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(54) Title: EPITOPE TAGS RECOGNIZED BY SPECIFIC BINDERS

(57) Abstract: The present invention provides peptides useful as epitope tags, which may be fused to a polypeptide of interest, as well as antibodies that specifically bind to these peptides. The peptides and/or antibodies can be used for detecting, immobilizing, isolating or purifying a molecule that is conjugated to such a peptide and/or antibody.

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EPITOPE TAGS RECOGNIZED BY SPECIFIC BINDERS

CROSS REFERENCE TO RELATED APPLICATIONS

[1] The present application claims the benefit of priority of European Patent Application No. 18193663.4 filed on 11 September 2018 and European Patent Application No. 19160485.9 filed on 4 March 2019, the contents of which are hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[2] The present invention provides peptides useful as epitope tags, which may be fused to a polypeptide of interest, as well as antibodies that specifically bind to these peptides. The peptides and/or antibodies can be used for detecting, immobilizing, isolating or purifying a molecule that is conjugated to such a peptide and/or antibody.

BACKGROUND

[3] Epitope tags play an important role in virtually every aspect of life sciences. They are, e.g., used in biotechnological applications in order to facilitate expression and purification of recombinant proteins (Waugh, D. S. Making the most of affinity tags. *Trends Biotechnol* **23,** 316–320 (2005)). In cell biology, epitope tags are often used to monitor the biogenesis or topology of a given protein of interest (POI) (Nooh, M. M. & Bahouth, S. W. Visualization and quantification of GPCR trafficking in mammalian cells by confocal microscopy. *Methods Cell Biol.* **142,** 67–78 (2017); Kocaoglu, O. & Carlson, E. E. Progress and prospects for small-molecule probes of bacterial imaging. *Nat Chem Biol* **12,** 472–478 (2016)). Tags have also been instrumental in immuno-precipitation of protein complexes to be studied with mass spectrometry techniques (Shi, Y. *et al.* A strategy for dissecting the architectures of native macromolecular assemblies. *Nat Methods* **12,** 1135–1138 (2015); Smits, A. H. & Vermeulen, M. Characterizing Protein-Protein Interactions Using Mass Spectrometry: Challenges and Opportunities. *Trends Biotechnol* **34,** 825–834 (2016). Over the years, at least a dozen of different tags evolved providing researchers with multiple tools for most scientific scenarios (Waugh, D. S. Making the most of affinity tags. *Trends Biotechnol* **23,** 316–320 (2005); Brizzard, B. Epitope tagging. *BioTechniques* **44,** 693–695 (2008)). A given tag might, however, perform extraordinarily well in a specific

application while failing completely in others. As a result, most researchers rely on a variety of tags to cover the range of required applications.

[4] It seems that a truly universal tag does not exist so far. One explanation could be the fact that most tags were found as byproducts while screening for binders (typically monoclonal antibodies) against naturally occurring proteins. This is, for example, true for the c-myc-tag (Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* **5,** 3610–3616 (1985)), the HA-tag (Field, J. *et al.* Purification of a RAS-responsive adenylyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. *Mol Cell Biol* **8,** 2159–2165 (1988)) or the Spot-tag® (Virant, D. *et al.* A peptide tag-specific nanobody enables high- quality labeling for dSTORM imaging. *Nat Commun* 1–14 (2018). doi:10.1038/s41467-018-03191-2, Braun, M. B. *et al.* Peptides in headlock - a novel high-affinity and versatile peptide-binding nanobody for proteomics and microscopy. *Sci Rep* **6,** 19211 (2016)). Typically, the tag is thus by default the minimal peptide that is efficiently recognized by the respective binder. As a consequence, the properties of such tags are predominantly defined by chance depending on the selected binder and they generally cannot be re-adjusted to the specific experimental needs or conditions. Alternatively, some tags have also been rationally designed for one specific application. For instance, the His-tag is ideally suited for a crude initial purification of recombinantly expressed proteins on metal ion chelate resins, which was the purpose it was developed for (Hochuli, E., Döbeli, H. & Schacher, A. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr.* **411,** 177–184 (1987)). However, due to the lack of high-affinity binders, the His-tag has so far not been used extensively for microscopic applications in cell biology.

[5] It is object of the invention to provide improved epitope tags and specific binding molecules thereto.

SUMMARY OF THE INVENTION

[6] The present invention relates to fusion protein comprising: (a) a peptide comprising the sequence of X1-X2-X3-X4-X5-X6-X7-X8-X9-R-L-X12-X13 (SEQ ID NO: 01), wherein X1 is G or S or T or P, X2 is R or G or A or E or P, X3 is L or V, X4 is E or Q, X5 is E or Q, X6 is E or Q, X7 is L or I or V, X8 is R or A or Q or E, X9 is R or A or Q or E, X12 is S or T or D or E or P or A or no amino acid, and wherein X13 is E or K or P or S or A or D or no amino acid; and (b) a polypeptide.

[7] The present invention also relates to an antibody that specifically binds to the peptide

comprised in the fusion protein of the invention.

[8] The present invention also relates to a fusion protein comprising a peptide that the antibody of the invention binds to.

[9] The present invention also relates to a complex comprising a fusion protein of the invention and an antibody of the invention.

[10] The present invention also relates to a nucleic acid encoding a fusion protein of the invention or an antibody of the invention.

[11] The present invention also relates to a vector comprising the nucleic acid of the invention.

[12] The present invention also relates to a host cell comprising a nucleic acid of the invention or a vector of the invention or expressing a fusion protein of the invention or the antibody of the invention.

[13] The present invention also relates to a use of an antibody of the invention for the detection, immobilization, isolation, or purification of a fusion protein of the invention.

[14] The present invention also relates to a method of detecting a fusion protein of the invention, comprising contacting the fusion protein with an antibody of the invention.

[15] The present invention also relates to a method of isolating the fusion protein of the invention, comprising contacting the fusion protein with an antibody of the invention. Where the fusion protein of the invention comprises an antibody moiety, the present invention also relates to a method of isolation of a specific target of the antibody moiety.

