SEQ ID NO: 170, and the FN3 domain comprises the amino acid sequence of SEQ ID NO: 173.

Methods of Using CD3 x PSMA-bispecific Antigen-binding Molecule or Bispecific Antigen-binding Fragments Thereof

[0027] Methods of using the described CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragments thereof are also disclosed. For example, the CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragments thereof may be useful in the treatment of a PSMA-overexpressing disease in a subject in need thereof. In some embodiments, the disease is cancer, preferably a PSMA-overexpressing cancer. In some embodiments, the PSMA-overexpressing disease is a prostatic intraepithelial neoplasia (PIN), a condition in which some prostate cells have begun to look and behave abnormally. In some embodiments, the cancers are primary and metastatic prostate cancers and other solid tumors, (e.g. breast, lung, bladder, kidney). In some embodiments, the cancers are prostate cancer, colorectal cancer, gastric cancer, clear cell renal carcinoma, bladder cancer, lung cancer, endometrial cancer or kidney cancer. In some embodiments, the PSMA-overexpressing cancer is associated with angiogenesis or vasculature of the cancer such as squamous cell carcinoma of the oral cavity, , gliomas and breast cancer.

[0028] In some embodiments, the cancer is a neovascular disorder such as, for example, a cancer characterized by solid tumor growth. Exemplary cancers with tumor vasculatures characterized by PSMA overexpression and amenable to treatment in accordance with the present invention include, for example, clear cell renal carcinoma (CCRCC), colorectal cancer, breast cancer, bladder cancer, lung cancer, and pancreatic cancer (see, e.g., Baccala et al., *Urology* 70:385.390, 2007 (expression of PSMA in CCRCC); Liu et al., *Cancer Res.* 57:3629-3634, 1997 (expression of PSMA in various non-prostate cancers, including renal, urothelial, lung, colon, breast, and adenocarcinoma to the liver); and Milowsky et al., *J. Clin. Oncol.* 25:540-547, 2007).

[0029] The described methods of treating PSMA-overexpressing disorders in a subject in need thereof include administering to the subject a therapeutically effective amount of a described CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof. In some embodiments, the subject is a mammal, preferably a human. Preferred embodiments are provided of methods for treating a subject having PSMA-overexpressing cancer by administering a therapeutically effective amount of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof to a patient in need thereof for a time sufficient to treat the cancer.

[0030] Further provided herein are methods for inhibiting growth or proliferation of cancer cells by administering a therapeutically effective amount of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof to inhibit the growth or proliferation of cancer cells.

[0031] Also provided herein are methods of redirecting a T cell to a PSMA-expressing cancer cell by administering a therapeutically effective amount of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof to redirect a T cell to a cancer.

CD3 x PSMA-bispecific Antigen-binding Molecule or Bispecific Antigen-binding Fragments Thereof Kits

[0032] Described herein are kits including the disclosed CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof or bispecific antigen-binding fragments thereof. The described kits may be used to carry out the methods of using the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof provided herein, or other methods known to those skilled in the art. In some embodiments the described kits may include the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof described herein and reagents for use in treating a PSMA expressing cancer. Accordingly, the described kits may include one or more of the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof, described herein, and a vessel for containing the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof when not in use, and/or instructions for use of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] Figure 1. Figure 1 shows biodistribution of untargeted ⁸⁹Zr-labeled FN3 domain following intravenous injection in male NSG mice.

[0034] Figure 2. Figure 2A shows the overall crystal structure of the P233FR9_H10 PSMA binding FN3 domain (H10) in complex with cynomolgous PSMA dimer, showing that H10 binds to the region near the PSMA active site. The zinc atoms (Zn) indicate the location of the PSMA active site. The N- and C-terminus of PSMA and H10 molecules are indicated for

one of the complexes. The approximate location of the cell membrane is indicated. Figure 2B shows the crystal structure of the H10 FN3 domain in complex with cynomolgous PSMA. The A, B, C, D, E, F and G beta strands in the H10 FN3 domain are shown. The negatively charged residues in the CD loop of H10 (residues W38, D39, D40, D41 and E43) that inserts into the positively charged entrance of the PSMA active site are shown. H10 residue numbering according to SEQ ID NO: 41. Figure 2C shows the crystal structure of the H10 FN3 domain in complex with cynomolgous PSMA. The H10 contact residues W38, D39, D40, D41 and E43 are shown in the Figure. Some of the cyno PSMA residues that contact H10 (R511, K514 and K545), coordinate the zinc atoms (H377, D387, E424, E425, D453, and H553) or compose the active site cavity (R536 and R534) are shown. H10 beta strands C, D, F and G are marked in the Figure. H10 and cynomolgous PSMA residue numbering is according to SEQ ID NO: 41 and 141, respectively.

[0035] Figure 3. Figure 3A shows a close view of the crystal structure combining site between the H10 FN3 domain and cynomolgous PSMA. The H10 FN3 domain contact residues A32, W36, W38-D41, E43, A44, V46, G64, P68, Y70, A72, W79, F81, P82, A85, and I86 are shown. The cyno PSMA contact residues Y460, K499-P502, P504, R511, K514, N540, W541, K545, F546, F488, K610, N613, and I614 are shown.. H10 and cynomolgous PSMA residue numbering is according to SEQ ID NO: 41 and 141, respectively. Figure 3B shows an interaction map between the H10 FN3 domain and cynomolgous PSMA contact residues. A distance cut-off of 4 Å was used to define the contact residues. FN3 domain and cyno PSMA residues are shown in gray and white boxes, respectively, van der Waals interactions are shown as dashed lines, and H-bonds are solid lines with arrows indicating backbone H bonds and pointing to the backbone atoms. Residue numbering is according to SEQ ID NO: 41 (H10) and SEQ ID NO: 141 (cyno PSMA).

[0036] Figure 4. Figure 4A shows the amino acid sequence alignment between human (h) and cynomolgous (c) PSMA extracellular domains. The H10 contact residues are underlined and in bold. The residues that differ between human and cynomolgus PSMA are shaded. All cyno PSMA residues interacting with H10 are conserved in human PSMA except for N613. Human PSMA ECD; SEQ ID NO: 143. Cyno PSMA ECD: SEQ ID NO: 32. Figure 4B shows the H10 FN3 domain residues in contact with cynomolgous PSMA. The contact residues are underlined and in bold. H10 amino acid sequence is shown in SEQ ID NO: 41.

[0037] Figure 5. Figure 5 shows the location of H10 FN3 domain residue N6, R11, T22, D25, A26, S52, E53, K62, and the N- and C-terminus, which are possible sites for chemical

conjugation, in the crystal structure of H10 bound to cynomolgous PSMA. The FN3 domain/PSMA contacting regions are shown in black. H10 beta strands C, D, F and G are marked in the Figure. Residue numbering is according to SEQ ID NO: 41 (H10).

[0038] Figure 6. Figure 6 shows the comparison of mean fluorescence intensity (MFI) of different tumor cell lines stained with anti PSMA PE-conjugated FN3 domain (black) and anti PSMA antibody-PE (white).

[0039] Figure 7. Figures 7A-7D show a series of the CellTracks Analyzer II browser images with different tumor cells stained with DAPI, anti cytokeratin-FITC, anti-CD45-APC and anti-PSMA FN3 domain-PE. The thumbnail images show, from right to left, PSMA-PE staining, CD45-APC signal, DAPI stained nuclei, Cytokeratin-FITC reactivity, and finally an overlay of the Cytokeratin-FITC & DAPI staining. A cell must have a nucleus, express cytokeratin and be negative for CD45 to be counted as a Circulating Tumor Cells (CTC). The CTC must have a positive signal for PSMA to be scored as PSMA positive CTC. Figure 7A shows the expression of PSMA on LNCaP cells. Figure 7B shows the expression of PSMA on 22Rv1 cells. Figure 7C shows the expression of PSMA on PC3 cells. Figure 7D shows the expression of PSMA on SKBR3 cells.

[0040] Figure 8. Figure 8 shows the amino acid sequence of SP34 with sequential numbering. CDRs in AbM definition are underlined. Ser230 is the last HC residue present in papain-cleaved Fab. Residues 231-455 are from IGHG3_MOUSE (mouse IgG3, isoform 2).

[0041] Figure 9. Figure 9 shows the variable domain of SP34 with key residues at VL/VH interface shown. Residues 38, 48, and 51 in VL (labeled) are in contact with CDR-H3.

[0042] Figure 10. Figure 10 shows the Human Framework Adaptation (“HFA”) variants for VH (SEQ ID NOS 233 and 184-187, respectively, in order of appearance) and VL (SEQ ID NOS 234 and 188-190, respectively, in order of appearance). The numbering is sequential; CDRs in the AbM definition are underlined; residues that differ from SP34 are highlighted in bold; back mutations in HFA variants are bold and underlined.

[0043] Figure 11. Figure 11 shows binding of SP34 HFA variants to primary Human T cells.

[0044] Figure 12. Figure 12 shows binding of SP34 HFA variants to Cynomolgus primary T cells.

[0045] Figure 13. Figure 13 shows that SP34 HFA variants activate primary human T cells in vitro. Negative controls are shown in white and positive controls are shown in black.

[0046] Figure 14. Figure 14 shows that SP34 HFA variants activate primary cynomolgus T cells in vitro. Negative controls are shown in white and positive controls are shown in black.

[0047] Figure 15. Figure 15 shows the correlation of binding and activation by SP34 HFA variants. Average binding and CD69 Mean Fluorescence Intensity (“MFI”) values for human (Figure 15A) and cynomolgus (Figure 15B) were plotted against each other.

[0048] Figure 16. Figure 16 shows the design of CD3 x PSMA-multispecific antigen-binding molecule constructs.

[0049] Figure 17. Figure 17 shows the results of the T-cell mediated cytotoxicity assay for the CD3 x PSMA-multispecific antigen-binding molecules on PSMA + LNCAP cells.

[0050] Figure 18. Figure 18 shows prevention of tumorigenesis of HEK293-PSMA xenografts treated with B219xCW6 Mabtyrin in PBMC humanized NSG mice. PBMC humanized mice bearing HEK293-PSMA cells were iv dosed with 0.004 mg/kg, 0.04 mg/kg and 0.4 mg/kg B219xCW6 (dosing indicated by arrows). Sc tumors were measured twice weekly and the results presented as the average tumor volume, expressed in mm³ ± standard error of the mean (SEM), of each group.

[0051] Figure 19. Figure 19 shows Body weight of PBMC-humanized NSG mice bearing HEK293-PSMA xenografts treated with B219xCW6 Mabtyrin. PBMC humanized NSG mice bearing HEK293-PSMA cells were iv dosed with 0.004 mg/kg, 0.04 mg/kg and 0.4 mg/kg B219xCW6 (dosing indicated by arrows). Body weights are presented as group means, expressed in g ± standard error of the mean (SEM).

[0052]

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Definitions

[0053] Various terms relating to aspects of the description are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definitions provided herein.

[0054] As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “a cell” includes a combination of two or more cells, and the like.

[0055] The term “about” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of up to $\pm 10\%$ from the specified value, as such variations are appropriate to perform the disclosed methods. Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0056] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0057] “Isolated” means a biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified from other biological components of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins that have been “isolated” thus include nucleic acids and proteins purified by standard purification methods. “Isolated” nucleic acids, peptides and proteins can be part of a composition and still be isolated if such composition is not part of the native environment of the nucleic acid, peptide, or protein. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids. An “isolated” CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof as used herein, is intended to refer to an isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof which is substantially free of other CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof having different antigenic specificities.

[0058] “Polynucleotide,” synonymously referred to as “nucleic acid molecule,” “nucleotides” or “nucleic acids,” refers to any polyribonucleotide or polydeoxyribonucleotide,

which may be unmodified RNA or DNA or modified RNA or DNA. “Polynucleotides” include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, “polynucleotide” refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. “Modified” bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, “polynucleotide” embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. “Polynucleotide” also embraces relatively short nucleic acid chains, often referred to as oligonucleotides.

[0059] The meaning of “substantially the same” can differ depending on the context in which the term is used. Because of the natural sequence variation likely to exist among heavy and light chains and the genes encoding them, one would expect to find some level of variation within the amino acid sequences or the genes encoding the antibodies or antigen-binding fragments described herein, with little or no impact on their unique binding properties (e.g., specificity and affinity). Such an expectation is due in part to the degeneracy of the genetic code, as well as to the evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, in the context of nucleic acid sequences, “substantially the same” means at least 65% identity between two or more sequences. Preferably, the term refers to at least 70% identity between two or more sequences, more preferably at least 75% identity, more preferably at least 80% identity, more preferably at least 85% identity, more preferably at least 90% identity, more preferably at least 91% identity, more preferably at least 92% identity, more preferably at least 93% identity, more preferably at least 94% identity, more preferably at least 95% identity, more preferably at least 96% identity, more preferably at least 97% identity, more preferably at least 98% identity, and more preferably at least 99% or greater identity. The percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The

percent identity between two nucleotide or amino acid sequences may e.g. be determined using the algorithm of E. Meyers and W. Miller, *Comput. Appl. Biosci* 4, 11-17 (1988) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences may be determined using the Needleman and Wunsch, *J. Mol. Biol.* 48, 444-453 (1970) algorithm.

[0060] The degree of variation that may occur within the amino acid sequence of a protein without having a substantial effect on protein function is much lower than that of a nucleic acid sequence, since the same degeneracy principles do not apply to amino acid sequences. Accordingly, in the context of an antibody or antigen-binding fragment, “substantially the same” means antibodies or antigen-binding fragments having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the antibodies or antigen-binding fragments described.

[0061] A “vector” is a replicon, such as plasmid, phage, cosmid, or virus in which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

[0062] A “clone” is a population of cells derived from a single cell or common ancestor by mitosis. A “cell line” is a clone of a primary cell that is capable of stable growth in vitro for many generations. In some examples provided herein, cells are transformed by transfecting the cells with DNA.

[0063] The terms “express” and “produce” are used synonymously herein, and refer to the biosynthesis of a gene product. These terms encompass the transcription of a gene into RNA. These terms also encompass translation of RNA into one or more polypeptides, and further encompass all naturally occurring post-transcriptional and post-translational modifications. The expression or production of an antibody or antigen-binding fragment thereof may be within the cytoplasm of the cell, or into the extracellular milieu such as the growth medium of a cell culture.

[0064] The term “treating” or “treatment” refers to any success or indicia of success in the attenuation or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement, remission, diminishing of symptoms or making the condition more tolerable to the patient, slowing in the rate of degeneration or decline, making the final point of degeneration less debilitating, improving a subject’s physical or mental well-being, or prolonging the length of survival. The treatment may be assessed by objective or subjective

parameters; including the results of a physical examination, neurological examination, or psychiatric evaluations.

[0065] An "effective amount" or "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount of a CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects.

[0066] "Antibody" refers to all isotypes of immunoglobulins (IgG, IgA, IgE, IgM, IgD, and IgY) including various monomeric, polymeric and chimeric forms, unless otherwise specified. Specifically encompassed by the term "antibody" are polyclonal antibodies, monoclonal antibodies (mAbs), and antibody-like polypeptides, such as chimeric antibodies and humanized antibodies.

[0067] Antigen-binding fragments are any proteinaceous structure that may exhibit binding affinity for a particular antigen. Antigen-binding fragments include those provided by any known technique, such as enzymatic cleavage, peptide synthesis, and recombinant techniques. Some antigen-binding fragments are composed of portions of intact antibodies that retain antigen-binding specificity of the parent antibody molecule. For example, antigen-binding fragments may comprise at least one variable region (either a heavy chain or light chain variable region) or one or more CDRs of an antibody known to bind a particular antigen. Examples of suitable antigen-binding fragments include, without limitation diabodies and single-chain molecules as well as Fab, F(ab')₂, Fc, Fabc, and Fv molecules, single chain (Sc) antibodies, individual antibody light chains, individual antibody heavy chains, chimeric fusions between antibody chains or CDRs and other proteins, protein scaffolds, heavy chain monomers or dimers, light chain monomers or dimers, dimers consisting of one heavy and one light chain, a monovalent fragment consisting of the VL, VH, CL and CH1 domains, or a monovalent antibody as described in WO2007059782, bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region, a Fd fragment consisting essentially of the V_H and C_{H1} domains; a Fv fragment consisting essentially of the VL and VH domains of a single arm of an antibody, a dAb fragment (Ward et al., Nature 341, 544-546 (1989)), which consists essentially of a VH domain and also called domain antibodies (Holt et al; Trends Biotechnol. 2003 Nov.;

21(11):484-90); camelid or nanobodies (Revets et al; Expert Opin Biol Ther. 2005 Jan.; 5(1):111-24); an isolated complementarity determining region (CDR), and the like. All antibody isotypes may be used to produce antigen-binding fragments. Additionally, antigen-binding fragments may include non-antibody proteinaceous frameworks that may successfully incorporate polypeptide segments in an orientation that confers affinity for a given antigen of interest, such as protein scaffolds. Antigen-binding fragments may be recombinantly produced or produced by enzymatic or chemical cleavage of intact antibodies. The phrase "an antibody or antigen-binding fragment thereof" may be used to denote that a given antigen-binding fragment incorporates one or more amino acid segments of the antibody referred to in the phrase. When used herein in the context of two or more antibodies or antigen-binding fragments, the term "competes with" or "cross-competes with" indicates that the two or more antibodies or antigen-binding fragments compete for binding. For some pairs of antibodies or antigen-binding fragments, competition or blocking in the assay of the Examples is only observed when one antibody is coated on the plate and the other is used to compete, and not vice versa. Unless otherwise defined or negated by context, the terms "competes with" or "cross-competes with" when used herein is also intended to cover such pairs of antibodies or antigen-binding fragments.

[0068] The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. The epitope may comprise amino acid residues directly involved in the binding and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked or covered by the specifically antigen binding peptide (in other words, the amino acid residue is within the footprint of the specifically antigen binding peptide).

[0069] "Immunospecific binding" or derivatives thereof when used in the context of antibodies, or antibody fragments, represents binding via domains encoded by immunoglobulin genes or fragments of immunoglobulin genes to one or more epitopes of a protein of interest, without preferentially binding other molecules in a sample containing a mixed population of molecules. Typically, an antibody binds to a cognate antigen with a K_d of less than about 1×10^{-8} M, as measured by a surface plasmon resonance assay or a cell binding assay. Phrases such as

“[antigen]-specific” antibody (e.g., CD3-specific antibody) are meant to convey that the recited antibody specifically binds the recited antigen.

[0070] The term “fibronectin type III (FN3) domain” (FN3 domain) as used herein refers to a domain occurring frequently in proteins including fibronectins, tenascin, intracellular cytoskeletal proteins, cytokine receptors and prokaryotic enzymes (Bork and Doolittle, Proc Nat Acad Sci USA 89:8990-8994, 1992; Meinke *et al.*, J Bacteriol 175:1910-1918, 1993; Watanabe *et al.*, J Biol Chem 265:15659-15665, 1990). Exemplary FN3 domains are the 15 different FN3 domains present in human tenascin C, the 15 different FN3 domains present in human fibronectin (FN), and non-natural synthetic FN3 domains as described for example in U.S. Pat. No. 8,278,419. Individual FN3 domains are referred to by domain number and protein name, e.g., the 3rd FN3 domain of tenascin (TN3), or the 10th FN3 domain of fibronectin (FN10).

[0071] “Centyrin” as used herein refers to a FN3 domain that is based on the consensus sequence of the 15 different FN3 domains present in human tenascin C.

[0072] The term “substituting” or “substituted” or “mutating” or “mutated” as used herein refers to altering, deleting, or inserting one or more amino acids or nucleotides in a polypeptide or polynucleotide sequence to generate a variant of that sequence.

[0073] The term “randomizing” or “randomized” or “diversified” or “diversifying” as used herein refers to making at least one substitution, insertion or deletion in a polynucleotide or polypeptide sequence.

[0074] “Variant” as used herein refers to a polypeptide or a polynucleotide that differs from a reference polypeptide or a reference polynucleotide by one or more modifications for example, substitutions, insertions or deletions.

[0075] The term “specifically binds” or “specific binding” as used herein refers to the ability of the FN3 domain of the invention to bind to a predetermined antigen with a dissociation constant (K_D) of about 1×10^{-6} M or less, for example about 1×10^{-7} M or less, about 1×10^{-8} M or less, about 1×10^{-9} M or less, about 1×10^{-10} M or less, about 1×10^{-11} M or less, about 1×10^{-12} M or less, or about 1×10^{-13} M or less. Typically the FN3 domain of the invention binds to a predetermined antigen (i.e. human PSMA) with a K_D that is at least ten fold less than its K_D for a nonspecific antigen (for example BSA or casein) as measured by surface plasmon resonance using for example a Proteon Instrument (BioRad). The isolated FN3 domain of the invention that specifically binds to human PSMA may, however, have cross-reactivity to other related antigens, for example to the same predetermined antigen from other species (homologs), such as *Macaca Fascicularis* (cynomolgous monkey, cyno) or *Pan troglodytes* (chimpanzee).

[0076] The term “library” refers to a collection of variants. The library may be composed of polypeptide or polynucleotide variants.

[0077] The term “stability” as used herein refers to the ability of a molecule to maintain a folded state under physiological conditions such that it retains at least one of its normal functional activities, for example, binding to a predetermined antigen such as human PSMA.

[0078] Human PSMA as used herein refers to the well known type II glycoprotein of about 100 kD with a short intracellular domain (residues 1-18), a transmembrane domain (residues 19-43) and an extracellular domain (residues 44-750). The amino acid sequence of the mature human PSMA is shown in SEQ ID NO: 144.

[0079] “Overexpress”, “overexpressed” and “overexpressing” as used herein interchangeably refer to a cancer or malignant cell that has measurably higher levels of PSMA on the surface compared to a normal cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. PSMA overexpression can be measured using well known assays, for example ELISA, immunofluorescence, flow cytometry or radioimmunoassay on live or lysed cells. Alternatively, or additionally, levels of PSMA nucleic acid molecules may be measured in the cell for example using fluorescent *in situ* hybridization, Southern blotting, or PCR techniques. PSMA is overexpressed when the level of PSMA on the surface of the cell is at least 1.5-fold higher when compared to the normal cell.

[0080] “Tencon” as used herein refers to the synthetic fibronectin type III (FN3) domain having the sequence shown in SEQ ID NO: 1 and described in U.S. Pat. Publ. No. US2010/0216708.

[0081] A “cancer cell” or a “tumor cell” as used herein refers to a cancerous, pre-cancerous or transformed cell, either *in vivo*, *ex vivo*, and in tissue culture, that has spontaneous or induced phenotypic changes that do not necessarily involve the uptake of new genetic material. Although transformation can arise from infection with a transforming virus and incorporation of new genomic nucleic acid, or uptake of exogenous nucleic acid, it can also arise spontaneously or following exposure to a carcinogen, thereby mutating an endogenous gene. Transformation/cancer is exemplified by, e.g., morphological changes, immortalization of cells, aberrant growth control, foci formation, proliferation, malignancy, tumor specific markers levels, invasiveness, tumor growth or suppression in suitable animal hosts such as nude mice, and the like, *in vitro*, *in vivo*, and *ex vivo* (Freshney, Culture of Animal Cells: A Manual of Basic Technique (3rd ed. 1994)).

[0082] “Inhibits growth” (e.g. referring to cells, such as tumor cells) refers to a measurable decrease in the cell growth *in vitro* or *in vivo* when contacted with a therapeutic or a combination of therapeutics or drugs when compared to the growth of the same cells grown in appropriate control conditions well known to the skilled in the art. Inhibition of growth of a cell *in vitro* or *in vivo* may be at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 99%, or 100%. Inhibition of cell growth may occur by a variety of mechanisms, for example by apoptosis, necrosis, or by inhibition of cell proliferation, or lysis of cells.

[0083] The term “vector” means a polynucleotide capable of being duplicated within a biological system or that can be moved between such systems. Vector polynucleotides typically contain elements, such as origins of replication, polyadenylation signal or selection markers that function to facilitate the duplication or maintenance of these polynucleotides in a biological system. Examples of such biological systems may include a cell, virus, animal, plant, and reconstituted biological systems utilizing biological components capable of duplicating a vector. The polynucleotide comprising a vector may be DNA or RNA molecules or a hybrid of these.

[0084] The term “expression vector” means a vector that can be utilized in a biological system or in a reconstituted biological system to direct the translation of a polypeptide encoded by a polynucleotide sequence present in the expression vector.

[0085] The term “polypeptide” or “protein” means a molecule that comprises at least two amino acid residues linked by a peptide bond to form a polypeptide. Small polypeptides of less than about 50 amino acids may be referred to as “peptides”.

[0086] “Valent” as used herein refers to the presence of a specified number of binding sites specific for an antigen in a molecule. As such, the terms “monovalent”, “bivalent”, “tetravalent”, and “hexavalent” refer to the presence of one, two, four and six binding sites, respectively, specific for an antigen in a molecule.

[0087] The term “in combination with” as used herein means that two or more therapeutics can be administered to a subject together in a mixture, concurrently as single agents or sequentially as single agents in any order.

[0088] “Synergy”, “synergism” or “synergistic” means more than the expected additive effect of a combination.

[0089] The term “ k_d ” (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{off} value.

[0090] The term “ k_a ” ($\text{M}^{-1} \text{sec}^{-1}$), as used herein, refers to the association rate constant of a particular antibody-antigen interaction.

[0091] The term " K_D " (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction.

[0092] The term " K_A " (M^{-1}), as used herein, refers to the association equilibrium constant of a particular antibody-antigen interaction and is obtained by dividing the k_a by the k_d .

[0093] The term "subject" refers to human and non-human animals, including all vertebrates, e.g., mammals and non-mammals, such as non-human primates, mice, rabbits, sheep, dogs, cats, horses, cows, chickens, amphibians, and reptiles. In many embodiments of the described methods, the subject is a human.

[0094] The term "sample" as used herein refers to a collection of similar fluids, cells, or tissues (e.g., surgically resected tumor tissue, biopsies, including fine needle aspiration), isolated from a subject, as well as fluids, cells, or tissues present within a subject. In some embodiments the sample is a biological fluid. Biological fluids are typically liquids at physiological temperatures and may include naturally occurring fluids present in, withdrawn from, expressed or otherwise extracted from a subject or biological source. Certain biological fluids derive from particular tissues, organs or localized regions and certain other biological fluids may be more globally or systemically situated in a subject or biological source. Examples of biological fluids include blood, serum and serosal fluids, plasma, lymph, urine, saliva, cystic fluid, tear drops, feces, sputum, mucosal secretions of the secretory tissues and organs, vaginal secretions, ascites fluids such as those associated with non-solid tumors, fluids of the pleural, pericardial, peritoneal, abdominal and other body cavities, fluids collected by bronchial lavage and the like. Biological fluids may also include liquid solutions contacted with a subject or biological source, for example, cell and organ culture medium including cell or organ conditioned medium, lavage fluids and the like. The term "sample," as used herein, encompasses materials removed from a subject or materials present in a subject.

[0095] The term "CD3" refers to the human CD3 protein multi-subunit complex. The CD3 protein multi-subunit complex is composed of 6 distinctive polypeptide chains. These include a CD3 γ chain (SwissProt P09693), a CD3 δ chain (SwissProt P04234), two CD3 ϵ chains (SwissProt P07766), and one CD3 ζ chain homodimer (SwissProt 20963), and which is associated with the T cell receptor α and β chain. The term "CD3" includes any CD3 variant, isoform and species homolog which is naturally expressed by cells (including T cells) or can be expressed on cells transfected with genes or cDNA encoding those polypeptides, unless noted.

[0096] A "CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof" is a multispecific molecule, optionally a CD3 x PSMA-bispecific antigen comprising: a FN3 domain; a light chain (LC); and a heavy chain (HC), wherein the FN3 domain forms a first antigen-binding site that specifically binds human prostate specific membrane antigen (PSMA), and the HC and the LC pair to form a second antigen-binding site that immunospecifically binds CD3, or a bispecific antigen-binding fragment thereof.

[0097] CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof, comprise two different antigen-binding regions, one of which binds specifically to the antigen PSMA and one of which binds specifically to CD3. A multispecific antibody can be a bispecific antibody, diabody, or similar molecule (see for instance *PNAS USA* 90(14), 6444-8 (1993) for a description of diabodies).

[0098] A "reference sample" is a sample that may be compared against another sample, such as a test sample, to allow for characterization of the compared sample. The reference sample will have some characterized property that serves as the basis for comparison with the test sample. For instance, a reference sample may be used as a benchmark for PSMA levels that are indicative of a subject having cancer. The reference sample does not necessarily have to be analyzed in parallel with the test sample, thus in some instances the reference sample may be a numerical value or range previously determined to characterize a given condition, such as PSMA levels that are indicative of cancer in a subject. The term also includes samples used for comparative purposes that are known to be associated with a physiologic state or disease condition, such as PSMA-expressing cancer, but that have an unknown amount of PSMA.

[0099] The term "progression," as used in the context of progression of PSMA-expressing cancer, includes the change of a cancer from a less severe to a more severe state. This may include an increase in the number or severity of tumors, the degree of metastasis, the speed with which the cancer is growing or spreading, and the like. For example, "the progression of colon cancer" includes the progression of such a cancer from a less severe to a more severe state, such as the progression from stage I to stage II, from stage II to stage III, etc.

[00100] The term "regression," as used in the context of regression of PSMA-expressing cancer, includes the change of a cancer from a more severe to a less severe state. This could include a decrease in the number or severity of tumors, the degree of metastasis, the speed with which the cancer is growing or spreading, and the like. For example, "the regression

of colon cancer” includes the regression of such a cancer from a more severe to a less severe state, such as the progression from stage III to stage II, from stage II to stage I, etc.

[00101] The term “stable” as used in the context of stable PSMA-expressing cancer, is intended to describe a disease condition that is not, or has not, changed significantly enough over a clinically relevant period of time to be considered a progressing cancer or a regressing cancer.

[00102] The embodiments described herein are not limited to particular methods, reagents, compounds, compositions or biological systems, which can, of course, vary.

[00103] In some embodiments, the antibodies or antigen-binding fragments are IgG, or derivatives thereof, e.g., IgG1, IgG2, IgG3, and IgG4 isotypes. In some embodiments wherein the antibody has an IgG1 isotype, the antibody contains L234A, L235A, and K409R substitution(s) in its Fc region. In some embodiments wherein the antibody has an IgG4 isotype, the antibody contains S228P, L234A, and L235A substitutions in its Fc region.

[00104] Polynucleotides encoding recombinant antigen-binding proteins also are within the scope of the disclosure. In some embodiments, the polynucleotides described (and the peptides they encode) include a leader sequence. Any leader sequence known in the art may be employed. The leader sequence may include, but is not limited to, a restriction site or a translation start site.

PSMA binding molecules

[00105] The FN3 domain of the invention may bind human, *Macaca Fascicularis* and/or *Pan troglodytes* PSMA with a dissociation constant (K_D) of less than about 1×10^{-7} M, for example less than about 1×10^{-8} M, less than about 1×10^{-9} M, less than about 1×10^{-10} M, less than about 1×10^{-11} M, less than about 1×10^{-12} M, or less than about 1×10^{-13} M as determined by surface plasmon resonance or the Kinexa method, as practiced by those of skill in the art. The measured affinity of a particular FN3 domain-antigen interaction can vary if measured under different conditions (e.g., osmolarity, pH). Thus, measurements of affinity and other antigen-binding parameters (e.g., K_D , K_{on} , K_{off}) are made with standardized solutions of protein scaffold and antigen, and a standardized buffer, such as the buffer described herein.

[00106] In another embodiment of the invention, the FN3 domain specifically binds human PSMA, wherein the FN3 domain inhibits human PSMA enzymatic activity. PSMA enzymatic activity may be measured using standard methods. For example, hydrolysis of a detectable or labeled PSMA substrate of PSMA may be used. Exemplary PSMA substrates that may be used are N-Acetyl Aspartyl Glutamate (NAAG), folate polyglutamate, methotrexate tri-

gamma glutamate, methotrexate di-gamma glutamate, pteroylpentaglutamate and derivatives thereof.

[00107] In some embodiments of the invention described herein, the FN3 domain comprises an amino acid sequence that is at least 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 41.

[00108] In some embodiments of the invention described herein, the FN3 domain comprises an amino acid sequence that has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 substitutions when compared to the amino acid sequence of SEQ ID NO: 41.

[00109] In some embodiments of the invention described herein, the FN3 domain that specifically binds human PSMA comprises a cysteine residue in at least one residue position corresponding to residue positions 6, 11, 22, 25, 26, 52, 53, 61 of SEQ ID NO 1, or at a C-terminus.

[00110] In some embodiments, the FN3 domain specifically competes for binding to human PSMA with the FN3 domain of SEQ ID NO: 41.

[00111] In some embodiments, the FN3 domain specifically binds to the region KKSPSPEFSGMPRISK (SEQ ID NO: 159) and NWETNKF (SEQ ID NO: 160) of human PSMA.

[00112] The human PSMA epitope bound by the FN3 domain of the invention includes some or all of the residues within the amino asequences shown in SEQ ID NO: 159 or SEQ ID NO: 160. In some embodiments disclosed herein, the epitope bound by the FN3 domain of the invention comprises at least one amino acid in the region KKSPSPEFSGMPRISK (SEQ ID NO: 159) and NWETNKF (SEQ ID NO: 160) of human PSMA (SEQ ID NO: 144). In some embodiments disclosed herein, the epitope bound by the FN3 domain of the invention comprises at least two, three, four, five, six or seven amino acids in the region KKSPSPEFSGMPRISK (SEQ ID NO: 159) and at least two, three, four, five or six amino acids in the region NWETNKF (SEQ ID NO: 160) of human PSMA (SEQ ID NO: 144).

[00113] In some embodiments disclosed herein, the FN3 domain of the invention binds human PSMA at residues K499, K500, S501, P502, P504, R511, K514, N540, W541, E542, N544, K545 and F546 (residue numbering according to SEQ ID NO: 144).

[00114] In some embodiments disclosed herein, the FN3 domain of the invention further binds human PSMA at residues R181, Y460, F488, K610 and/or I614.

[00115] The crystal structure of the FN3 domain P233FR9_H10 was solved in complex with cynoPSMA. As the contact residues between human and cyno PSMA are identical except

for one residue, it is expected that P233FR9_H10 will bind human PSMA at the same epitope residues than what it binds cyno PSMA.

Methods of Treatment

[00116] The subjects for administration of the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof, as described herein, include patients at high risk for developing a particular disorder characterized by PSMA overexpression as well as patients presenting with an existing such disorder. Typically, the subject has been diagnosed as having the disorder for which treatment is sought. Further, subjects can be monitored during the course of treatment for any change in the disorder (e.g., for an increase or decrease in clinical symptoms of the disorder).

[00117] In prophylactic applications, pharmaceutical compositions or medicants are administered to a patient susceptible to, or otherwise at risk of, a particular disorder in an amount sufficient to eliminate or reduce the risk or delay the onset of the disorder. In therapeutic applications, compositions or medicants are administered to a patient suspected of, or already suffering from such a disorder in an amount sufficient to cure, or at least partially arrest, the symptoms of the disorder and its complications. An amount adequate to accomplish this is referred to as a therapeutically effective dose or amount. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient response (e.g., inhibition of inappropriate angiogenesis activity) has been achieved. Typically, the response is monitored and repeated dosages are given if the desired response starts to fade.

[00118] To identify subject patients for treatment according to the methods of the invention, accepted screening methods may be employed to determine risk factors associated with specific disorders or to determine the status of an existing disorder identified in a subject. Such methods can include, for example, determining whether an individual has relatives who have been diagnosed with a particular disorder. Screening methods may also include, for example, conventional work-ups to determine familial status for a particular disorder known to have a heritable component. For example, various cancers are also known to have certain inheritable components. Inheritable components of cancers include, for example, mutations in multiple genes that are transforming (e.g., Ras, Raf, EGFR, cMet, and others), the presence or absence of certain HLA and killer inhibitory receptor (KIR) molecules, or mechanisms by which cancer cells are able to modulate immune suppression of cells like NK cells and T cells, either directly or indirectly (see, e.g., Ljunggren and Malmberg, *Nature Rev. Immunol.* 7:329-339,

2007; Boyton and Altmann, *Clin. Exp. Immunol.* 149:1-8, 2007). Toward this end, nucleotide probes can be routinely employed to identify individuals carrying genetic markers associated with a particular disorder of interest. In addition, a wide variety of immunological methods are known in the art that are useful to identify markers for specific disorder. For example, various ELISA immunoassay methods are available and well-known in the art that employ monoclonal antibody probes to detect antigens associated with specific tumors. Screening can be implemented as indicated by known patient symptomology, age factors, related risk factors, etc. These methods allow the clinician to routinely select patients in need of the methods described herein for treatment. In accordance with these methods, targeting pathological, PSMA-expressing cells can be implemented as an independent treatment program or as a follow-up, adjunct, or coordinate treatment regimen to other treatments.

[00119] In some methods described herein, the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof of the invention may be used to treat a subject with prostate cancer in combination with a second therapeutic.

[00120] In some methods described herein, the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof may be used to treat a subject who is resistant or has acquired resistance to a treatment with a second therapeutic.

[00121] The second therapeutic may be an approved drug for the treatment of prostate cancer, such as Abiraterone Acetate (Zytiga), Bicalutamide, Cabazitaxel, Casodex (Bicalutamide), Degarelix, Docetaxel, Enzalutamide, Goserelin Acetate, Jevtana (Cabazitaxel), Leuprolide Acetate, Lupron (Leuprolide Acetate), Lupron Depot (Leuprolide Acetate), Lupron Depot-3 Month (Leuprolide Acetate), Lupron Depot-4 Month (Leuprolide Acetate), Lupron Depot-Ped (Leuprolide Acetate), Mitoxantrone Hydrochloride, Prednisone, Provenge (Sipuleucel-T), Radium 223 Dichloride, Sipuleucel-T, Taxotere (Docetaxel), Viadur (Leuprolide Acetate), Xofigo (Radium 223 Dichloride), Xtandi (Enzalutamide) or Zoladex (Goserelin Acetate) (source: National Cancer Institute).

[00122] Various qualitative and/or quantitative methods may be used to determine if a subject is resistant, has developed or is susceptible to developing a resistance to treatment. Symptoms that may be associated with resistance include, for example, a decline or plateau of the well-being of the patient, an increase in the size of a tumor, arrested or slowed decline in growth of a tumor, and/or the spread of cancerous cells in the body from one location to other organs, tissues or cells. Re-establishment or worsening of various symptoms associated with cancer may also be an indication that a subject has developed or is susceptible to developing

resistance to treatment, such as anorexia, cognitive dysfunction, depression, dyspnea, fatigue, hormonal disturbances, neutropenia, pain, peripheral neuropathy, and sexual dysfunction. The symptoms associated with cancer may vary according to the type of cancer. For example, symptoms associated with prostate cancer may include trouble passing or frequent urge to pass urine, painful urination, blood in the urine or semen, nagging pain in the pelvis, back and/or hips. Symptoms associated with lung cancer may include persistent cough, coughing up blood, shortness of breath, wheezing chest pain, loss of appetite, losing weight without trying and fatigue. One skilled in oncology may readily identify symptoms associated with a particular cancer type.

Administration/ Pharmaceutical Compositions

[00123] The invention provides for pharmaceutical compositions of the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof, and a pharmaceutically acceptable carrier. For therapeutic use, the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof may be prepared as pharmaceutical compositions containing an effective amount of the domain or molecule as an active ingredient in a pharmaceutically acceptable carrier. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the active compound is administered. Such vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. For example, 0.4% saline and 0.3% glycine can be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by conventional, well-known sterilization techniques (*e.g.*, filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating and coloring agents, etc. The concentration of the molecules of the invention in such pharmaceutical formulation can vary widely, *i.e.*, from less than about 0.5%, usually at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on required dose, fluid volumes, viscosities, etc., according to the particular mode of administration selected. Suitable vehicles and formulations, inclusive of other human proteins, *e.g.*, human serum albumin, are described, for example, in *e.g.* Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, See especially pp. 958-989.

[00124] Thus the mode of administration for therapeutic use of the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof of the invention may be any suitable route that delivers the agent to the host, such as parenteral administration, e.g., intradermal, intramuscular, intraperitoneal, intravenous or subcutaneous, pulmonary; transmucosal (oral, intranasal, intravaginal, rectal), using a formulation in a tablet, capsule, solution, powder, gel, particle; and contained in a syringe, an implanted device, osmotic pump, cartridge, micropump; or other means appreciated by the skilled artisan, as well known in the art. Site specific administration may be achieved by for example intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracerebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intracardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravascular, intravesical, intralesional, vaginal, rectal, buccal, sublingual, intranasal, or transdermal delivery.

[00125] Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 ml sterile buffered water, and between about 1 ng to about 100 mg, e.g. about 50 ng to about 30 mg or more preferably, about 5 mg to about 25 mg, of the CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof.

[00126] CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof of the invention may be administered to a patient by any suitable route, for example parentally by intravenous (IV) infusion or bolus injection, intramuscularly or subcutaneously or intraperitoneally. IV infusion can be given over as little as 15 minutes, but more often for 30 minutes, 60 minutes, 90 minutes or even 2 or 3 hours. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention may also be injected directly into the site of disease. The dose given to a patient having a cancer is sufficient to alleviate or at least partially arrest the disease being treated ("therapeutically effective amount") and may be sometimes 0.1 to 10 mg/kg body weight, for example 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mg/kg, but may even higher, for example 15, 20, 30, 40, 50, 60, 70, 80, 90 or 100 mg/kg. A fixed unit dose may also be given, for example, 50, 100, 200, 500 or 1000 mg, or the dose may be based on the patient's surface area, e.g., 400, 300, 250, 200, or 100 mg/m². Usually between 1 and 8 doses, (e.g., 1, 2, 3, 4, 5, 6, 7 or 8) may be administered to treat cancer, but 10, 12, 20 or more doses may be given. Administration of the CD3 x PSMA-

multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof of the invention may be repeated after one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, five weeks, six weeks, seven weeks, two months, three months, four months, five months, six months or longer. Repeated courses of treatment are also possible, as is chronic administration. The repeated administration may be at the same dose or at a different dose.

[00127] For example, a pharmaceutical composition of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof for intravenous infusion may be made up to contain about 200 ml of sterile Ringer's solution, and about 8 mg to about 2400 mg, about 400 mg to about 1600 mg, or about 400 mg to about 800 mg of the PSMA binding FN3 domains for administration to a 80 kg patient. Methods for preparing parenterally administrable compositions are well known and are described in more detail in, for example, "Remington's Pharmaceutical Science", 15th ed., Mack Publishing Company, Easton, PA.

[00128] The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention may be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional protein preparations and art-known lyophilization and reconstitution techniques can be employed.

[00129] The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention may be administered to a subject in a single dose or the administration may be repeated, e.g. after one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, five weeks, six weeks, seven weeks, two months or three months. The repeated administration can be at the same dose or at a different dose. The administration can be repeated once, twice, three times, four times, five times, six times, seven times, eight times, nine times, ten times, or more.

[00130] The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention may be administered in combination with a second therapeutic agent as described above simultaneously, sequentially or separately.

[00131] The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention, optionally in combination with the second therapeutic agent may be administered together with any form of radiation therapy including external beam radiation, intensity modulated radiation therapy (IMRT) and any form of

radiosurgery including Gamma Knife, Cyberknife, Linac, and interstitial radiation (e.g. implanted radioactive seeds, GliaSite balloon), and/or with surgery.

[00132] With particular regard to treatment of solid tumors, protocols for assessing endpoints and anti-tumor activity are well-known in the art. While each protocol may define tumor response assessments differently, the RECIST (Response evaluation Criteria in solid tumors) criteria is currently considered to be the recommended guidelines for assessment of tumor response by the National Cancer Institute (see Therasse et al., *J. Natl. Cancer Inst.* 92:205-216, 2000). According to the RECIST criteria tumor response means a reduction or elimination of all measurable lesions or metastases. Disease is generally considered measurable if it comprises lesions that can be accurately measured in at least one dimension as ≥ 20 mm with conventional techniques or ≥ 10 mm with spiral CT scan with clearly defined margins by medical photograph or X-ray, computerized axial tomography (CT), magnetic resonance imaging (MRI), or clinical examination (if lesions are superficial). Non-measurable disease means the disease comprises of lesions < 20 mm with conventional techniques or < 10 mm with spiral CT scan, and truly non-measurable lesions (too small to accurately measure). Non-measurable disease includes pleural effusions, ascites, and disease documented by indirect evidence.

[00133] The criteria for objective status are required for protocols to assess solid tumor response. Representative criteria include the following: (1) Complete Response (CR), defined as complete disappearance of all measurable disease; no new lesions; no disease related symptoms; no evidence of non-measurable disease; (2) Partial Response (PR) defined as 30% decrease in the sum of the longest diameter of target lesions (3) Progressive Disease (PD), defined as 20% increase in the sum of the longest diameter of target lesions or appearance of any new lesion; (4) Stable or No Response, defined as not qualifying for CR, PR, or Progressive Disease. (See Therasse et al., *supra*.)

[00134] Additional endpoints that are accepted within the oncology art include overall survival (OS), disease-free survival (DFS), objective response rate (ORR), time to progression (TTP), and progression-free survival (PFS) (see *Guidance for Industry: Clinical Trial Endpoints for the Approval of Cancer Drugs and Biologics*, April 2005, Center for Drug Evaluation and Research, FDA, Rockville, Md.)

[00135] Pharmaceutical compositions can be supplied as a kit comprising a container that comprises the pharmaceutical composition as described herein. A pharmaceutical composition can be provided, for example, in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Alternatively,

such a kit can include a dry-powder disperser, liquid aerosol generator, or nebulizer for administration of a pharmaceutical composition. Such a kit can further comprise written information on indications and usage of the pharmaceutical composition.

[00136] In some embodiments, expression of the fusion protein is placed under control of a powerful constitutive promoter, such as the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, beta-actin, human myosin, human hemoglobin, human muscle creatine, and others. In addition, many viral promoters function constitutively in eukaryotic cells and are suitable for use with the described embodiments. Such viral promoters include without limitation, Cytomegalovirus (CMV) immediate early promoter, the early and late promoters of SV40, the Mouse Mammary Tumor Virus (MMTV) promoter, the long terminal repeats (LTRs) of Maloney leukemia virus, Human Immunodeficiency Virus (HIV), Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV), and other retroviruses, and the thymidine kinase promoter of Herpes Simplex Virus.

[00137] Vectors described herein may contain one or more Internal Ribosome Entry Site(s) (IRES). Inclusion of an IRES sequence into fusion vectors may be beneficial for enhancing expression of some proteins. In some embodiments the vector system will include one or more polyadenylation sites (e.g., SV40), which may be upstream or downstream of any of the aforementioned nucleic acid sequences. Vector components may be contiguously linked, or arranged in a manner that provides optimal spacing for expressing the gene products (i.e., by the introduction of “spacer” nucleotides between the ORFs), or positioned in another way. Regulatory elements, such as the IRES motif, may also be arranged to provide optimal spacing for expression.

[00138] The vectors may comprise selection markers, which are well known in the art. Selection markers include positive and negative selection markers, for example, antibiotic resistance genes (e.g., neomycin resistance gene, a hygromycin resistance gene, a kanamycin resistance gene, a tetracycline resistance gene, a penicillin resistance gene), glutamate synthase genes, HSV-TK, HSV-TK derivatives for ganciclovir selection, or bacterial purine nucleoside phosphorylase gene for 6-methylpurine selection (Gadi et al., *7 Gene Ther.* 1738-1743 (2000)). A nucleic acid sequence encoding a selection marker or the cloning site may be upstream or downstream of a nucleic acid sequence encoding a polypeptide of interest or cloning site.

[00139] The vectors described herein may be used to transform various cells with the genes encoding the described antibodies or antigen-binding fragments. For example, the vectors may be used to generate fusion protein-producing cells. Thus, another aspect features host cells

transformed with vectors comprising a nucleic acid sequence encoding fusion proteins, such as the fusion proteins described and exemplified herein.

[00140] Numerous techniques are known in the art for the introduction of foreign genes into cells and may be used to construct the recombinant cells for purposes of carrying out the described methods, in accordance with the various embodiments described and exemplified herein. The technique used should provide for the stable transfer of the heterologous gene sequence to the host cell, such that the heterologous gene sequence is heritable and expressible by the cell progeny, and so that the necessary development and physiological functions of the recipient cells are not disrupted. Techniques which may be used include but are not limited to chromosome transfer (e.g., cell fusion, chromosome mediated gene transfer, micro cell mediated gene transfer), physical methods (e.g., transfection, spheroplast fusion, microinjection, electroporation, liposome carrier), viral vector transfer (e.g., recombinant DNA viruses, recombinant RNA viruses) and the like (described in Cline, 29 *Pharmac. Ther.* 69-92 (1985)). Calcium phosphate precipitation and polyethylene glycol (PEG)-induced fusion of bacterial protoplasts with mammalian cells may also be used to transform cells.

[00141] Cells suitable for use in the expression of the fusion proteins described herein are preferably eukaryotic cells, more preferably cells of plant, rodent, or human origin, for example but not limited to NSO, CHO, CHOK1, perC.6, Tk-ts13, BHK, HEK293 cells, COS-7, T98G, CV-1/EBNA, L cells, C127, 3T3, HeLa, NS1, Sp2/0 myeloma cells, and BHK cell lines, among others. In addition, expression of antibodies may be accomplished using hybridoma cells. Methods for producing hybridomas are well established in the art.

[00142] Cells transformed with expression vectors described herein may be selected or screened for recombinant expression of the antibodies or antigen-binding fragments described herein. Recombinant-positive cells are expanded and screened for subclones exhibiting a desired phenotype, such as high level expression, enhanced growth properties, or the ability to yield proteins with desired biochemical characteristics, for example, due to protein modification or altered post-translational modifications. These phenotypes may be due to inherent properties of a given subclone or to mutation. Mutations may be effected through the use of chemicals, UV-wavelength light, radiation, viruses, insertional mutagens, inhibition of DNA mismatch repair, or a combination of such methods.

Multispecific Isolated CD3 x PSMA-bispecific Antigen-binding Molecule or Bispecific Antigen-binding Fragments Thereof.

[00143] Preferred isolated CD3 x PSMA-bispecific antigen-binding molecules or bispecific antigen-binding fragments thereof are provided in Table 21.

[00144] Different formats of bispecific antibodies have been described and were recently reviewed by Chames and Baty (2009) Curr Opin Drug Disc Dev 12: 276.

[00145] In some embodiments, the bispecific antibody of the present invention is a diabody, a cross-body, or a bispecific antibody obtained via a controlled Fab arm exchange as those described in the present invention.

[00146] In some embodiments, the bispecific antibodies include IgG-like molecules with complementary CH3 domains to force heterodimerisation; recombinant IgG-like dual targeting molecules, wherein the two sides of the molecule each contain the Fab fragment or part of the Fab fragment of at least two different antibodies; IgG fusion molecules, wherein full length IgG antibodies are fused to an extra Fab fragment or parts of Fab fragment; Fc fusion molecules, wherein single chain Fv molecules or stabilized diabodies are fused to heavy-chain constant-domains, Fc-regions or parts thereof; Fab fusion molecules, wherein different Fab-fragments are fused together; ScFv- and diabody-based and heavy chain antibodies (e.g., domain antibodies, nanobodies) wherein different single chain Fv molecules or different diabodies or different heavy-chain antibodies (e.g. domain antibodies, nanobodies) are fused to each other or to another protein or carrier molecule.

[00147] In some embodiments, IgG-like molecules with complementary CH3 domains molecules include the Triomab/Quadroma (Trion Pharma/Fresenius Biotech), the Knobs-into-Holes (Genentech), CrossMAbs (Roche) and the electrostatically-matched (Amgen), the LUZ-Y (Genentech), the Strand Exchange Engineered Domain body (SEEDbody)(EMD Serono), the Biclonic (Merus) and the DuoBody (Genmab A/S).

[00148] In some embodiments, recombinant IgG-like dual targeting molecules include Dual Targeting (DT)-Ig (GSK/Domantis), Two-in-one Antibody (Genentech), Cross-linked Mabs (Karmanos Cancer Center), mAb2 (F-Star) and CovX-body (CovX/Pfizer).

[00149] In some embodiments, IgG fusion molecules include Dual Variable Domain (DVD)-Ig (Abbott), IgG-like Bispecific (InnClone/Eli Lilly), Ts2Ab (MedImmune/AZ) and BsAb (Zymogenetics), HERCULES (Biogen Idec) and TvAb (Roche).

[00150] In some embodiments, Fc fusion molecules include to ScFv/Fc Fusions (Academic Institution), SCORPION (Emergent BioSolutions/Trubion, Zymogenetics/BMS),

Dual Affinity Retargeting Technology (Fc-DART) (MacroGenics) and Dual(ScFv).sub.2-Fab (National Research Center for Antibody Medicine--China).

[00151] In some embodiments, Fab fusion bispecific antibodies include F(ab)₂ (Medarex/AMGEN), Dual-Action or Bis-Fab (Genentech), Dock-and-Lock (DNL) (ImmunoMedics), Bivalent Bispecific (Biotechnol) and Fab-Fv (UCB-Celltech). ScFv-, diabody-based and domain antibodies include but are not limited to Bispecific T Cell Engager (BITE) (Micromet, Tandem Diabody (Tandab) (Affimed), Dual Affinity Retargeting Technology (DART) (MacroGenics), Single-chain Diabody (Academic), TCR-like Antibodies (AIT, ReceptorLogics), Human Serum Albumin ScFv Fusion (Merrimack) and COMBODY (Epigen Biotech), dual targeting nanobodies (Ablynx), dual targeting heavy chain only domain antibodies.

[00152] Full length bispecific antibodies of the invention may be generated for example using Fab arm exchange (or half molecule exchange) between two mono specific bivalent antibodies by introducing substitutions at the heavy chain CH3 interface in each half molecule to favor heterodimer formation of two antibody half molecules having distinct specificity either in vitro in cell-free environment or using co-expression. The Fab arm exchange reaction is the result of a disulfide-bond isomerization reaction and dissociation-association of CH3 domains. The heavy-chain disulfide bonds in the hinge regions of the parent mono specific antibodies are reduced. The resulting free cysteines of one of the parent monospecific antibodies form an inter heavy-chain disulfide bond with cysteine residues of a second parent mono specific antibody molecule and simultaneously CH3 domains of the parent antibodies release and reform by dissociation-association. The CH3 domains of the Fab arms may be engineered to favor heterodimerization over homodimerization. The resulting product is an isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof having two Fab arms or half molecules which each bind a distinct epitope, i.e. an epitope on PSMA and an epitope on CD3.

[00153] "Homodimerization" as used herein refers to an interaction of two heavy chains having identical CH3 amino acid sequences. "Homodimer" as used herein refers to an antibody having two heavy chains with identical CH3 amino acid sequences.

[00154] "Heterodimerization" as used herein refers to an interaction of two heavy chains having non-identical CH3 amino acid sequences. "Heterodimer" as used herein refers to an antibody having two heavy chains with non-identical CH3 amino acid sequences.

[00155] The "knob-in-hole" strategy (see, e.g., PCT Int. Publ. No. WO 2006/028936) may be used to generate full length bispecific antibodies. Briefly, selected amino acids forming the interface of the CH3 domains in human IgG can be mutated at positions affecting CH3 domain interactions to promote heterodimer formation. An amino acid with a small side chain (hole) is introduced into a heavy chain of an antibody specifically binding a first antigen and an amino acid with a large side chain (knob) is introduced into a heavy chain of an antibody specifically binding a second antigen. After co-expression of the two antibodies, a heterodimer is formed as a result of the preferential interaction of the heavy chain with a "hole" with the heavy chain with a "knob". Exemplary CH3 substitution pairs forming a knob and a hole are (expressed as modified position in the first CH3 domain of the first heavy chain/modified position in the second CH3 domain of the second heavy chain): T366Y/F405A, T366W/F405W, F405W/Y407A, T394W/Y407T, T394S/Y407A, T366W/T394S, F405W/T394S and T366W/T366S_L368A_Y407V.

[00156] Other strategies such as promoting heavy chain heterodimerization using electrostatic interactions by substituting positively charged residues at one CH3 surface and negatively charged residues at a second CH3 surface may be used, as described in US Pat. Publ. No. US2010/0015133; US Pat. Publ. No. US2009/0182127; US Pat. Publ. No. US2010/028637 or US Pat. Publ. No. US2011/0123532. In other strategies, heterodimerization may be promoted by the following substitutions (expressed as modified position in the first CH3 domain of the first heavy chain/modified position in the second CH3 domain of the second heavy chain): L351Y_F405A_Y407V/T394W, T366I_K392M_T394W/F405A_Y407V, T366L_K392M_T394W/F405A_Y407V, L351Y_Y407A/T366A_K409F, L351Y_Y407A/T366V_K409F_Y407A/T366A_K409F, or T350V_L351Y_F405A_Y407V/T350V_T366L_K392L_T394W as described in U.S. Pat. Publ. No. US2012/0149876 or U.S. Pat. Publ. No. US2013/0195849.

[00157] In addition to methods described above, isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention may be generated in vitro in a cell-free environment by introducing asymmetrical mutations in the CH3 regions of two mono specific homodimeric antibodies and forming the bispecific heterodimeric antibody from two parent monospecific homodimeric antibodies in reducing conditions to allow disulfide bond isomerization according to methods described in Int. Pat. Publ. No. W02011/131746. In the methods, the first monospecific FN3 domain and a monospecific bivalent antibody (e.g., anti-CD3 antibody) are engineered to have certain

substitutions at the CH3 domain that promotes heterodimer stability; the antibodies are incubated together under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide bond isomerization; thereby generating the bispecific antibody by Fab arm exchange. The incubation conditions may optimally be restored to non-reducing conditions. Exemplary reducing agents that may be used are 2-mercaptoethylamine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione, tris (2-carboxyethyl)phosphine (TCEP), L-cysteine and beta-mercaptoethanol, preferably a reducing agent selected from the group consisting of: 2-mercaptoethylamine, dithiothreitol and tris (2-carboxyethyl)phosphine. For example, incubation for at least 90 min at a temperature of at least 20° C in the presence of at least 25 mM 2-MEA or in the presence of at least 0.5 mM dithiothreitol at a pH from 5-8, for example at pH of 7.0 or at pH of 7.4 may be used.

[00158] Also provided herein are methods for killing a PMSA-expressing cell by administering to a patient in need thereof a multispecific antibody which binds said PMSA and is able to recruit T cells to kill said PMSA-expressing cell (i.e., T cell redirection). Any of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention may be used therapeutically.

[00159] In a preferred embodiment, isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the invention are used for the treatment of a hyperproliferative disorder in a mammal. In a more preferred embodiment, one of the pharmaceutical compositions disclosed above, and which contains a multispecific antibody or antibody fragment of the invention, is used for the treatment of a hyperproliferative disorder in a mammal. In one embodiment, the disorder is a cancer.

[00160] Similarly, further provided herein is a method for inhibiting the growth of selected cell populations comprising contacting PMSA-expressing target cells, or tissue containing such target cells, with an effective amount of a isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof of the present invention, either alone or in combination with other cytotoxic or therapeutic agents. In preferred embodiments, the multispecific antigen-binding molecule is an isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof. The method for inhibiting the growth of selected cell populations can be practiced *in vitro*, *in vivo*, or *ex vivo*.

Kits

[00161] Also provided herein are includes kits, e.g., comprising a described multispecific antibody or antigen-binding fragment thereof and instructions for the use of the

isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof for killing of particular cell types. In preferred embodiments, the multispecific isolated CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragments thereof as described herein, and more preferably isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragments thereof. The instructions may include directions for using the multispecific antibody or antigen-binding fragment thereof in vitro, in vivo or ex vivo.

[00162] Typically, the kit will have a compartment containing the isolated CD3 x PSMA-multispecific antigen-binding molecule or multispecific antigen-binding fragment thereof. The multispecific antibody or antigen-binding fragment thereof may be in a lyophilized form, liquid form, or other form amendable to being included in a kit. The kit may also contain additional elements needed to practice the method described on the instructions in the kit, such a sterilized solution for reconstituting a lyophilized powder, additional agents for combining with the multispecific antibody or antigen-binding fragment thereof prior to administering to a patient, and tools that aid in administering the multispecific antibody or antigen-binding fragment thereof to a patient.

EXAMPLES

[00163] The following examples are provided to supplement the prior disclosure and to provide a better understanding of the subject matter described herein. These examples should not be considered to limit the described subject matter. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be apparent to persons skilled in the art and are to be included within, and can be made without departing from, the true scope of the invention.

Reagents and Constructs:

[00164] The extracellular domains of cynomolgus (cyno monkey protein database ref# EHH56646.1, SEQ ID NO: 32) and chimpanzee (Uniprot, Ref#H2Q3K5, SEQ ID NO: 33) PSMA were cloned into the pUnder expression vector along with a 6His and Avi tag. Proteins were transiently expressed in 293HEK-expi cells. Supernatants were harvested and clarified by centrifugation. The proteins were purified using a two-step purification process: 1) IMAC purification with a HisTrap HP column and 2) size exclusion purification (Superdex 200) where the elution buffer is DPBS containing Mg^{2+} , Ca^{2+} , and 0.5mM $ZnCl_2$ to stabilize PSMA

dimerization. Fractions containing the protein of interest were pooled and protein concentration was determined by A280.

[00165] The gene encoding *S. aureus* sortase A was produced by DNA2.0 and subcloned into pJexpress401 vector (DNA2.0) for expression under the T5 promoter. The sortase construct for soluble expression is lacking the N-terminal domain of the natural protein consisting of 25 amino acids since this domain is membrane associated (Ton-That et al., Proc Natl Acad Sci U S A 96: 12424-12429, 1999). The sortase was expressed as N-terminal His6-tag (HHHHHH, SEQ ID NO:34) followed by a TEV protease site for tag removal (ENLYFQS, SEQ ID NO: 54), resulting in the sortase having the amino acid sequence of SEQ ID NO: 52. The sortase protein used also includes 5 mutations sequence that are reported to increase the catalytic efficiency of the enzyme when compared to the wild type proteins (SEQ ID NO: 53) (Chen et al., Proc Natl Acad Sci U S A 108: 11399-11404, 2011). The plasmid was transformed into E.coli BL21 Gold cells (Agilent) for expression. A single colony was picked and grown in Luria Broth (Teknova) supplemented with kanamycin and incubated 18 h at 37°C 250RPM. 250 mL of Terrific Broth (Teknova), supplemented with kanamycin, was inoculated from these subcultures and grown at 37°C for ~4 h while shaking. Protein expression was induced with 1 mM IPTG, and the protein was expressed for 18 h at 30°C. Cells were harvested by centrifugation at 6000 g and stored at -20°C until purification. The frozen cell pellet was thawed for 30 min at room temperature and suspended in BugBusterHT protein extraction reagent (EMD Millipore) supplemented with 1 uL per 30 mL of recombinant lysozyme (EMD Millipore) at 5ml per gram of cell paste and incubated for 30 minutes on a shaker at room temperature. The lysate was clarified by centrifugation at 74,600 g for 30 min.

[00166] The supernatant was applied onto a gravity column packed with 3 mL of Qiagen Superflow Ni-NTA resin pre-equilibrated with buffer A (50 mM sodium phosphate buffer, pH 7.0 containing 0.5 M NaCl and 10 mM imidazole). After loading, the column was washed with 100 mL of Buffer A. The protein was eluted with Buffer A supplemented with 250 mM imidazole and loaded on a preparative gel-filtration column, TSK Gel G3000SW 21.5 x 600 mm (Tosoh) equilibrated in PBS (Gibco). The gel-filtration chromatography was performed at room temperature in PBS at flow rate 10 ml/min using an AKTA-AVANT chromatography system. Purified sortase was then digested with TEV protease to remove the His6 tag. 28 mgs of sortase was incubated in 10 mLs with 3000 units of AcTEV protease (Invitrogen) in the supplied buffer supplemented with 1 mM DTT for 2 hours at 30°C. The tagless sortase was purified with Ni-NTA resin. The reaction was exchanged into TBS buffer (50 mM Tris pH 7.5, 150 mM NaCl)

using PD-10 columns (GE Healthcare) and applied onto a gravity column packed with 0.5 mL of Qiagen Superflow Ni-NTA resin pre-equilibrated with buffer A. The flowthrough was collected and the resin was washed with 3 mL of buffer A which was added to the flowthrough. This flowthrough was concentrated to ~ 0.5 mL in an Amicon 15 concentrator with 10 kDa cutoff (EMD Millipore). Additional TBS buffer was added and the sample was concentrated again (repeated twice) to exchange the buffer to TBS. 1/3rd volume of 40% glycerol was added (final concentration of 10% glycerol), and the sortase was stored at -20°C for short term use or -80°C for long term.

EXAMPLE 1. Construction of Tencon Libraries with Randomized Loops

[00167] Tencon (SEQ ID NO: 1) is an immunoglobulin-like scaffold, fibronectin type III (FN3) domain, designed from a consensus sequence of fifteen FN3 domains from human tenascin-C (Jacobs *et al.*, Protein Engineering, Design, and Selection, 25:107-117, 2012; U.S. Pat. No. 8,278,419). The crystal structure of Tencon shows six surface-exposed loops that connect seven beta-strands. These loops, or selected residues within each loop, can be randomized in order to construct libraries of fibronectin type III (FN3) domains that can be used to select novel molecules that bind to specific targets.

[00168] Tencon:

Lpapknlvvsevtedsrlrlswtapdaafdsfliqyqesekvgeainltvpgsersydlgtlkgpteytvsygyvgkgghrsnplsaeftt (SEQ ID NO 1):

[00169] Various libraries were generated using the tencon scaffold and various design strategies. In general, libraries TCL1 and TCL2 produced good binders. Generation of TCL1 and TCL2 libraries are described in detail in Int. Pat. Publ. No. WO2014081944A2.

Construction of TCL1 Library

[00170] A library designed to randomize only the FG loop of Tencon (SEQ ID NO: 1), TCL1, was constructed for use with the *cis*-display system (Jacobs *et al.*, Protein Engineering, Design, and Selection, 25:107-117, 2012). In this system, a single-strand DNA incorporating sequences for a Tac promoter, Tencon library coding sequence, RepA coding sequence, *cis*-element, and ori element is produced. Upon expression in an *in vitro* transcription/translation system, a complex is produced of the Tencon-RepA fusion protein bound in *cis* to the DNA from which it is encoded. Complexes that bind to a target molecule are then isolated and amplified by polymerase chain reaction (PCR), as described below.

[00171] Construction of the TCL1 library for use with *cis*-display was achieved by successive rounds of PCR to produce the final linear, double-stranded DNA molecules in two halves; the 5' fragment contains the promoter and Tencon sequences, while the 3' fragment contains the *repA* gene and the *cis*- and *ori* elements. These two halves are combined by restriction digest in order to produce the entire construct. The TCL1 library was designed to incorporate random amino acids only in the FG loop of Tencon, KGGHRSN (SEQ ID NO: 55). NNS codons were used in the construction of this library, resulting in the possible incorporation of all 20 amino acids and one stop codon into the FG loop. The TCL1 library contains six separate sub-libraries, each having a different randomized FG loop length, from 7 to 12 residues, in order to further increase diversity.

[00172] TCL1 library (SEQ ID NO: 2)

LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSEFLIQYQSEKVGGEAINLTVPGSERSYDLTG
LKPGTEYTVSIYGVX₇₋₁₂PLSAEFTT; wherein X₁, X₂, X₃, X₄, X₅, X₆, X₇ is any amino acid;
and X₈, X₉, X₁₀, X₁₁ and X₁₂ are any amino acid or deleted.

Construction of TCL2 Library

[00173] TCL2 library was constructed in which both the BC and the FG loops of Tencon were randomized and the distribution of amino acids at each position was strictly controlled. Table 6 shows the amino acid distribution at desired loop positions in the TCL2 library. The designed amino acid distribution had two aims. First, the library was biased toward residues that were predicted to be structurally important for Tencon folding and stability based on analysis of the Tencon crystal structure and/or from homology modeling. For example, position 29 was fixed to be only a subset of hydrophobic amino acids, as this residue was buried in the hydrophobic core of the Tencon fold. A second layer of design included biasing the amino acid distribution toward that of residues preferentially found in the heavy chain HCDR3 of antibodies, to efficiently produce high-affinity binders (Birtalan *et al.*, J Mol Biol 377:1518-28, 2008; Olson *et al.*, Protein Sci 16:476-84, 2007). Towards this goal, the “designed distribution” in Table 5 refers to the distribution as follows: 6% alanine, 6% arginine, 3.9% asparagine, 7.5% aspartic acid, 2.5% glutamic acid, 1.5% glutamine, 15% glycine, 2.3% histidine, 2.5% isoleucine, 5% leucine, 1.5% lysine, 2.5% phenylalanine, 4% proline, 10% serine, 4.5% threonine, 4% tryptophan, 17.3% tyrosine, and 4% valine. This distribution is devoid of methionine, cysteine, and STOP codons.

[00174] TCL2 library (SEQ ID NO: 3)

LPAPKNLVVSEVTEDSLRLSWX₁X₂X₃X₄X₅X₆X₇X₈SFLIQYQESEKVGAINLTVPGSERS
YDLTGLKPGTEYTVSIYGVX₉X₁₀X₁₁X₁₂X₁₃SX₁₄X₁₅LSAEFTT; wherein X₁ is Ala, Arg, Asn,
Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₂ is Ala, Arg, Asn,
Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₃ Ala, Arg, Asn,
Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₄ is Ala, Arg, Asn,
Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₅ is Ala, Arg, Asn,
Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₆ is Ala, Arg, Asn,
Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₇ is Phe, Ile, Leu,
Val or Tyr; X₈ is Asp, Glu or Thr; X₉ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₁₀ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₁₁ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₁₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₁₃ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₁₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; and X₁₅ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu,
Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val.

[00175] Table 5.

Residue Position*	WT residues	Distribution in the TCL2 library
22	T	designed distribution
23	A	designed distribution
24	P	50% P + designed distribution
25	D	designed distribution
26	A	20% A + 20% G + designed distribution
27	A	designed distribution
28	F	20% F, 20% I, 20% L, 20% V, 20% Y
29	D	33% D, 33% E, 33% T
75	K	designed distribution
76	G	designed distribution
77	G	designed distribution
78	H	designed distribution
79	R	designed distribution
80	S	100% S
81	N	designed distribution
82	P	50% P + designed distribution

[00176] *residue numbering is based on Tencon sequence of SEQ ID NO: 1

[00177] Subsequently, these libraries were improved by various ways, including building of the libraries on a stabilized Tencon framework (U.S. Pat. No. 8,569,227) that incorporates substitutions E11R/L17A/N46V/E86I (Tencon27; SEQ ID NO: 4) when compared to the wild type tencon as well as altering of the positions randomized in the BC and FG loops. Tencon27 is described in Int. Pat. Appl. No. WO2013049275. From this, new libraries designed to randomize only the FG loop of Tencon (library TCL9), or a combination of the BC and FG loops (library TCL7) were generated. These libraries were constructed for use with the cis-display system (Odegrip et al., Proc Natl Acad Sci U S A 101: 2806-2810, 2004). The details of this design are shown below:

[00178] Stabilized Tencon (Tencon27) (SEQ ID NO: 4)

LPAPKNLVVSRVTEDSARLSWTAPDAAFD SFLIQYQESEKVG EAI VLTVP G SERSYDLTG
LKPGTEYTVSIYGVKGGHRSNPLSAIFTT

[00179] TCL7 (randomized FG and BC loops) (SEQ ID NO: 5)

LPAPKNLVVSRVTEDSARLSW $X_1X_2X_3X_4X_5X_6X_7X_8X_9$ FD SFLIQYQESEKVG EAI VLTVP G
SERSYDLTGLKPGTEYTVSIYGV $X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}$ SNPLSAIFTT; wherein
 $X_1, X_2, X_3, X_4, X_5, X_6, X_{10}, X_{11}, X_{12}, X_{13}, X_{14}, X_{15}$ and X_{16} is A, D, E, F, G, H, I, K, L, N, P, Q, R,
S, T, V, W or Y; and $X_7, X_8, X_9, X_{17}, X_{18}$ and X_{19} , is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T,
V, W, Y or deleted.

[00180] TCL9 (randomized FG loop) (SEQ ID NO: 6)

LPAPKNLVVSRVTEDSARLSWTAPDAAFD SFLIQYQESEKVG EAI VLTVP G SERSYDLTG
LKPGTEYTVSIYGV $X_1X_2X_3X_4X_5X_6X_7X_8X_9$ $X_{10}X_{11}X_{12}$ SNPLSAIFTT; $X_1, X_2, X_3, X_4, X_5, X_6$
and X_7 , is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; and X_8, X_9, X_{10}, X_{11} and X_{12} is A,
D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted.

[00181] For library construction, DNA fragments encoding randomized BC loops (lengths 6-9 positions) or FG loops (lengths 7-12 positions) were synthesized using Slonomics technology (Sloning Biotechnology GmbH) so as to control the amino acid distribution of the library and to eliminate stop codons. Two different sets of DNA molecules randomizing either the BC loop or the FG loops were synthesized independently and later combined using PCR to produce the full library product.

Construction of FG loop libraries (TCL9)

[00182] A set of synthetic DNA molecules consisting of a 5' Tac promoter followed by the complete gene sequence of Tencon with the exception of randomized codons in the FG loop was produced (SEQ ID NOs: 26-31). For FG loop randomization, all amino acids except cysteine and methionine were encoded at equal percentages. The lengths of the diversified portion are such that they encode for 7, 8, 9, 10, 11, or 12 amino acids in the FG loop. Sub-libraries of each length variation were synthesized individually at a scale of 2 μ g and then amplified by PCR using oligos Sloning-FOR (SEQ ID NO: 9) and Sloning-Rev (SEQ ID NO: 10).

[00183] The 3' fragment of the library is a constant DNA sequence containing elements for display, including a PspOMI restriction site, the coding region of the repA gene, and the cis- and ori elements. PCR reactions were performed to amplify this fragment using a plasmid (pCR4Blunt) (Invitrogen) as a template with M13 Forward and M13 Reverse primers. The resulting PCR products were digested by PspOMI overnight and gel-purified. To ligate the 5' portion of library DNA to the 3' DNA containing repA gene, 2 pmol (~540ng to 560ng) of 5' DNA was ligated to an equal molar (~1.25 μ g) of 3' repA DNA in the presence of NotI and PspOMI enzyme and T4 ligase at 37°C overnight. The ligated library product was amplified by using 12 cycles of PCR with oligos POP2250 (SEQ ID NO: 11) and DigLigRev (SEQ ID NO: 12). For each sub-library, the resulting DNA from 12 PCR reactions were combined and purified by Qiagen spin column. The yield for each sub-library of TCL9 ranged from 32-34 μ g.

Construction of FG/BC Loop libraries (TCL7)

[00184] The TCL7 library provides for a library with randomized Tencon BC and FG loops. In this library, BC loops of lengths 6-9 amino acids were mixed combinatorially with randomized FG loops of 7-12 amino acids in length. Synthetic Tencon fragments BC6, BC7, BC8, and BC9 (SEQ ID No. 13-16) were produced to include the Tencon gene encoding for the N-terminal portion of the protein up to and including residue VX such that the BC loop is replaced with either 6, 7, 8, or 9 randomized amino acids. These fragments were synthesized prior to the discovery of L17A, N46V and E83I mutations (CEN5243) but these mutations were introduced in the molecular biology steps described below. In order to combine this fragment with fragments encoding for randomized FG loops, the following steps were taken.

[00185] First, a DNA fragment encoding the Tac promoter and the 5' sequence of Tencon up to the nucleotide encoding for amino acid A17 (130mer-L17A, SEQ ID No. 17) was

produced by PCR using oligos POP2222ext (SEQ ID No. 18) and LS1114 (SEQ ID No. 19). This was done to include the L17A mutation in the library (CEN5243). Next, DNA fragments encoding for Tencon residues R18-V75 including randomized BC loops were amplified by PCR using BC6, BC7, BC8, or BC9 as a templates and oligos LS1115 (SEQ ID No. 20) and LS1117 (SEQ ID No. 21). This PCR step introduced a BsaI site at the 3' end. These DNA fragments were subsequently joined by overlapping PCR using oligos POP2222ext and LS1117 as primers. The resulting PCR product of 240bp was pooled and purified by Qiagen PCR purification kit. The purified DNA was digested with BsaI-HF and gel purified.

[00186] Fragments encoding the FG loop were amplified by PCR using FG7, FG8, FG9, FG10, FG11, and FG12 (SEQ ID No. 26-31) as templates with oligonucleotides SDG10 (SEQ ID No. 22) and SDG24 (SEQ ID No. 23) to incorporate a BsaI restriction site and N46V and E86I variations (CEN5243).

[00187] The digested BC fragments and FG fragments were ligated together in a single step using a 3-way ligation. Four ligation reactions in the 16 possible combinations were set up, with each ligation reaction combining two BC loop lengths with 2 FG loop lengths. Each ligation contained ~300 ng of total BC fragment and 300 ng of the FG fragment. These 4 ligation pools were then amplified by PCR using oligos POP2222 (SEQ ID No. 24) and SDG28 (SEQ ID No. 25). 7.5 µg of each reaction product were then digested with NotI and cleaned up with a Qiagen PCR purification column. 5.2 µg of this DNA, was ligated to an equal molar amount of RepA DNA fragment (~14 µg) digested with PspOMI and the product amplified by PCR using oligos POP2222.

EXAMPLE 2: Generation of Tencon Libraries Having Alternative Binding Surfaces

[00188] The choice of residues to be randomized in a particular library design governs the overall shape of the interaction surface created. X-ray crystallographic analysis of an FN3 domain containing scaffold protein selected to bind maltose binding protein (MBP) from a library in which the BC, DE, and FG loops were randomized was shown to have a largely curved interface that fits into the active site of MBP (Koide et al., Proc Natl Acad Sci U S A 104: 6632-6637, 2007). In contrast, an ankyrin repeat scaffold protein that was selected to bind to MBP was found to have a much more planar interaction surface and to bind to the outer surface of MBP distant from the active (Binz et al., Nat Biotechnol 22: 575-582, 2004). These results suggest that the shape of the binding surface of a scaffold molecule (curved vs. flat) may dictate what target proteins or specific epitopes on those target proteins are able to be bound effectively

by the scaffold. Published efforts around engineering protein scaffolds containing FN3 domains for protein binding has relied on engineering adjacent loops for target binding, thus producing curved binding surfaces. This approach may limit the number of targets and epitopes accessible by such scaffolds.