[16] The present invention also relates to a kit comprising a nucleic acid or a nucleic acid expression construct encoding a peptide as comprised in a fusion protein of the invention and optionally an antibody of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[17] **Fig. 1: Interaction of ALFAST and ALFA^{PE} with NbALFAST**

[18] **A**, Sketch of proteins used for ALFA binding assays. In this figure, the ALFA tag used in shGFP2 fusions can either be ALFAST (SEQ ID NOs: 05-07) or ALFA^{PE} (SEQ ID NO: 33). **B**, 20µl ALFA SelectorST resin presenting NbALFAST (SEQ ID NO: 133) was saturated with a GFP variant (shGFP2, Frey and Görlich; Cell. 2018 Jun 28;174(1):202-217.e9. doi: 10.1016/j.cell.2018.05.045) fused to ALFAST or ALFA^{PE} at different locations (internal (left); N-terminus (middle) or C-terminus (right)). After washing 4 times with PBS, the beads were suspended in a 10-fold excess of PBS containing 200µM ALFAST peptide (Ac-PSRLEEELRRRLTEP-Amide, SEQ ID NO: 179) and gently mixed at room temperature. At indicated time points, specific elution from the beads was quantified using the GFP

fluorescence released into the supernatant. Shown are mean fluorescence values from three independent experiments performed in parallel and standard deviations for each time point. Efficient peptide elution of ALFA^{PE} fusions is observed already after 20-30 min at room temperature. In contrast, all GFP variants fused to ALFAST remained tightly bound to the resin. Note that the elution kinetics is largely independent of the localization of the respective ALFA tag variant within the fusion protein. **C**, 10µl of ALFA SelectorST resin saturated with either ALFAST-shGFP2 (top row) or ALFA^{PE}-shGFP2 (bottom row) were transferred into 8-well PCR strips. After removing remaining liquid, the beads were incubated with 100µl of the indicated substances for 60 min at room temperature. Photos were taken after sedimentation of the beads. Asterisks (*) indicate conditions known to lead to a partial or complete loss of GFP fluorescence.

[19] Fig. 2 Pull-down of ALFA-tagged target proteins and protein complexes from complex lysates using ALFA SelectorST.

[20] **A**, shGFP2 N-terminally fused to ALFAST (SEQ ID NO: 07; ALFAST-sfGFP2, left) or ALFA^{PE} (SEQ ID NO: 33; ALFA^{PE}-GFP, right). Proteins were over-expressed in E.coli and purified by Nickel-affinity chromatography via their C-terminal His_6-tag followed by gel filtration on a Superdex 75 size exclusion column. **B+C**, To obtain defined input material for one-step affinity purifications using the ALFA SelectorST, *E.coli* (**B**) or HeLa (**C**) mock extracts were blended with 3µM of the respective substrate. Mock lysate served as a specificity control. 1mL of each lysate/substrate mixture was incubated with 25µl of ALFA-SelectorST comprising the NbALFAST (SEQ ID NO: 133) for 1h at 4°C. After washing 4 times with 1mL of PBS, bound proteins were eluted two times for 10 min with 25µl of 200 µM of ALFAST peptide (SEQ ID NO: 179) in PBS at room temperature. Proteins remaining on the beads were afterwards eluted with SDS sample buffer. 0.5 µL (**B**) or 1.5 µL (**C**) of input and non-bound fractions were analyzed by SDS-PAGE (12%) and Coomassie staining. Shown eluate fractions correspond to the material eluted from 1 µl of ALFA SelectorST resin. Note that protein tagged with either ALFAST tag or ALFA^{PE} tag can be specifically pulled down under native conditions using ALFA SelectorST. Highly efficient and specific elution of proteins fused to the ALFA^{PE} tag can be accomplished under native conditions by competition with free ALFAST peptide. Further note that the proteins purified from either lysate using the ALFA SelectorST resin contain significantly less impurities than the respective substrate proteins purified by conventional two-step chromatography.

[21] **D**, Left: Sketch of the YfgM-PpiD complex. Right: Non-tagged (-) (SEQ ID NO: 198) or C-terminally ALFA^{PE} tagged YfgM (+) (SEQ ID NO: 197) was expressed in a

yfgM deletion stain. Membrane protein complexes were solubilized using 1% DDM from total lysate. YfgM-ALFA^{PE}-containing complexes were purified in a single step using the ALFA SelectorST affinity resin comprising the nanobody of SEQ ID NO: 133. A serum raised against the YfgM-PpiD complex recognized both, PpiD and YfgM, in the input fractions. ALFA SelectorST specifically immunoprecipitated the native protein complex of YfgM-ALFA^{PE} and its interaction partner PpiD.

[22] **Fig. 3: Nanobody-based detection of ALFAST-tagged proteins in immunofluorescence applications**

[23] **A:** Sketch of NbALFAST bound to ALFAST tags (left) or ALFA^{PE} tags (right). Given are ALFA tag sequences used for tagging at various positions (N-terminal ALFAST tag: SEQ ID NO: 05, internal ALFAST tag: SEQ ID NO: 06, C-terminal ALFAST tag: SEQ ID NO: 07)

[24] **B:** Sequence of NbALFAST (NbALFA clone 1G5; SEQ ID NO: 133). Grey boxes indicate CDRs 1-3 (AbM definition, SEQ ID NOs: 115-117).

[25] **C:** COS-7 cells transfected with Tom70-EGFP-ALFAST (upper row) or Tom70-EGFP-ALFA^{PE} (lower row) were fixed with 4% paraformaldehyde. Staining with NbALFAST coupled to AbberiorStar635P (FluoTag-X2 anti-ALFA AbberiorStar635P) was performed after permeabilization. First column: FluoTag-X2 anti-ALFA; second column: target detection using the intrinsic EGFP fluorescence; third column: overlay incl. DAPI stain; fourth column: Sketch of target proteins detected by fluorescently labeled NbALFAST All scale bars: 20µm.

[26] **D:** N-terminally ALFAST-tagged Vimentin (upper row) or ALFA^{PE}-tagged Vimentin (lower row) was detected with FluoTag-X2 anti-ALFA AbberiorStar635P after fixation with 4% paraformaldehyde (PFA), 2% glutaraldehyde (GA), or 100% Methanol (MeOH). Right column: Sketch of ALFA-tagged vimentins detected by fluorescently labeled NbALFAST.

[27] **E:** Intrabody-based detection of ALFAST-tagged proteins. COS-7 cells were co-transfected with an NbALFAST-mScarlet-I fusion and ALFAST-tagged target proteins. Target proteins were detected via EGFP fluorescence (for TOM70-EGFP-ALFAST) or immunofluorescence using FluoTag anti-ALFA AbberiorStar635P (for ALFAST-FLAG-Vimentin). In parallel, NbALFAST-mScarlet-I was detected by the red mScarlet-I fluorescence. Note the excellent co-localization between the target protein (left column) and the mScarlet-I signal (middle column).

[28] **Fig. 4: ALFA-tagged proteins can be detected by fluorescently labeled**

NbALFAST regardless of its localization within the fusion protein

[29] COS-7 cells were transfected with constructs encoding proteins fused to an ALFA tag at their N-termini (ALFAST-FLAG-Vimentin or ALFA^{PE}-FLAG-Vimentin; A), or within individual protein-domains (EGFP-ALFAST-myc-TM; B). Cells were fixed with 4% PFA and stained as indicated. For A and B, cells were permeabilized with 0.1% TritonX-100; for C, cells were stained under non-permeabilizing conditions. TM: transmembrane domain. Sketches illustrate the topology of substrates and detection by fluorescently labeled NbALFAST (FluoTag-X2 anti-ALFA).

[30] **Fig. 5: GFP fused to N- or C-terminal ALFAST tags show normal intracellular localization**

[31] 3T3 cells were transiently transfected with EGFP fusions harboring N- or C-terminal ALFAST tags. Non-tagged EGFP from pEGFP-N1 served as a control. The localization of the respective EGFP variants was analyzed on 6-7 individual images for each construct. Together 120-130 cells were imaged per construct and the localization of EGFP was analyzed. In general, each EGFP construct displayed a distribution across the cytosol and the nucleus. Cells were distributed into three groups (”slightly nuclear”, “uniform” and “other”) according to the observed nucleocytoplasmic localization of EGFP. Standard deviations were deduced from values obtained from individual images. Differences between the localizations of tagged and non-tagged EGFP variants were statistically insignificant (Student’s t-test).

[32] **Fig. 6: Western-blot and dot-blot detection of ALFA-tagged target proteins using fluorescently labeled NbALFAST (FluoTag-X2 anti-ALFA)**

[33] **A**, COS-7 cells transfected with ALFAST-FLAG-Vimentin or ALFA^{PE}-FLAG-Vimentin were lysed in SDS buffer. Cells transfected with an irrelevant plasmid served as a control. Lysates corresponding to the same number of cells were analyzed by SDS-PAGE and Western-Blot. The vimentin fusion proteins were visualized with NbALFAST coupled to IRDye800 (FluoTag-X2 anti-ALFA IRDye800). Tubulin served as a loading control and was detected by a mouse anti-Tubulin followed by a FluoTag-X2 anti-Mouse coupled to IRDye680. Complete lanes are shown in Fig. 7A.

[34] **B,** Sketch of recombinant E.coli maltose-binding protein (MBP) harboring multiple epitope tags (FLAG, HA, myc and ALFAST) used for experiment shown in C and D.

[35] **C,** Dilution series of the protein sketched in B were spotted onto nitrocellulose membranes. Established monoclonal antibodies (anti-FLAG M2 – Sigma #F1804, anti-myc 9E10 – SynapticSystems #343 011, anti-HA F-7 – SantaCruz #sc-7392) were used in

combination with a secondary anti-mouse IgG IRDye800CW (Li-Cor #925-32210, dilution 1:1000) to detect FLAG, myc and HA-tag, respectively. The ALFAST tag was detected using a FluoTag-X2 anti-ALFA directly coupled to IRDye800CW. The nanobody and all primary antibodies were used at 2.7nM final concentration, which is well within the range recommended by the suppliers. The complete experiment including internal controls is shown in Fig. 7B.

[36] **D,** Quantification of signals obtained in C, displayed in a double logarithmic plot. Lines represent linear fits to the obtained values. Even without signal amplification by a secondary antibody, signals obtained by NbALFAST were 3- to >10-times stronger than by established reagents recognizing epitope tags. At the same time, detection with NbALFAST was 10-fold more sensitive and showed an excellent linearity over ~3 orders of magnitude.

[37] **Fig. 7: Highly sensitive Western-blot and dot-blot detection of ALFA-tagged target proteins using fluorescently labeled NbALFAST (FluoTag-X2 anti-ALFA)**

[38] **A,** Same experiment as shown in Fig. 6A. Here, however, complete lanes are shown. Note that in the absence of any vector encoding an ALFAST tagged protein, only very minor bands (*) can be detected using fluorescently labeled NbALFA (FluoTag-X2 anti-ALFA).

[39] **B,** Same experiment as shown in Fig. 6C. In addition to the data presented in Fig. 6C, detection of MBP by a combination of rabbit polyclonal serum recognizing MBP (SynapticSystems) and an anti-rabbit IgG IRDye680RD (Li-Cor #925-68071) is shown as an internal loading control. Overlays show MBP signals in red and signals corresponding to epitope tags in green.

[40] **Fig. 8: Interaction of ALFAST-tagged proteins with ALFA SelectorST and ALFA Selector^{PE} resins.**

[41] **A**; Sketch of ALFA Selector resins bound to shGFP2-ALFAST. In this sketch, the ALFA Selector resin could be ALFA SelectorST or ALFA Selector^{PE}.

[42] **B and C;** Peptide elution from NbALFA-coupled affinity resins. Agarose-based resins coupled to NbALFAST (SEQ ID NO: 133; ALFA SelectorST, left) or an NbALFA^{PE} mutant (SEQ ID NO: 134; ALFA Selector^{PE}, right) were charged with shGFP2 harboring a C-terminal ALFAST tag. To estimate off-rates, the resins were suspended PBS containing an excess of free ALFAST peptide and incubated at 25°C. Control reactions were carried out without peptide. At indicated time points, shGFP2 released from the resin was quantified. **B** shows mean fluorescence readings of three experiments as well as standard deviations for each time point. Lines represent fits to a single exponential. Efficient peptide

elution of shGFP2-ALFAST from ALFA Selector^{PE} was observed already after 15-20min at room temperature. In contrast, peptide elution from ALFA SelectorST was inefficient even after prolonged incubation. In the absence of free ALFAST peptide during elution, the ALFAST-tagged target protein remained tightly bound to both resins. A photo was taken upon UV illumination after 3h of elution (**C**).