[00189] Tencon and other FN3 domains contain two sets of CDR-like loops lying on the opposite faces of the molecule, the first set formed by the BC, DE, and FG loops, and the second set formed by the AB, CD, and EF loops. The two sets of loops are separated by the beta-strands that form the center of the FN3 structure. If the image of the Tencon is rotated by 90 degrees, an alternative surface can be visualized. This slightly concave surface is formed by the CD and FG loops and two antiparallel beta- strands, the C and the F beta-strands, and is herein called the C-CD-F-FG surface. The C-CD-F-FG surface can be used as a template to design libraries of protein scaffold interaction surfaces by randomizing a subset of residues that form the surface. Beta-strands have a repeating structure with the side chain of every other residue exposed to the surface of the protein. Thus, a library can be made by randomizing some or all surface exposed residues in the beta strands. By choosing the appropriate residues in the beta-strands, the inherent stability of the Tencon scaffold should be minimally compromised while providing a unique scaffold surface for interaction with other proteins.

[00190] Library TCL14 (SEQ ID NO: 7), was designed into Tencon27 scaffold (SEQ ID NO: 4).

[00191] A full description of the methods used to construct this library is described in US. Pat. Publ. No. US2013/0226834.

[00192] TCL14 library (SEQ ID NO: 7):
LPAPKNLVVSRVTEDSARLSWTAPDAAFD₁SFX₁IX₂YX₃EX₄X₅X₆X₇GEAIVLTVPGSERSY
DLTGLKPGTEYX₈VX₉IX₁₀GVKGGX₁₁X₁₂SX₁₃PLSAIFTT; wherein X₁, X₂, X₃, X₄, X₅, X₆,
X₁₀, X₁₁, X₁₂ and X₁₃ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y, C or M.

[00193] The two beta strands forming the C-CD-F-FG surface in Tencon27 have a total of 9 surface exposed residues that could be randomized; C-strand: S30, L32, Q34, Q36; F-strand: E66, T68, S70, Y72, and V74, while the CD loop has 6 potential residues: S38, E39, K40, V41, G42, and E43 and the FG loop has 7 potential residues: K75, G76, G77, H78, R79, S80, and N81. Select residues were chosen for inclusion in the TCL14 design due to the larger theoretical size of the library if all 22 residues were randomized.

[00194] Thirteen positions in Tencon were chosen for randomizing: L32, Q34 and Q36 in C-strand, S38, E39, K40 and V41 in CD-loop, T68, S70 and Y72 in F-strand, H78, R79, and

N81 in FG-loop. In the C and F strands S30 and E66 were not randomized as they lie just beyond the CD and FG loops and do not appear to be as apparently a part of the C-CD-F-FG surface. For the CD loop, G42 and E43 were not randomized as glycine, providing flexibility, can be valuable in loop regions, and E43 lies at the junction of the surface. The FG loop had K75, G76, G77, and S80 excluded. The glycines were excluded for the reasons above while careful inspection of the crystal structures revealed S80 making key contacts with the core to help form the stable FG loop. K75 faces away from the surface of the C-CD-F-FG surface and was a less appealing candidate for randomization. Although the above mentioned residues were not randomized in the original TCL14 design, they could be included in subsequent library designs to provide additional diversity for de novo selection or for example for an affinity maturation library on a select TCL14 target specific hit.

[00195] Subsequent to the production of TCL14, 3 additional Tencon libraries of similar design were produced. These two libraries, TCL19, TCL21 and TCL23, are randomized at the same positions as TCL14 (see above) however the distribution of amino acids occurring at these positions is altered (Table 6). TCL19 and TCL21 were designed to include an equal distribution of 18 natural amino acids at every position (5.55% of each), excluding only cysteine and methionine. TCL23 was designed such that each randomized position approximates the amino acid distribution found in the HCDR3 loops of functional antibodies (Birtalan et al., J Mol Biol 377: 1518-1528, 2008) as described in Table 6. As with the TCL21 library, cysteine and methionine were excluded.

[00196] A third additional library was built to expand potential target binding surface of the other libraries library. In this library, TCL24, 4 additional Tencon positions were randomized as compared to libraries TCL14, TCL19, TCL21, and TCL23. These positions include N46 and T48 from the D strand and S84 and I86 from the G strand. Positions 46, 48, 84, and 86 were chosen in particular as the side chains of these residues are surface exposed from beta-strands D and G and lie structurally adjacent to the randomized portions of the C and F strand, thus increasing the surface area accessible for binding to target proteins. The amino acid distribution used at each position for TCL24 is identical to that described for TCL19 and TCL21 in Table 6.

[00197] TCL24 Library (SEQ ID NO: 8)

LPAPKNLVVSRVTEDSARLSWTAPDAAFD₁SFX₁IX₂YX₃EX₄X₅X₆X₇GEAIX₈LX₉VPGSERSYDLTGLKPGTEYX₁₀VX₁₁IX₁₂GVKGGX₁₃X₁₄SX₁₅PLX₁₆AX₁₇FTT; wherein X₁, X₂, X₃, X₄,

X₅, X₆, X₁₀, X₁₁, X₁₂, X₁₃, X₁₄, X₁₅, X₁₆ and X₁₇ are A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V Y or W.

[00198] Table 6. Amino acid frequency (%) at each randomized position for TCL21, TCL23, and TCL24.

Table 6.

<u>Amino Acid</u>	<u>TCL19</u>	<u>TCL21</u>	<u>TCL23</u>	<u>TCL24</u>
Ala	5.6	5.6	6.0	5.6
Arg	5.6	5.6	6.0	5.6
Asn	5.6	5.6	3.9	5.6
Asp	5.6	5.6	7.5	5.6
Cys	0.0	0.0	0.0	0.0
Gln	5.6	5.6	1.5	5.6
Glu	5.6	5.6	2.5	5.6
Gly	5.6	5.6	15.0	5.6
His	5.6	5.6	2.3	5.6
Ile	5.6	5.6	2.5	5.6
Leu	5.6	5.6	5.0	5.6
Lys	5.6	5.6	1.5	5.6
Met	0.0	0.0	0.0	0.0
Phe	5.6	5.6	2.5	5.6
Pro	5.6	5.6	4.0	5.6
Ser	5.6	5.6	10.0	5.6
Thr	5.6	5.6	4.5	5.6
Trp	5.6	5.6	4.0	5.6
Tyr	5.6	5.6	17.3	5.6
Val	5.6	5.6	4.0	5.6

Generation of TCL21, TCL23, and TCL24 libraries

[00199] The TCL21 library was generated using Colibra library technology (Isogenica) in order to control amino acid distributions. TCL19, TCL23, and TCL24 gene fragments were generated using Slonomics technology (Morphosys) to control amino acid distributions. PCR was used to amplify each library following initial synthesis followed by ligation to the gene for RepA in order to be used in selections using the CIS-display system (Odegrip et al., Proc Natl Acad Sci U S A 101: 2806-2810, 2004) as described above for the loop libraries.

EXAMPLE 3: Selection of Fibronectin Type III (FN3) Domains That Bind PSMA**Plate-based selections**

[00200] CIS-display was used to select PSMA binding FN3 domains from the TCL7, TCL9, TCL19, and TCL21 libraries. For in vitro transcription and translation (ITT), 3 µg of library DNA were incubated at 30°C with 0.1 mM complete amino acids, 1X S30 premix components, and 15 µL of S30 extract (Promega) in a total volume of 50 µL. After 1 hour, 375 µL of blocking solution (1x TBS pH 7.4, 0.01% I-block (Life Technologies, #T2015), 100ug/ml herring sperm DNA) was added and reactions were incubated on ice for 15 minutes. ITT reactions were incubated with recombinant proteins, chimpanzee (pan 229) or cynomolgus monkey PSMA (pan 230), or cynomolgus monkey PSMA-Fc fusion (pan 231), which were immobilized on anti-human PSMA antibody (Lifespan Bioscience, catalog # LC-C150527) coated 96-well Maxisorb plates. Unbound library members were removed by successive washes with TBST and TBS. After washing, DNA was eluted from the target protein by heating to 85°C for 10 minutes and amplified by PCR for further rounds of panning. High affinity binders were isolated by successively lowering the concentration of target PSMA during each round from 400 nM to 100 nM and increasing the washing stringency.

[00201] Following panning, selected FN3 domains were amplified by PCR, subcloned into a pET vector modified to include a ligase independent cloning site, and transformed into BL21-GOLD (DE3) (Stratagene) cells for soluble expression in E. coli using standard molecular biology techniques. A gene sequence encoding a C-terminal poly-histidine tag was added to each FN3 domain to enable purification and detection. Cultures were grown to an optical density of 0.6-0.8 in TB medium supplemented with 100 µg/mL carbenicillin in 1-mL 96-well blocks at 37°C before the addition of IPTG to 1 mM, at which point the temperature was reduced to 30°C. Cells were harvested approximately 16 hours later by centrifugation and frozen at -20°C. Cell lysis was achieved by incubating each pellet in 0.6 mL of BugBuster® HT lysis buffer (Novagen EMD Biosciences) with shaking at room temperature for 45 minutes.

Bead-based selections

[00202] FN3 domains were also selected using a bead-based capture setup. ITT reactions were prepared as described above and then incubated with biotinylated recombinant proteins, chimpanzee or cynomolgus monkey PSMA. The biotinylated recombinant proteins and the bound library members were captured on neutravidin or streptavidin coated magnetic beads.

Unbound library members were removed by successive washes with TBST and TBS. After washing, DNA was eluted from the target protein by heating to 85°C for 10 minutes and amplified by PCR for further rounds of panning. High affinity binders were isolated by successively lowering the concentration of target PSMA during each round from 400 nM to 100 nM and increasing the washing stringency.

Off-rate selections

[00203] Outputs from the fifth round of bead-based selection were subjected to four rounds of off-rate selection. After the ITT reactions were incubated with biotinylated recombinant chimpanzee or cynomolgus monkey proteins, the proteins and the bound library members were captured on neutravidin or streptavidin coated magnetic beads, and washed in TBST extensively, the bound complexes were washed in 5 μ M cold recombinant PSMA proteins for 1 hour. Then the ITT bound to beads were washed extensively in TBST and TBS before being eluted. The biotinylated target antigen concentration was stepped down from 25 nM in rounds 6 and 7 to 2.5 nM in rounds 8 and 9. Selection outputs from rounds 7 and 9 were subcloned into the modified pET15 vector for expression and screening.

Affinity maturation library selection

[00204] An affinity maturation library (TCL25) based on the sequence of clone P229CR9P819-H11 (SEQ ID NO: 40) was generated using Slonomics technology at Morphosys (Munich, Germany) in which positions 23-30 from the BC loop and positions 78-83 from the FG loop were randomized. Maintenance of target binding in the library was achieved by doping nucleotides encoding the parent amino acid (from P229CR9P819-H11) at a target frequency of 65% at each randomized position. The remaining 35% of nucleotides were designed to contain a mixture of codons encoding for an equal probability of all other 20 natural amino acids, with the exception of cysteine and methionine which were not included. Table 7 shows the design of the TCL25 maturation library. In the table, numbers in parenthesis represent the percentage of molecules in the library designed to contain the corresponding amino acid at each position. This doping scheme (65% parent at 14 positions) generates a theoretical distribution of molecules containing mostly 3, 4, 5, 6, or 7 changes as compared to the parent molecule.

Table 7.

Position	Parent Amino Acid	Amino Acid Distribution (%)
23	Asp	(2.05), arg (2.05), asn (2.05), asp (65), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
24	Ile	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (65), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
25	Asp	ala (2.05), arg (2.05), asn (2.05), asp (65), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
26	Glu	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (65), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
27	Gln	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (65), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
28	Arg	ala (2.05), arg (65), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
29	Asp	ala (2.05), arg (2.05), asn (2.05), asp (65), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
30	Trp	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (65), val (2.05)
78	Tyr	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(65), trp (2.05), val (2.05)
79	His	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (65), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
80	Val	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (65)
81	Tyr	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(65), trp (2.05), val (2.05)
82	Arg	ala (2.05), arg (65), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (2.05), thr (2.05), tyr(2.05), trp (2.05), val (2.05)
83	Ser	ala (2.05), arg (2.05), asn (2.05), asp (2.05), gln (2.05), glu (2.05), gly (2.05), his (2.05), ile (2.05), leu (2.05), lys (2.05), phe (2.05), pro (2.05), ser (65), thr (2.05), tyr(2.05), trp (2.05), val (2.05)

[00205] CIS-display was used to select PSMA binding FN3 domains from TCL25 library. The ITT reactions were incubated with biotinylated recombinant proteins, chimpanzee or cyno monkey PSMA. The biotinylated recombinant proteins and the bound library members were captured on neutravidin or streptavidin coated magnetic beads. Unbound library members were removed by successive washes with TBST and TBS. After washing, DNA was eluted from the target protein by heating to 85°C for 10 minutes and amplified by PCR for further rounds of panning. FN3 domain binders were isolated by successively lowering the concentration of target PSMA during each round from 400 nM to 100 nM and increasing the washing stringency.

[00206] Outputs from the second round selection were subjected to four rounds of off-rate selection. After the ITT reactions were incubated biotinylated recombinant PSMA proteins, the proteins and the bound library members were captured on neutravidin or streptavidin coated magnetic beads, and washed in TBST extensively, the bound complexes were washed in 5 μ M cold recombinant PSMA proteins for 1 hour. Then the ITT bound to beads were washed extensively in TBST and TBS before being eluted. The biotinylated target antigen concentration was stepped down from 25 nM in rounds 3 and 4 to 2.5 nM in rounds 5 and 6. Selection outputs from rounds 7 and 9 were subcloned into the modified pET15 vector for expression and screening.

Biochemical Screening for FN3 Domains that Bind PSMA

[00207] Neutravidin-coated plates were blocked for 1h in Starting Block T20 (Pierce) and then coated with biotinylated PSMA (using same antigen as in panning) or negative control for 1h. Plates were rinsed with TBST and diluted lysate was applied to plates for 1h. Following additional rinses, wells were treated with HRP-conjugated anti-FN3 domain antibody (PAB25) for 1h and then assayed with POD (Roche). FN3 domains with signals at least 10-fold above background were selected for further analysis.

Size Exclusion Chromatography Analysis

[00208] Size exclusion chromatography was used to determine the aggregation state of PSMA binding FN3 domains. Aliquots (10 μ L) of each purified FN3 domain were injected onto a Superdex 75 5/150 column (GE Healthcare) at a flow rate of 0.3 mL/min in a mobile phase of PBS pH 7.4. Elution from the column was monitored by absorbance at 280 nm. Wild type Tencon was included in each run as a control. Agilent ChemStation software (Rev. B. 04.02)

was used to analyse the elution profiles. Only those proteins with elution profiles similar to that of wild type protein in the same run were considered for further characterization.

High-throughput Expression, Conjugation and Purification of FN3 Domains

[00209] Isolated clones from unique hits identified by biochemical binding ELISA were combined into a single hit plate for growth in 96-well block plates; clones grew in 1mL cultures (LB media supplemented with kanamycin for selection) at 37°C overnight with shaking. For protein expression in 96-block plates, 1mL TB media supplemented with kanamycin was inoculated with 50uL of the overnight culture and grown at 37°C with continual shaking at 300rpm until OD₆₀₀=0.6-1. Once the target OD was reached, protein expression was induced with addition of IPTG to 1mM; plates were transferred to 30°C (300rpm) for overnight growth. Overnight cultures were centrifuged to harvest the cells; bacterial pellets were stored at -80°C until ready for use. Both positive and negative controls were included in replicate on every plate.

[00210] For conjugation to the sortase tag, bacterial pellets were thawed, resuspended and lysed in BugBusterHT (EMD Catalog #70922) supplemented with recombinant human lysozyme (EMD, Catalog # 71110). Lysis proceeded at room temperature with gentle agitation, after which the plate was transferred to a 42°C to precipitate host proteins. Debris was pelleted by centrifugation, and supernatants were transferred to a new block plate for sortase-catalyzed labeling. A master mix containing Gly3-vc-MMAF (Concortis), tagless SortaseA, and sortase buffer (Tris, sodium chloride, and calcium chloride) was prepared at a 2X concentration and added in equal volume to the lysate supernatants. The labeling reaction proceeded for two hours at room temperature, after which proteins were purified using a Ni-NTA multi-trap HP plate (GE Catalog #28-4009-89). Protein conjugates were recovered by step elution with imidazole-containing elution buffer (50mM Tris pH7.5, 500 mM NaCl, 250 mM imidazole), filter sterilized and used directly for cell based cytotoxicity assays.

High-throughput Cytotoxicity Assay of FN3 Domain-drug Conjugates

[00211] 96-well black tissue culture-coated plates (BD/Corning Catalog # 353219) were seeded with LNCaP FGC cells (ATCC, Catalog #CRL-1740) at a density of 10,000 cells/well in assay media (phenol red-free RPMI (Life Technologies Catalog #11835-030) supplemented with 5% fetal bovine serum). Seeded plates were incubated overnight at 37°C with 5% CO₂ to allow for cell attachment. Twenty-four hours later, CDCs were diluted in assay media (1:100, 1:300, 1:1000, or 1:3000) and applied directly to LNCaP cells. LNCaP cells then

incubated at 37°C, 5%CO₂ for 66-72h. Cell toxicity was assessed using CellTiter-Glo reagent (Promega, Catalog #G7571); 100 µL prepared reagent was added directly to treated wells and incubated for ten minutes with gentle shaking, protected from light. Luminescence was measured using a SpectraMax M5 plate reader. Values were normalized to untreated controls and selected for further analysis if more than 50% toxicity was achieved.

EXAMPLE 4: Characterization of anti-PSMA FN3 Domains

Large-scale Expression and Purification

[00212] Gene sequences encoding FN3 Domain mutants were discovered through panning and cloned into the pET15b vector for expression under the T7 promoter or produced by DNA2.0 and subcloned into pJexpress401 vector (DNA2.0) for expression under the T5 promoter. The resulting plasmids were transformed into E.coli BL21 Gold (Agilent) or BL21DE3 Gold (Agilent) for expression. A single colony was picked and grown in Luria Broth (Teknova) supplemented with kanamycin and incubated 18 h at 37°C 250RPM. One liter Terrific Broth (Teknova), supplemented with kanamycin, was inoculated from these subcultures and grown at 37°C for 4 h while shaking. Protein expression was induced with 1 mM IPTG, once the optical density at the absorption of 600 nm reached 1.0. The protein was expressed for 4 h at 37°C or 18 h at 30°C. Cells were harvested by centrifugation at 6000 g and stored at -20°C until purification. The frozen cell pellet (~15-25 g) was thawed for 30 min at room temperature and suspended in BugBusterHT protein extraction reagent (EMD Millipore) supplemented with 0.2 mg/ml recombinant lysozyme (Sigma) at 5ml per gram of cell paste and incubated for 1 h on a shaker at room temperature. The lysate was clarified by centrifugation at 74 600 g for 25 min. The supernatant was applied onto a 5 ml Qiagen Ni-NTA cartridge immersed in ice at a flow rate of 4 ml/min using an AKTA AVANT chromatography system. All other Ni-NTA chromatography steps were performed at flow rate 5ml/min. The Ni-NTA column was equilibrated in 25.0 ml of 50 mM Tris-HCl buffer, pH 7.0 containing 0.5 M NaCl and 10 mM imidazole (Buffer A). After loading, the column was washed with 100 ml of Buffer A, followed by 100 ml of 50 mM Tris-HCl buffer, pH7.0 containing 10 mM imidazole, 1% CHAPS and 1% n-octyl-β-D-glucopyranoside detergents, and 100 ml Buffer A. The protein was eluted with Buffer A supplemented with 250 mM imidazole and loaded on a preparative gel-filtration column, TSK Gel G3000SW 21.5 x 600 mm (Tosoh) equilibrated in PBS (Gibco). The gel-

filtration chromatography was performed at room temperature in PBS at flow rate 10 ml/min using an AKTA-AVANT chromatography system.

Determination of Thermal Stability

[00213] Thermal stability was measured by capillary DSC. Each sample was diluted in PBS pH 7.4 to a concentration of 1 mg/ml. Melting temperatures were measured for these samples using a VP-DSC instrument equipped with an autosampler (MicroCal, LLC). Samples were heated from 10 to 95°C or 100°C at a rate of 1°C per minute. A buffer only scan was completed between each sample scan in order to calculate a baseline for integration. Data were fit to a two-state unfolding model following subtraction of the buffer only signal. Reversibility of thermal denaturation was determined by repeating the scan for each sample without removing it from the cell.

Selective Cytotoxicity of anti-PSMA FN3 Domain Drug Conjugates on PSMA+ Cells

[00214] FN3 domains were conjugated to vc-MMAF through either cysteine-maleimide chemistry (Brinkley, Bioconjugate Chemistry 3: 2-13, 1992) or using the sortase reaction described above. Cytotoxicity of FN3 domain-vcMMAF conjugates was assessed in LNCaP, VCAP, MDA-PC-2B, and PC3 cells *in vitro*. Cells were plated in 96well black plates for 24h and then treated with variable doses of FN3 domain-vcMMAF conjugates. Cells were allowed to incubate with FN3 domain drug conjugates (FDDCs) for 66-72h. CellTiterGlo was used to assess toxicity, as described above. Luminescence values were imported into Excel, from which they were copied and pasted into Prism for graphical analysis. Data were transformed using $X = \text{Log}(x)$, then analyzed using nonlinear regression, applying a 3-parameter model to determine IC_{50} .

[00215] Table 8 summarizes the unique hits identified through panning, spanning multiple sequence families. FN3 domains exhibited thermal stabilities between 55° to 85°C and were cytotoxic to LNCaP cells when conjugated to vcMMAF, with IC_{50} values from 22.6-0.38 nM. Table 9, 10 and 11 shows the BC, C, CD, F and FG loop amino acid sequences of select clones. Table 12 shows the amino acid sequences of the clones.

[00216] Table 8.

Clone ID	SEQ ID NO:	Antigen species	LNCaP IC ₅₀ (nM)	T _m (°C)
P229CR5P819_H11	40	Chimp	20.7	78.1
P258AR6P1071_G03	35	Cyno	5.8	83.1
P258AR6P1070_A05	36	Cyno	4.6	83
P258AR6P1071_F04	37	Cyno	5.4	80.8
P258AR6P1070_F09	38	Cyno	0.9	79.8
P258AR6P1071_D02	39	Cyno	0.8	78.5
P234CR9_H01	46	Cyno	22.6	74.1
P234CR9_A7	45	Cyno	8.8	ND
P233FR9_H10	41	Chimp	0.4	65.5
P233FR9P1001_D9	44	Chimp	1.4	58.1
P233FR9P1001_B5-5	42	Chimp	0.5	65
P233FR9P1001_H3-1	43	Chimp	0.4	64.5

[00217] Table 9.

Clone ID		BC loop		C loop	
Clone ID	SEQ ID NO:	Sequence	SEQ ID NO:	Sequence	SEQ ID NO:
P229CR5P819_H11	40	DIDEQRDW	56	FDSFLIQYQE	63
P258AR6P1071_G03	35	DIDEQRDW	56	FDSFLIQYQE	63
P258AR6P1070_A05	36	TIDEQRDW	57	FDSFLIQYQE	63
P258AR6P1071_F04	37	VIDEQRDW	58	FDSFLIQYQE	63
P258AR6P1070_F09	38	TIDEQRDW	57	FESFLIQYQE	64
P258AR6P1071_D02	39	AIDEQRDW	59	FESFLIQYQE	64
P234CR9_H01	46	EWVVI PGD	60	FDSFLIQYQE	63
P234CR9_A7	45	GEQFTI	61	FDSFLIQYQE	63
P233FR9_H10	41	TAPDAA	62	FDSFAIGYWE	65
P233FR9P1001_D9	44	TAPDAA	62	FDSFPIGYWE	66
P233FR9P1001_B5-5	42	TAPDAA	62	FDSFTIGYWE	67
P233FR9P1001_H3-1	43	TAPDAA	62	FDSFPIGYWE	66

[00218] Table 10.

Clone ID		CD loop		F loop	
Clone ID	SEQ ID NO:	Sequence	SEQ ID NO:	Sequence	SEQ ID NO:
P229CR5P819_H11	40	SEKVGE	68	TEYTVSIYGV	70
P258AR6P1071_G03	35	SEKVGE	68	TEYTVSIYGV	70
P258AR6P1070_A05	36	SEKVGE	68	TEYTVSIYGV	70
P258AR6P1071_F04	37	SEKVGE	68	TEYTVSIYGV	70
P258AR6P1070_F09	38	SEKVGE	68	TEYTVSIYGV	70
P258AR6P1071_D02	39	SEKVGE	68	TEYTVSIYGV	70
P234CR9_H01	46	SEKVGE	68	TEYTVSIYGV	70
P234CR9_A7	45	SEKVGE	68	TEYTVSIYG	71
P233FR9_H10	41	WDDDGE	69	TEYPVYIAGV	72
P233FR9P1001_D9	44	WDDDGE	69	TEYWVYIAGV	73
P233FR9P1001_B5-5	42	WDDDGE	69	TEYPVYIAGV	72
P233FR9P1001_H3-1	43	WDDDGE	69	TEYHVYIAGV	74

[00219] Table 11.

Clone ID		FG loop	
Clone ID	SEQ ID NO:	Sequence	SEQ ID NO:
P229CR5P819_H11	40	YHVYRSSN	75
P258AR6P1071_G03	35	YHVYRSSN	76
P258AR6P1070_A05	36	YHVYRSSN	76
P258AR6P1071_F04	37	YHVYRSSN	76
P258AR6P1070_F09	38	YHVYRSSN	76
P258AR6P1071_D02	39	YHVYRSSN	76
P234CR9_H01	46	VNSGQWNDTSN	77
P234CR9_A7	45	ASGYEWFHAFGSSN	78
P233FR9_H10	41	KGGQWSF	79
P233FR9P1001_D9	44	KGGQWSF	79
P233FR9P1001_B5-5	42	KGGQWSF	79
P233FR9P1001_H3-1	43	KGGQWSF	79

[00220] Table 12.

Clone		
Clone ID	SEQ ID NO:	Sequence
P229CR5P819_H11	40	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_G03	35	LPAPKNLVVSRVTEDSARLSWDIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1070_A05	36	LPAPKNLVVSRVTEDSARLSWTIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_F04	37	LPAPKNLVVSRVTEDSARLSWVIDEQRDWFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1070_F09	38	LPAPKNLVVSRVTEDSARLSWTIDEQRDWFESFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_D02	39	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFESFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVYHVYRSNPLSAI FTT
P234CR9_H01	46	LPAPKNLVVSRVTEDSARLSWEWWVIPGDFDSFL IQYQESEKVGGEAIVLTVPGSERSYDLTGLKPGTE YTVSIYGVVNSGQWNDTSNPLSAI FTT
P234CR9_A7	45	LPAPKNLVVSRVTEDSARLSWGEQFTIFDSFLIQ YQESEKVGGEAIVLTVPGSERSYDLTGLKPGTEYT VSIYGASGYEWFHAFGSSNPLSAI FTT
P233FR9_H10	41	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYP VYIAGVKGGQWSFPLSAI FTT

P233FR9P1001_D 9	44	LPAPKKNLVVSRVTEDSARLSWTAPDAAFDSFPIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYW VYIAGVKGGQWSFPLSAIFTT
P233FR9P1001_B 5-5	42	LPAPKKNLVVSRVTEDSARLSWTAPDAAFDSFTIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYP VYIAGVKGGQWSFPLSAIFTT
P233FR9P1001_H 3-1	43	LPAPKKNLVVSRVTEDSARLSWTAPDAAFDSFPIG YWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYH VYIAGVKGGQWSFPLSAIFTT

[00221] Select drug conjugates were tested across a panel of cell lines. Table 13 shows the IC₅₀ values for several FN3 domains conjugated to vcMMAF. Data represent averages between one and nine curve fits. Data are presented as average $\pm$ SEM. CDCs were most potent in LNCaP cells, a line known to express high levels of PSMA. CDCs were also active in MDA-PCA-2B and VCAP cells, prostate cancer lines with lower levels of PSMA. No activity was observed in PC3 cells, a PSMA negative cell line, demonstrating selectivity.

[00222] Table 13.

Clone	SEQ ID NO:	Cytotoxicity Assays of FN3 domain-Drug-Conjugates			
		LNCaP cells IC ₅₀ (nM)	MDA-PCA-2B cells IC ₅₀ (nM)	VCAP cells IC ₅₀ (nM)	PC3 cells IC ₅₀ (nM)
P233FR9P1001-H3-1	43	0.4	4.6 $\pm$ 1.2	15.2 $\pm$ 1.0	>500
P234CR9_H01	46	22.6	150.8 $\pm$ 4.4	401.0 $\pm$ 130.0	>500
P233FR9_H10	41	0.5 $\pm$ 0.1	5.8 $\pm$ 2.3	25.9 $\pm$ 15.0	>500
P229CR5P819_H11	40	9.3 $\pm$ 1.9	106.8 $\pm$ 13.6	231.0 $\pm$ 38.0	>500

EXAMPLE 5: Engineering of anti-PSMA FN3 Domains

Cysteine Scan

[00223] Genes encoding anti-PSMA FN3 Domain, P233FR9_10 with cysteine residues introduced at various positions in the protein were obtained from DNA2.0 and used to express and purify proteins as described above. The resulting FN3 domains were evaluated for thermal

stability (with and without vcMMAF conjugate) and LNCaP cytotoxicity, as described above. Results are summarized in Table 14.

[00224] Table 14:

Clone ID	SEQ ID NO:	Cysteine location*	Thermal stability (NEM capped)	Thermal Stability (vcMMAF conjugated)	LNCaP cytotoxicity (IC ₅₀ ; nM)
P233FR9_H10(c-term)	47	c-terminal	TBD	TBD	ND
P233FR9_H10(K62 C)	51	K62	56.91	54.03	0.69
P233FR9_H10(R11 C)	50	R11	65.72	63.58	0.40
P233FR9_H10(E53 C)	49	E53	66.75	65.98	0.66
*Residue numbering according to SEQ ID NO: 41					

EXAMPLE 6: Imaging Biodistribution of Untargeted FN3 Domains

[00225] A FN3 domain with no specific binding to a target antigen engineered to contain a cysteine at position 62 was conjugated to DOTA and then a zirconium-89 radioisotope at IsoTherapeutics Group, LLC (Angleton, TX). Castrated male NSG mice were (Jackson laboratories) were anesthetized with 1.5% isoflurane and imaged in a Siemens Inveon microPET/CT. Mice were administered approximately 0.2 mCi [⁸⁹Zr] FN3 Domain (SEQ ID 51) via tail vein injection (made up to a 1 mg/kg dose with cold FN3 domain) and imaged continuously for the first 60 minutes, and then at 3, 6 and 24 hrs post injection of the FN3 domain.

[00226] Three-dimensional PET images were reconstructed using a 2D ordered-subsets expectation maximization algorithm (Siemens Healthcare, Knoxville, TN) into a 768 x 768 x 512 tomographic volume, with voxel size 0.107 mm x 0.107 mm x 0.107 mm. Images were processed and analyzed using PMOD v3.0 software (PMOD Technologies, Zurich, Switzerland). A cylinder of known activity was scanned in the PET scanner to provide a cross-calibration between injected dose measured by the dose calibrator, and counts per voxel in the PET images. Each PET image was co-registered to the CT image, to provide anatomical reference, using PMOD image fusion software. Regions of interest (ROI) were drawn around every 4th section for each tissue being analyzed. Mean counts per voxel were derived, and converted percentage injected dose per gram of body weight, and using the correction factor derived from the

calibration cylinder of known activity. All measures of radioactivity were corrected for decay, using the known half-life of Zr-89 (78.41 hours).

[00227] Figure 1 shows the tissue distribution of radiolabeled FN3 domain over time. Rapid accumulation in the kidney and bladder is observed, with only limited accumulation in the liver, suggesting that FN3 domains are cleared through the kidneys.

EXAMPLE 7: Crystal Structure of anti-PSMA P233FR9-H10 in Complex with Cyno PSMA

[00228] The His-tagged P233FR9-H10 FN3 domain (called herein as H10 FN3 domain) was expressed in *E. coli* and purified using affinity and size-exclusion chromatography. The FN3 domain was received in dPBS, pH 7.2.

[00229] The cynomolgus PSMA extracellular domain as a C-terminal fusion to the huIgG1 Fc domain was expressed in GnTI⁻ cells and purified by affinity and size-exclusion chromatography. The fusion protein was received in dPBS, 0.5 mM ZnCl₂, pH 7.2. Then, the Fc domain was removed with a Prescission protease treatment followed by affinity and size-exclusion chromatography. The isolated cynomolgus PSMA (cynoPSMA) extracellular domain was stored in dPBS, 0.5 mM ZnCl₂, pH 7.2.

[00230] The H10 FN3 Domain/cynoPSMA complex was prepared by mixing cynoPSMA with H10 FN3 domain at a molar ratio of 1:3 (excess FN3 domain) while dialyzing for 48 h at 4°C against 20 mM Hepes pH 7.0, 0.5 mM ZnCl₂. The complex was then eluted from a monoS column with a gradient of 48-68 mM NaCl, 20 mM Hepes pH 7.5, 10% glycerol and concentrated to 3.4 mg/mL. Crystals suitable for X-diffraction were obtained from 25% PEG 3 kDa, 0.2 M NH₄Cl, 0.1 M Na Acetate pH 4.5 using the sitting drop vapor-diffusion method at 20°C.

[00231] For X-ray data collection, the crystal was soaked for a few seconds in a cryo-protectant solution containing mother liquor supplemented with 20% glycerol, and then frozen in liquid nitrogen. X-ray diffraction data were collected with a Dectris Pilatus 6M Pixel Array detector at the beamline 17-ID of the Advanced Photon Source (APS) at Argonne National Laboratory. Diffraction data were processed with the program HKL2000 (Otwinowski & Minor, 1997). X-ray data statistics are given in Table 15.

[00232] The structure was solved by molecular replacement (MR) with Phaser (Read, 2001). The search models for MR were the crystal structures of human PSMA (PDB code 2C6G) and the structure of P114AR7P94-A3 W33A FN3 domain. The structures were refined with

PHENIX (Adams et al, 2004) and model adjustments were carried out using COOT (Emsley & Cowtan, 2004). All other crystallographic calculations were performed with the CCP4 suite of programs (CCP4, 1994). All molecular graphics were generated with PyMol (DeLano, 2002). The structure refinement statistics are given in Table 15.

[00233] Table 15.

PS42	
<i>Crystal data</i>	
Crystallization solution	
0.1M Buffer	Acetate pH 4.5
Precipitant	25% PEG 3kDa
Additive	0.2M NH ₄ Cl
Space group	P2 ₁ 2 ₁ 2
Complex/asym.unit	2
Unit cell	
<i>a</i> (Å)	84.0
<i>b</i> (Å)	109.9
<i>c</i> (Å)	261.6
V _m (Å ³ /Da)	3.32
Solvent content (%)	63
<i>X-ray data*</i>	
Resolution (Å)	50.00-2.80
High Resolution Shell (Å)	(2.85-2.80)
Measured reflections	335,467
Unique reflections	57,166
Completeness (%)	93.2 (69.3)
Redundancy	5.9 (4.4)
Rsym (%)	25.1 (64.1)
<I/σ>	6.4 (1.6)
<i>Refinement</i>	
Resolution (Å)	40.0-2.8
Number of reflections	57,063
Number of all atoms	12,330
Number of waters	8
Rfactor (%)	25.14
Rfree (%)	31.28
RMSD	
bond lengths (Å)	0.003
bond angles (°)	0.998
Average B-factor (Å²)	78.9
Ramachandran Plot	
favored region (%)	94.9
allowed region (%)	4.8
outliers (%)	0.3

[00234] *Values for high resolution shell are in parenthesis.

[00235] The structure of the homodimeric cynoPSMA includes residues 57-750, corresponding to the protease (residues 57-116 and 352-590), apical (residues 117-351) and helical (residues 591-750) domains, and eight of eleven possible N-linked glycans (in Asn-76, -121, -140, -195, -459, -476, -613, and -638) per dimer subunit. The cynoPSMA active site is

located at the interface between the three domains and it contains two zinc atoms coordinated by histidine (H377 and H553) and glutamate/aspartate (D387, catalytic E424, E425, and D453) residues and a water molecule. The H10 FN3 domain (SEQ ID NO: 41) structure contains residues 2-92. H10 residues are numbered sequentially according to SEQ ID NO: 41. cynoPSMA residues are numbered according to the full length cyno PSMA sequence of SEQ ID NO: 141. The mature cynoPSMA (without signal peptide) starts at residue 44 of SEQ ID NO: 141.

[00236] There is one cynoPSMA homodimer in the asymmetric unit with one H10 FN3 domain bound to each PSMA subunit (Figure 2A). The two FN3 domain/PSMA complexes are structurally very similar as indicated by the root mean square deviation (r.m.s.d.) of 0.72 Å for the superposition of all equivalent atoms in the PSMA subunits. Also, there is a high degree of structural similarity between human and cynomolgus PSMA and absence of large conformational changes induced by the FN3 domain binding, as indicated by a r.m.s.d of 0.5 Å for the C α atom superposition between the cynoPSMA molecule in the FN3 domain complex and unbound human PSMA (PDB code 2OOT, structure at 1.6Å resolution). An interesting feature is that the loop region 541-547 is visible only in the cynomolgus protein due to stabilization of the loop conformation through interactions with the FN3 domain.

[00237] The FN3 domain/PSMA combining site is well defined by the $2F_{\text{obs}}-F_{\text{calc}}$ electron density map, which allows reliable positioning of the binding residues. Only the interactions between the B and C chains (PSMA and FN3 domain chains, respectively) are described in the next section.

[00238] The H10 FN3 domain binds to a region near the PSMA active site (Figure 2A) and covers a cynoPSMA area of about 1,170 Å². Specifically, the FN3 domain recognizes cynoPSMA residues in the protease (Y460, F488, K499-P502, P504, R511, K514, N540-E542, and N544-F546), apical (residue R181), and helical (residues K610, N613, and I614) domains as shown in Figures 3 and 4.

[00239] The face of the FN3 domain four-stranded β -sheet packs onto the PSMA surface with the CD loop deeply inserted into the active site entrance (Figure 2B and 2C). Specifically, the H10 FN3 domain residues involved in PSMA binding are located in the C (A32 and G34), D (V46), F (G64, P68, Y70, and A72), and G (S84-I86) β -strands and the CD (W36, W38-D41, E43, and A44) and FG loops (W79, F81, and P82). Residues D39, D40, D41, and E43 confer a negative charge to the FN3 domain CD loop and these residues are inserted into the ~20 Å deep, positively charged, funnel that leads to the zinc ions in the active site, likely

blocking substrate entrance into the funnel and PSMA enzymatic activity (Figure 2B and 2C). However, the FN3 domain does not interact directly with the zinc ions or their coordinating residues.

[00240] Conserved PSMA residues W541, Y460, F488, P502 and P504 form an aromatic cluster across the combining site with FN3 domain residues W36, P68, Y70, W79, F81, and P82 (Figure 3A). Conserved R511 is in a central location of the combining site and H bonds Y70, a central residue of the FN3 domain four-stranded β -sheet. Figure 3B shows a cartoon of the paratope and epitope residues.

[00241] Human and cynomolgus PSMA are 97% identical, and, except for a S613N change, all residues interacting with H10 are conserved between the two species (Figure 4). The S613N change results in N613 glycosylation in cynoPSMA and the gain of van der Waals contacts between the carbohydrate and FN3 domain residues E66, I86, T88 (F and G β -strands) that will not be present in the human enzyme.

FN3 Domain Residues for Conjugation

[00242] Various H10 FN3 domain residues outside the combining site can be modified for conjugation of small molecules (toxic payloads) without disrupting PSMA binding or FN3 domain fold. Cysteines were already placed and conjugated to payloads at the C-terminus (after the His-tag) and at positions R11, E53, and K62 and all of these variants demonstrated similarly potent cytotoxicity. In addition, residues T22, D25, and A26 in the BC loop, terminal residue N6, and S52 in the DE loop are potentially good sites for mutagenesis followed by chemical conjugation (Figure 5). These solvent exposed residues are away from the FN3 domain /PSMA interface and located in structurally flexible regions.