[43] **D;** Resistance towards stringent washing steps. ALFA Selector variants described in B were charged with either ALFAST-shGFP2 or shGFP2-ALFAST and incubated with a 10-fold volume of the indicated substances for 1h at 25°C with shaking. Without further washing steps, photos were taken upon UV illumination after sedimentation of the beads.

[44] **E;** Resistance towards non-physiological pH. Similar to D. Here, however, the resin was washed to remove non-bound material after incubating at indicated pH for 30min. Photos were taken after re-equilibration in PBS to allow for recovery of the GFP fluorescence.

[45] **Fig. 9: Pull-down of ALFAST-tagged target proteins and protein complexes from complex lysates using ALFA SelectorST and ALFA Selector^{PE}.**

[46] **A;** Input protein used for experiments described in (B and C).

[47] **B and C;** One-step affinity purifications using the ALFA Selector Resins. *E.coli* (A) or HeLa (B) lysates blended with 3µM purified ALFAST-tagged shGFP2 (A) were incubated with ALFA SelectorST, ALFA Selector^{PE} or an analogous resin without immobilized sdAb (Selector Control). After washing with PBS, the resins were incubated with 200µM ALFAST peptide for 20min. Proteins remaining on the beads were eluted with SDS sample buffer. Indicated fractions were analyzed by SDS-PAGE and Coomassie staining. Shown eluate fractions correspond to the material eluted from 1µl of resin.

[48] **D;** Pull-down of a native *E.coli* YfgM-PpiD inner membrane protein complex using the ALFA Selector^{PE}. Left: Sketch of the YfgM-PpiD membrane protein complex. Right: A *yfgM* deletion strain was complemented with either C-terminally ALFAST-tagged (left panel) or untagged YfgM (right panel; control reaction) expressed from a low-copy vector. Membrane protein complexes were solubilized from total lysate using DDM. Complexes containing YfgM-ALFAST were purified in a single step using ALFA Selector^{PE} affinity resin and eluted under native conditions using 200µM ALFAST peptide. Samples corresponding to 1/800 of the input and non-bound material or 1/80 of eluate fractions were resolved by SDS page and analyzed by Western-blot. A rabbit serum raised against the YfgM-PpiD complex (Götzke et al., YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)) recognized both, PpiD and YfgM,

in the input fractions. ALFA Selector^{PE} specifically immunoprecipitated the native protein complex comprising ALFAST-tagged YfgM and its interaction partner PpiD. In the control reaction (no ALFAST tag on YfgM), both proteins were absent in the eluate.

[49] Fig. 10: Peptide elution of ALFAST-tagged GFPs from ALFA Selector resins.

[50] 20µl ALFA SelectorST (presenting NbALFAST, SEQ ID NO: 133) or ALFA Selector^{PE} (presenting NbALFA^{PE}, SEQ ID NO: 134) were charged with shGFP2-ALFAST (A), bdSUMO-ALFAST-shGFP2 (B) or ALFAST-shGFP2 (C). After washing with PBS, the beads were suspended in a 10-fold excess of PBS containing 200µM free ALFAST peptide and gently mixed at 25°C. Control reactions were carried out without peptide. At indicated time points, specific elution from the beads was quantified using the GFP fluorescence released into the supernatant. Shown are mean fluorescence readings of three experiments as well as standard deviations for each time point. Lines represent fits to a single exponential. Half times are given for peptide elution from ALFA Selector^{PE} only. For all substrate proteins, peptide elution from ALFA SelectorST was inefficient even after prolonged incubation. In the absence of ALFAST peptide, the ALFAST-tagged target proteins remained tightly bound to both resins. Fig. 10A recapitulates data shown in Fig. 8B and is repeated here to allow for a direct comparison. Left panels: Sketch illustrating the experimental setup. The ALFA Selector resin could be ALFA SelectorST or ALFA Selector^{PE}; middle panels: Experiments performed with ALFA SelectorST; right panel: Experiments performed with ALFA Selector^{PE}.

[51] Fig. 11: X-ray structure of NbALFAST bound to ALFAST peptide

[52] A-C; Views on the NbALFAST-ALFAST peptide structure. **A,** view on the N-terminus of the ALFAST peptide; **B,** side view on the ALFAST peptide; **C,** view on the C-terminus of the ALFAST peptide. NbALFAST is illustrated in light grey with side chains represented as lines. Residues contacting the ALFAST peptide are represented by sticks. The ALFAST peptide is depicted in dark grey with side chains shown as sticks. The ALFAST peptide was used with N-terminal acetylation and C-terminal amidation (SEQ ID NO: 179).

[53] D; Sequence of NbALFAST (SEQ ID NO: 133). As in Fig. 3B, boxes indicate CDRs 1-3 (SEQ ID NOs:115-117). Residues directly contacting the ALFAST peptide are boxed. Residues in filled boxes were mutated to reduce the affinity for the ALFAST peptide.

[54] Fig. 12: Isolation of naïve lymphocytes using an ALFA-tagged nanobody recognizing CD62L.

[55] Total human PBMCs were left untreated (before sorting) or isolated using an ALFA Selector^{PE} resin loaded with an ALFA-tagged anti-human CD62L nanobody (after

sorting). A sketch of the affinity purification strategy is shown in (a). Cells were stained with an anti-CD62L antibody and analyzed by flow cytometry (b). The same cells as in (b) were stained with antibodies directed against CD3, CD19 and CD62L, and analyzed by flow cytometry (c). A forward scatter/side scatter gate was set on lymphocytes in all analyses.

DETAILED DESCRIPTION

[56] In order to overcome some of the shortcomings of the state of the art, the inventors of the present application created a small epitope tag recognized by a high-affinity nanobody. Such system may allow analysis of a protein's function in multiple aspects comprising but not limited to the analysis of its localization, analysis of its interaction partners by purification from lysates or *in-vivo* manipulations including induced protein mislocalization or depletion using a minimal set of recombinant constructs and cell lines without unintended interference with the protein of interest's physiological function.