[00243] Furthermore, both N- and C-terminal regions are free for fusions with other protein domains. The N-terminus is oriented towards the PSMA protease domain and reachable with a fusion linker, while the also accessible C-terminus goes towards the PSMA helical domain. The optimal linker length to the FN3 domain fusion partner will depend on the structure of the fusion partner and location of its binding site on the target molecule.

Mechanism of action

[00244] The H10 FN3 domain is a candidate for targeted delivery of payloads (toxic small molecules, nucleic acid, etc.) into prostate cancer cells due to internalization of the FN3

domain /PSMA complex. Furthermore, the H10 FN3 domain is a candidate for redirection of immune cells to prostate cancer cells when in a multispecific format.

[00245] H10 FN3 domain is likely to also inhibit the enzymatic activity of PSMA, which may contribute to decreased cell fitness and survival. The FN3 domain /cynoPSMA structure shows the FN3 domain bound to the entrance of the active site, which might prevent substrate interaction with PSMA through steric occlusion and direct competition for the binding site.

EXAMPLE 8: Generation of Additional anti-PSMA FN3 Domain Variants

[00246] Select anti-PSMA FN3 domain were further engineered to improve properties of the parental FN3 domain. Which, FN3 domains binding to PSMA were generated using libraries described above, and tested for their binding to PSMA.

[00247] Table 16 shows the amino acid sequences of the generated molecules.

[00248] Table 16.

Clone ID	SEQ ID NO:	Sequence
P258AR6P1071_D02_v 1	75	LPAPKNLVVSRVTEDSARLSWAADQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 2	76	LPAPKNLVVSRVTEDSARLSWAIAEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 3	77	LPAPKNLVVSRVTEDSARLSWAIDAQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 4	78	LPAPKNLVVSRVTEDSARLSWAIDEARDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 5	79	LPAPKNLVVSRVTEDSARLSWAIDEQADWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 6	80	LPAPKNLVVSRVTEDSARLSWAIDEQRAWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 7	81	LPAPKNLVVSRVTEDSARLSWAIDEQRDAFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 8	82	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAIFTT
P258AR6P1071_D02_v 9	83	LPAPKNLVVSRVTEDSARLSWAIDEQRDWFE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG

		LKPGTEYTVSIYGVYAVYRSNPLSAI FTT
P258AR6P1071_D02_v 10	84	LPAPKNLVVSRVTEDSARLSWAIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHAYRSNPLSAI FTT
P258AR6P1071_D02_v 11	85	LPAPKNLVVSRVTEDSARLSWAIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVARSNPLSAI FTT
P258AR6P1071_D02_v 12	86	LPAPKNLVVSRVTEDSARLSWAIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYASNPLSAI FTT
P258AR6P1071_D02_v 13	87	LPAPKNLVVSRVTEDSARLSWAIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_D02_v 14	88	LPAPKNLVVSRVTEDSARLSWDIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_D02_v 15	89	LPAPKNLVVSRVTEDSARLSWAIDEQRDWF SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_D02_v 16	90	LPAPKNLVVSRVTEDSARLSWAIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSSNPLSAI FTT
P258AR6P1071_D02_v 17	91	LPAPKNLVVSRVTEDSARLSWDIDEQRDWF SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSNPLSAI FTT
P258AR6P1071_D02_v 18	92	LPAPKNLVVSRVTEDSARLSWDIDEQRDWE SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSSNPLSAI FTT
P258AR6P1071_D02_v 19	93	LPAPKNLVVSRVTEDSARLSWAIDEQRDWF SFLIQYQESEKVGGEAIVLTVPGSCRSYDLTG LKPGTEYTVSIYGVYHVYRSSNPLSAI FTT
P233FR9_H10_v1	94	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYRVYIAGVKGGQWSFPLSAI FTT
P233FR9_H10_v2	95	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYKVYIAGVKGGQWSFPLSAI FTT
P233FR9_H10_v3	96	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYEVYIAGVKGGQWSFPLSAI FTT
P233FR9_H10_v4	97	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAI FTT
P233FR9_H10_v5	98	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYDVYIAGVKGGQWSFPLSAI FTT
P233FR9_H10_v6	99	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF AIGYWEWDDDGEAIVLTVPGSCRSYDLTGLK PGTEYAVYIAGVKGGQWSFPLSAI FTT

P233FR9_H10_v7	100	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYGVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v8	101	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYVVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v9	102	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYLVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v10	103	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYIVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v11	104	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYFVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v12	105	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYWVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v13	106	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYNVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v14	107	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYQVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v15	108	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYSVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v16	109	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYTVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v17	110	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYYVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v18	111	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AI A YWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v19	112	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AI S YWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v20	113	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDTDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT
P233FR9_H10_v21	114	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWSDSDEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQWSFPLSAIFTT

P233FR9_H10_v22	115	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY YEW DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ WS FPLSAIFTT
P233FR9_H10_v23	116	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY F EWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ WS FPLSAIFTT
P233FR9_H10_v24	117	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY L EWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ WS FPLSAIFTT
P233FR9_H10_v25	118	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY WEY DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ WS FPLSAIFTT
P233FR9_H10_v26	119	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY WEF DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ WS FPLSAIFTT
P233FR9_H10_v27	120	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY WEL DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ WS FPLSAIFTT
P233FR9_H10_v28	121	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY WEW DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ YS FPLSAIFTT
P233FR9_H10_v29	122	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY WEW DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ F SFPLSAIFTT
P233FR9_H10_v30	123	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGY WEW DDDDGEAIVLTVPGSCRSYDLTGLK PGTEYPVYIAGVKGGQ LS FPLSAIFTT
P233FR9P1001-H3- 1_v1	124	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE RIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQ WS FPLSAIFTT
P233FR9P1001-H3- 1_v2	125	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE KIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQ WS FPLSAIFTT
P233FR9P1001-H3- 1_v3	126	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE EIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQ WS FPLSAIFTT
P233FR9P1001-H3- 1_v4	127	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE HIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQ WS FPLSAIFTT
P233FR9P1001-H3- 1_v5	128	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE DIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQ WS FPLSAIFTT
P233FR9P1001-H3- 1_v6	129	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE AIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQ WS FPLSAIFTT
P233FR9P1001-H3-	130	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSE

1_v7		GIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v8	131	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF VIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v9	132	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF LIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v10	133	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF IIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v11	134	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF FIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v12	135	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF WIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v13	136	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF NIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v14	137	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF QIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v15	138	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF SIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v16	139	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF TIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT
P233FR9P1001-H3- 1_v17	140	LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF YIGYWEWDDDDGEAIVLTVPGSCRSYDLTGLK PGTEYHVYIAGVKGGQWSFPLSAIFTT

EXAMPLE 9: Detection of PSMA Expression on Tumor Cells Using anti PSMA FN3 domain Conjugated to Fluorescent Dye

[00249] This example shows the detection of PSMA present on cells with anti PSMA FN3 domain conjugated to a fluorescent dye. C-terminally His-tagged anti PSMA FN3 domain P233FR9_H10 (SEQ ID NO:49) with a free cysteine at amino acid 53 was conjugated to R-phycoerythrin (PE) (Prozyme catalog # PB31). The PE was activated using sulfo-SMCC (Pierce catalog # 22122) for 60 min, and activated PE was separated from free sulfo-SMCC by gel filtration chromatography using Sephadex G25 and PBS/EDTA buffer. The FN3 domain was reduced using TCEP (Sigma, cat. # 646547) for 30 min. The reduced FN3 domain was separated from free TCEP by gel filtration chromatography using Sephadex G25 and PBS/EDTA buffer.

The activated PE was covalently coupled to the reduced FN3 domain for 90 min followed by quenching with N-Ethylmaleimide (Sigma catalog# 04260) for 20 min. The “PE-conjugated FN3 domain” was purified by size-exclusion chromatography (SEC) using a Tosoh TSKgel G3000SW column in 100 mM sodium phosphate, 100 mM sodium sulfate, 0.05% sodium azide, pH 6.5 on an AKTA explorer FPLC (General Electric).

[00250] The PE-conjugated FN3 domain was tested for sensitivity and specificity using PSMA positive and negative cell lines by flow cytometry and CellSearch Circulating Tumor Cell (CTC) assay. The following prostate cell lines were purchased from ATCC and used to validate the specificity of the anti-PSMA FN3 domain: LNCaP (high PSMA expression), 22Rv1 (low PSMA expression) and PC3 (no PSMA expression).

Detection of PSMA on cell lines by Flow Cytometry

[00251] Prostate cell lines were harvested using standard cell culture procedures. The cells (~30,000) were stained in 0.1ml of PBS containing 1% bovine serum albumin (BSA) with PE-conjugated FN3 domain for 20 minutes. Anti PSMA antibody-PE conjugate from Biolegend (clone LNI-17 catalog # 342504) was used as a positive control. After the incubation, 3ml of PBS/BSA buffer was added and unbound PE conjugate was removed by centrifugation at 800g for 5 minutes. The supernatant was aspirated and the cells were resuspended in 0.3ml of PBS/BSA. The samples were analyzed by BD Biosciences FACSCalibur. The mean fluorescent intensity (MFI) of PSMA staining from each cell line was determined and compared to MFI with anti PSMA antibody. The MFI is directly related to PSMA expression level with higher MFI from high PSMA expressing cell line. Figure 6 shows the MFI values from different cell lines detected with anti PSMA PE-conjugated FN3 domain in comparison to MFI values with anti PSMA antibody-PE.

[00252] The results show that anti-PSMA PE-conjugated FN3 domain binds to PSMA positive cell lines and does not bind nonspecifically to PSMA negative cells. The MFI is higher with high PSMA expressing cell line (LNCaP) compared to low MFI with low PSMA expressing cell line (22Rv1) as expected. The MFI with PSMA negative cell line (PC3) is close to the background signal. In addition, the performance of FN3 domain-PE in binding to different cell lines is similar to anti-PSMA antibody-PE, as similar MFI values were obtained with both FN3 domain and antibody conjugates. This example shows that anti PSMA PE-conjugated FN3 domain shows sensitivity and specificity in the detection of PSMA on tumor cells.

Detection of PSMA by Circulating Tumor Cell Assay

[00253] The above results were further confirmed by testing anti-PSMA PE-conjugated FN3 domain in a CELLSEARCH assay to detect and enumerate circulating tumor cells (CTCs) from 7.5ml of blood. Circulating tumor cell enumeration using the CELLSEARCH system (Janssen Diagnostics, Raritan, NJ, USA) was carried out according to the manufacturer's protocol and training. The CellSearch assay uses anti-EpCAM conjugated to ferrofluid magnetic particles to capture and anti-cytokeratin specific to cytokeratins 8, 18 and 19 conjugated to fluorescein to visualize CTCs. The CELLSEARCH assay uses AutoPrep for sample preparation and CELLTRACKS Analyzer II® (CTA II) for the analysis. The CTA II is a four color semi-automated fluorescent microscope and uses 3 colors to identify and enumerate CTCs. The fourth color on CTA II is available to phenotype CTCs with additional markers of interest. In this example, tissue cultured tumor cells were spiked into normal blood to mimic CTCs in blood. Approximately 500 tumor cells (LNCaP, 22Rv1, PC3 or SKBR3 cells) were spiked into 7.5ml of normal donor blood collected in a CellSave tube (Janssen Diagnostics). The breast cancer cell line (SKBR3) was also used as PSMA negative cell line. The samples were processed on the AutoPrep using CELLSEARCH CXC kit and anti PSMA PE-conjugated FN3 domain as a marker. The AutoPrep sample preparation system enriches tumor cells by capturing tumor cells using anti EpCAM ferrofluid. The CTC enriched samples were stained with a nucleic acid dye (DAPI) to identify nucleated cells, anti-cytokeratin antibody conjugated to fluorescein isothiocyanate (FITC) to identify tumor cells, and anti-leukocyte antibody conjugated to allophycocyanin (APC) to identify leukocytes. The sample was processed to a final volume of 0.32ml and was transferred to a sample chamber while inside the MagNest® cell presentation device. The MagNest® device presents the magnetically labeled cells for analysis by the CELLTRACKS Analyzer II®. The samples were analyzed using CTAII to enumerate CTCs and detect PSMA on CTCs. The analyzer automatically analyzes samples and presents candidate tumor cells which are positive for DAPI and cytokeratin as thumbnail images for the review. The results from tumor cells stained with anti PSMA PE-conjugated FN3 domain in CELLSEARCH assay are shown in Figure 7.

[00254] Figure 7A shows the expression of PSMA on LNCaP tumor cells and 100% of these cells are positive for PSMA. Low PSMA expressing cell line (22Rv1) is 26% positive for PSMA (Figure 7B). On the other hand, PSMA negative cell lines (PC3-9 and SKBR3) are negative for PSMA (Figure 7C and 7D). These results are consistent with flow cytometry results.

This example shows that anti PSMA FN3 domain can be used to detect PSMA expression on CTCs and further confirms the sensitivity and specificity of anti PSMA FN3 domain.

EXAMPLE 10: Crystal Structure of an anti-CD3 Fab

[00255] The crystal structure of the SP34 Fab was determined at 2.1 Å resolution. It revealed the complete amino acid sequence and identified the possible mouse germlines from which the SP34 mAb was derived. The structure was used to guide human framework adaptation.

Materials

[00256] SP34 mAb, mouse IgG3/lambda isotype, was purchased from BD Biosciences Pharmingen (San Diego, CA), Cat. No. 556611. According to the technical data sheet, it was purified from tissue culture supernatant by affinity chromatography and stored at 4 °C. The Fab fragment was produced by papain digestion of mAb (Pierce, Cat # 44985, Thermofisher) and was separated from Fc using Nab Protein A Plus Spin column (Pierce, Cat # 44985, Thermofisher) according to manufacturer's protocol. The Fab was further purified on a MonoS column (GE Healthcare) equilibrated with 20 mM MES, pH 6.5 (buffer A). Elution was performed with buffer A in 13-28 % gradient of 1 M NaCl in 50 column volumes. Fractions corresponding to the main peak were pooled, concentrated to 9.2 mg/mL and used for crystallization.

Crystallization

[00257] Crystallization was carried out by the vapor diffusion method at 20 °C using an Oryx4 robot (Douglas Instruments) and a Mosquito robot (TTP Labtech). The experiments were composed of equal volumes of protein and reservoir solution in a sitting drop format in 96-well Corning 3550 plates. The initial screening was performed with the PEGs kit (Qiagen) and in-house screens IH1 and IH2. MMS optimization using the Fab seeds obtained after initial screening from IH2 screen produced a number of crystals under various conditions. The Fab crystal used for X-ray analysis was obtained from 12% PEG 3350, 0.2 M K/Na tartrate (pH 7.4), 3% isopropanol and 3% dioxane (no buffer). Crystal data are given in Table 17.

[00258] Table 17 Crystal Data, X-ray data and refinement statistics

Crystal data	
Space group	P21

Unit cell axes	55.14, 141.23, 61.29	
Unit cell angles (°)	90, 99.02, 90	
Molecules/asym.unit	2	
V _m (Å ³ /Da)	2.48	
Solvent content (%)	50	
X-ray data		
Resolution (Å)	30–2.1	(2.15–2.10)*
No. measured reflections	179,420	(11,506)
No.unique reflections	53,483	(3,667)
Completeness (%)	98.9	(92.5)
Redundancy	3.4	(3.1)
R-merge	0.038	(0.393)
<I/σ>	18.7	(3.8)
B-factor (Wilson) (Å ²)	45.4	
Refinement		
Resolution (Å)	15-2.1	
No. refls used in refinement	52,212	
No. all atoms	6,886	
No water molecules	219	
R-factor (%)	20.5	
R-free (%)	26.2	
RMSD bond lengths (Å)	0.008	
RMSD bond angles (°)	1.2	
RMSD B-factor main-chain (Å ²)	2.7	
Mean B-factor (Å ²)	53.7	

[00259] * Numbers in parentheses are for the highest resolution shell.

X-ray data collection and structure determination

[00260] For X-ray data collection, one crystal was soaked for a few seconds in the mother liquor supplemented with 20% glycerol and flash frozen in liquid nitrogen. Diffraction data were collected at the Advanced Photon Source (Argonne, IL) IMCA beamline using a Pilatus CCD detector. Diffraction intensities were detected over a 180° crystal rotation with 0.5

sec exposures per half-degree image and were processed with the program XDS [Kabsch W. 2010. XDS. Acta Crystallogr. D66:125-132.]. X-ray data statistics are given in Table 17.

[00261] The structure was solved by molecular replacement using a Fab model constructed from mouse anti-Thomsen-Friedenreich Antigen antibody Jaa-F11 (PDB 3gnm), which is a IgG3/kappa isotype. All crystallographic calculations were performed with the CCP4 suite of programs [1994, Acta Crystallogr. D50:760-763.]. Model adjustments were carried out using the program COOT [Emsley P, and Cowtan K. 2004. Acta Crystallogr. D60:2126-2132.]. The refinement statistics are given in Table 17.

[00262] Germline information guided sequence determination for SP34. X-ray data allowed identification of several somatic mutations, as well as the entire sequence of CDR-H3, which is not part of the germline. Ambiguities of assigning D/N, E/Q, T/V were resolved, where possible, on the basis of H-bond networks and atomic B-factors, which in some cases can differentiate between atoms C, N and O.

[00263] Somatic mutations were identified in positions 40, 97 and 98 of VL and in positions 35, 55, 56, 57 and 80 of VH.

[00264] Five disulfides were observed in the Fab structure as expected: 22-90 and 137-196 in the light chain, 22-98 and 152-207 in the heavy chain, and an interchain disulfide 214(L)-140(H).

Interaction Between Fabs in the Crystal

[00265] In the crystal, Fab molecules pack head-to-tail, so that the CDRs of one Fab bind the C-terminal portion of the heavy chain of the other Fab. The C-terminus fits the deep crevice between VL and VH in a dead-end mode. The terminal carboxyl group of S230 forms hydrogen bonds to N35 and R50 of VH and W98 of VL. This leaves no room for an extra residue and indicates that the papain cleavage of mAb occurred between S230 and T231 of the hinge region.

Putative paratope

[00266] The conformation of the CDRs in the present structure and the mode of the C-terminus recognition described above allowed selection of residues that are most likely involved in antigen binding. These are the following:

[00267] CDR-L1: Y34; CDR-L2: none; CDR-L3: W93;

[00268] CDR-H1: T31, Y32, A33; CDR-H2: R50, R52, Y55, N56; CDR-H3: N103, G105, S107, Y108, S110

[00269] The majority of interactions likely occur at VH with major contributions from CDR-H2 and CDR-H3.

[00270] The sequence of SP34 is shown in Figure 8 (SEQ ID NO:160 and 161), with residues 231 – 455 derived from IGHG3_MOUSE (mouse IgG3, isoform 2).

EXAMPLE 11: Human Framework Adaptation of anti-CD3 Antibody SP34

[00271] Anti-CD3 murine antibody SP34 was humanized by the Human Framework Adaptation method (Fransson, et al, JMB, 2010). Four different heavy chains were combined with three different light chains to produce 12 humanized variants.

SP34 Humanization and Affinity Maturation

Selection of Human Germlines

[00272] SP34 was humanized using the Human Framework Adaptation (HFA) method [16]. A matrix of four human heavy and three light v region sequences were selected for testing. Selection of human germlines was based solely on the overall sequence similarity to SP34 in the framework region (FR). Neither the CDR sequences, nor their length or canonical structures, were considered in this selection.

[00273] The closest matches for the heavy chain are human GLs IGHV3-72 and IGHV3-73. Another GL, IGHV3-23 was selected because of its high frequency of occurrence in the human B-cell repertoire.

[00274] The closest matches for the light chain are human lambda GLs IGLV7-43 (aka 7a), IGLV7-46 (aka 7b) and IGLV1-51 (aka 1b). IGLV7-46 is virtually identical to IGLV7-43, but has an advantage of Ala at position 2, i.e. as in SP34.

[00275] Selected J-regions are the following: IGHJ1 for the heavy chain; IGLJ3 for the lambda light chain.

Back mutations

[00276] Based on the crystal structure of SP34, a model of the HFA variants was built. The model revealed several FR positions in VL with potential clashes, most notably positions Val38, Gly48 and Gly51 (Figure 9). Mouse residues must be retained at all three positions (aka

‘back mutations’) in order to preserve the conformation of CDR-H3. These mutations were added into the maturation plan.

[00277] The Asn at position 57 of the heavy chain does not have good side chain density in the structure. It also sits in the middle of CDR-H2 and points away from the typical binding site. Based upon this analysis, it may not contribute to binding significantly. In addition, the backbone geometry sits in a region most favorable for a Gly residue in the Ramachadran plot. Thus it was truncated to Gly in the maturation plan to allow necessary flexibility and potentially improve stability (by reducing non-glycine related locat structural strain) while not impacting binding.

[00278] There were several other considerations made in the affinity maturation design. First, human GLs IGLV7-46 and IGLV7-43 introduce a Trp at position 59 with an unwanted oxidation potential. Two other GLs have Gly at this position, which corresponds to the mouse sequence. Therefore, Gly59 was preserved in both IGLV7-46 and IGLV7-43 variants. Finally, Ala at position 49 of VH is essential. Also, the residue at position 99 (Val in SP34) may impact antigen binding. To test these positions, back mutations were introduced in some variants (Figure 10).

HFA matrix

[00279] The HFA matrix is composed of four variants of VH and three variants of VL (Figure 10). For the purpose of HFA, AbM CDR definition is used (short CDR-H2, long CDR-L1).

The variants for VH:

[00280] CD3H141 (SEQ ID NO:163): IGHV3-72*01 with mouse CDRs+ Gly49Ala
EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNN
YATYYAASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSWFAY
WGQGTLVTVSS

[00281] CD3H142 (SEQ ID NO:164): IGHV3-23*01 with mouse CDRs+ Ser49Ala
EVQLLES GGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNN
YATYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKHGNFGNSYVSWFAY
WGQGTLVTVSS

[00282] CD3H143 (SEQ ID NO:165): IGHV3-23*01 with mouse CDRs+ Ser49Ala,
Ala99Val

EVQLLESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNN
YATYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCVKHGNFGNSYVSWFAY
WGQGTLVTVSS

[00283] CD3H144(SEQ ID NO:166): IGHV3-73*01 with mouse CDRs + Asn57Gly
EVQLVESGGGLVQPGGSLKLSCAASGFTFNTYAMNWVRQASGKGLEWVGRIRSKYNG
YATYYAASVKGRFTISRDDSKNTAYLQMNSLKTEDTAVYYCTR HGNFGNSYVSWFAY
WGQGTLVTVSS

The variants for VL:

[00284] CD3L63 (SEQ ID NO:167): IGLV7-46*01 with mouse CDRs +
F38V,A48G,Y51G,W59G
QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAP
GTPARFSGSLLGGKAALTL SGAQPEDEAEYYCALWYSNLWVFGGGGTKLTVL

[00285] CD3L64 (SEQ ID NO:168): IGLV1-51*01 with mouse CDRs + Y38V, L48G,
Y51G
QSVLTQPPSVSAAPGQKVTISCRSSTGAVTTSNYANWVQQLPGTAPKGLIGGTNKRAPGI
PDRFSGSKSGTSATLGITGLQTGDEADYYCALWYSNLWVFGGGGTKLTVL

[00286] CD3L66 (SEQ ID NO:169): IGLV7-43*01 with mouse CDRs +
F38V,A48G,Y51G,W59G
QTVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAP
GTPARFSGSLLGGKAALTL SGVQPEDEAEYYCALWYSNLWVFGGGGTKLTVL

[00287] Table 18 Matrix of CD3 Heavy and Light chains
(All were prepared with IgG1-AA Fc containing L234A, L235A, and F405L)

[00288] Table 18.

	CD3L63 (LV7-46/W59G) SEQ ID NO:167	CD3L64 (LV1-51) SEQ ID NO:168	CD3L66 (LV7-43/W59G) SEQ ID NO: 169
CD3H141 (HV3-72 + G49A) SEQ ID NO: 163	CD3B143	CD3B144	CD3B146
CD3H142 (HV3-23 + S49A) SEQ ID NO:164	CD3B147	CD3B148	CD3B150
CD3H143 (HV3-23 +S49A,A99V) SEQ ID NO: 165	CD3B151	CD3B152	CD3B154

CD3H144 (VH3-73 with G49) SEQ ID NO:166	CD3B155	CD3B156	CD3B158
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[00289] Amino acid sequences were back-translated to DNA and cDNA was prepared using gene synthesis techniques (U.S. Pat. No. 6,670,127; U.S. Pat. No. 6,521,427). Heavy chain (HC) v regions were subcloned onto human IgG1-AA Fc containing L234A, L235A, and F405L mutations using an in-house expression vector with the CMV promoter using standard molecular biology techniques. Light chain (LC) variable regions were subcloned onto a human Lambda (λ) constant regions using an in-house expression vector with the CMV promoter using standard molecular biology techniques. Resulting plasmids were transfected into Expi293F cells (Invitrogen) and mAbs were expressed. Purification was by standard methods using a Protein A column (hiTrap MAbSelect SuRe column). After elution, the pools were dialyzed into D-PBS, pH 7.2.

[00290] Table 19. The VH and VL sequences of the antibodies are shown below:

mAb	HC	VH Amino Acid sequence	SEQ ID NO:	LC	VL Amino Acid sequence	SEQ ID NO:
CD3B1 43	CD3H 141	EVQLVESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYAASVKGRFTISR DSKNSLYLQMN SLKT EDTAVYYCARHGN FEG NSYVSWFAYWGQGT LTVTSS	163	CD3L 63	QAVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167
CD3B1 44	CD3H 141	EVQLVESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYAASVKGRFTISR DSKNSLYLQMN SLKT EDTAVYYCARHGN FEG NSYVSWFAYWGQGT LTVTSS	163	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 46	CD3H 141	EVQLVESGGGLVQPG GSLRLSCAASGFTFN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT	163	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR	169

		YYAASVKGRFTISR DSKNSLYLQMN SLKT EDTAVYYCARHGN FNG NSYVSWFAYWGQGT L VTVSS			APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	
CD3B1 47	CD3H 142	EVQLLES GGGLVQPG GSLRLS CAASGFTFN TYAMNW VRQAPGKGL EWVARIR SKYNNYAT YYADSVKGRFTISR D NSKNTLYLQMN SLRA EDTAVYYCAKHGN FNG NSYVSWFAYWGQGT L VTVSS	164	CD3L 63	QAVVTQE PSLTVSP GGTVTLT CRSSTGA VTTSNYANWVQQK P GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167
CD3B1 48	CD3H 142	EVQLLES GGGLVQPG GSLRLS CAASGFTFN TYAMNW VRQAPGKGL EWVARIR SKYNNYAT YYADSVKGRFTISR D NSKNTLYLQMN SLRA EDTAVYYCAKHGN FNG NSYVSWFAYWGQGT L VTVSS	164	CD3L 64	QSVLTQP PPSVSAA P GQKVTIS CRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 50	CD3H 142	EVQLLES GGGLVQPG GSLRLS CAASGFTFN TYAMNW VRQAPGKGL EWVARIR SKYNNYAT YYADSVKGRFTISR D NSKNTLYLQMN SLRA EDTAVYYCAKHGN FNG NSYVSWFAYWGQGT L VTVSS	164	CD3L 66	QTVVTQE PSLTVSP GGTVTLT CRSSTGA VTTSNYANWVQQK P GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	169
CD3B1 51	CD3H 143	EVQLLES GGGLVQPG GSLRLS CAASGFTFN TYAMNW VRQAPGKGL EWVARIR SKYNNYAT YYADSVKGRFTISR D NSKNTLYLQMN SLRA EDTAVYYCVKHGN FNG NSYVSWFAYWGQGT L VTVSS	165	CD3L 63	QAVVTQE PSLTVSP GGTVTLT CRSSTGA VTTSNYANWVQQK P GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167

CD3B1 52	CD3H 143	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCVKHGNGF NSYVSWFAYWGQGT LTVSS	165	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 54	CD3H 143	EVQLLES GGGGLVQPG GSLRLSCAASGFTN TYAMNWVRQAPGKGL EWVARIRSKYNNYAT YYADSVKGRFTISR NSKNTLYLQMNSLRA EDTAVYYCVKHGNGF NSYVSWFAYWGQGT LTVSS	165	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	169
CD3B1 55	CD3H 144	EVQLVES GGGGLVQPG GSLKLSCAASGFTN TYAMNWVRQASGKGL EWVGRIRSKYNGYAT YYAASVKGRFTISR DSKNTAYLQMNSLKT EDTAVYYCTRHGNGF NSYVSWFAYWGQGT LTVSS	166	CD3L 63	QAVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGAQPED EAEYYCALWYSNLW VFGGGTKLTVL	167
CD3B1 56	CD3H 144	EVQLVES GGGGLVQPG GSLKLSCAASGFTN TYAMNWVRQASGKGL EWVGRIRSKYNGYAT YYAASVKGRFTISR DSKNTAYLQMNSLKT EDTAVYYCTRHGNGF NSYVSWFAYWGQGT LTVSS	166	CD3L 64	QSVLTQPPSVSAAP GQKVTISCRSSTGA VTTSNYANWVQQLP GTAPKGLIGGTNKR APGIPDRFSGSKSG TSATLGITGLQTGD EADYYCALWYSNLW VFGGGTKLTVL	168
CD3B1 58	CD3H 144	EVQLVES GGGGLVQPG GSLKLSCAASGFTN TYAMNWVRQASGKGL EWVGRIRSKYNGYAT YYAASVKGRFTISR DSKNTAYLQMNSLKT EDTAVYYCTRHGNGF NSYVSWFAYWGQGT LTVSS	166	CD3L 66	QTVVTQEPSLTVSP GGTVTLTCRSSTGA VTTSNYANWVQQKP GQAPRGLIGGTNKR APGTPARFSGSLLG GKAALTLSGVQPED EAEYYCALWYSNLW VFGGGTKLTVL	169

[00291] A monospecific anti-CD3 antibody CDB143 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 163 and the VL of SEQ ID NO: 167 and an IgG1 constant region with L234A, L235A, F405L substitution. A monospecific anti-CD3 antibody CDB144 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 163 and the VL of SEQ ID NO: 168 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB146 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 163 and the VL of SEQ ID NO: 169) and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB147 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 164 and the VL of SEQ ID NO: 167) and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB148 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 164 and the VL of SEQ ID NO: 168 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB150 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 164 and the VL of SEQ ID NO: 169 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB151 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 165 and the VL of SEQ ID NO: 167 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB152 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 165 and the VL of SEQ ID NO: 168 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB154 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 165 and the VL of SEQ ID NO: 169 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB155 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 166 and the VL of SEQ ID NO: 167 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB156 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 166 and the VL of SEQ ID NO: 168 and an IgG1 constant region with L234A, L235A, and F405L substitutions. A monospecific anti-CD3 antibody CDB158 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 166 and the VL of SEQ ID NO: 169 and an IgG1 constant region with L234A, L235A, and F405L substitutions.

EXAMPLE 12: Endogenous Cell Binding of the Humanized anti-CD3 Hits to Primary T Cells

[00292] The resulting panel of anti-CD3 antibodies was tested for binding against cell-surface CD3 ϵ on primary human T cells. To do this, binding of antibodies from expression supernatants was visualized using a polyclonal anti-human secondary antibody and analyzed by flow cytometry. Briefly, binding of anti-CD3 antibodies to cell-surface CD3 ϵ was assessed by flow cytometry using primary Human T lymphocytes purified by negative selection (Biological Specialty, Colmar, USA). Expression supernatants or purified antibodies were normalized to 10 μ g/ml in media or FACS buffer (BD BioSciences), respectively. 2x10⁵ cells were aliquoted into wells of a 96 well round-bottomed plate (CoStar) for labeling. Antibodies in expression supernatant were added to cells and incubated for 45 min at 4 °C. Following centrifugation at 1300rpm for 3 min and removal of supernatant, 50 μ L of anti-human IgG (H+L) Alexa Fluor 647 secondary antibody (Life technologies Inc.) was incubated with the cells at a final concentration of 10 μ g/mL for 30 min at 4 °C away from direct light. Following washing and resuspension in 30 μ L FACS buffer (BD BioSciences). Sample collection was performed on an Intellicyt HTFC system using ForeCyt software. Viable single cells were gated prior to analysis of binding using the green or red fixable live/dead dyes (Life Technologies Inc.) and forward/side scatter area and height parameters, respectively. Graphs were generated in GraphPad Prism version 5 using mean fluorescence intensity values.

[00293] Although a titration series was run, an intermediate concentration is presented in Figure 13 for clarity. Two in-house phage-derived antibodies with the same Fc region as the therapeutic antibodies were used as controls: G11 (HC SEQ ID NO:176, LC SEQ ID NO:177), a non-cyno cross-reactive, agonistic antibody was used as a positive control and CD3B94 (HC-SEQ ID NO:178, LC – SEQ ID NO:179) a non-binder / non-agonistic antibody was used to assess non-specific binding. The commercial SP34 antibody was not used as a comparator in this assay since it is a mouse antibody and the use of a different secondary detection reagent would have prohibited direct comparison with the variants tested.

[00294] The data demonstrates an array of binding potential within the panel of humanized anti-CD3 hits, with two antibodies (CD3B144, CD3B152) showing complete loss of binding to human T cells. The remaining antibodies showed a range of binding potential that could be broadly split into strong and weak binders using G11 binding as an arbitrary threshold. Using these parameters, seven strong binders and seven weak binders were identified from the panel of variants (Figure 13).

[00295] Binding analysis of the anti-CD3 hits to primary cynomolgus CD4⁺ T cells was then tested in order to assess the retention of cross-reactivity. Purified CD4⁺ T cells from the peripheral blood of cynomolgus monkeys (Zen Bio, Triangle Research Park, USA) were used. Assay protocols were similar to those described above. Since G11 does not cross-react with cynomolgus CD3 ϵ , CD3B124, an in-house chimeric SP34-derived antibody having the VH and VL of SP34 with murine framework and a human IgG1 Fc was used as a positive control in this assay (Figure 14). Interestingly, several variants showed decreased binding potential compared to that seen with human cells. This included the strong binders CD3B150, CD3B151 and CD3B154, in which binding was reduced, and several weak binders where binding could no longer be detected over background. This loss of binding was not related to a specific immunoglobulin chain, suggesting that the combination of heavy and light chains played a role in the loss of cross-reactivity. Together, these assays allowed the identification of variants that retained species cross-reactivity between human and cynomolgus CD3 ϵ .

EXAMPLE 13: Functional Analysis of the Humanized anti-CD3 Hits in Primary T Cells

[00296] Binding analysis demonstrated that the panel of humanized anti-CD3 hits showed a range of binding potential to human and cynomolgus T-cells. To investigate the capacity of each variant to induce activation in via CD3 ϵ crosslinking, primary T-cells were cultured overnight in the presence of bead-conjugated antibody. The following day, cells were harvested and labeled with an anti-CD69 antibody to measure activation (Figure 13). Humanized anti-CD3 antibodies were bound to protein A coated magnetic beads (SpheroTech, Lake forest, USA) by overnight incubation with antibody at 10 μ g/mL. The following day, 2×10^5 primary human T cells were plated in round-bottomed cell culture plates in triplicate and 2×10^5 coated beads were added. Following overnight culture at 37 °C, cells were harvested and labeled with anti-CD69 Alexa Fluor 488 antibody (clone FN50; Biolegend) to assess the up-regulation of this activation marker. Sample collection and analysis were performed as described above for binding. Several negative controls were run, including T-cells alone, T-cells with non-coated beads, and T-cells with isotype control (CD3B94)-coated beads. All of these showed similar mean fluorescence intensity values comparable to unstained T-cells indicating that background was low in this assay. Several positive controls were run for comparison, including OKT3 (US5929212) and commercially available SP34-2 antibody.

[00297] The humanized anti-CD3 hits were then tested for their capacity to activate primary cynomolgus CD4⁺ T cells (Zen Bio, Triangle Research Park, USA) in the same assay

(Figure 14). The FN50 anti-CD69 antibody has been described as being cross-reactive with non-human protein and could therefore be used to test activation of these cells.

[00298] The human and cynomolgus activation data correlated with the binding data in that the panel of hits displayed a range of activation potentials. A number of the strong binders showed the capacity to activate human T-cells to an equivalent or greater extent when compared to commercially available SP34-2. Several variants showed activation potential that was lower compared SP34-2, whereas some binders did not show evidence of CD69 stimulation. The inability to activate was only seen in the variants that showed no or weak binding and all strong binders showed some level of activation, suggesting a correlation between binding and activation potentials for both human (Figure 15A) and cynomolgus (Figure 15B).

EXAMPLE 15. Preparation of the Multispecific Antigen-binding Molecule in a Bispecific Format in IgG4 S228P, L234A, L235A

[00299] Bispecific PSMA x CD3 multispecific antigen-binding molecules were generated by combining a CD3 mAb arm with and without FN3 domain fusion and a monospecific PSMA Fn3 domain FC fusion in *in-vitro* Fab arm exchange (as described in WO2011/131746). In order to produce a bispecific isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA, the anti-PSMA FN3 domain (P233FR9-H10, SEQ ID 41) was produced as a fusion protein with an anti-CD3 mAb in multiple orientations to assess the effects of such orientations on the ability of these molecules to activate and target cytotoxic T-cells to PSMA expressing cell lines.

Expression of CD3 mAb B219

[00300] One of the monospecific CD3 antibodies, CDB146, was expressed as IgG4, having Fc substitutions S228P, F234A, L235A F405L and R409K (numbering according to EU index) in their Fc regions. The monospecific antibody was produced by transient transfection in HEK293 cells.

[00301] A monospecific anti-CD3 antibody B219 was generated comprising the VH and VL regions having the VH of SEQ ID NO: 163 and the VL of SEQ ID NO: 169 and an IgG4 constant region with S228P, L234A, L235A, F405L and R409K substitutions. The monospecific anti-CD3 antibody B219 comprises the light chain amino acid sequence of SEQ ID NO:170 and the heavy chain amino acid sequence of SEQ ID NO:171.

Design of PSMA FN3 Domain Fusions

[00302] Constructs were designed to produce either monovalent CD3 interactions: B219 (SEQ ID NOs: 170,171) X CW5 (SEQ ID NO:172), B219(SEQ ID NOs: 170,171) X CW6(SEQ ID NO:173), B221(SEQ ID NOs:170,174) X CW5 (SEQ ID NO:172) or bivalent CD3 interactions: B221(SEQ ID NOs:170,174). Likewise molecules capable of monovalent PSMA interactions: B219(SEQ ID NOs: 170,171) X CW6(SEQ ID NO:173, B219(SEQ ID NOs: 170,171)X CW5(SEQ ID NO:172), or bivalent PSMA interactions: (B221(SEQ ID NOs:170,174), B221(SEQ ID NOs:170,175) XCW5(SEQ ID NO:172) were designed. A final variation was the location of the FN3 domain relative to the heavy chain, with both N-terminal and C-terminal fusions produced. A (GGGGS)₂ linker (SEQ ID NO:175) was incorporated between P233FR9-H10 and the heavy chain constant region in all molecules. A cartoon diagram of the designed isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA is shown in Figure 16.

[00303] Table 20. Location and valence of FN3 domain fusions

Molecule	Fusion form to Fc or heavy chain	PSMA binding	CD3 binding	Heavy chain1 SEQ ID	FN3 domain FC fusion SEQ ID	Light Chain SEQ ID
B219XCW5	C-terminal	monovalent	monovalent	171	172	170
B219XCW6	N-terminal	monovalent	monovalent	171	173	170
B221XCW5	C-terminal	bivalent	monovalent	174	172	170
B221	C-terminal	bivalent	bivalent	174	174	170

[00304] Amino acid sequences were back-translated to DNA and cDNA was prepared using gene synthesis techniques (U.S. Pat. No. 6,670,127; U.S. Pat. No. 6,521,427) and proteins were expressed using an in-house expression vector with the CMV promoter using standard molecular biology techniques. Heavy chain (HC) v regions were subcloned onto human IgG4-AA Fc containing L234A, L235A, and F405L mutations using an in-house expression vector with the CMV promoter using standard molecular biology techniques. Light chain (LC) variable regions were subcloned onto a human Lambda (λ) constant regions using an in-house expression vector with the CMV promoter using standard molecular biology techniques. Resulting plasmids were transfected into Expi293F cells (Invitrogen) and mAbs were expressed. Purification was by standard methods using a Protein A column (hiTrap MAbSelect SuRe column). After elution, the pools were dialyzed into D-PBS, pH 7.2.

[00305] An anti-PSMA FN3 domain fusion CW5 was generated comprising the anti-PSMA FN3 domain P233FR9-H10 (SEQ ID NO:41) connected with a linker (SEQ ID NO: 175) to the C-terminus of an IgG4 constant region with S228P, L234A and L235A substitutions, the amino acid sequence of the fusion comprising SEQ ID NO: 172. An anti-PSMA FN3 domain fusion CW6 was generated comprising the anti-PSMA FN3 domain P233FR9-H10 (SEQ ID NO:41) connected with a linker (SEQ ID NO: 175) to the N-terminus of an IgG4 constant region with S228P, L234A and L235A substitutions, the amino acid sequence of the fusion comprising SEQ ID NO: 173. An isolated bispecific, anti-PSMA X CD3 antigen-binding molecule that binds the antigens CD3 and PSMA, B221, was generated comprising the anti-CD3 mAb B219 comprising the VH and VL regions having the VH of SEQ ID NO: 163 and the VL of SEQ ID NO: 169 and an IgG4 constant region with S228P, L234A, L235A, F405L and K409R substitutions fused at the C-terminus of the heavy chain by a linker (SEQ ID NO:175) to the anti-PSMA isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA P233FR9-H10 (SEQ ID NO:41), the amino acid sequence of the anti-PSMA X CD3 isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA heavy chain comprising SEQ ID NO: 174 and the light chain comprising SEQ ID NO:170.

[00306] Isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA proteins B219XCW5, B219XCW6 and B221XCW5 were prepared by partial reduction and controlled Fab arm exchange using combinations of parent molecules as described in Table 17. Briefly, the parental molecules were mixed at about 1 – 20 mg/ml at a molar ratio of 1.08:1 B219/B221 to W5/W6, and 2-MEA (stock solution of 750 mM in PBS) was added to a final concentration of 75 mM. Reactions were incubated at 5 hr at 31 °C, followed by dialysis into PBS. The protein was then collected, sterile filtered and purity and concentration measured by absorbance at 280 nm, cation exchange HPLC, size exclusion HPLC and SDS-PAGE.

[00307] A bispecific, anti-PSMA X CD3 isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA B219XCW5 was generated comprising the CW5 anti-PSMA FN3 domain fusion arm comprising the amino acids sequence of SEQ ID NO: 172 paired with the B219 anti-CD3 heavy and light chains comprising the VL and VH amino acid sequences of SEQ ID NOs: 163 and 169. A bispecific, anti-PSMA X CD3 isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA B219XCW6 was generated comprising the CW6 anti-PSMA FN3 domain fusion arm comprising the amino acids sequence of SEQ ID NO: 173 paired with the B219 anti-CD3 heavy and light chains comprising the amino acid sequences of SEQ ID NOs: 171 and 170. A bispecific, anti-PSMA X CD3

isolated multispecific antigen-binding molecule B221XCW5 was generated comprising the CW5 anti-PSMA FN3 domain fusion arm comprising the amino acid sequence of SEQ ID NO: 172 paired with the B221 anti-CD3 X anti-PSMA FN3 domain heavy chain fusion comprising the amino acid sequence of SEQ ID NO:174 and a light chain comprising the amino acid sequences of SEQ ID NO: 170

[00308] Table 21.

Molecule		Amino Acid Sequence
B219XCW5	Heavy chain 1 B219 HC (SEQ ID NO:171)	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQ APGKGLEWVARIRSKYNNYATYYAASVKGRFTISRDDSK NSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSWFAYWGQ GTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPC PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREP QVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTTPVLDSDGSFLLYSKLTVDKSRWQEGNVFS CSVMHEALHNHYTQKSLSLSLGK
	Light Chain 1 B219 LC (SEQ ID NO:170)	QTVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQ QKPGQAPRGLIGGTNKRAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCALWYSNLWVFGGGTKLTVLGQPKAAPS VTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSS PVKAGVETTTTPSKQSNNKYAASSYLSLTPEQWKSHRYS CQVTHEGSTVEKTVAPTECS
	FN3 domain C- terminal FC fusion CW5 (SEQ ID NO:172)	GSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYR VSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQ EGNVFSCSVMHEALHNHYTQKSLSLSLGKGGGGSGGGGS MLPAPKNLVVSRVTEDSARLSWTAPDAAFD SFAIGYWEW DDDGEAIVLTVPGSERSYDLTGLKPGTEYPVYIAGVKGG QWSFPLSAIFTT
B219XCW6	Heavy chain 1 B219 HC (SEQ ID	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQ APGKGLEWVARIRSKYNNYATYYAASVKGRFTISRDDSK NSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSWFAYWGQ GTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPC

	NO:171)	PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREP QVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQEGNVFS CSVMHEALHNHYTQKSLSLGLGK
	Light Chain 1 B219 LC (SEQ ID NO:170)	QTVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQ QKPGQAPRGLIGGTNKRAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCALWYSNLWVFGGGTKLTVLGQPKAAPS VTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSS PVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRYS CQVTHEGSTVEKTVAPTECS
	FN3 domain N- terminal FC fusion CW6 (SEQ ID NO:173)	MLPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEW DDDGEAIVLTVPGSERSYDLTGLKPGTEYPVYIAGVKGG QWSFPLSAIFTTGGGGSGGGGSCPPCPAPEAAGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDG VEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVL DSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYT QKSLSLGLGK
B221XCW5	Centryn C- terminal Heavy chain fusion B221 (SEQ ID NO:174)	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQ APGKGLEWVARIRSKYNNYATYYAASVKGRFTISRDDSK NSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSWFAYWGQ GTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPC PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREP QVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQEGNVFS CSVMHEALHNHYTQKSLSLGLGKGGGGSGGGGSMLPAPK NLVVSRTVEDSARLSWTAPDAAFDSFAIGYWEWDDDGEA IVLTVPGSERSYDLTGLKPGTEYPVYIAGVKGGQWSFPL SAIFTT
	Light Chain 1 B219 LC	QTVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQ QKPGQAPRGLIGGTNKRAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCALWYSNLWVFGGGTKLTVLGQPKAAPS

	(SEQ ID NO:170)	VTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSS PVKAGVETTTTPSKQSNNKYAASSYLSLTPEQWKSHRSYS CQVTHEGSTVEKTVAPTECS
	FN3 domain N-terminal FC fusion CW6 (SEQ ID NO:173)	MLPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEW DDDGEAIVLTVPGSERSYDLTGLKPGTEYPVYIAGVKGG QWSFPLSAIFTTGGGGSGGGGSCPPCPAPEAAGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDG VEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKGLPSSIEKTIISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVL DSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYT QKSLSLSLGK

EXAMPLE 16: Evaluation of Bispecific Isolated Multispecific Antigen-binding Molecules that Bind the Antigens CD3 and PSMA in Functional Cell Killing Assay

[00309] A 24 hour standard chromium release assay was used to determine isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA function in killing PSMA positive LNCAP tumor targets (ATCC CRL-1740). Frozen normal donor pan-T cells (All Cells Cat# PB900-1F) were pre-activated with 1µg/ml of OKT3 (US5929212) and 20U/ml of IL2 (Peprotech Cat#200-02) for 12-24 hours. T cells were then washed and co-cultured with LNCAP tumor target cells in RPMI labeled with chromium (Perkin-Elmer, Cat#NEZ030S001MC) at a 5:1 effector to target ratio. Isolated multispecific antigen-binding molecules that bind the antigens CD3 and PSMA were added so that the final concentration in the well at the highest dose was 10µg/ml and followed a 7-point titration curve using 1:20 dilutions and incubated for 18-24 hours. The culture supernatant was harvested and read on a gamma-counter. As a control for spontaneous release of chromium from target cells, targets were cultured with media only and supernatant was collected. Maximum release was determined by the addition of Triton-X-100 to lyse all targets. The counts per minute were collected using a gamma counter and used in the following formula to determine % cytotoxicity. All samples were run in triplicate.

[00310] The calculation for % Cytotoxicity = (experimental counts-spontaneous release) (maximum release-spontaneous release) x 100%.

[00311] % cytotoxicity is calculated for each triplicate and plotted at a mean % cytotoxicity +SEM of the triplicates.

[00312] Figure 17 shows a representative graph of two independent experiments. The data demonstrates that all CD3B219xPSMA isolated CD3 x PSMA-bispecific antigen-binding molecule configurations resulted in tumor cell killing with B219XCW6 being the most potent followed by B221 and B219xCW5. Table 22 below indicates the EC50 for each isolated CD3 x PSMA-bispecific antigen-binding molecule, as well as the 95% confidence range for these EC50s and R squared values.

[00313] Table 22.

	EC50	EC50 95% Confidence Range	R2
B221	3.713E-13	7.105e-015 to 1.990e-011	0.916
B219xCW5	9.877E-13	3.393e-013 to 2.876e-012	0.9926
B219xCW6	1.066E-14	0.0 to 1.289e-009	0.9475
B221xCW5	2.526E-12	7.070e-013 to 9.031e-012	0.9908

[00314] Example 17: Anti-tumor efficacy of Mabtyrin, B219xCW6, in tumorigenesis prevention of HEK293-PSMA xenografts in PBMC-humanized NSG mice

[00315] [00312] Efficacy of B219xCW6 was evaluated by prevention of tumorigenesis (prophylactic model) of HEK293-PSMA human xenografts using inoculated human donor peripheral blood mononuclear cells (PBMC) in male NSG mice (NOD.Cg-Prkdcscid IL2rgtmlWjl/SzJ, Jackson Laboratories, Bar Harbor, ME). Mice were injected intravenously (iv) in the lateral tail vein with 1×10^7 human PBMCs 7 days (day -7) prior to tumor cell implantation. Mice were implanted subcutaneously (sc) on day 0 with 1×10^7 HEK293-PSMA cells in the right hind flank. Beginning on the day of tumor implantation PBS (phosphate buffered saline) control and B219xCW6 were administered iv at 0.004 mg/kg, 0.04 mg/kg, 0.4 mg/kg (equivalent to 0.1 μ g, 1 μ g and 10 μ g per 25 gram mouse, respectively), q2d-q3d for a total of 5 doses on days 0, 3, 5, 7 and 10.

[00316] [00313] Tumor volume was calculated using the formula: Tumor Volume (mm³) = $(a \times b^2/2)$; where 'a' represents the length, and 'b' the width of the tumor as determined by caliper measurements], and monitored twice weekly throughout the study. Percent tumor growth inhibition (TGI) was defined as the difference between mean tumor volumes of the treated and control (PBS) groups, calculated as $TGI = [((TV_c - TV_t)/TV_c) \times 100]$ where TV_c is the mean tumor volume of a given control group and TV_t is the mean tumor volume of the treated

group. As defined by NCI criteria, $\geq 60\%$ TGI is considered biologically significant (Johnson JL, et al (2001) Br J Cancer 84(10):1424-31). Animals were removed from studies when a maximum tumor volume of 1500 mm³ was reached.

[00317] [00314] Engraftment of human PBMC eventually leads to graft versus host disease (GVHD) in the mice, where the engrafted donor T cells become activated and infiltrate the host tissues, leading to body weight loss, organ failure, and inevitably, death. To monitor the onset and severity of GVHD, body weight was recorded twice weekly and expressed in grams (g). Percent body weight change was calculated using the formula: Body weight change = $[(B_t - B_0)/B_0] \times 100$ where B_t is the body weight on a given day of study and B_0 is the body weight at the initiation of treatment. Animals with sustained body weight loss greater than 20% of the initial body weight were considered moribund and removed from the study.

[00318] [00315] Statistical significance was evaluated using a 1-way ANOVA with multiple comparisons using Dunnett's multiple comparisons test using Graph Pad Prism software (version 6). Additional statistical analyses for indicated studies can be found in the laboratory notebook.

[00319] [00316] All in vivo studies were performed in accordance with The Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Janssen R & D, Spring House, PA.

[00320] [00317] B219xCW6 Mabtyrin treatment effectively delayed tumorigenesis and inhibited tumor growth of implanted HEK293-PSMA cells (Figure 18). Small but palpable HEK293-PSMA tumors were detectable in seven of eight mice in the PBS treated group on study day 16 (6 days post last therapeutic treatment), whereas there were no tumors in all eight mice of the 0.4 mg/kg CD3B250 treated group. Two out of eight mice had palpable tumors in the 0.04 mg/kg B219xCW6 treatment group and one out of eight mice had a small tumor in the 0.004 mg/kg B219xCW6 group. Tumor growth inhibition was assessed 32 days following cessation of treatment (day 42 post-tumor implantation), when there were still 7 or 8 animals per group. Tumor growth in the high dosed B219xCW6 (0.4 mg/kg, n=8) treated group was inhibited by 85% compared to PBS-treated controls ($p < 0.001$). The mid dose of B219xCW6 (0.04 mg/kg) inhibited tumor growth in a statistically significant fashion (TGI=67%, n=7) vs. PBS control, ($p < 0.0001$). Lastly, the lowest dose, 0.004 mg/kg, of B219xCW6 was also efficacious inhibiting HEK293-PSMA tumor growth by 78% ($p < 0.0001$) compared to control PBS treated tumors.

[00321] Animal groups receiving PBMCs eventually succumb to progressive GVHD, however body weight loss was slight in the current study (Figure 19). Only one mouse in the 1

ug B219xCW6 group had significant body weight and succumbed to GVHD on day 30. By day 42, the majority of the tumors in the PBS treated group had exceeded the 1500 mm³ tumor volume endpoint, at which time the study was terminated.

Sequences

SEQ ID No. 1= Original Tencon Sequence

LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSFLIQYQESEKVGGEAINLTVPGSERSY
DLTGLKPGTEYTVSIYGVKGGHRSNPLSAEFTT

SEQ ID No. 2= TCL1 library

LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSFLIQYQESEKVGGEAINLTVPGSERSY
DLTGLKPGTEYTVSIYGV(X)₇₋₁₂PLSAEFTT; wherein X₁, X₂, X₃, X₄, X₅, X₆, X₇ is
any amino acid; and X₈, X₉, X₁₀, X₁₁ and X₁₂ are any amino acid or deleted

SEQ ID No. 3=TCL2 library

LPAPKNLVVSEVTEDSLRLSWX₁X₂X₃X₄X₅X₆X₇X₈SFLIQYQESEKVGGEAINLTVPG
SERSYDLTGLKPGTEYTVSIYGVX₉X₁₀X₁₁X₁₂X₁₃SX₁₄X₁₅LSAEFTT; wherein X₁ is
Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;
X₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or
Val; X₃ Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp,
Tyr or Val; X₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser,
Thr, Trp, Tyr or Val; X₅ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe,
Pro, Ser, Thr, Trp, Tyr or Val; X₆ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys,
Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₇ is Phe, Ile, Leu, Val or Tyr; X₈ is Asp, Glu or Thr;
X₉ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or
Val; X₁₀ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp,
Tyr or Val; X₁₁ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser,
Thr, Trp, Tyr or Val; X₁₂ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe,
Pro, Ser, Thr, Trp, Tyr or Val; X₁₃ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu,
Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; X₁₄ is Ala, Arg, Asn, Asp, Glu, Gln, Gly, His,
Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val; and X₁₅ is Ala, Arg, Asn, Asp, Glu,
Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val.

SEQ ID No. 4= Stabilized Tencon

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFLIQYQESEKVGGEAIVLTVPGSERS
YDLTGLKPGTEYTVSIYGVKGGHRSNPLSAIFTT

SEQ ID No. 5= TCL7 (FG and BC loops)

LPAPKNLVVSRVTEDSARLSWX₁X₂X₃X₄X₅X₆X₇X₈X₉FDSFLIQYQESEKVGGEAIVL
TVPGSERSYDLTGLKPGTEYTVSIYGVX₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈X₁₉SNPLSAIF
TT; wherein X₁, X₂, X₃, X₄, X₅, X₆, X₁₀, X₁₁, X₁₂, X₁₃, X₁₄, X₁₅ and X₁₆ are A, D, E, F, G,
H, I, K, L, N, P, Q, R, S, T, V, W or Y; and X₇, X₈, X₉, X₁₇, X₁₈ and X₁₉, are A, D, E, F,
G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted.

SEQ ID No. 6= TCL9 (FG loop)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFLIQYQESEKVGGEAIVLTVPGSERS
YDLTGLKPGTEYTVSIYGV X₁X₂X₃X₄X₅X₆X₇X₈X₉ X₁₀X₁₁X₁₂SNPLSAIFTT;

wherein $X_1, X_2, X_3, X_4, X_5, X_6$ and X_7 , is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; and X_8, X_9, X_{10}, X_{11} and X_{12} is A, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, Y or deleted.

SEQ ID No. 7= TCL14 library

LPAPKNLVVSRVTEDSARLSWTAPDAAFD $SFX_1IX_2YX_3EX_4X_5X_6X_7$ GEAIVLTVPG
SERSYDLTGLKPGTEY $X_8VX_9IX_{10}GVKGGX_{11}X_{12}SX_{13}$ PLSAIFTT; wherein $X_1, X_2,$
 $X_3, X_4, X_5, X_6, X_7, X_8, X_9, X_{10}, X_{11}, X_{12}$ and X_{13} are A, C, D, E, F, G, H, I, K, L, M, N, P,
Q, R, S, T, V, W or Y

SEQ ID No. 8= TCL24 Library

TCL24 Library (SEQ ID NO: 8)

LPAPKNLVVSRVTEDSARLSWTAPDAAFD $SFX_1IX_2YX_3EX_4X_5X_6X_7$ GEAIX $_8LX_9VP$
GSERSYDLTGLKPGTEY $X_{10}VX_{11}IX_{12}GVKGGX_{13}X_{14}SX_{15}PLX_{16}AX_{17}$ FTT; wherein
 $X_1, X_2, X_3, X_4, X_5, X_6, X_{10}, X_{11}, X_{12}, X_{13}, X_{14}, X_{15}, X_{16}$ and X_{17} , are A, D, E, F, G, H, I,
K, L, N, P, Q, R, S, T, V, Y or W.

SEQ ID No. 9 = Sloning-FOR

GTGACACGGCGGTTAGAAC

SEQ ID No. 10 = Sloning-REV

GCCTTTGGGAAGCTTCTAAG

SEQ ID No. 11 = POP2250

CGGCGGTTAGAACGCGGCTACAATTAATAC

SEQ ID No. 12 = DigLigRev

CATGATTACGCCAAGCTCAGAA

SEQ ID No. 13 = BC9

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
TTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNNNN
NNNNNNNNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAA
AAGTTGGTGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGAC
CTGACCGGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTCT
TAGAAGCTTCCCAAAGGC

SEQ ID No. 14 = BC8

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG

TTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNNNN
 NNNNNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAG
 TTGGTGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTG
 ACCGGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTCTTAG
 AAGCTTCCCAAAGGC

SEQ ID No. 15 = BC7

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
 GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
 AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
 TTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNNNN
 NNNNNNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTG
 GTGAAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACC
 GGTCTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTCTTAGAAG
 CTCCCAAAGGC

SEQ ID No. 16 = BC6

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
 GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
 AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
 TTGTTTCTGAAGTTACCGAAGACTCTCTGCGTCTGTCTTGGNNNNNNNNNNNN
 NNNNNNTTYGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
 AAGCGATCAACCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
 CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTCTTAGAAGCTT
 CCCAAAGGC

SEQ ID No. 17 = 130mer-L17A

CGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCTGTTGAC
 AATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCA
 CACAGGAAACAGGATCTACCATGCTG

SEQ ID No. 18 = POP222ext

CGG CGG TTA GAA CGC GGC TAC AAT TAA TAC

SEQ ID No. 19 = LS1114

CCA AGA CAG ACG GGC AGA GTC TTC GGT AAC GCG AGA AAC AAC CAG
 GTT TTT CGG CGC CGG CAG CAT GGT AGA TCC TGT TTC

SEQ ID No. 20 = LS1115

CCG AAG ACT CTG CCC GTC TGT CTT GG

SEQ ID No. 21 = LS1117

CAG TGG TCT CAC GGA TTC CTG GTA CTG GAT CAG GAA AGA GTC GAA

SEQ ID No. 22 = SDG10

CATGCGGTCTCTTCCGAAAAAGTTGGTGAAGCGATCGTCCTGACCGTTCCGG
GT

SEQ ID No. 23 = SDG24

GGTGGTGAAGATCGCAGACAGCGGGTTAG

SEQ ID No. 24 = POP2222

CGGCGGTTAGAACGCGGCTAC

SEQ ID No. 25 = SDG28

AAGATCAGTTGCGGCCGCTAGACTAGAACCGCTGCCACCGCCGGTGGTGAAG
ATCGCAGAC

SEQ ID No. 26 = FG12

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
TTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGGACCGCGCCGGAC
GCGGCGTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
AAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCA
CCACCGGCGGTCAACCATCACCATGGCAGCGGTTCTAGTCTAGCGGC
CGCAACTGATCTTGGC

SEQ ID No. 27 = FG11

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
TTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGGACCGCGCCGGAC
GCGGCGTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
AAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCAC
CGGCGGTCAACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCA
ACTGATCTTGGC

SEQ ID No. 28 = FG10

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
TTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGGACCGCGCCGGAC

GCGGCGTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
 AAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
 CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNNNN
 NNNNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGG
 CGGTCACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACT
 GATCTTGGC

SEQ ID No. 29 = FG9

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
 GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
 AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
 TTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGGACCGCGCCGGAC
 GCGGCGTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
 AAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
 CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNNNN
 NNNNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGGCGG
 TCACCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACTGAT
 CTTGGC

SEQ ID No. 30 = FG8

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
 GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
 AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
 TTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGGACCGCGCCGGAC
 GCGGCGTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
 AAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
 CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNNNN
 NNNNNNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGGCGGTCA
 CCATCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACTGATCTTG
 GC

SEQ ID No. 31 = FG7

GTGACACGGCGGTTAGAACGCGGCTACAATTAATACATAACCCCATCCCCCT
 GTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAAC
 AATTTACACAGGAAACAGGATCTACCATGCTGCCGGCGCCGAAAAACCTGG
 TTGTTTCTCGCGTTACCGAAGACTCTGCGCGTCTGTCTTGGACCGCGCCGGAC
 GCGGCGTTCGACTCTTTCCTGATCCAGTACCAGGAATCTGAAAAAGTTGGTG
 AAGCGATCGTGCTGACCGTTCCGGGTTCTGAACGTTCTTACGACCTGACCGGT
 CTGAAACCGGGTACCGAATACACCGTTTCTATCTACGGTGTTNNNNNNNNNN
 NNNNNNNNNNNNTCTAACCCGCTGTCTGCGATCTTCACCACCGGCGGTCA
 TCACCATCACCATGGCAGCGGTTCTAGTCTAGCGGCCGCAACTGATCTTGGC

SEQ ID No. 32 = PSMW1 (N'-AviTag-HisTag-GS-Cyno PSMA_ECD)

KSSSEATNITPKHNMKAFDELKAENIKKFLHNFTQIPHLAGTEQNFQLAKQIQSQ
 WKEFGLDSVELTHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPAGYENV
 DIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMKINCSGKIVIARYGKVFR

GNKVKNAQLAGATGVILYSDPDDYFAPGVKSYPDGWNLPGGGVQQRGNILNLNG
 AGDPLTPGYPAN EYAYRRGMAEAVGLPSIPVHPIGYDDAQKLLEKMGGASPD
 SWRGS LKVPYNVGP GFTGNFSTQKV KMHIHSTSEVTRIYNVIGTLRGAVEPDRY
 VILGGHRDSWVFGGIDPQSGAAVVHEIVRSFGMLKKEGWRPRRTILFASWDAEE
 FLLGSTEWAEENSRLQERGVAYINADSSIEGNYTLRVDCTPLMYSLVYNLTKE
 LESPDEGFEGKSLYESWTKKSPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRAR
 YTKNWETNKFSSYPLYHSVYETYELVEKFYDPMFKYHLTV AQVRGGMV FELAN
 SVVLPFDCRDYAVVLRKYADKIYNISMKHPQEMKTYSVSFDSLFS AVKNFTEIAS
 KFSERLRDFDKSNPILLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYAPSSH NK
 YAGESFPGIYDALFDIESKVDPSQAWGEVKRQISIAFTTVQAAAETLSEVA

SEQ ID No. 33 = PSMW8 (N'-AviTag-HisTag-GS-Chimp PSMA_ECD)

KSSNEATNITPKHNMK AFLDELKAENIKKFLYNFTQIPHLAGTEQNFQLAKQIQS
 QWKEFG LDSVELAHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPPGYENV
 LDIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMKINCSGKIVIARYGKVF
 RGNKVKNAQLAGAKGVILYSDPADYFAPGVKSYPDGWNLPGGGVQQRGNILNLN
 GAGDPLTPGYPAN EYAYRHGIAEAVGLPSIPVHPIGYDDAQKLLEKMGGASPD
 SWRGS LKVPYNVGP GFTGNFSTQKV KMHIHSTNEVTRIYNVIGTLRGAVEPDRY
 VILGGHRDSWVFGGIDPQSGAAVVHEIVRSFGTLKKEGWRPRRTILFASWDAEEF
 GLLGSTEWAEENSRLQERGVAYINADSSIEGNYTLRVDCTPLMYSLVYNLTKE
 KSPDEGFEGKSLYESWTKKSPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARY
 TKNWETNKFSGYPLYHSVYETYELVEKFYDPMFKYHLTV AQVRGGMV FELANS
 IVLPFDCRDYAVVLRKYADKIYNISMKHPQEMKTYSVSFDSLFS AVKNFTEIAS
 FTERLQDFDKSNPILLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYAPSSH NK
 YAGESFPGIYDALFDIESKVDPSKAWGDVVKRQISVAAFTVQAAAETLSEVA

SEQ ID NO. 34 hexahistidine tag

HHHHHH

SEQ ID No. 35 = P258AR6P1071_G03

LPAPKNLVVSRVTEDSARLSWDIDEQRDWFDSFLIQYQESEKVG EAIVLTVP GSE
 RSYDLTGLKPGTEYTVSIYGVYHVYRSNPLSAIFTT

SEQ ID No. 36 = P258AR6P1070_A05

LPAPKNLVVSRVTEDSARLSWTIDEQRDWFDSFLIQYQESEKVG EAIVLTVP GSE
 RSYDLTGLKPGTEYTVSIYGVYHVYRSNPLSAIFTT

SEQ ID No. 37 = P258AR6P1071_F04

LPAPKNLVVSRVTEDSARLSWVIDEQRDWFDSFLIQYQESEKVG EAIVLTVP GSE
 RSYDLTGLKPGTEYTVSIYGVYHVYRSNPLSAIFTT

SEQ ID No. 38 = P258AR6P1070_F09

LPAPKNLVVSRVTEDSARLSWTIDEQRDWFESFLIQYQESEKVG EAIVLTVP GSE
 RSYDLTGLKPGTEYTVSIYGVYHVYRSNPLSAIFTT

SEQ ID No. 39 = P258AR6P1071_D02

LPAPKNLVVSRVTEDSARLSWAIDEQRDWFESFLIQYQESEKVGGEAIVLTVPGSE
RSYDLTGLKPGTEYTVSIYGVYHVYRSNPLSAIFTT

SEQ ID No. 40 = P229CR5P819_H11

LPAPKNLVVSRVTEDSARLSWDIDEQRDWFDSFLIQYQESEKVGGEAIVLTVPGSE
RSYDLTGLKPGTEYTVSIYGVYHVYRSSNPLSAIFTT

SEQ ID No. 41 = P233FR9_H10

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEWDDDGEAIVLTVPGSER
SYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 42 = P233FR9P1001_B5-5

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFTIGYWEWDDDGEAIVLTVPGSER
SYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 43 = P233FR9P1001_H3-1

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFPIGYWEWDDDGEAIVLTVPGSER
SYDLTGLKPGTEYHVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 44 = P233FR9P1001_D9

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFPIGYWEWDDDGEAIVLTVPGSER
SYDLTGLKPGTEYWVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 45 = P234CR9_A07

LPAPKNLVVSRVTEDSARLSWGEQFTIFDSFLIQYQESEKVGGEAIVLTVPGSERSY
DLTGLKPGTEYTVSIYGASGYEWFHAFGSSNPLSAIFTT

SEQ ID No. 46 = P234CR9_H01

LPAPKNLVVSRVTEDSARLSWEWWVIPGDFDSFLIQYQESEKVGGEAIVLTVPGSE
RSYDLTGLKPGTEYTVSIYGVVNSGQWNDTSNPLSAIFTT

SEQ ID No. 47 = P233FR9_H10 (cterm cys)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEWDDDGEAIVLTVPGSER
SYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTTC

SEQ ID No. 48 = P233FR9_H10 (K62C)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEWDDDGEAIVLTVPGSER
SYDLTGLCPGTEYPVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 49 = P233FR9_H10 (E53C)

LPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEWDDDGEAIVLTVPGSCR
SYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 50 = P233FR9_H10 (R11C)

LPAPKNLVVSCVTEDSARLSWTAPDAAFDSFAIGYWEWDDDGEAIVLTVPGSER
SYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTT

SEQ ID No. 51 = untargeted FN3 domain (K62C)

LPAPKNLVVSEVTEDSARLSWTAPDAAFDSFLIQYQSEKVGGEAIVLTVPGSERS
YDLTGLCPGTEYTVSIYGVKGGHRSNPLSAIFTTGGHHHHHH

SEQ ID No. 52 = Sortase A

MSHHHHHHSSGENLYFQSKPHIDNYLHDKDKDEKIEQYDKNVKEQASKDKKQQ
AKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATREQLNRGVSF AEENESLDDQNISI
AGHTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRNVKPTAVEVLD
EQKGKDKQLTLITCDDYNEETGVWETRKIFVATEVK

SEQ ID No. 53 = tagless Sortase A

SKPHIDNYLHDKDKDEKIEQYDKNVKEQASKDKKQQAKPQIPKDKSKVAGYIEI
PDADIKEPVYPGPATREQLNRGVSF AEENESLDDQNISIAGHTFIDRPNYQFTNLK
AAKKGSMVYFKVGNETRKYKMTSIRNVKPTAVEVLDEQKGKDKQLTLITCDDY
NEETGVWETRKIFVATEVK

SEQ ID NO: 54 TEV protease cleavage site

ENLYFQS

SEQ ID NO: 55 FG loop of Tencon

KGGHRSN

SEQ ID NO: 56 BC loop

DIDEQRDW

SEQ ID NO: 57 BC loop

TIDEQRDW

SEQ ID NO: 58 BC loop

VIDEQRDW

SEQ ID NO: 59 BC loop

AIDEQRDW

WO 2016/179518

PCT/US2016/031260

SEQ ID NO: 60 BC loop

EWWVIPGD

SEQ ID NO: 61 BC loop

GEQFTI

SEQ ID NO: 62 BC loop

TAPDAA

SEQ ID NO: 63 C loop

FDSFLIQYQE

SEQ ID NO: 64 C loop

FESFLIQYQE

SEQ ID NO: 65 C loop

FDSFAIGYWE

SEQ ID NO: 66 C loop

FDSFPIGYWE

SEQ ID NO: 67 C loop

FDSFTIGYWE

SEQ ID NO: 68 CD loop

SEKVGE

SEQ ID NO: 69 CD loop

WDDDGE

SEQ ID NO: 70 F loop

TEYTVSIYGV

SEQ ID NO: 71 F loop

TEYTVSIYG

SEQ ID NO: 72 F loop

TEYPVYIAGV

WO 2016/179518

PCT/US2016/031260

SEQ ID NO: 73 F loop

TEYWVYIAGV

SEQ ID NO: 74 F loop

TEYHVYIAGV

SEQ ID NOs: 75-140 are above in the tables

SEQ ID NO: 141 full length cynoPSMA

MWNLLHETDSAVATARRPRWLCAGALVLAGGFFLLGFLFGWFIKSSSEATNITP
 KHNMKAFDELKAENIKKFLHNFTQIPHLAGTEQNFQLAKQIQSQWKEFGLDSV
 ELTHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPAGYENVSDIVPPFSAFSP
 QGMPEGDLVYVNYARTEDFFKLERDMKINCSGKIVIARYGKVFRGNKVKNAQL
 AGATGVILYSDPDDYFAPGVKSYPDGWNLPGGGVQRGNILNLNGAGDPLTPGYP
 ANEYAYRRGMAEAVGLPSIPVHPIGYDDAQKLLEKMGGASPDSSWRGSLKVP
 YNVGPGFTGNFSTQKVKMHIHSTSEVTRIYNVIGTLRGAVEPDRYVILGGHRDS
 WVFGGIDPQSGAAVVHEIVRSFGMLKKEGWRPRRTILFASWDAEEFGLLGSTEW
 AEENSRLQERGVAYINADSSIEGNYTLRVDCTPLMYSLVYNLTKELESPDEGFE
 GKSLYESWTKKSPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARYTKNWETN
 KFSSYPLYHSVYETYELVEKFYDPMFKYHLTVAQVRGGMVFELANSVLPFDCR
 DYAVVLRKYADKIYNISMKHPQEMKTYSVSFDSLFSAVKNFTEIASKFSERLRDF
 DKSNPILLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYAPSSHNKY

>142 linker

AEAAAKEAAAKEAAAKEAAAKEAAAKAAA

>143 human PSMA ECD

KSSNEATNITPKHNMKAFDELKAENIKKFLYNFTQIPHLAGTEQNFQLAKQIQS
 QWKEFGLDSVELAHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPPGYENV
 SDIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMKINCSGKIVIARYGKVF
 RGNKVKNAQLAGAKGVILYSDPADYFAPGVKSYPDGWNLPGGGVQRGNILNLN
 GAGDPLTPGYPANEYAYRRGIAEAVGLPSIPVHPIGYDDAQKLLEKMGGASPPDS
 SWRGSLKVPYNVGPFTGNFSTQKVKMHIHSTNEVTRIYNVIGTLRGAVEPDRY
 VILGGHRDSWVFGGIDPQSGAAVVHEIVRSFGTLKKEGWRPRRTILFASWDAEEF
 GLLGSTEWAEENSRLQERGVAYINADSSIEGNYTLRVDCTPLMYSLVHNLTKEL
 KSPDEGFEGKSLYESWTKKSPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARY
 TKNWETNKFSGYPLYHSVYETYELVEKFYDPMFKYHLTVAQVRGGMVFELANS
 IVLPFDCRDYAVVLRKYADKIYSISMKHPQEMKTYSVSFDSLFSAVKNFTEIASKF
 SERLQDFDKSNPIVLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYAPSSHNKYA
 GESFPGIYDALFDIESKVDPSKAWGEVKRQIYVAAFTVQAAAETLSEVA

>144 human FL PSMA with signal sequence

MWNLLHETDSAVATARRPRWLCAGALVLAGGFFLLGFLFGWFIKSSNEATNITP
 KHNMKAFDELKAENIKKFLYNFTQIPHLAGTEQNFQLAKQIQSQWKEFGLDSV

ELAHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPPGYENVSDIVPPFSAFSP
 QGMPEGDLVYVNYARTEDFFKLERDMKINCSGKIVIARYGKVFRGNKVKNAQL
 AGAKGVILYSDPADYFAPGVKSYPDGWNLPGGGVQRGNILNLNGAGDPLTPGY
 PANEYAYRRGIAEAVGLPSIPVHPIGYYDAQKLLEKMGGSAAPPDSSWRGSLKVP
 YNVGPGFTGNFSTQKVKMHIHSTNEVTRIYNVIGTLRGAVEPDRYVILGGHRDS
 WVFGGIDPQSGAAVVHEIVRSFGTLKKEGWRPRRTILFASWDAEEFGLLGSTEW
 AEENSRLQERGVAYINADSSIEGNYTLRVDCTPLMYSLVHNLTKELKSPDEGFE
 GKSLYESWTKKSPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARYTKNWETN
 KFSGYPLYHSVYETYELVEKFYDPMFKYHLTVAQVRGGMVFELANSIVLPFDCR
 DYAVVLRKYADKIYSISMKHPQEMKTYSVSFDLSFSAVKNFTEIASKFSERLQDF
 DKSNPVLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYAPSSHNKYAGESFP
 YDALFDIESKVDPSKAWGEVKRQIYVAAFTVQAAAETLSEVA

>145 3rd FN3 domain of tenascin C

DAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSI
 GNLKPDTEYEVSLSISRRGDMSSNPAKETFTT

>146 Fibcon

LDAPTDLQVTNVTDTTSITVSWTPPSATITGYRITYTPSNGPGEPKELTVPPSSTSVT
 ITGLTPGVEYVVSLYALKDNQESPLVGTQTT

>147 10th FN3 domain of fibronectin

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSKS
 TATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRT

>148

GSGS

>149

GGGSGGGS

>150

GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG

>151

APAP

>152

APAPAPAPAP

>153

APAPAPAPAPAPAPAPAPAP

>154

APAPAPAPAPAPAPAPAPAPAPAPAPAPAPAPAPAP

>155 Albumin variant

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCV
ADESAENCDSLHTLFGDKLCTVATLRETYGEMADCCAKQEPPERNECFLQHKD
DNPNLRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRY
KAAFTECCQAADKAACLLPKLDELRLDEGKASSAKQRLKCASLQKFGERAFAKAW
AVARLSQRFPKAEFAEVSKLVTDLT KVHTECCHGDLLECADDRADLAKYICENQ
DSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAK
DVFLGMFLY EYARRHPDYSVLLRLAKTYETTLKCCAAADPHECYAKVFDE
FKPLVEEPQNLIKQNCLEFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLG
KVGSKCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNR
RPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKA
TKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQAALGL

>156 cDNA H10

CTGCCAGCCCCGAAGAATTTGGTCGTTTCCCGTGTCACTGAGGACTCTGCACG
TCTGAGCTGGACCGCACCGGACGCGGGCGTTTCGACAGCTTTGCAATCGGCTAC
TGGGAGTGGGATGATGACGGCGAGGCCATTGTGCTGACCGTTCCGGGTAGCG
AGCGCAGCTACGATCTGACCGGTCTGAAGCCGGGTACGGAATATCCGGTGTA
TATTGCGGGCGTGAAGGGTGGCCAGTGGAGCTTCCCGCTGAGCGCGATCTTT
ACCACC

>157 cDNA P258AR6P1071_D02

CTGCCGGCTCCGAAAAACCTGGTCGTTTCCCGTGTCACTGAAGATTCTGCACG
CTTGAGCTGGGCGATCGACGAGCAGCGTGACTGGTTTGAGAGCTTCCTGATT
CAGTATCAAGAATCGGAAAAAGTTGGCGAGGCCATCGTGCTGACCGTTCCGG
GTAGCGAGCGCAGCTATGATCTGACGGGTCTGAAGCCAGGCACCGAGTATAC
GGTGAGCATTTACGGTGTCTACCATGTGTACCGTAGCAATCCGCTGAGCGCG
ATCTTCACCACC