[57] In view of the limitations on the current epitope tags, the inventors of the present application decided to address the problem and find the features that an ideal epitope tag system should posses. A truly versatile tag should be small to minimize the potential side effect (Kocaoglu, O. & Carlson, E. E. Progress and prospects for small-molecule probes of bacterial imaging. *Nat Chem Biol* **12,** 472–478 (2016)). It is preferably monomeric in order to minimize artifactual oligomerization of the tagged proteins. It should also be electroneutral to avoid adding net charges to the tagged proteins; at the same time it should be soluble (Esposito, D. & Chatterjee, D. K. Enhancement of soluble protein expression through the use of fusion tags. *Curr. Opin. Biotechnol.* **17,** 353–358 (2006)). An ideal tag should not affect the native structure, topology or localization of the tagged protein (Stadler, C. *et al.* Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. *Nat Methods* **10,** 315–323 (2013); Hoffmann, C. *et al.* A FlAsH-based FRET approach to determine G protein–coupled receptor activation in living cells. *Nat Methods* **2,** 171–176 (2005)). In addition, the tag should be well expressed in eukaryotic and prokaryotic hosts and should be resistant towards proteolytic degradation. Ideally, it should be resistant to fixation and its sequence should be absent in common model organisms to avoid non-intended detection of endogenous host proteins.

[58] Similar than for an ideal epitope tag, its corresponding binder should also have several characteristic to make the tag detection ideal. For example, the binder should be small in order to have easy access to crowded regions and provide the best binding affinity for the different applications. Current sophisticated applications (e.g. live *in-vivo* imaging) need a

specific and genetically accessible probe with high affinity to the tag, which should be able to autonomously fold *in vivo* in various host organisms. For biochemical applications, however, the preferred binder should preferentially have intermediate affinity in order to allow for a competitive elution of the immuno-precipitated material under native conditions. When assessing commonly used epitope tags existing thus far, one applying the state of the art will ultimately need to sacrifice at least one of the mentioned features (see Table 1 below). To manufacture an epitope tag with ultimate versatility fulfilling all of the mentioned boundary conditions, the inventors of the present application have recognized that the most straightforward approach is to design it *de novo*.

[59] Table 1: Properties of common epitope tag systems

		FLAG-tag[10]	**HA-tag[11]**	**myc-tag[12]**	**Twin-Strep-tag[8]**	**polyHis-tag[9]**	**Spot-tag[13]**	**Epitope tag of the present invention comprising the core sequence of SEQ ID NO: 4**
Properties of tag	Size (amino acids)	8	9	10	28	3-10	12	13-15
	Mass (kDa)	1.1	1.1	1.2	2.9	0.4-1.4	1.4	~1.8
	Charge at pH7.0	-3	-2	-3	0.2	0.3-1	1.1	0
	pKi	3.5	0	3.5	8.4	14	12.1	8.1
	Physical size (nm)	2.2	2.5	2.8	>6	0.8-2.8	3.3	2.0
	Water solubility	+	poor	+	+	poor	+	+
	Structured in solution	–	–	–	–	–	–	+[14]
	Stable	(+)[15]	(+)[16]	+	+	+	+	+
	Fixation resistant[7]	–	+	–	–	+	+	+
	Unique within model organisms	+	–	–	(+)[2]	(–)[3]	(–)[5]	+

	Possible localizations[18]	N, M, C	N, M, C	N, M, C	N, C	N, C	N, C	N, M, C
Properties of binder	Name	M1, M2, M5	F-7, 12CA5	9E10	StrepTactin-XT	Ni^{2+}/Co^{2+}-chelate	Spot-nanobody	$NbALFA^{ST}$
	Type	mAb	mAb	mAb	Protein	Inorganic	sdAb	sdAb
	Size (kDa)	~150	~150	~150	~60	n.a.	~15/30(6)	~15
	No. of polypeptides	4	4	4	4	n.a.	1	1
	No of binding sites	2	2	2	2	1	1-2 (6)	1
	Affinity	n.d.	n.d.	n.d.	~60pM[17]	n.d.[4]	~6nM	~10pM
	Genetically accessible	–	–	–	+	–	+	+
Applications	Protein purification	+	+	+	+	+	+	+
	Imaging	+[1]	+[1]	+[1]	n.d.	–	(+)(6)	+
	In-vivo applications	–	–	–	–	–	–	+

n.a.: not applicable

n.d.: no data available

mAb: monoclonal antibody

sdAb: single-domain antibody

[1] Multiple tags are often used in tandem for optimal performance (Hernan, R., Heuermann, K. & Brizzard, B. Multiple epitope tagging of expressed proteins for enhanced detection. *BioTechniques* **28,** 789–793 (2000); Ross-Macdonald, P., Sheehan, A., Roeder, G. S. & Snyder, M. A multipurpose transposon system for analyzing protein production, localization, and function in Saccharomyces cerevisiae. *Proc Natl Acad Sci USA* **94,** 190–195 (1997); Sharrock, R. A. & Clack, T. Heterodimerization of type II phytochromes in Arabidopsis. *Proc Natl Acad Sci USA* **101,** 11500–11505 (2004); Graumann, J. *et al.* Applicability of tandem affinity purification MudPIT to pathway proteomics in yeast. *Mol. Cell Proteomics* **3,** 226–237 (2004))

[2] Binder also recognizes biotinylated proteins

[3] Binder recognizes endogenous proteins with multiple accessible histidines.

[4] Depends on chelate and polyHis-tag used

[5] Binder also recognizes endogenous β-catenin

[6] Binder needs to be dimerized for high-profile imaging applications (Virant, D. *et al.* A

peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. *Nat Commun* 1–14 (2018). doi:10.1038/s41467-018-03191-2)

[7]Fixation by amine-reactive fixatives and cross-linkers; deduced from sequence

[8]Schmidt, T. G. M. *et al.* Development of the Twin-Strep-tag® and its application for purification of recombinant proteins from cell culture supernatants. *Protein Expr. Purif.* **92,** 54–61 (2013)

[9]Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **258,** 598–599 (1975); Hochuli, E., Döbeli, H. & Schacher, A. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr.* **411,** 177–184 (1987)

[10]Hopp, T. P. *et al.* A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification. *Nat Biotechnol* **6,** 1204–1210 (1988)

[11]Wilson, I. A. *et al.* The structure of an antigenic determinant in a protein. *Cell* **37,** 767–778 (1984)

[12]Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* **5,** 3610–3616 (1985)

[13]Virant, D. *et al.* A peptide tag-specific nanobody enables high- quality labeling for dSTORM imaging. *Nat Commun* 1–14 (2018). doi:10.1038/s41467-018-03191-2; Braun, M. B. *et al.* Peptides in headlock - a novel high-affinity and versatile peptide-binding nanobody for proteomics and microscopy. *Sci Rep* **6,** 19211 (2016)

[14] Petukhov, M. *et al.* Design of stable alpha-helices using global sequence optimization. *J. Pept. Sci.* **15,** 359–365 (2009)

[15] Hunter, M. R., Grimsey, N. L. & Glass, M. Sulfation of the FLAG epitope is affected by co-expression of G protein-coupled receptors in a mammalian cell model. *Sci Rep* **6,** 27316 (2016)

[16] Schembri, L. *et al.* The HA tag is cleaved and loses immunoreactivity during apoptosis. *Nat Methods* **4,** 107–108 (2007)

[17]https://www.iba-lifesciences.com/tl_files/ProteinProductionAssays/5-Immobilization/DynamicBiosensors-Application-Note-StrepTactinXT-switchSENSE.pdf

[18] N: N-terminus; M: in between two folded domains; C: C-terminus.

[60] With this clear objective inventors of the present application designed the epitope tags described herein. The epitope tags of the invention preferably consist about 8 to 25 amino acids and are collectively called ALFA tags.

[61] The inventors of the application decided to take a de-novo approach for

generating a new epitope tag. The epitope tag according to the invention is a small, monomeric epitope tag of preferably ≤15aa with minimal size. The sequence is preferably uncharged and hydrophilic at physiological pH and most preferably, it is devoid of residues prone to be modified by amine-reactive fixatives and cross-linkers. The size stands in contrast to larger tags, such as, e.g., a FLAG tag trimer, which is commonly used in a 3x tandem in order to increase avidity, or even larger fluorescent protein. Further, the epitope tag of the present invention has no counterpart in eukaryotic or prokaryotic sequence databanks, which minimizes the risk of cross-binding to native structures. This stands e.g. in contrast to the SPOT-tag®, which is described in WO 2017/085086 A1.

[62] A further advantage of the epitope tag of the invention is that it is compatible to common amine-reactive fixatives (paraformaldehyde (PFA), glutaraldehyde (GA)), unlike e.g. the Myc tag. It is also compatible with methanol fixation. A further advantage of the epitope tag of the present invention is that it is not restricted in terms of localization (N, C or in between proteins), unlike the EPEA tag, which is described in WO 2011/147890 A1.

[63] Without wishing to be bound by theory, it is believed that the epitope tag according to the invention forms a stable alpha-helical structure. Formation of a stable alpha helix is believed to be advantageous over differently folded, non-folded or non-stably folded structures, because certain antibodies, such as single domain antibodies (sdAb) or nanobodies are believed to prefer defined three-dimensional surfaces for binding. In the past, it has been very hard to generate single domain antibodies binding to natively unfolded peptides, which resulted in sdAb with weak to moderate binding affinities only. The inventors of the present application therefore focused on providing epitope tags that are believed to form small alpha-helices that are stably folded in solution, because such structures are the smallest entities forming stable secondary structures as a monomer. Without wishing to be bound by theory, it is believed that the alpha-helical structure efficiently and spontaneously refolds even after exposure to harsh chemical treatment. Further it is believed that due to its helical structure, the tag is smaller than most unstructured linear epitope tags. In addition, the epitope tags of the invention can be placed at the N- or C-terminus of a target protein or even in between two folded protein domains without compromising proper targeting and folding of target proteins.

[64] Finding the ideal binder(s), however, proved to be challenging. While conventional antibodies would indeed fulfill most requirements, their large size makes them suboptimal for the current super-resolution microscopy (Fornasiero, E. F. & Opazo, F. Super-resolution imaging for cell biologists: Concepts, applications, current challenges and developments. *Bioessays* **37,** 436–451 (2015); Mikhaylova, M. *et al.* Resolving bundled

microtubules using anti-tubulin nanobodies. *Nat Commun* **6,** 7933 (2015) and cannot be encoded genetically to target intracellular targets in living cells. The inventors of the present application therefore chose to develop a camelid single-domain antibody (sdAb, also know as nanobody (Muyldermans, S. Nanobodies: Natural Single-Domain Antibodies. *Annu Rev Biochem* (2013). doi:10.1146/annurev-biochem-063011-092449) fulfilling the set criteria. For this, a novel in-house selection method called "Celline" allowed the inventors to generate alpaca-derived sdAb with exceedingly high affinity in a very timely manner. The antibodies of the invention in combination with the epitope tags of the invention proved to be ideal for imaging and intracellular detection of ALFAST-tagged target proteins and allowed very efficient and clean immuno-precipitations.

[65] The present invention also provides high-affinity antibodies for the epitope tag of the invention. Some of the antibodies are monovalent sdAb-based binders. Monovalent binding stands in contrast to epitope tags that are bound by conventional antibodies. While the SPOT-tag® is bound by an sdAb, the SPOT-tag®- binding sdAb is employed as a dimer for some applications of the SPOT-tag® in order to increase avidity. Utilization of monovalent antibodies has the advantage that cluster formation can be prevented. It is further believed that due to the alpha-helical structure of the epitope tag, the inventors of the present invention were able to generate single domain antibodies that bind to the epitope tag of the invention with a K_d in the range of about 10 pM, which is approximately a ~1000-fold higher affinity than comparable epitope tag/sdAb systems, as e.g. described in WO 2017/085086 A1 or WO 2011/147890 A1.

[66] For some of the epitope tags of the present invention, such as SEQ ID NOs:05-07, it was virtually impossible to efficiently separate the antibody comprising the sequence of SEQ ID NO:133 from the tag under native conditions. In some cases, this might be limiting for the application of SEQ ID NOs:05-07 for purification of native proteins and their interacting partners. Solving the crystal structure of the high affinity complex of the antibody of SEQ ID NO: 133 and the peptide of SEQ ID NO: 179 allowed the inventors to map the interaction determinants in detail and to engineer a new versions of the single-domain antibody that allows a competitive elution of ALFAST-tagged target proteins and interacting partners under native conditions. An exemplary antibody for this purpose comprises the amino acid sequence of SEQ ID NO: 134.