>158 cDNA

P233FR9P1001_H3-1

CTGCCAGCCCCGAAAACTTAGTTGTCTCCCGCGTGACCGAAGATTCTGCTCG
TCTGAGCTGGACTGCACCGGACGCGGGCGTTTCGACAGCTTTCCGATTGGCTACT
GGGAGTGGGATGATGACGGTGAAGCGATCGTGCTGACCGTTCCGGGTAGCGA
GCGTAGCTATGACCTGACGGGTTTGAAACCTGGTACCGAGTATCACGTTTAC
ATTGCGGGCGTCAAGGGTGGCCAGTGGTCGTTCCCGCTGAGCGCAATCTTTA
CGACC

>159 PSMA epitope

KKSPSPEFSGMPRISK

>160 PSMA epitope

NWETNKF

>161 SP34 Light Chain

QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTN
KRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNLWVFGGGTKLTV
LGQPKSSPSVTLFPPSSEETNKATLVCTITDFYPGVVTVDWKVDGTPVTQGME
TTQPSKQSNNKYMASSYLTLTARAWERHSSYSCQVTHEGHTVEKSLSRADCS

>162 SP34 Heavy Chain

EVKLLESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRS
KYNKYATYYADSVKDRFTISRDDSQSILYLQMNNLKTEDTAMYYCVRHGNFGN
SYVSWFAYWGQGLVTVSAATTTAPSVYPLVPGCSDTSGSSVTLGCLVKGYFPE
PVTVKWNYGALSSGVRTVSSVLQSAFYSLSSLVTVPSSTWPSQTVICNVAHPASK
TELIKRIEPRIPKPSTPPGSSCPPGNILGGPSVFIFPPKPKDALMISLTPKVTCVVVDV
SEDDPDVHVSFVDNKEVHTAWTQPREAQYNSTFRVVSALPIQHQDWMRGKE
FKCKVNNKALPAPIERTISKPKGRAQTPQVYTIPPPREQMSKKKVSLTCLVTNFFS
EASVEWERNGELEQDYKNTPPILDSGTYFLYSKLTVDTDSWLQGEIFTCVSVH
EALHNHHTQKNLSRSPGK

>163 CD3H141

EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRS
KYNKYATYYAASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGN
SYVSWFAYWGQGLVTVSS

>164 CD3H142

EVQLLESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRS
KYNKYATYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKHGNFGN
SYVSWFAYWGQGLVTVSS

>165 CD3H143

EVQLLESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRS
KYNKYATYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCVKHGNFGN
SYVSWFAYWGQGLVTVSS

>166 CD3H144

EVQLVESGGGLVQPGGSLKLSCAASGFTFNTYAMNWVRQASGKGLEWVGRIRS
KYNKYATYYAASVKGRFTISRDDSKNTAYLQMNSLKTEDTAVYYCTRHNFGN
SYVSWFAYWGQGLVTVSS

>167 CD3L63

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTN
KRAPGTPARFSGSLLGGKAALTLSGAQPEDEAEYYCALWYSNLWVFGGGTKLT
VL

>168 CD3L64

QSVLTQPPSVSAAPGQKVTISCRSSTGAVTTSNYANWVQQLPGTAPKGLIGGTNK
RAPGIPDRFSGSKSGTSATLGITGLQTGDEADYYCALWYSNLWVFGGGTKLTVL

>169 CD3L66

QTVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTN
KRAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCALWYSNLWVFGGGTKLT
VL

>170. B219 Light Chain

QTVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTN
KRAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCALWYSNLWVFGGGTKLT
VLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAG
VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

>171. B219 Heavy chain

EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRS
KYNKYATYYAASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGN
SYVSWFAYWGQGLTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEP
VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVDPKPSN
TKVDKRVESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKE
YKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQEGNVFSCSV
MHEALHNHYTQKSLSLGLK

>172. CW5 C-terminal FN3 domain FC fusion

GSCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWY
VDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSS
IEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFLLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKS
LSLSLGGKGGGGSGGGGSMPLPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYW
EWDDDGEAIVLTVPGSERSYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTT

>173. CW6 N-terminal FN3 domain FC fusion

MLPAPKNLVVSRVTEDSARLSWTAPDAAFDSFAIGYWEWDDDGEAIVLTVPGSE
RSYDLTGLKPGTEYPVYIAGVKGGQWSFPLSAIFTTGGGGSGGGGSCPPCPAPEA
AGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAK
TKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ

PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDS DGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLK

>174. B221 Heavy Chain

EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRS
KYN NYATYYAASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGN
SYVSWFAYWGQGLTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEP
VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGKTYTCNV DHKPSN
TKVDKRVESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKE
YKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFLLYSKLTVDKSRWQEGNVFSCSV
MHEALHNHYTQKSLSLGLKGGGGSGGGGSM LPA PKNLVVSRTEDSARLSWT
APDAAFDSFAIGYWEWDDDGEAIVLTVPGSERSYDLTGLKPGTEYPPVYIAGVKG
GQWSFPLSAIFTT

>175

GGGGSGGGGS

>176 G11 mAb heavy chain

EVQLLES GGG LVQPGGSLRL SCAASGFTFS SYGMSWVRQA PGKGLEWWSG
INGGGGSKYYADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKTS
AQRFDYWGQG TLTVTVSSASTKGPSVFPLAP SSKSTSGGTA ALGCLVKDYF
PEPVT VSWNS GALTSGVHTF PAVLQSSGLYSLSSVTVPS SSLGTQTYIC
NVNHKPSNTK VDKKVEPKSC DKTHTCPPCP APELLGGPSVFLFPPKPKDT
LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK
PREEQYNSTYRVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK
GQPREPQVYT LPPSRDELTKNQVSLTCLVK GFYPSDIAVE WESNGQPENN
YKTTPPVLD S DGSFFLYSKL TVDKSRWQQGNVFSCSVMHE ALHNHYTQKS
LSLSPGK

>177 G11 mAb light chain

DIQMTQSPSS LSASVGDRVT ITCRASQSIG SYLNWYQQKP GKAPKLLIYA
ASSLQSGVPSRFS GSGSGTD FTLTISSLQP EDFATYYCQQ SYSLPLTFGQ
GTKVEIKRTV AAPSVFIFPPSDEQLKSGTA SVVCLLNNFY PREAKVQWKV
DNALQSGNSQ ESVTEQDSKD STYLSSTLTLSKADYEKHK VYACEVTHQG
LSSPVTKSFN RGEC

>178 CD3B94 mAb heavy chain

QVQLVQSGAE VKKPGSSVKV SCKASGGTFS SYAISWVRQA PGQGLEWMGG
IPIFGTANYAQKFQGRVTI TADESTSTAY MELSSLRSED TAVYYCARDP
ARLYSYFDY WGQGLTVTVSSASTKGPSVF PLAPSSKSTS
GGTAALGCLVKDYFPEPVT VSWNSGALTSG VHTFPAVLQSSGLYSLSSV
TVPSSSLGTQTYICNVNHKP SNTKVDKKVE PKSCDKTHTC
PPCPAPEAAGGPSVFLFPPK PKDTLMISRT PEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG

KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRDELTKNQVSLT
CLVKGFYPSD IAVEWESNGQPENNYKTTPP VLDS DGSFLLYSKLTVDKSR
WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K

>179 CD3B94 mAb light chain

DIQMTQSPSS LSASVGDRVIT ITCRASQSISSYLNWYQQKPGKAPKLLIYA
ASSLQSGVPSRFSGSGSGTDFTLTISSLQP
EDFATYYCQQSYSTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKV DNALQSGNSQESVTEQDSKD
STYLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFN

What is Claimed:

1. An isolated CD3 x PSMA-bispecific antigen-binding molecule comprising:

- a. an FN3 domain;
- b. a light chain (LC); and
- c. a heavy chain (HC),

wherein the FN3 domain forms a first antigen-binding site that specifically binds human PSMA, and the HC and the LC pair form a second antigen-binding site that immunospecifically binds CD3, or a bispecific antigen-binding fragment thereof.

2. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1, wherein the PSMA comprises SEQ ID NO: 144.

3. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1, wherein the PSMA is SEQ ID NO: 144.

4. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1, wherein the FN3 domain cross-reacts with *Macaca fascicularis* PSMA or with *Pan troglodytes* PSMA.

5. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 4, wherein *Macaca fascicularis* PSMA comprises SEQ ID NO: 32.

6. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 4, wherein the *Pan troglodytes* PSMA comprises SEQ ID NO: 33.

7. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1, wherein:

- a) the FN3 domain is based on Tencon sequence of SEQ ID NO: 1 or Tencon27 sequence of SEQ ID NO: 4, the SEQ ID NO: 1 or SEQ ID NO: 4 optionally having substitutions at residue positions 11, 14, 17, 37, 46, 73, and/or 86; or

- b) the FN3 domain is isolated from a library comprising the sequence of SEQ ID NOs: 2, 3, 5, 6, 7, or 8.
8. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claims 1 - 7, wherein the FN3 domain has a cysteine residue in at least one residue position corresponding to residue positions 6, 11, 22, 25, 26, 52, 53, 62 of SEQ ID NO: 1, or at a C-terminus.
9. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any one of of claims 1 to 8, wherein the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof comprises an IgG1, IgG2, IgG3, or IgG4 isotype.
10. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 9, the CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof comprises an IgG4 isotype.
11. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any one of claims 1 to 10, wherein the FN3 domain inhibits human PSMA enzymatic activity.
12. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of claims 1 to 12, wherein the FN3 domain comprises SEQ ID NO: 41.
13. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of claims 1 to 13, wherein the FN3 domain is SEQ ID NO: 41.
14. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of claims 1 to 14, wherein the FN3 domain binds to the epitopes KKSPSPEFSGMPRISK (SEQ ID NO: 159) and NWETNKF (SEQ ID NO: 160) of human PSMA.
15. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of claims 1 to 15, wherein the FN3 domain comprises an amino acid sequence that is at least 89% identical to the amino acid sequence of SEQ ID NO: 41, or that

has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 substitutions when compared to the amino acid sequence of SEQ ID NO: 41.

16. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of claims 1 to 16, further comprising a methionine at the N-terminus of the FN3 domain.

17. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the FN3 domain comprises:

- a. the amino acid sequence of SEQ ID NO: 40;
- b. the amino acid sequence of SEQ ID NO: 35;
- c. the amino acid sequence of SEQ ID NO: 36;
- d. the amino acid sequence of SEQ ID NO: 37;
- e. the amino acid sequence of SEQ ID NO: 38;
- f. the amino acid sequence of SEQ ID NO: 39;
- g. the amino acid sequence of SEQ ID NO: 46;
- h. the amino acid sequence of SEQ ID NO: 45;
- i. the amino acid sequence of SEQ ID NO: 41;
- j. the amino acid sequence of SEQ ID NO: 44;
- k. the amino acid sequence of SEQ ID NO: 42; or,
- l. the amino acid sequence of SEQ ID NO: 43.

18. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the FN3 domain comprises:

- a. the amino acid sequence of SEQ ID NO: 47;
- b. the amino acid sequence of SEQ ID NO: 51;
- c. the amino acid sequence of SEQ ID NO: 50; or,
- d. the amino acid sequence of SEQ ID NO: 49.

19. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the FN3 domain comprises:

- a. the amino acid sequence of SEQ ID NO: 75;
- b. the amino acid sequence of SEQ ID NO: 76;
- c. the amino acid sequence of SEQ ID NO: 77;
- d. the amino acid sequence of SEQ ID NO: 78;
- e. the amino acid sequence of SEQ ID NO: 79;
- f. the amino acid sequence of SEQ ID NO: 80;
- g. the amino acid sequence of SEQ ID NO: 81;
- h. the amino acid sequence of SEQ ID NO: 82;
- i. the amino acid sequence of SEQ ID NO: 83;
- j. the amino acid sequence of SEQ ID NO: 84;
- k. the amino acid sequence of SEQ ID NO: 85;
- l. the amino acid sequence of SEQ ID NO: 88;
- m. the amino acid sequence of SEQ ID NO: 87;
- n. the amino acid sequence of SEQ ID NO: 88;
- o. the amino acid sequence of SEQ ID NO: 89;
- p. the amino acid sequence of SEQ ID NO: 90;
- q. the amino acid sequence of SEQ ID NO: 91;
- r. the amino acid sequence of SEQ ID NO: 92;
- s. the amino acid sequence of SEQ ID NO: 93;
- t. the amino acid sequence of SEQ ID NO: 94;
- u. the amino acid sequence of SEQ ID NO: 95;
- v. the amino acid sequence of SEQ ID NO: 96;

- w. the amino acid sequence of SEQ ID NO: 97;
- x. the amino acid sequence of SEQ ID NO: 98;
- y. the amino acid sequence of SEQ ID NO: 99;
- z. the amino acid sequence of SEQ ID NO: 100;
- aa. the amino acid sequence of SEQ ID NO: 101;
- bb. the amino acid sequence of SEQ ID NO: 102;
- cc. the amino acid sequence of SEQ ID NO: 103;
- dd. the amino acid sequence of SEQ ID NO: 104;
- ee. the amino acid sequence of SEQ ID NO: 105;
- ff. the amino acid sequence of SEQ ID NO: 106;
- gg. the amino acid sequence of SEQ ID NO: 107;
- hh. the amino acid sequence of SEQ ID NO: 108;
- ii. the amino acid sequence of SEQ ID NO: 109;
- jj. the amino acid sequence of SEQ ID NO: 110;
- kk. the amino acid sequence of SEQ ID NO: 111;
- ll. the amino acid sequence of SEQ ID NO: 112;
- mm. the amino acid sequence of SEQ ID NO: 113;
- nn. the amino acid sequence of SEQ ID NO: 114;
- oo. the amino acid sequence of SEQ ID NO: 115;
- pp. the amino acid sequence of SEQ ID NO: 116;
- qq. the amino acid sequence of SEQ ID NO: 117;
- rr. the amino acid sequence of SEQ ID NO: 118;
- ss. the amino acid sequence of SEQ ID NO: 119;
- tt. the amino acid sequence of SEQ ID NO: 120;

- uu. the amino acid sequence of SEQ ID NO: 121;
 - vv. the amino acid sequence of SEQ ID NO: 122;
 - ww. the amino acid sequence of SEQ ID NO: 123;
 - xx. the amino acid sequence of SEQ ID NO: 124;
 - yy. the amino acid sequence of SEQ ID NO: 125;
 - zz. the amino acid sequence of SEQ ID NO: 126;
 - aaa. the amino acid sequence of SEQ ID NO: 127;
 - bbb. the amino acid sequence of SEQ ID NO: 128;
 - ccc. the amino acid sequence of SEQ ID NO: 129;
 - ddd. the amino acid sequence of SEQ ID NO: 130;
 - eee. the amino acid sequence of SEQ ID NO: 131;
 - fff. the amino acid sequence of SEQ ID NO: 132;
 - ggg. the amino acid sequence of SEQ ID NO: 133;
 - hhh. the amino acid sequence of SEQ ID NO: 134;
 - iii. the amino acid sequence of SEQ ID NO: 135;
 - jjj. the amino acid sequence of SEQ ID NO: 136;
 - kkk. the amino acid sequence of SEQ ID NO: 137;
 - lll. the amino acid sequence of SEQ ID NO: 138;
 - mmm. the amino acid sequence of SEQ ID NO: 139; or,
 - nnn. the amino acid sequence of SEQ ID NO: 140.
20. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the LC comprises:
- a. the amino acid sequence of SEQ ID NO: 167;
 - b. the amino acid sequence of SEQ ID NO: 168; or,

c. the amino acid sequence of SEQ ID NO: 169.

21. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the HC comprises:

- a. the amino acid sequence of SEQ ID NO: 163;
- b. the amino acid sequence of SEQ ID NO: 164;
- c. the amino acid sequence of SEQ ID NO: 165; or,
- d. the amino acid sequence of SEQ ID NO: 166.

22. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein

a. the HC comprises the amino acid sequence of SEQ ID NO: 163 and the LC comprises the amino acid sequence of SEQ ID NO: 167;

b. HC comprises: the amino acid sequence of SEQ ID NO: 163 and the LC comprises: the amino acid sequence of SEQ ID NO: 168;

c. HC comprises: the amino acid sequence of SEQ ID NO: 163 and the LC comprises: the amino acid sequence of SEQ ID NO: 169;

d. HC comprises: the amino acid sequence of SEQ ID NO: 164 and the LC comprises: the amino acid sequence of SEQ ID NO: 167;

e. HC comprises: the amino acid sequence of SEQ ID NO: 164 and the LC comprises: the amino acid sequence of SEQ ID NO: 168;

g. HC comprises: the amino acid sequence of SEQ ID NO: 164 and the LC comprises: the amino acid sequence of SEQ ID NO: 169;

h. HC comprises: the amino acid sequence of SEQ ID NO: 165 and the LC comprises: the amino acid sequence of SEQ ID NO: 167;

i. HC comprises: the amino acid sequence of SEQ ID NO: 165 and the LC comprises: the amino acid sequence of SEQ ID NO: 168;

j. HC comprises: the amino acid sequence of SEQ ID NO: 165 and the LC comprises: the amino acid sequence of SEQ ID NO: 169;

k. HC comprises: the amino acid sequence of SEQ ID NO: 166 and the LC comprises: the amino acid sequence of SEQ ID NO: 167;

l. HC comprises: the amino acid sequence of SEQ ID NO: 166 and the LC comprises: the amino acid sequence of SEQ ID NO: 168;

f. HC comprises: the amino acid sequence of SEQ ID NO: 166 and the LC comprises: the amino acid sequence of SEQ ID NO: 169; or

g. HC comprises: the amino acid sequence of SEQ ID NO: 171 and the LC comprises: the amino acid sequence of SEQ ID NO: 170.

23. A method for treating a subject having a PSMA-overexpressing cancer, said method comprising:

administering a therapeutically effective amount of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any one of claims 1 to 18 to said subject for a time sufficient to treat the cancer.

24. A method for inhibiting growth or proliferation of PSMA-overexpressing cancer cells in a subject in need thereof, said method comprising:

administering a therapeutically effective amount of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of one claims 1 to 18 to said subject.

25. A method of redirecting a T cell to a PSMA-overexpressing cancer cell in a subject in need thereof, said method comprising:

administering a therapeutically effective amount of the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any of claims 1 to 18 to said subject.

26. The method of claim 23, 24, or 25 wherein the cancer is a solid tumor.

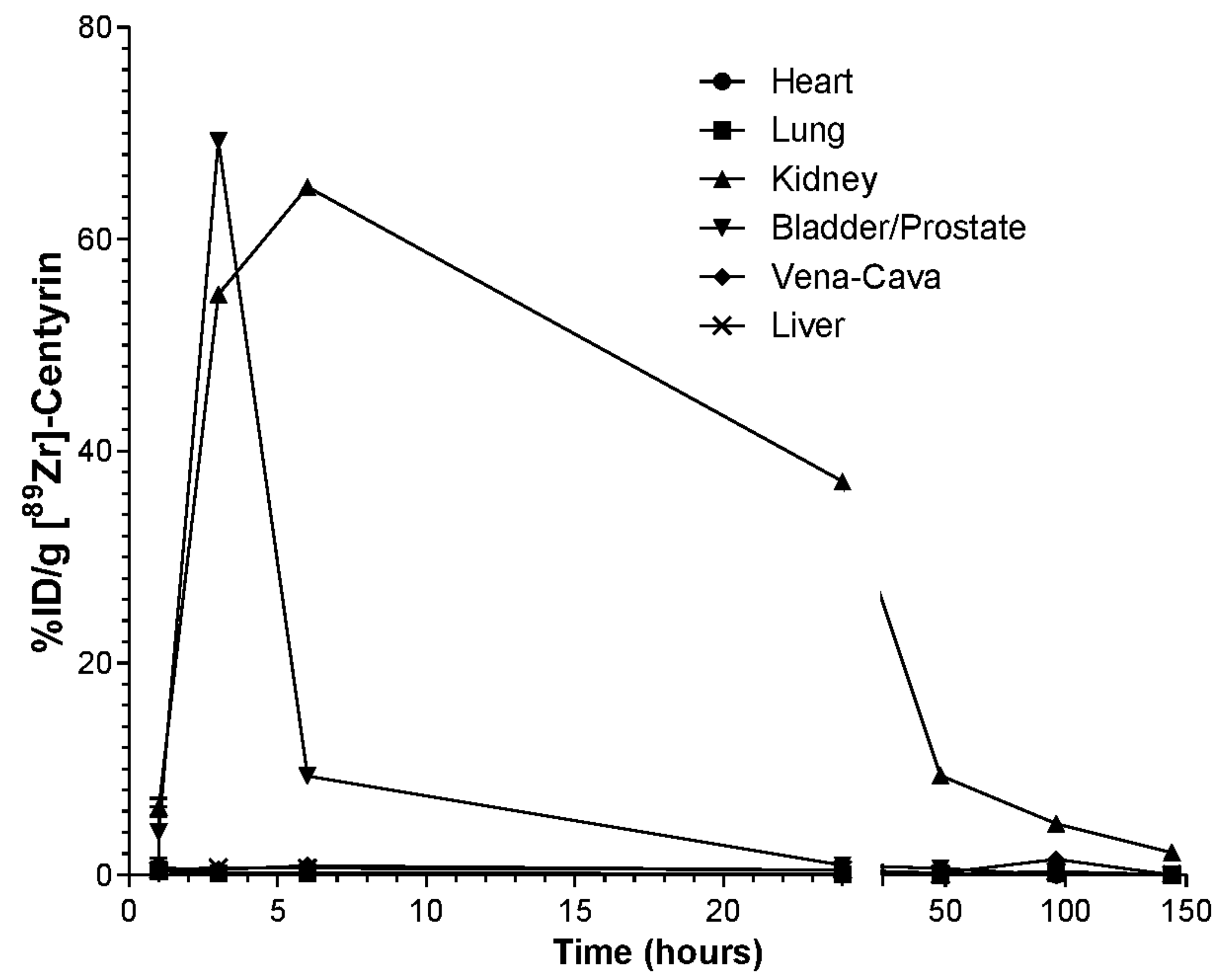
27. The method of claim 23, 24, or 25 wherein the cancer is a neovascular disorder.

28. The method of claim 23, 24, or 25 wherein the cancer is prostate cancer, colorectal cancer, gastric cancer, clear cell renal carcinoma, bladder cancer, lung cancer, squamous cell carcinoma, gliomas, breast cancer or kidney cancer.

29. The method of claim 27 wherein the cancer is prostate cancer.
30. The method of claim 23, 24, or 25 further comprising administering a second therapeutic agent.
31. The method of claim 30 wherein the second therapeutic agent is a chemotherapeutic agent or a targeted anti-cancer therapy.
32. The method of claim 31 wherein the chemotherapeutic agent or a targeted anti-cancer therapy is Abiraterone Acetate (Zytiga), Bicalutamide, Cabazitaxel, Casodex (Bicalutamide), Degarelix, Docetaxel, Enzalutamide, Goserelin Acetate, Jevtana (Cabazitaxel), Leuprolide Acetate, Lupron (Leuprolide Acetate), Lupron Depot (Leuprolide Acetate), Lupron Depot-3 Month (Leuprolide Acetate), Lupron Depot-4 Month (Leuprolide Acetate), Lupron Depot-Ped (Leuprolide Acetate), Mitoxantrone Hydrochloride, Prednisone, Provenge (Sipuleucel-T), Radium 223 Dichloride, Sipuleucel-T, Taxotere (Docetaxel), Viadur (Leuprolide Acetate), Xofigo (Radium 223 Dichloride), Xtandi (Enzalutamide) or Zoladex (Goserelin Acetate).
33. The method of any of claims 30 to 32 wherein the second therapeutic agent is administered to said subject simultaneously with, sequentially, or separately from the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof.
34. A pharmaceutical composition comprising the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any one of claims 1 to 18 and a pharmaceutically acceptable carrier.
35. A kit comprising the isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any one of claims 1 to 18 and packaging for the same.
36. A fusion protein comprising:
 - a. a heavy chain;
 - b. a FN3 domain;
 - c. a linker.
37. The fusion protein of claim 36, wherein the heavy chain Fc region is IgG4 PAA.

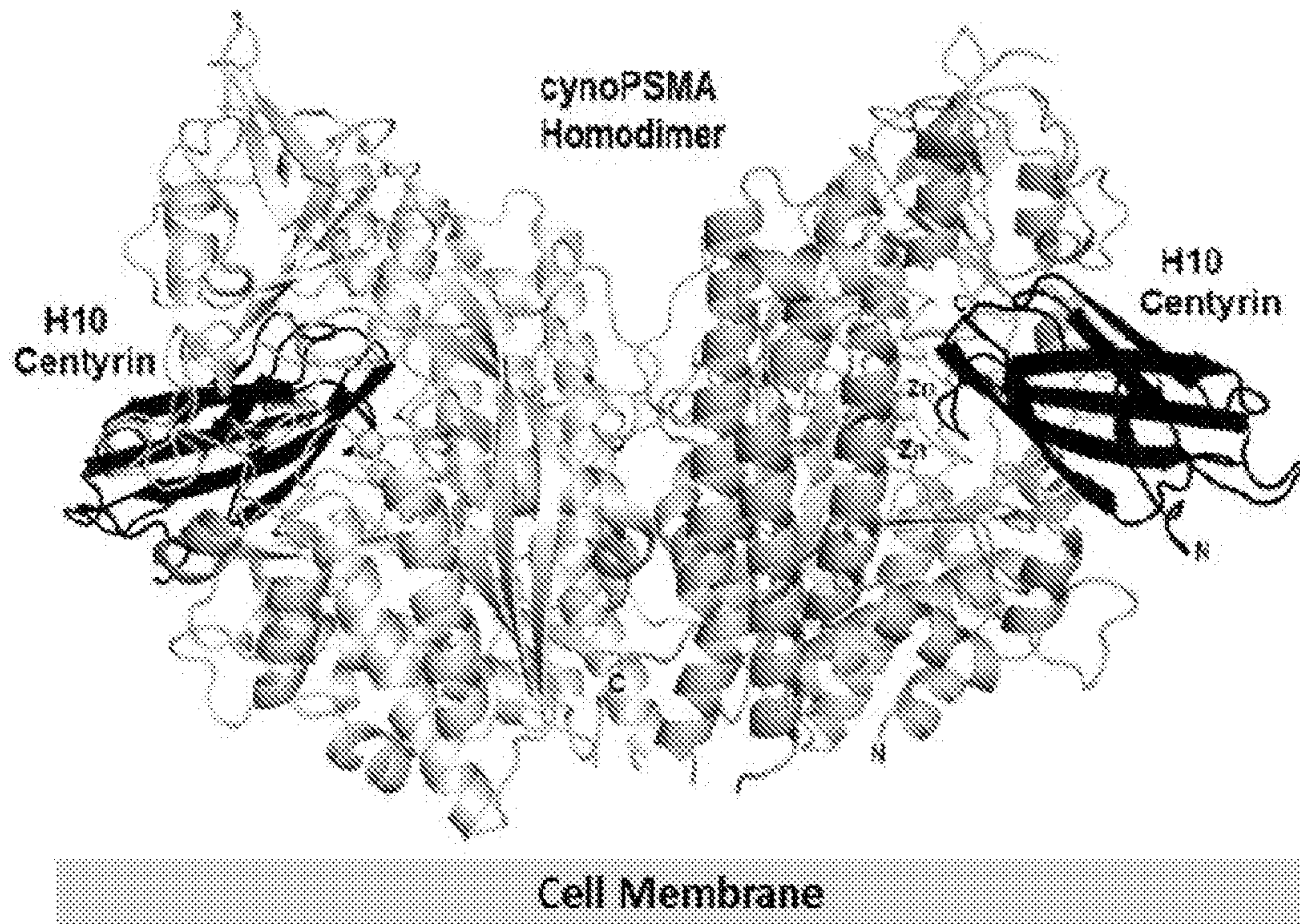
38. A fusion protein comprising:
- a. a Fc region;
 - b. a FN3 domain;
 - c. a linker.
40. The fusion protein of claim 38, wherein the linker and FN3 domain are attached to the amino-terminus of the Fc region.
41. The fusion protein of claim 38, wherein the linker and FN3 domain are attached to the carboxyl-terminus of the Fc region.
42. The fusion protein of any one of claims 36-41, wherein the linker comprises the amino acid sequence of SEQ ID NO: 175.
43. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 171, the light chain comprises the amino acid sequence of SEQ ID NO: 170, and FN3 domain comprises the amino acid sequence of SEQ ID NO: 172.
44. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 171, the light chain comprises the amino acid sequence of SEQ ID NO: 170, and FN3 domain comprises the amino acid sequence of SEQ ID NO: 173.
45. The isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of claim 1 wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 174, the light chain comprises the amino acid sequence of SEQ ID NO: 170, and FN3 domain comprises the amino acid sequence of SEQ ID NO: 173.
46. A pharmaceutical composition comprising isolated CD3 x PSMA-bispecific antigen-binding molecule or bispecific antigen-binding fragment thereof of any one of claims 43 to 45 and a pharmaceutically acceptable carrier.
47. An isolated synthetic polynucleotide encoding the fusion protein of any one of claims 36 to 42.

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Fig. 1

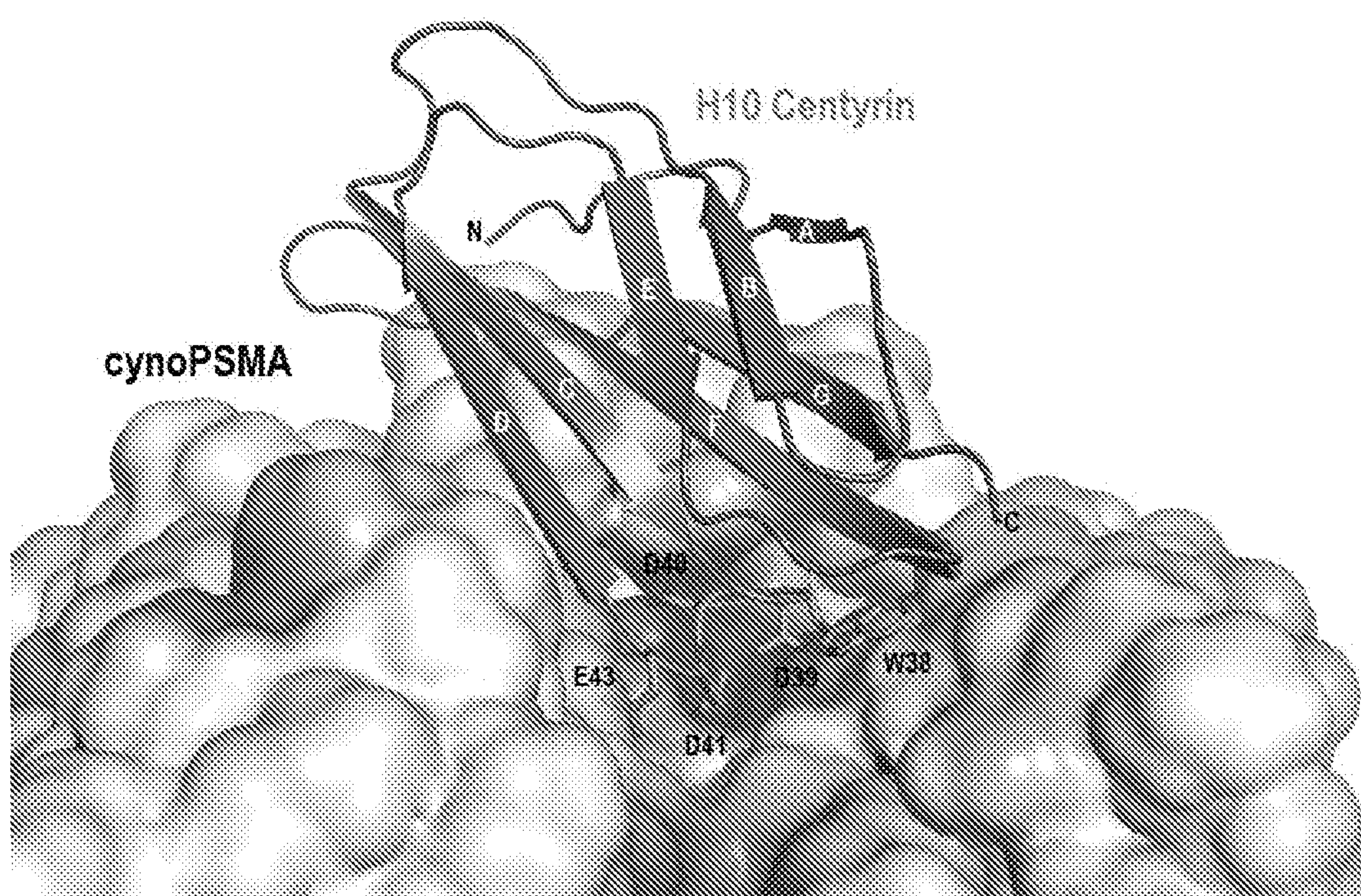
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Fig. 2A



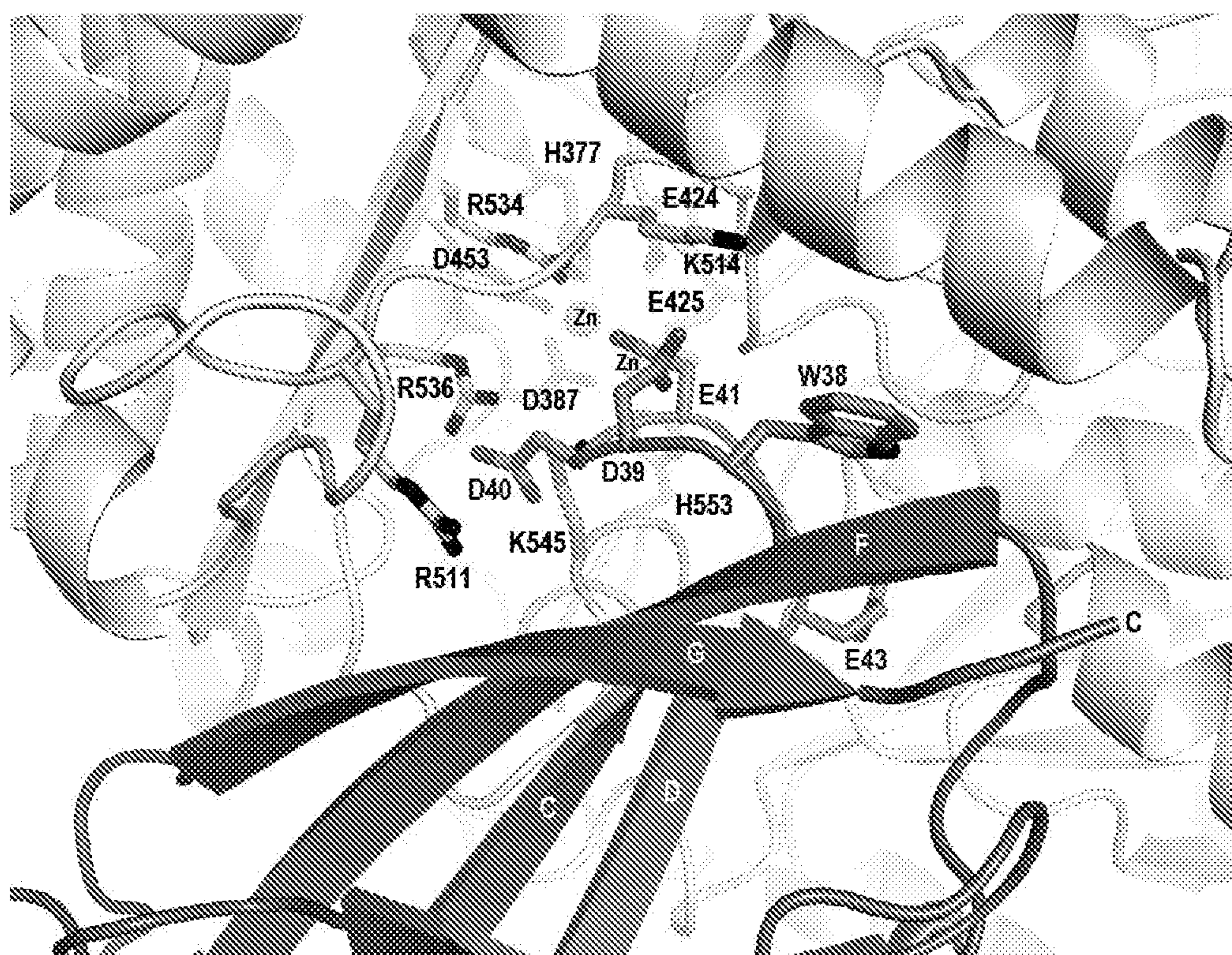
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Fig. 2B