[67] The epitope tag and antibody system presented here is suited for an exceptionally broad range of applications ranging from biotechnology to cell biology. A single tag can therefore simultaneously replace a great variety of traditional epitope tags.

[68] The present invention therefore relates to a fusion protein comprising (a) a peptide comprising the sequence of X1-X2-X3-X4-X5-X6-X7-X8-X9-R-L-X12-X13 (SEQ ID NO: 01), wherein X1 is G or S or T or P, X2 is R or G or A or E or P, X3 is L or V, X4 is E or Q, X5 is E or Q, X6 is E or Q, X7 is L or I or V, X8 is R or A or Q or E, X9 is R or A or Q or E, X12 is S or T or D or E or P or A or no amino acid, and wherein X13 is E or K or P or S or A or D or no amino acid; and (b) a polypeptide. In the fusion protein of the invention, the peptide may serve the purpose of an epitope tag.

[69] The term "peptide" as used herein refers to a linear series of amino acids connected one to the other preferably by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics, with proteogenic amino acids being preferred. A “proteinogenic amino acid” is an amino acid that can be incorporated biosynthetically into proteins during translation. Currently, there are 22 known genetically encoded (proteinogenic) amino acids, 20 in the standard genetic code and an additional 2 that can be incorporated by special translation mechanisms. The “peptide” as used herein preferably comprises no more than about 50 amino acids.

[70] The term “polypeptide” as used herein usually refers to a peptide having at least about 30, at least about 40, or at least about 50 amino acids. The term “protein” as used herein comprises one or more polypeptides.

[71] The term “fusion protein” as used herein refers to a polypeptide or protein comprising two or more subunits. At least one of the subunits is preferably a protein or polypeptide, and at least one of the subunits is preferably a peptide. Within the fusion protein, these subunits may be linked by covalent or non-covalent linkage. Preferably, the fusion protein is a translational fusion between the two or more subunits. The translational fusion may be generated by genetically engineering the coding nucleotide sequence for one subunit in a reading frame with the coding nucleotide sequence of a further subunit. Subunits may be interspersed by a linker.

[72] If one or more of the subunits is part of a protein (complex) that consists of more than one polypeptide chain, the term “fusion protein” may also refer to the protein comprising the fused sequences and all other polypeptide chain(s) of the protein (complex).

[73] As used herein, an “epitope tag” refers to a stretch of amino acids to which a specific antibody or proteinaceous molecule with antibody-like function can be raised. Such an epitope tag may allow for specifically identifying and/or tracking of the tagged polypeptide

or protein that may be present in a living organism or cultured cells. Detection of the tagged molecule can be achieved using a number of different techniques. Examples of such techniques include: immunohistochemistry, immunoprecipitation, flow cytometry, immunofluorescence microscopy, electron microscopy, ELISA, immunoblotting ("Western blot"), and affinity chromatography. The epitope tag adds a known epitope (antibody binding site) on the subject polypeptide, to provide binding of a known and often high-affinity antibody. An epitope tag may also be used for isolation and/or purification of the tagged molecule, e.g. by pull-down applications.

[74] In the fusion protein of the invention, the peptide, i.e. the epitope tag, may be located at any position of the fusion protein. The peptide may be fused to the N-terminus or the C-terminus of the polypeptide. Alternatively, the peptide may be fused to the polypeptide at a position between the N-terminus and the C-terminus of the polypeptide. As an illustrative example, the peptide may be fused in between two domains of the polypeptide.

[75] The polypeptide comprised in the fusion protein may have a stable fold that is independent from the presence or absence of the peptide. This means that the peptide preferably does not alter or interfere with the native structure of the polypeptide.

[76] The peptide itself has preferably an alpha-helical structure. A solved crystal structure of an antibody having the sequence of SEQ ID NO: 133 in complex with the ALFAST peptide comprising the sequence of Ac-PSRLEEELRRRLTEP-Amide (SEQ ID NO: 179) shows that the epitope tag binds to the nanobody as a stably folded alpha helix. The structure is believed to explain the extraordinarily tight binding. An "alpha helical structure" as used herein refers to a secondary structure in the form of an alpha helix. It is preferred that the alpha helical secondary structure of the peptide is independent from a fusion partner. This means that the peptide is preferably capable of forming an alpha helical secondary structure in physiological buffers if the peptide is in form of an isolated peptide and also if the peptide is part of the fusion protein.

[77] The peptide may be specifically recognized by a camelid VHH domain comprising the CDR sequences GVTISALNAMAMG (SEQ ID NO: 115), AVSERGNAM (SEQ ID NO: 116), and LEDRVDSFHDY (SEQ ID NO: 117). The peptide may further be specifically recognized by other antibodies described herein.

[78] In the fusion protein of the invention, the peptide may be fused to the polypeptide either by direct fusion or through a linker. A "linker" as used herein joins together two or more subunits of a fusion protein as described herein. The linkage can be covalent. A preferred covalent linkage is via a peptide bond, such as a peptide bond between

amino acids. A preferred linker is a peptide linker. Said linker preferably comprises one or more amino acids, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acids. Preferred peptide linkers include glycine-serine (GS) linkers, glycosylated GS linkers, and proline-alanine-serine polymer (PAS) linkers. A GS linker may be a $(G_4S)_3$ linker as described in SEQ ID NO: 159.

[79] The polypeptide comprised in the fusion protein of the invention may comprise at least one protein domain. A "protein domain" as used herein refers to a part of a given protein sequence and (tertiary) structure that can function and/or exist independently of the rest of the protein chain. The protein domain preferably forms a compact three-dimensional structure and often can be independently stable and folded. A protein domain may further form a functional unit. The polypeptide comprised in the fusion protein may comprise more than one protein domain, such as 2, 3, 4, or even more protein domains. A preferred location for the peptide may be outside of the protein domain. This can be N-terminal or C-terminal of the at least one protein domain of the polypeptide, or in between two protein domains of the polypeptide. The polypeptide comprised in the fusion protein of the invention may be a globular protein, and membrane protein, a fibrous protein, or a natively unfolded protein, or a subunit or domain of the globular protein, membrane protein, fibrous protein, or natively unfolded protein.