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Fig. 2C



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Fig. 3A

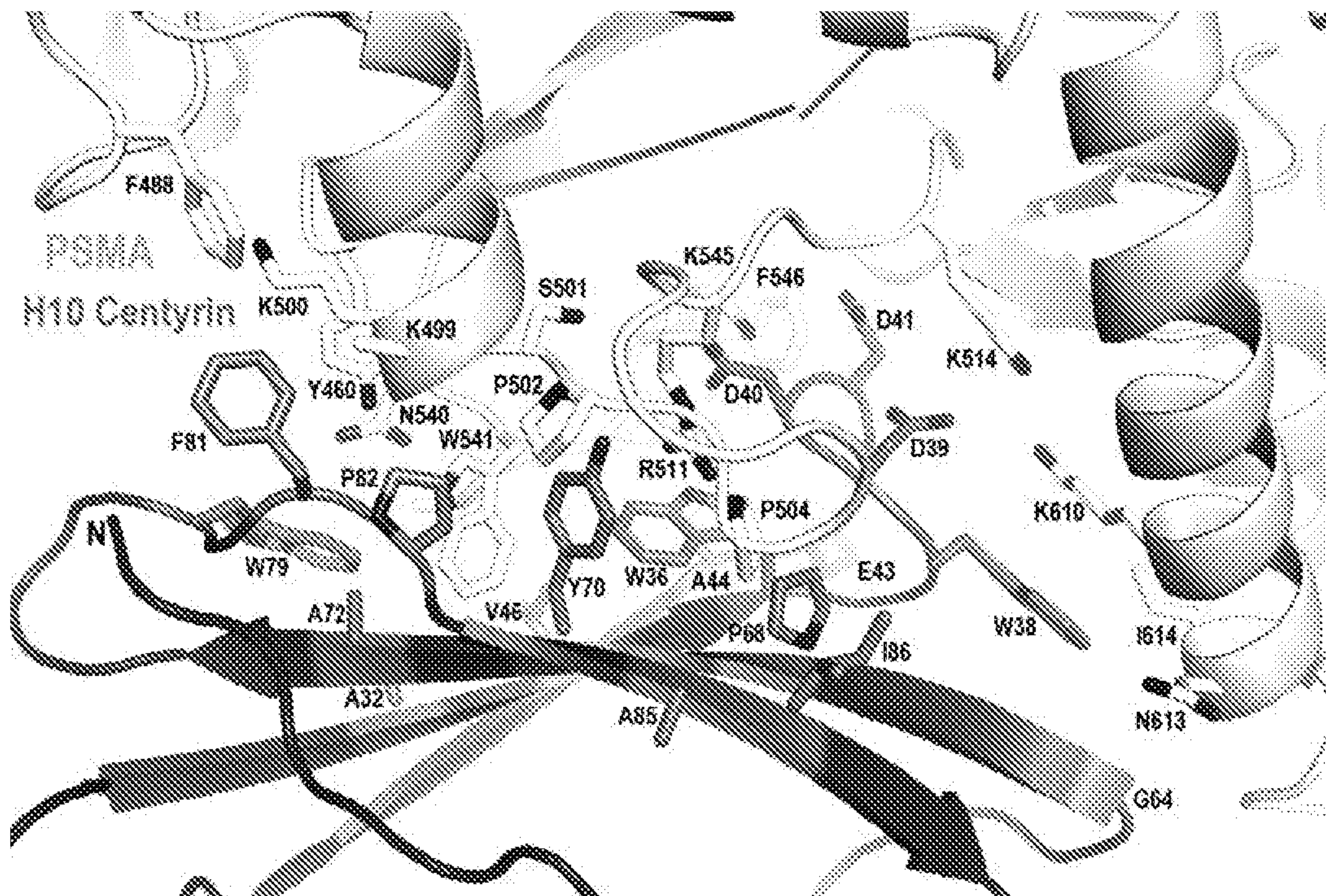
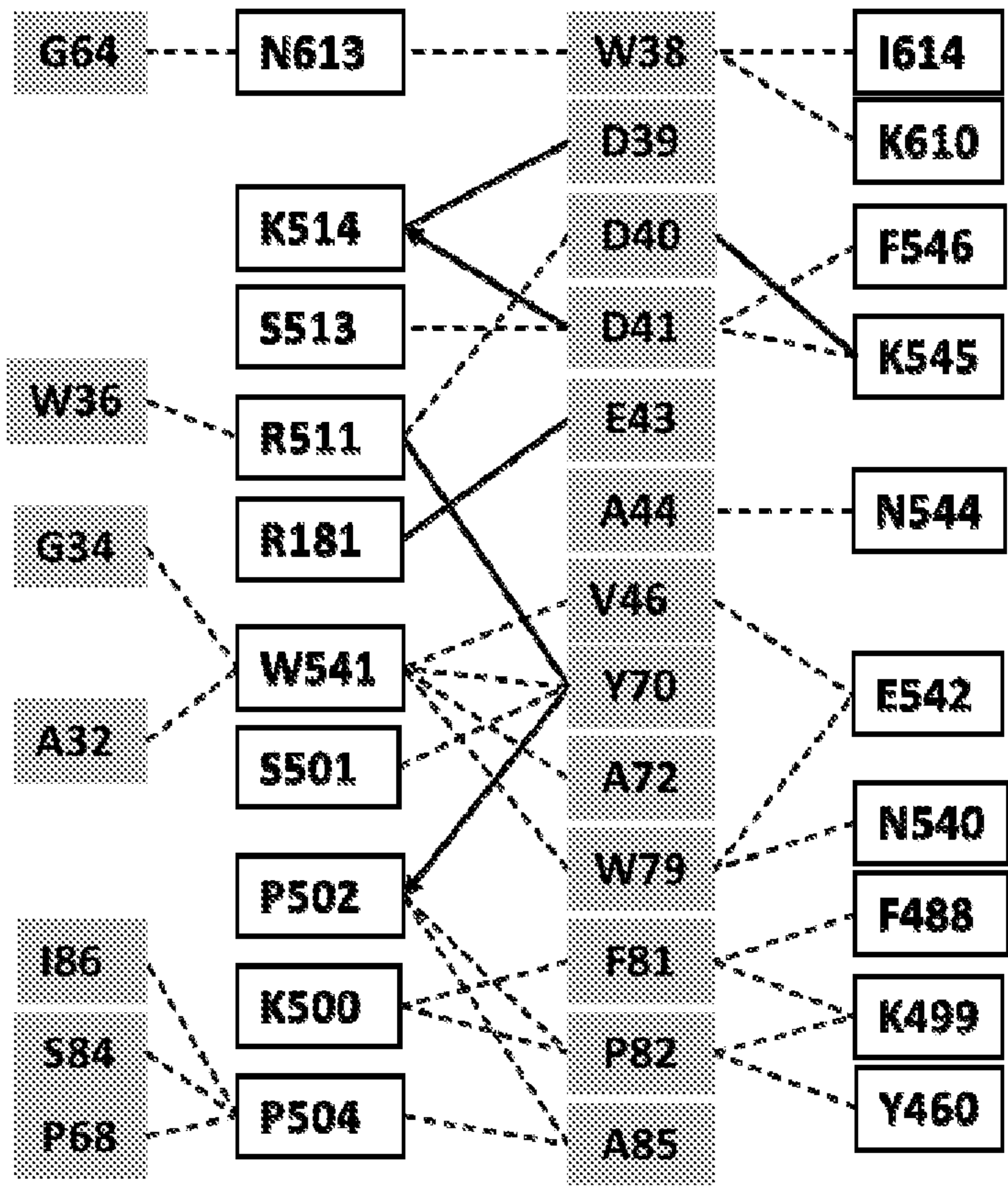


Fig. 3B



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Fig. 4A

h	KSSNEATNITPKHNMKAFLDELKAENIKKFLYNFTQIPHLAGTEQNFQLA
c	KSSSEATNITPKHNMKAFLDELKAENIKKFLHNFTQIPHLAGTEQNFQLA
h	KQIQSQWKEFGLDSVELAHYDVLLSYPNKTHPNYISIINEDGNEIFNTSL
c	KQIQSQWKEFGLDSVELTHYDVLLSYPNKTHPNYISIINEDGNEIFNTSL
h	FEPPPPGYENVSDIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMK
c	FEPPPAGYENVSDIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMK
h	INCSGKIVVIARYGKVFRGNKVKNAQLAGAKGVILYSDPADYFAPGVKSYP
c	INCSGKIVVIARYGKVFRGNKVKNAQLAGATGVILYSDPDDYFAPGVKSYP
h	DGWNLPGGGVQQRGNILNLNGAGDPLTPGYPANAYARRGTAEAVGLPSIP
c	DGWNLPGGGVQQRGNILNLNGAGDPLTPGYPANAYARRGMAEAVGLPSIP
h	VHPIGYYYDAQKLLLEKMGGSAFPDSSWRGSLKVPYNVGPFGFTGNFSTQKVK
c	VHPIGYYYDAQKLLLEKMGGSASPDPSSWRGSLKVPYNVGPFGFTGNFSTQKVK
h	MHIHSTNEVTRIYNVIGTLRGAVEPDRYVILGGHRDSWVFGGIDPQSGAA
c	MHIHSTSEVTRIYNVIGTLRGAVEPDRYVILGGHRDSWVFGGIDPQSGAA
h	VVHEIVRSFGTLKKEGWRPRRTILFASWDAEEFGLLGSTEWAEENSRLLO
c	VVHEIVRSFGMLKKEGWRPRRTILFASWDAEEFGLLGSTEWAEENSRLLO
h	ERGVAYINADSSIEGNYTLRVDCTPLMYSLVHNLTKELEKSPDEGFEGKSL
c	ERGVAYINADSSIEGNYTLRVDCTPLMYSLVYNLTKELESPDEGFEGKSL
h	YESWTKKSPSPPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARYTKNWET
c	YESWTKKSPSPPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARYTKNWET
h	NKFSGYPLYHSVYETYELVEKFYDPMFKYHLLTVAQVRGGMVFELANSTVL
c	NKFSGYPLYHSVYETYELVEKFYDPMFKYHLLTVAQVRGGMVFELANSVVL
h	PFDCRDYAVVLRKYADKIYSISMKHPQEMKTYSVSFDSLFSVKNFTEIA
c	PFDCRDYAVVLRKYADKIYNISMKHPQEMKTYSVSFDSLFSVKNFTEIA
h	SKFSERLQDFDKSNPIVLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYA
c	SKFSERLRDFDKSNPIVLRMMNDQLMFLERAFIDPLGLPDRPFYRHVIYA
h	PSSHNKYAGESFPGIYDALFDIESKVDPSKAWGEVKRQIYVAAFTVQAAA
c	PSSHNKYAGESFPGIYDALFDIESKVDPSQAWGEVKRQISIAFTVQAAA
h	ETLSEVA
c	ETLSEVA

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Fig. 4B

1
LPAPKNLVVSRVTEDSARLSWTAPDAAFDSF**A****G****Y****W****E****W****D****D****D****G****E****A****I****V**LTVP

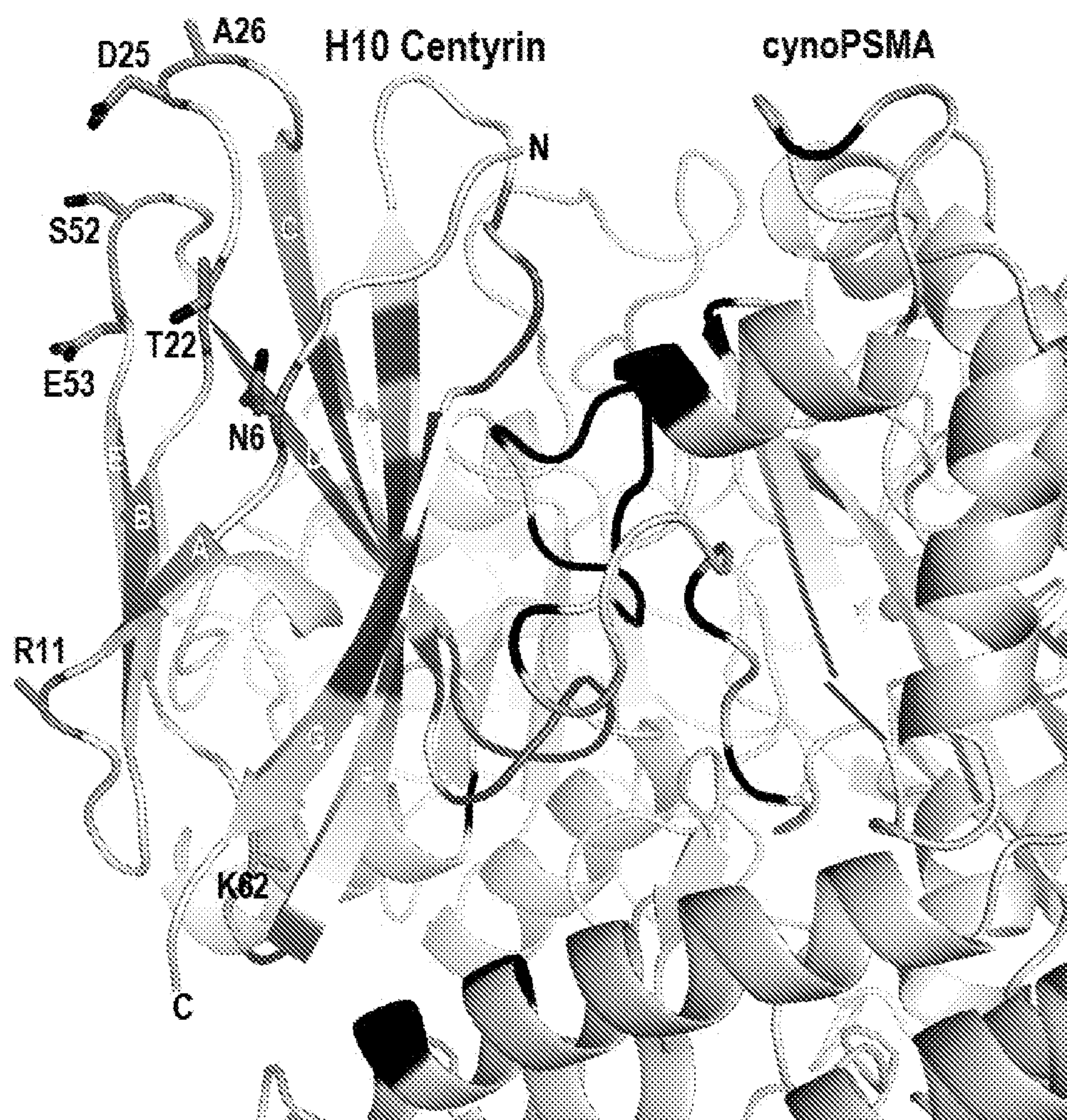
50

51
GSERSYDLTGLK**P****G****T****E****Y****P****V****Y****I****A****G****V****K****G****G****Q****W****S****F****P****L****S****A****I**FTT

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Fig. 5



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Fig. 6

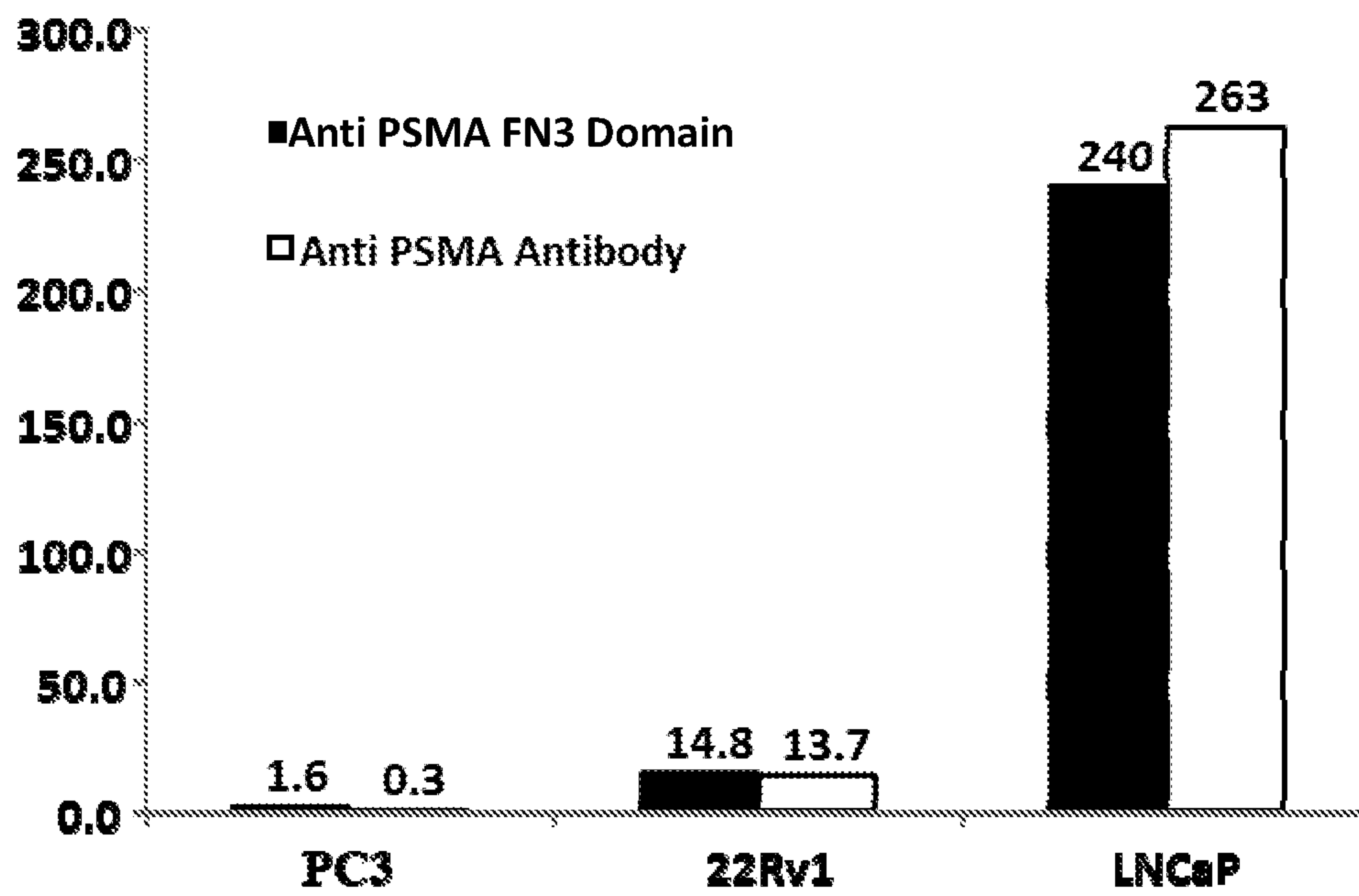


Fig. 7A

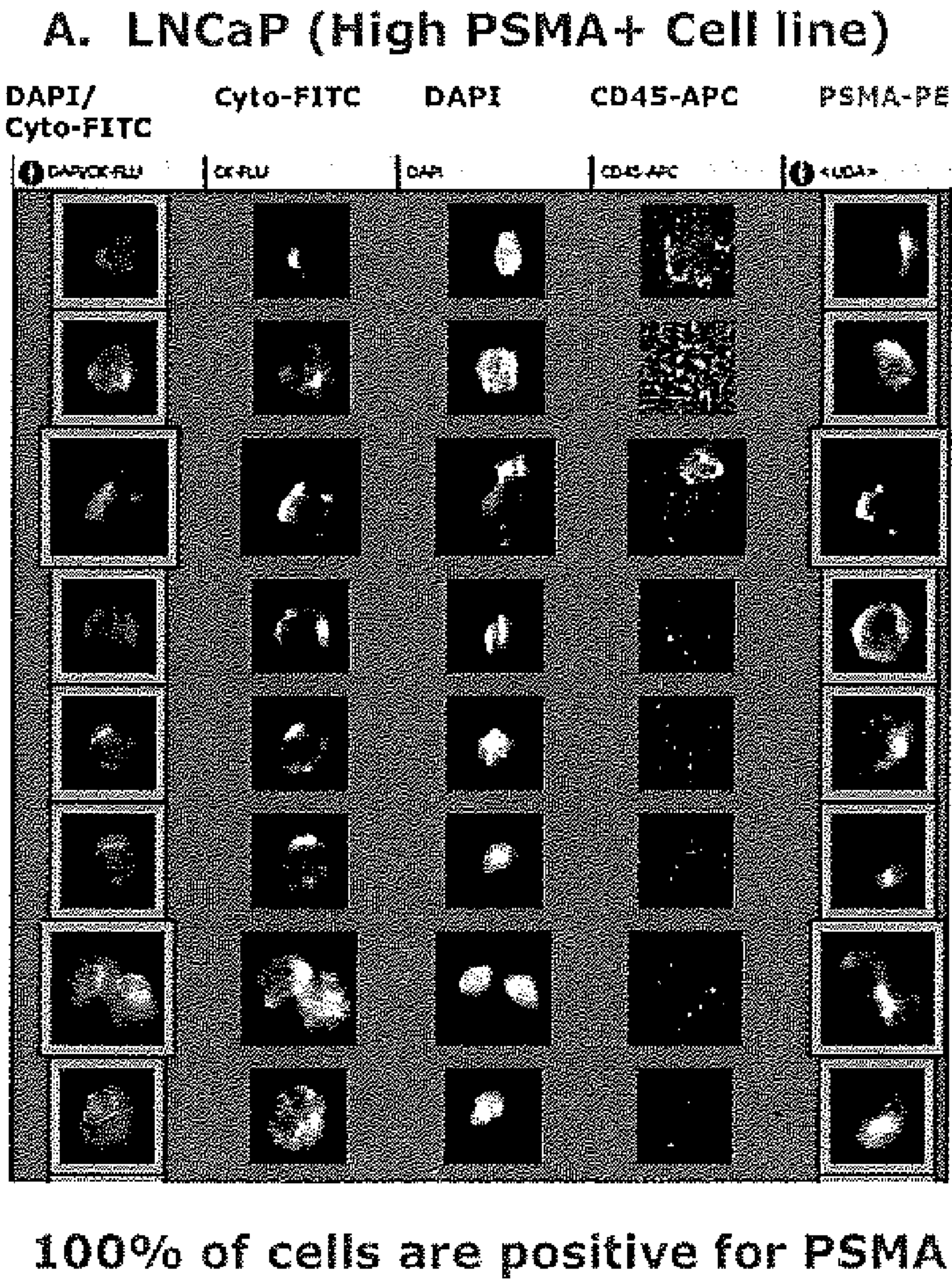
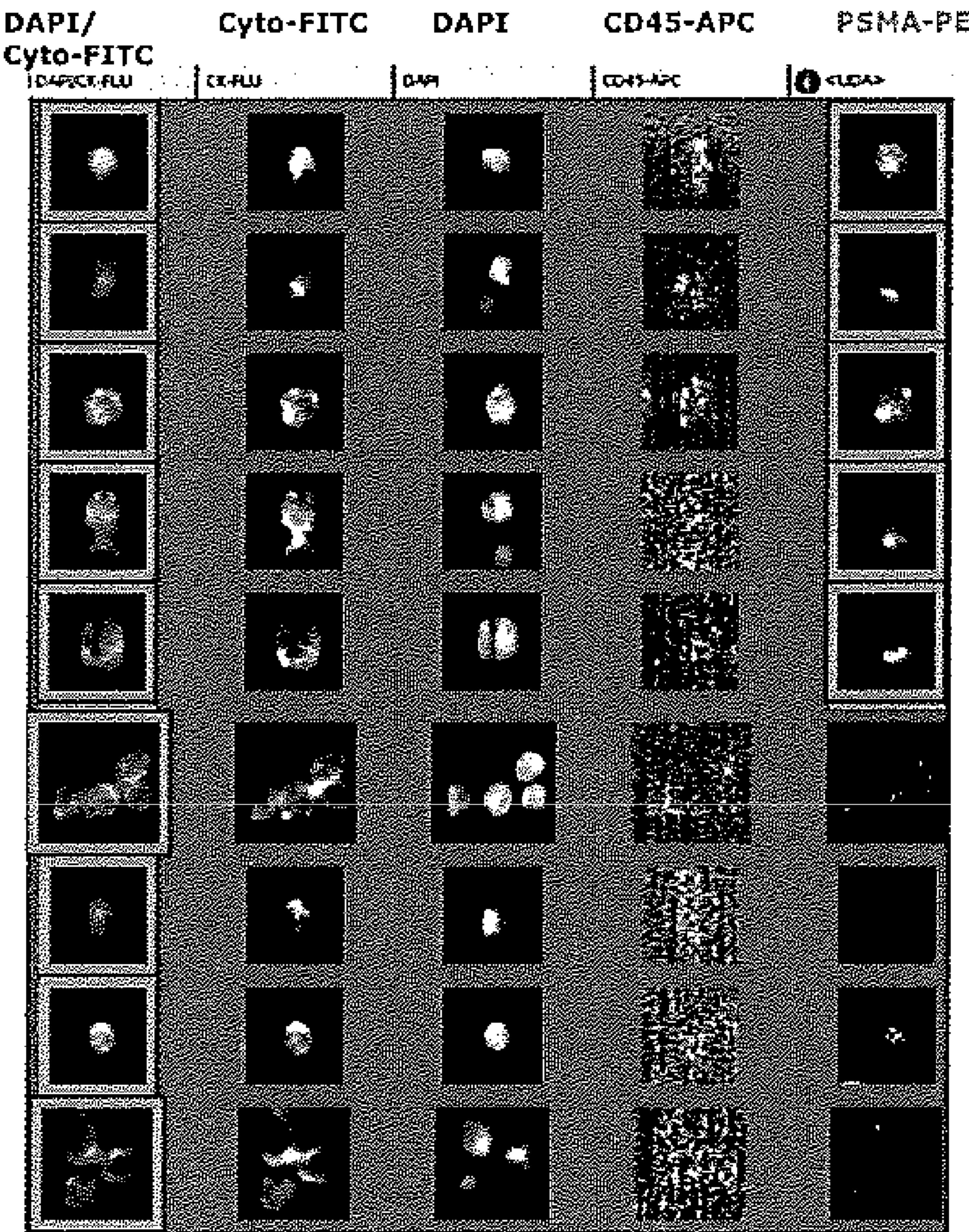


Fig. 7B

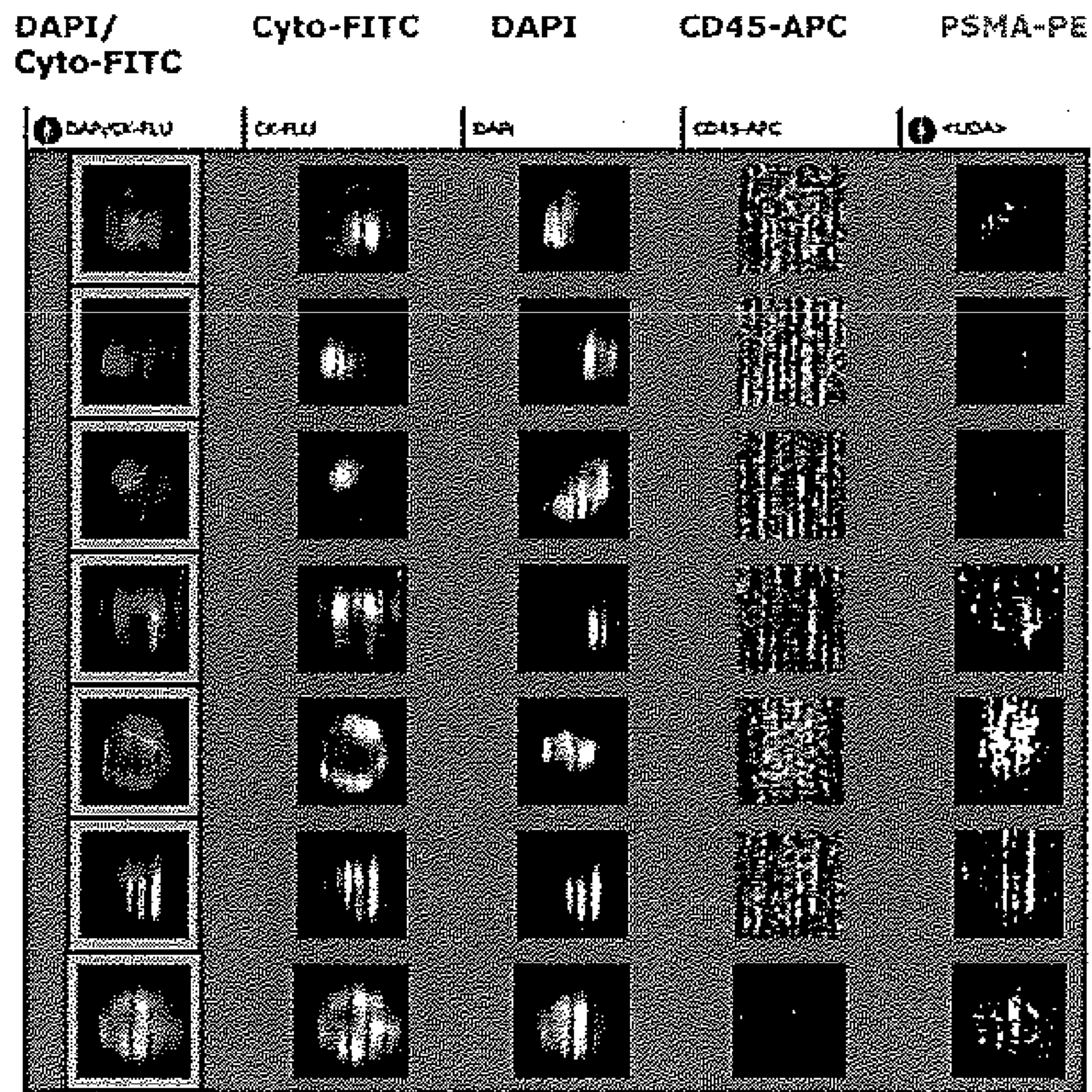
B. 22Rv1 (Low PSMA+ Cell line)



26% of cells are positive for PSMA

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Fig. 7C

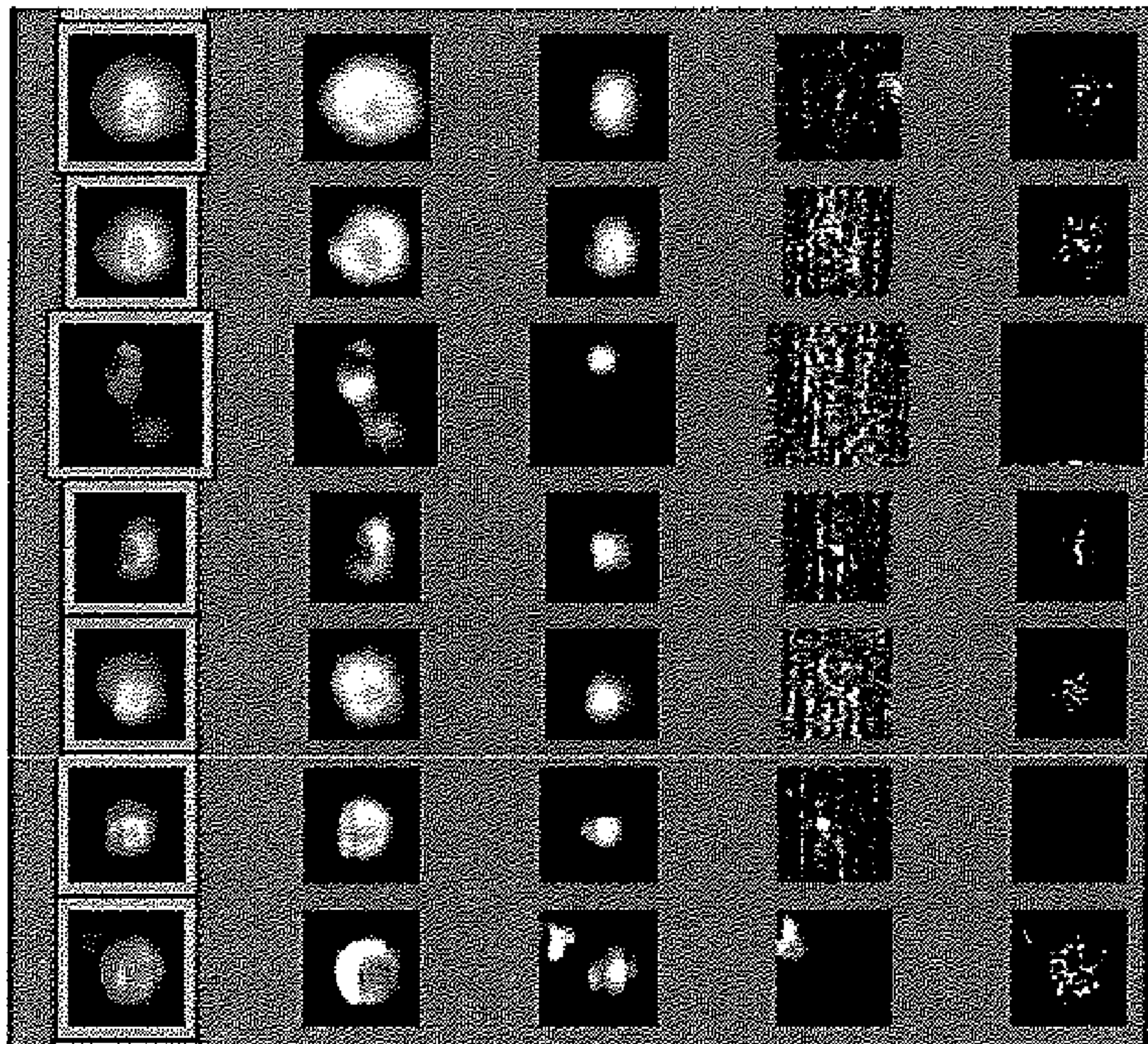
C. PC3 (PSMA- Cell line)**0% of cells are positive for PSMA**

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Fig. 7D

D. SKBR3 (PSMA- Cell line)

DAPI/
Cyto-FITC Cyto-FITC DAPI CD45-APC PSMA-PE



0% of cells are positive for PSMA

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Fig. 8

CDRs in AbM definition are underlined [Abhinandan KR, Martin AC. 2008. *Mol. Immunol.* 45, 3832-3839.]. Ser230 is the last HC residue present in papain-cleaved Fab. Residues 231-455 are from IGHG3_MOUSE (mouse IgG3, isoform 2).

Light Chain (SEQ ID NO:160)

```

      10      20      30      40      50      60
      . | . | . | . | . |
QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNKRAPGV
      70      80      90     100     110     120
      . | . | . | . | . |
PARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNLWVFGGTKLTVLGQPKSSPSVTL
     130     140     150     160     170     180
      . | . | . | . | . |
FPPSSEETNKATLVCTITDFYPGVVTVDWKVDGTPVTQGMETTQPSKQSNKYMASSY
     190     200     210
      . | . | . |
LTLTARAWERHSSYSCQVTHEGHTVEKSLSRADCS

```

Heavy Chain (SEQ ID NO:161)

```

      10      20      30      40      50      60
      . | . | . | . | . |
EVKLLESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYAT
     70     80     90    100    110    120
      . | . | . | . | . |
YYADSVKDRFTISRDDSQSILYLQMNNLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTL
     130    140    150    160    170    180
      . | . | . | . | . |
VTVSAATTTAPSVYPLVPGCSDTSGSSVTLGCLVKGYFPEPVTVKWNYGALSSGVRTVSS
     190    200    210    220    230    240
      . | . | . | . | . |
VLQSAFYSLSSLVTVPSSTWPSQTVICNVAHPASKTELIKRIEPRIPKPSTPPGSSCPPG
     250    260    270    280    290    300
      . | . | . | . | . |
NILGGPSVFIFPPKPKDALMISLTPKVTCVVVDVSEDDPDVHVSFVDNKEVHTAWTQPR
     310    320    330    340    350    360
      . | . | . | . | . |
EAQYNSTFRVVSALPIQHQDWMRGKEFKCKVNNKALPAPIERTISKPKGRAQTPQVYTIP
     370    380    390    400    410    420
      . | . | . | . | . |
PPREQMSKKKVSILTCLVTNFFSEAI SVEWERNGELEQDYKNTPPILDSGTYFLYSKLTV
     430    440    450
      . | . |
DTDSWLQGEIFTCSVVHEALHNHHTQKNLSRSPGK

```

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Fig. 9

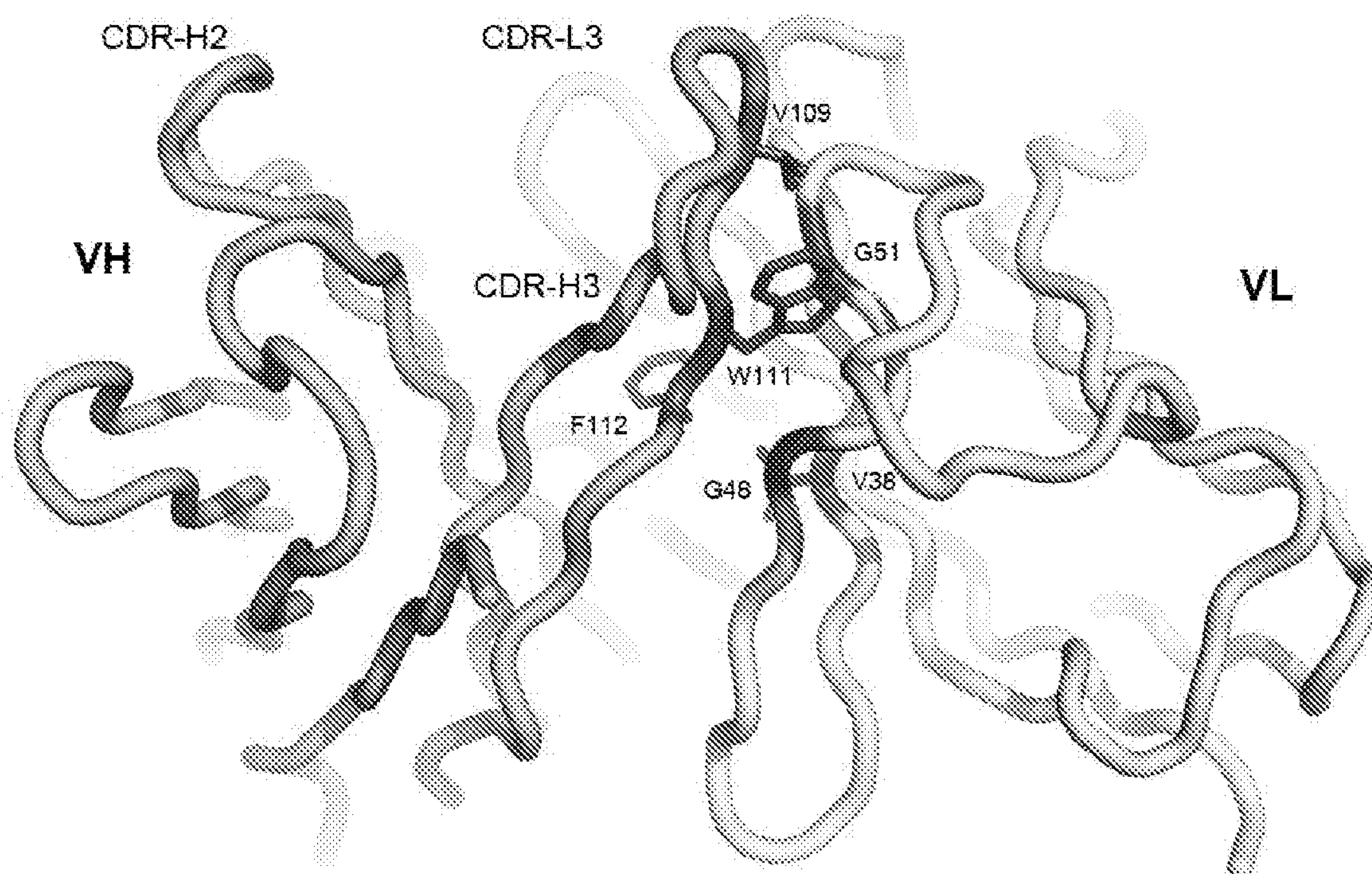


Fig. 10

VH

	10	20	30	40	50	60
						
sp34	EVKLL ES GGGLVQ PK GS LK LS CA AS GFT FNTYAMNWVRQ AP G K GLEW VAR IRSKYNNYATY					
CD3H141	EV QL V ES GGGLVQ P G GS LR LS CA AS GFT FNTYAMNWVRQ AP G K GLEW VA IRSKYNNYATY					
CD3H142	EV QL L ES GGGLVQ P G GS LR LS CA AS GFT FNTYAMNWVRQ AP G K GLEW VA IRSKYNNYATY					
CD3H143	EV QL L ES GGGLVQ P G GS LR LS CA AS GFT FNTYAMNWVRQ AP G K GLEW VA IRSKYNNYATY					
CD3H144	EV QL V ES GGGLVQ P G GS LK LS CA AS GFT FNTYAMNWVRQ AS G K GLEW VG IRSKYNGYATY					

	70	80	90	100	110	120
						
sp34	YADSVKDRFTISRDDS QS ILYLQ M N N LKTEDTAMYYCVRHG N FGNSYVSWFAYWGQ G TLVTVSA					
CD3H141	YA AS V K GRFTISRDDS SK NSLYLQ M N S LKTEDTAVYYC AR HG N FGNSYVSWFAYWGQ G TLVTVSS					
CD3H142	YADSV K GRFTISR D N SK N TLYLQ M N S L RA EDTAVYYC AK HG N FGNSYVSWFAYWGQ G TLVTVSS					
CD3H143	YADSV K GRFTISR D N SK N TLYLQ M N S L RA EDTAVYYC VK HG N FGNSYVSWFAYWGQ G TLVTVSS					
CD3H144	YA AS V K GRFTISRDDS SK NTAYLQ M N S LKTEDTAVYYC TR HG N FGNSYVSWFAYWGQ G TLVTVSS					

VL

	10	20	30	40	50	60
						
sp34	QAVVTQES-ALTTSPGETVTTLT CR SSTGAVTT S NYAN W VQ E KPDHLFTGLIG T GNKR AP GV					
CD3L63	QAVVTQ EP - SLT VSPGGT VT TLT CR SSTGAVTT S NYAN W V Q Q K P G Q AP R G L I G TGNKR AP GT					
CD3L64	Q S V L TQ PP - S V S A A P G Q K V T I S C R S S T G A V T T S N Y A N W V Q L P G T A P K G L I G TGNKR AP G I					
CD3L66	Q T V V TQ EP - SLT VSPGGT VT TLT CR SSTGAVTT S NYAN W V Q Q K P G Q AP R G L I G TGNKR AP GT					

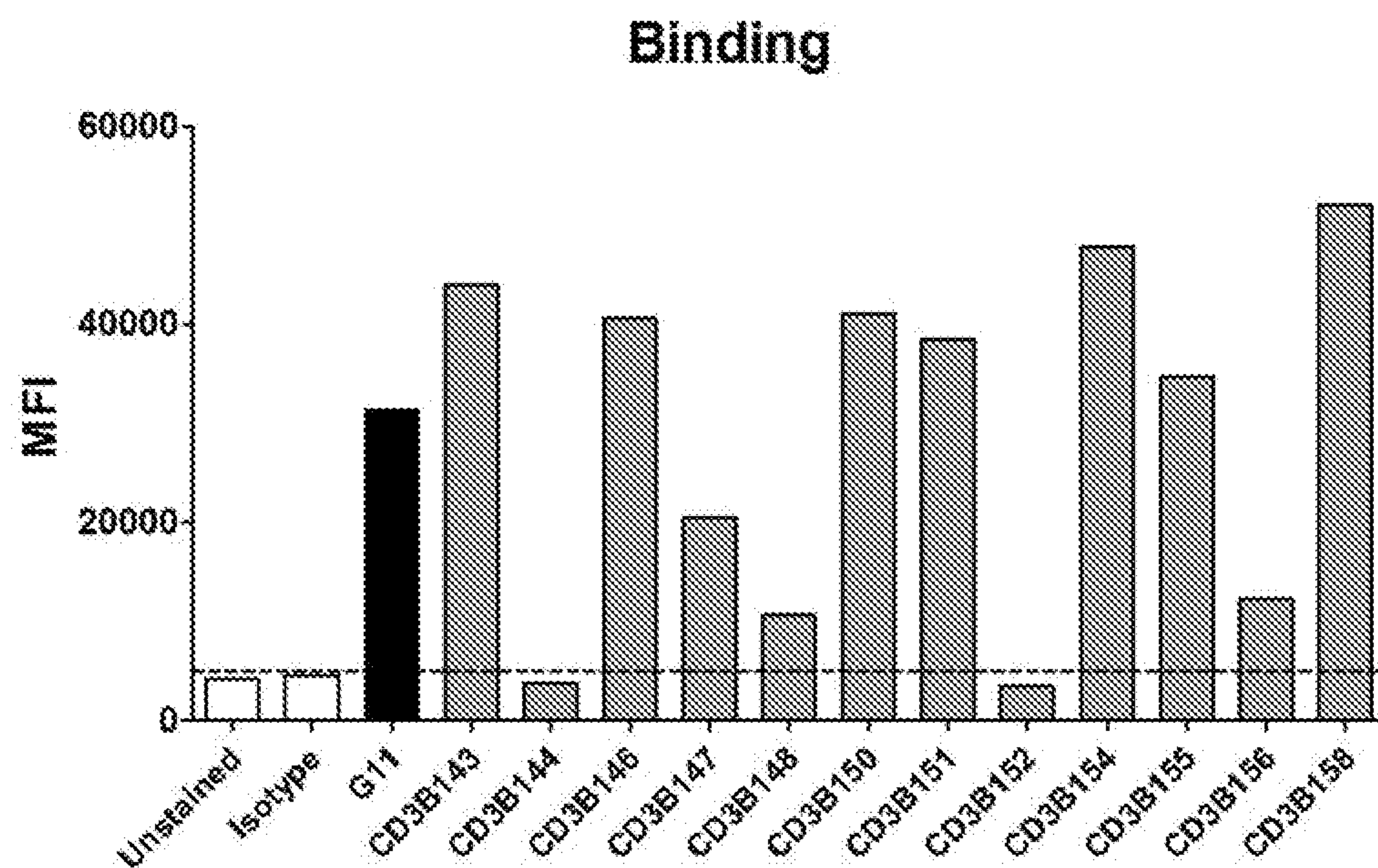
	70	80	90	100	110
					
sp34	PARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNLWVF G GGTKLTVL				
CD3L63	PARFSGSL L G G KAA L T L S GAQ P ED E A E Y Y CALWYSNLWVF G GGTKLTVL...				
CD3L64	PDRFSGS K S G T S A T L G I T G L Q T G D E A D Y Y CALWYSNLWVF G GGTKLTVL...				
CD3L66	PARFSGSL L G G KAA L T L S G V Q P ED E A E Y Y CALWYSNLWVF G GGTKLTVL...				

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Fig. 11

Binding of SP34 HFA variants to primary Human T cells.

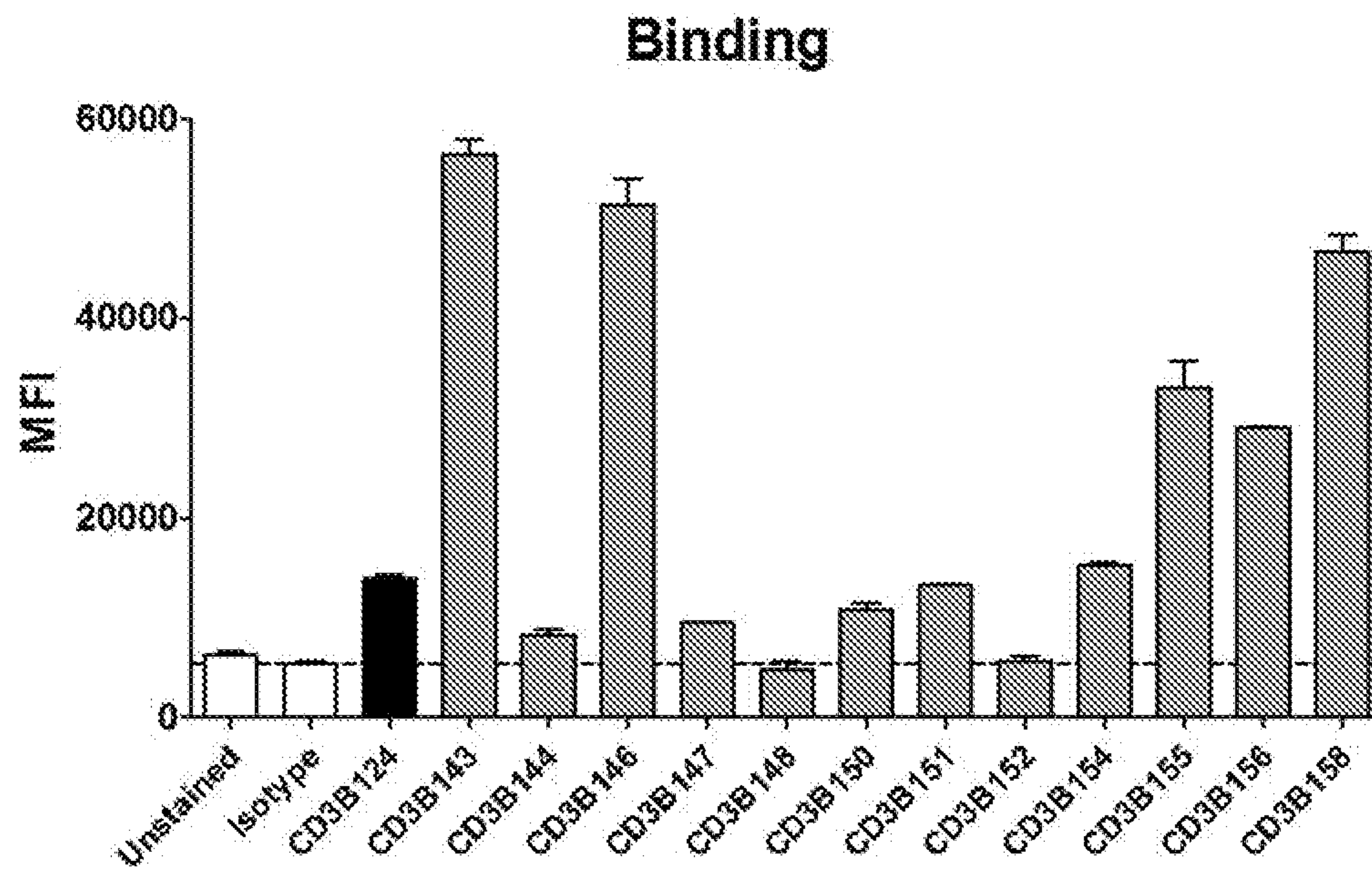
Primary antibodies were used at 12.5 µg/mL.



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Fig. 12

Binding of SP34 HFA variants to primary Cynomolgous primary T cells

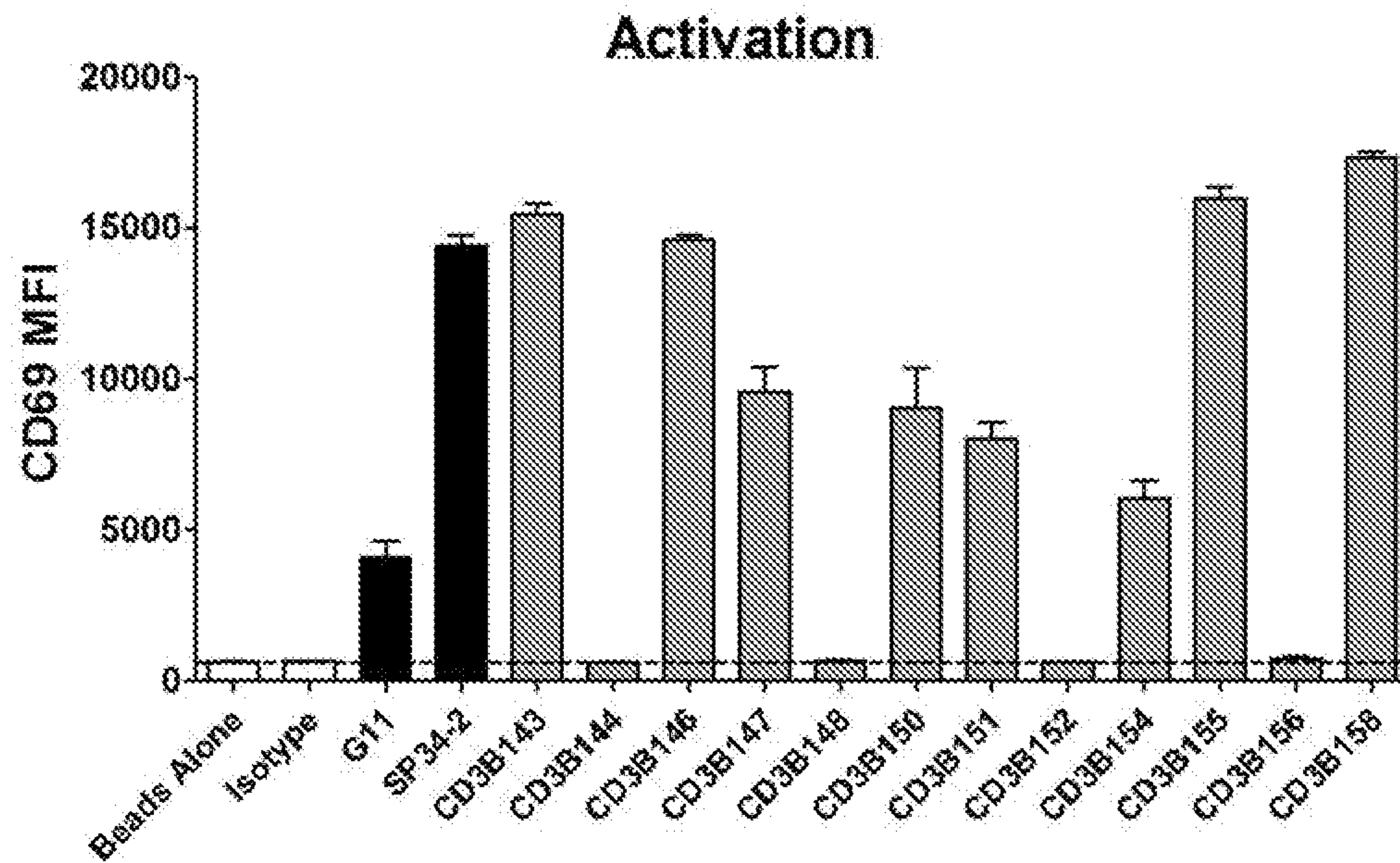


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Fig. 13

SP34 HFA variants Activate primary Human T cells *in vitro*.

Negative and positive controls are shown in white and black, respectively.

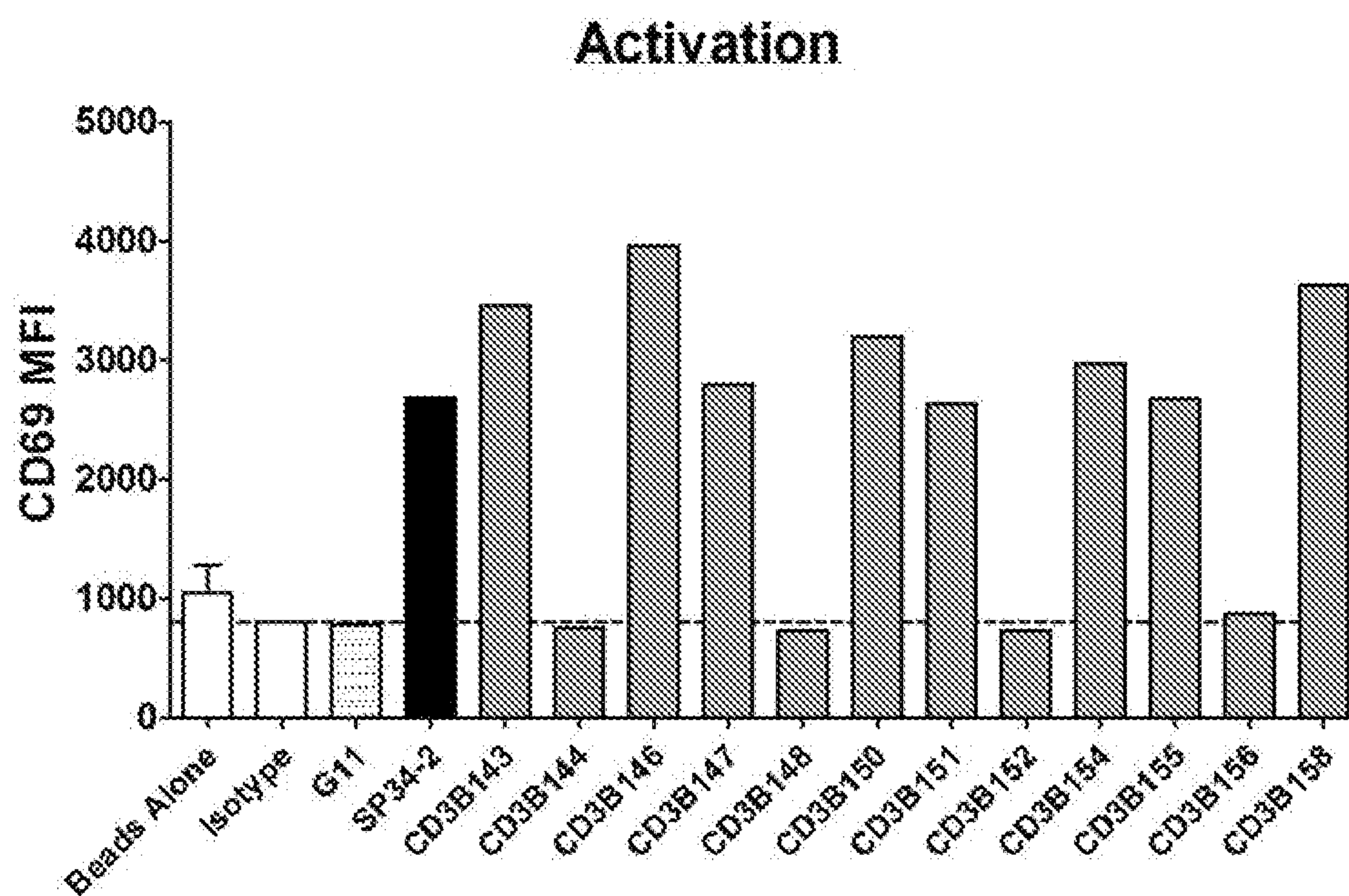


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Fig. 14

SP34 HFA variants Activate primary Cynomolgous T cells *in vitro*.

Negative and positive controls are shown in gray and green, respectively.



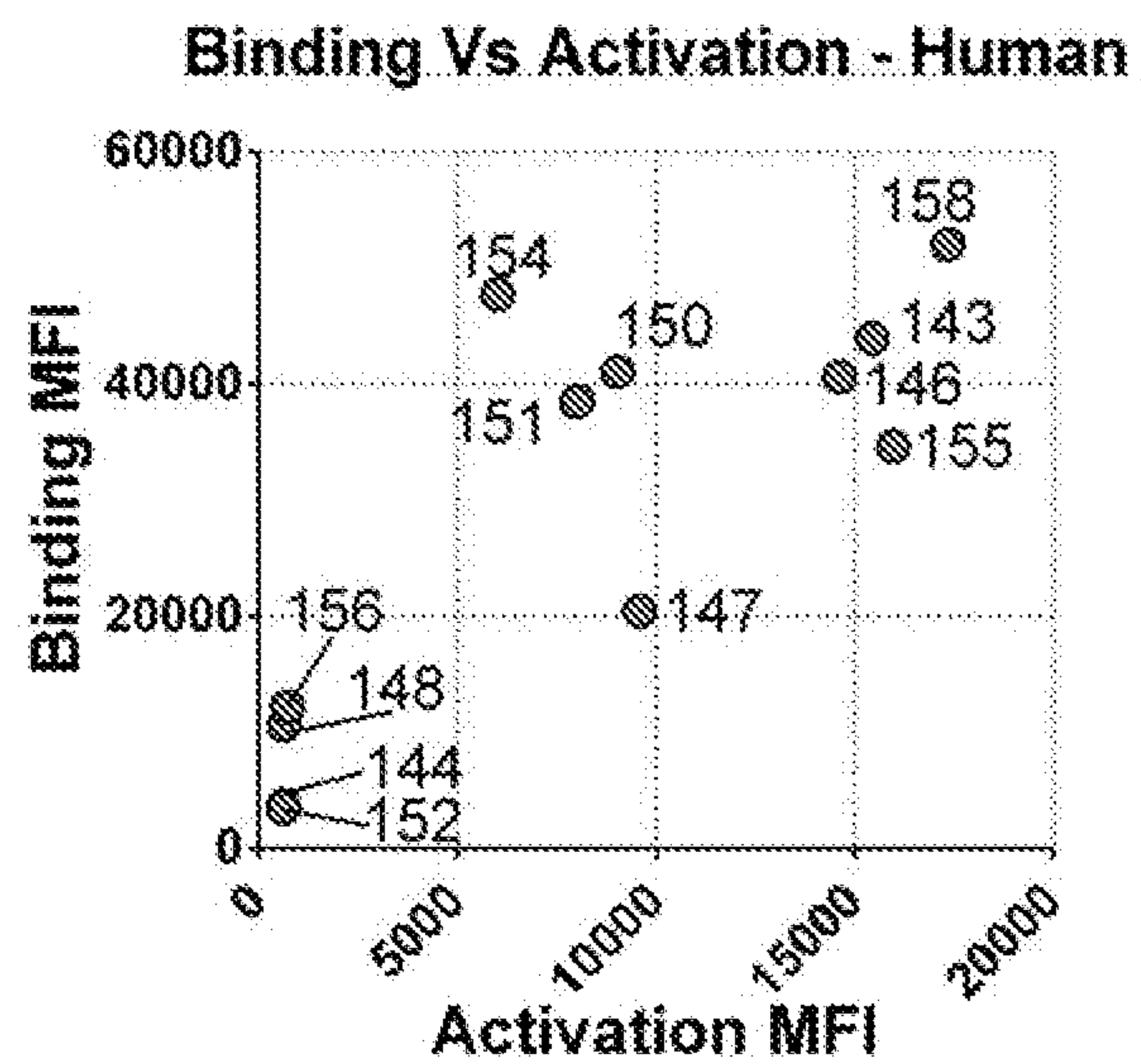
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Figure 15

Correlation of binding and activation by SP34 HFA variants.

Average binding and CD69 MFI values for human (a) and cynomolgus (b) were plotted against each other.

a) Human



b) Cynomolgous monkey

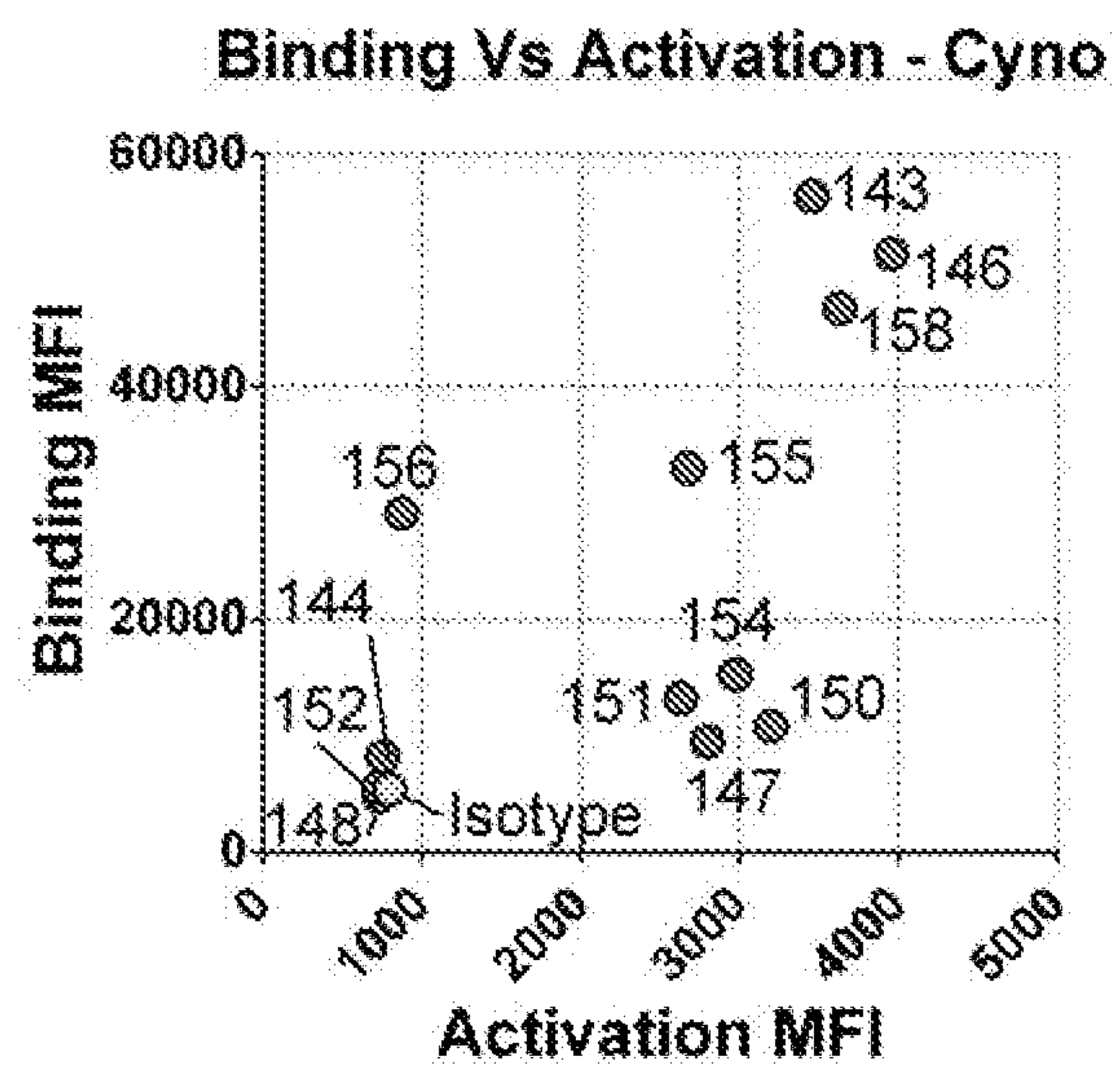
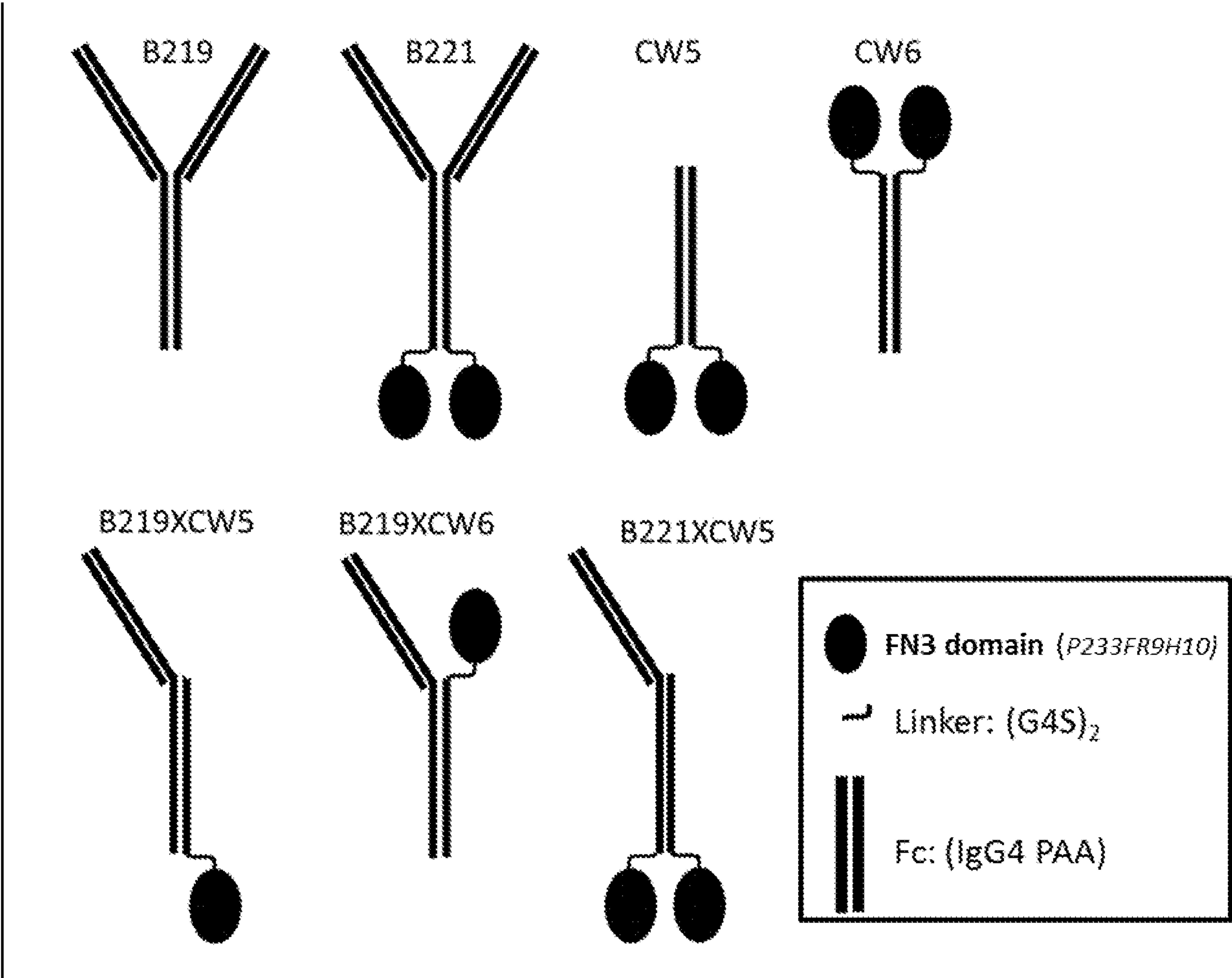


Fig. 16

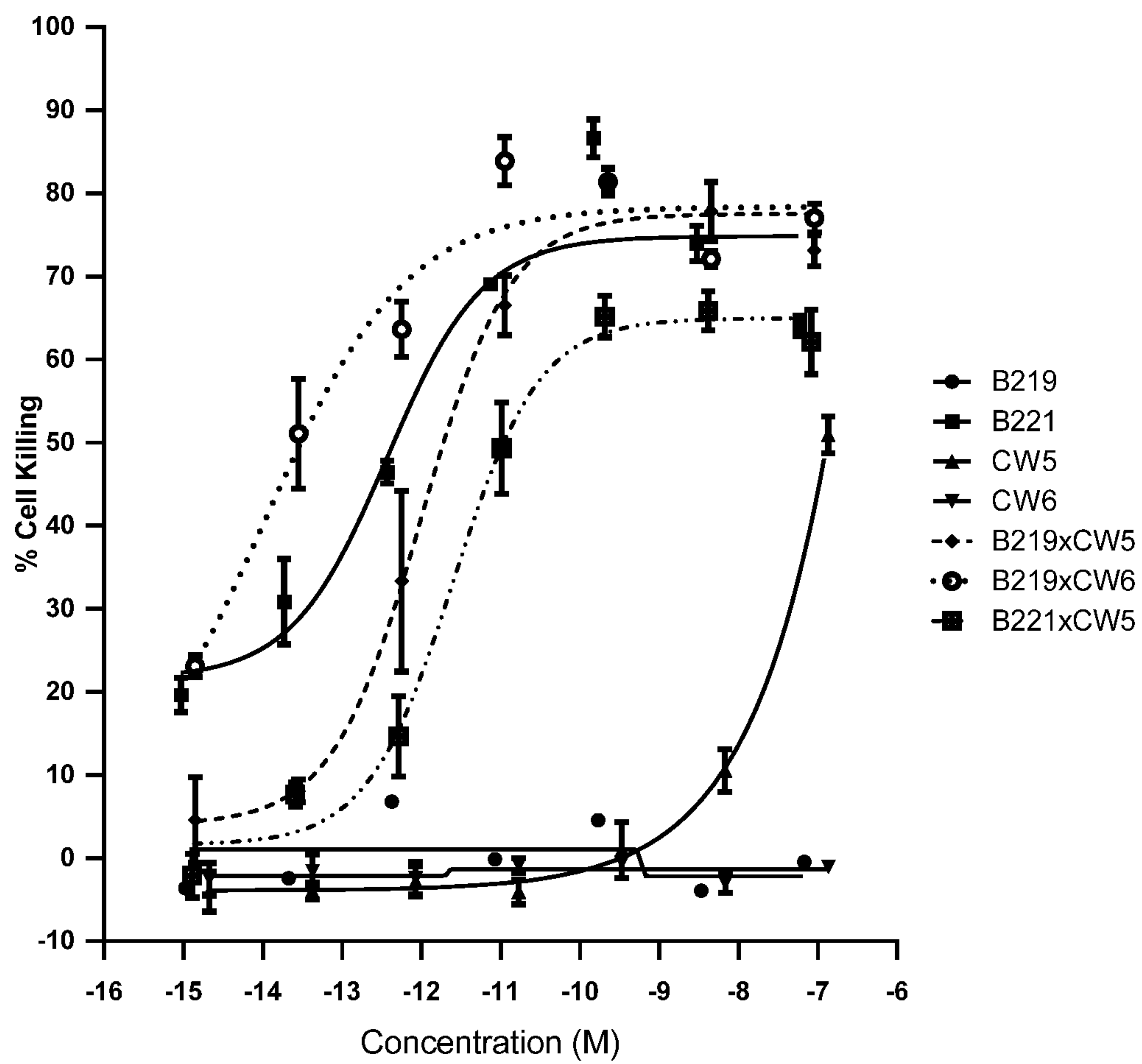
Design of CD3 X PSMA multispecific antigen-binding molecule constructs.



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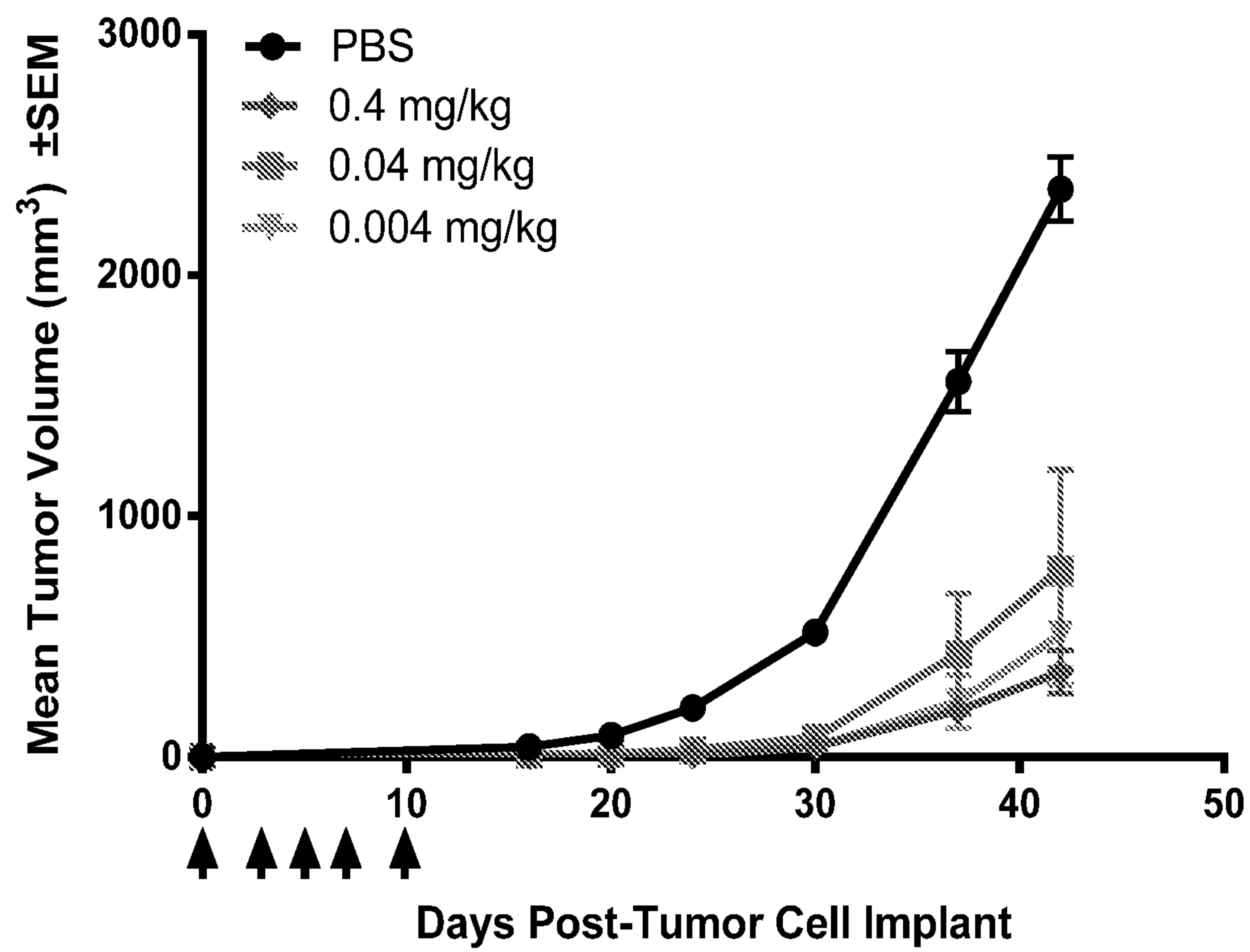
Fig. 17

T-cell mediated cytotoxicity assay



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Fig. 18



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Fig. 19

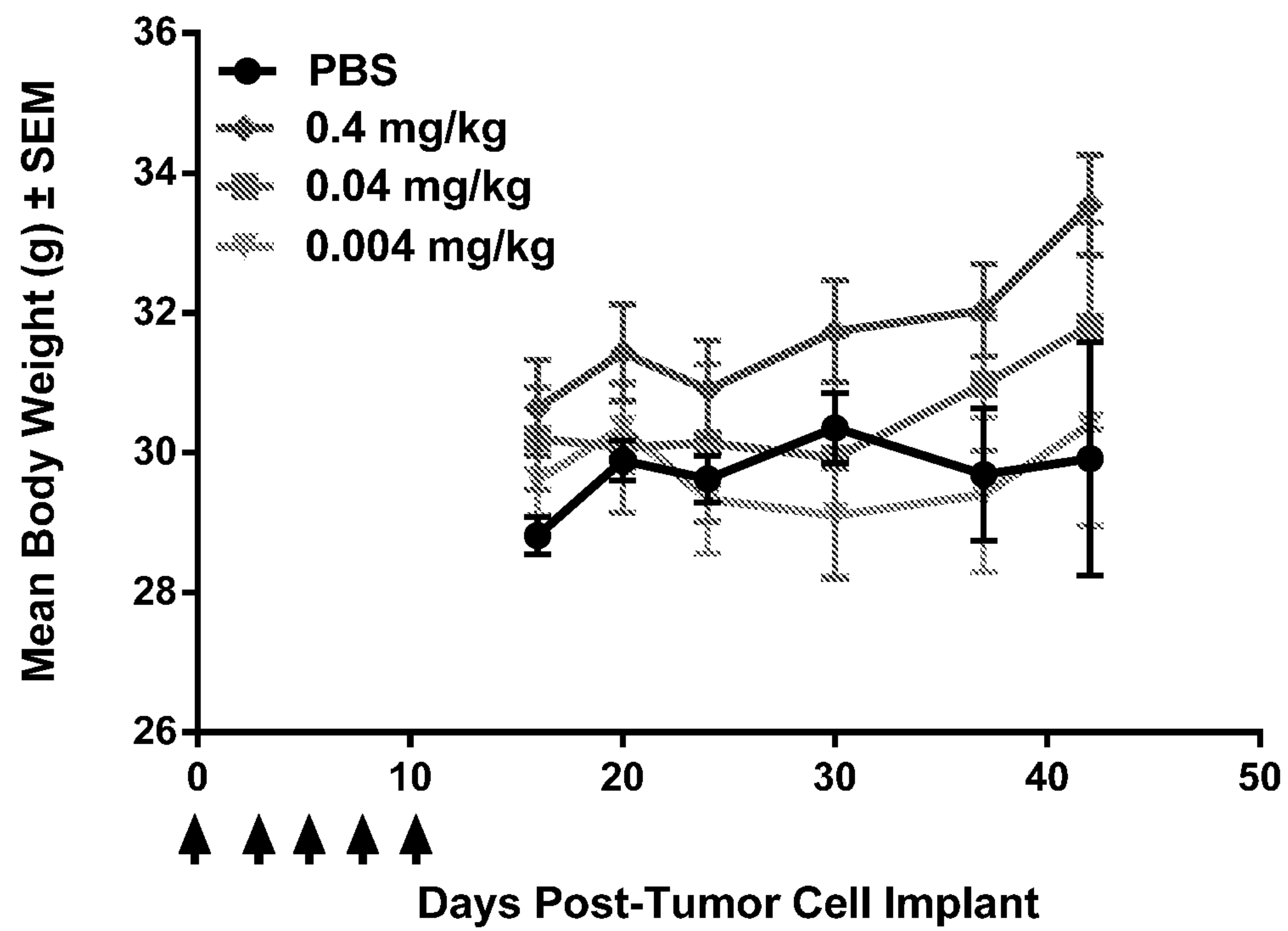


Fig. 1