[80] The peptide comprised in the fusion protein of the invention may have a length of about 8 to about 25 amino acids, preferably about 10 to about 18 amino acids, preferably about 12 to about 17 amino acids, preferably 12 to 15 amino acids. The polypeptide comprised in the fusion protein of the invention may have a length of at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids. The polypeptide may be polypeptide or protein that naturally occurs in a cell that expresses the fusion protein of the invention.

[81] The fusion protein of the invention may comprise a peptide comprising the sequence of X1-X2-L-E-X5-E-X7-R-R-R-L-X12-X13 (SEQ ID NO: 02), wherein X1 is G or S or P or T, X2 is R or G or P, X5 is E or Q, X7 is L or I, X12 is S or T or P or A or D or E, and wherein X13 is P or A or S or A or D or E or no amino acid. This sequence defines the core structure of the peptide and may further comprise up to two additional amino acids at the N terminus and up to two additional amino acids at the C terminus. Such additional amino acids at the ends of the core structure of the peptide usually do not necessarily influence the secondary structure of the of the peptide or specific binding of the peptide to an antibody specific for the peptide, but may serve as linker structures in the fusion protein. Accordingly,

type and number of the additional amino acids may depend on the location of the peptide in the fusion protein and may vary depending on whether the peptide is located N-terminal or C-terminal or somewhere in between of the polypeptide. A peptide comprising the sequence of SEQ ID NO: 02 may have a $T_{1/2}$ of at least about 2 min for dissociation from a single domain antibody comprising the sequence of SEQ ID NO: 133, e.g. when measured in an assay as essentially described in Example 1. The peptide may have a K_d of about 30 nM or less for the binding to the single domain antibody comprising the sequence of SEQ ID NO: 133.

[82] The peptide comprised in the fusion protein of the invention may comprise N-terminal of X1 the amino acids Xa-Xb, wherein Xa is D or S or G or M or P or no amino acid and Xb is S or D or P or M or R or G no amino acid.

[83] The peptide comprised in the fusion protein of the invention may comprise C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or S or P or D or A or E or K or no amino acid, and Xz is S or P or no amino acid.

[84] The fusion protein of the invention may comprise a peptide comprising as a core structure the sequence of X1-X2-L-E-X5-E-L-R-R-R-L-X12-X13 (SEQ ID NO: 03), wherein X1 is S or T, X2 is R or G, X5 is E or Q, X12 is T or D or E, and wherein X13 is A or D or E or no amino acid. Such a peptide may have a T1/2 of at least about 100 min for dissociation from a single domain antibody comprising the sequence of SEQ ID NO: 133, e.g. when measured in an assay as essentially described in Example 1. The peptide may have a K_d of about 1 nM or less for the binding to the single domain antibody comprising the sequence of SEQ ID NO: 133. Such a peptide may comprise the sequence of S-R-L-E-E-E-L-R-R-R-L-T-E (SEQ ID NO: 04) or a variant thereof, wherein the variant has as compared to (SEQ ID NO: 04) 1 to 5 mutations selected from the group consisting of: S1→T, R2→G, E5→Q, T12→D, and T12→E, E13→A, E13→D, and deletion of E13. The variant may have as compared to SEQ ID NO: 04 following mutations: (a) S1→T and E13→A; (b) R2→G; (c) R2→G and E5→Q; (d) R2→G, E5→Q and E13→A; (e) R2→G, E5→Q, and T12→D, and E13→A; (f) R2→G, E5→Q, and T12→E, and E13→A; (g) T12→D and E13→A; (h) T12→E and E13→A; (i) and E13→A; (j) and E13→D; or (k) deletion of E13.

[85] A peptide comprising as a core structure the sequence SEQ ID NO: 03 may comprise N-terminal of X1 the amino acids Xa-Xb, wherein Xa is S or G or M or P or no amino acid, and Xb is R or G or S or P or M or no amino acid. Xa-Xb may be selected from the group consisting of P, M-P, G-R, P-G, P-S, S-P, G-P, S-P, M, and M-S, preferably P or M-P. Such a peptide may also comprise C-terminal of X13 the amino acids Xy-Xz, wherein is P or D or A or no amino acid, and Xz is P or S or no amino acid. Xy-Xz may be selected from

the group consisting of no amino acid, P, D-P, A, and A-S, preferably no amino acid or P. The peptide may comprise a combination of Xa-Xb and Xy-Xz selected from the group consisting of: (a) M-P and P; (b) P and P; and (c) P and no amino acid.

[86] A peptide comprised in the fusion protein of the invention may have the core structure of SEQ ID NO: 03 and may comprise a sequence selected from the group consisting of:

(a) MPSRLEEELRRRLTEP (SEQ ID NO: 05);
(b) PSRLEEELRRRLTEP (SEQ ID NO: 06);
(c) PSRLEEELRRRLTE (SEQ ID NO: 07);
(d) GRSRLEEELRRRLTA (SEQ ID NO: 08);
(e) PGSRLEEELRRRLTAP (SEQ ID NO: 09);
(f) PSTRLEEELRRRLTAP (SEQ ID NO: 10);
(g) SPSRLEEELRRRLTAP (SEQ ID NO: 11);
(h) SPSRLEEELRRRLDAP (SEQ ID NO: 12);
(i) SPSRLEEELRRRLEAP (SEQ ID NO: 13);
(j) SPSRLEEELRRRLTDP (SEQ ID NO: 14);
(k) SPSRLEEELRRRLTEP (SEQ ID NO: 15);
(l) SPSRLEEELRRRLTADP (SEQ ID NO: 16);
(m) SPSGLEEELRRRLTEP (SEQ ID NO: 17);
(n) GPSRLEEELRRRLT (SEQ ID NO: 18);
(o) GPSRLEEELRRRLTA (SEQ ID NO: 19);
(p) GPSRLEEELRRRLTAA (SEQ ID NO: 20);
(q) GPSRLEEELRRRLTAAS (SEQ ID NO: 21);
(r) SPSGLEQELRRRLTAP (SEQ ID NO: 22);
(s) SPSGLEQELRRRLDAP (SEQ ID NO: 23);
(t) SPSGLEQELRRRLEAP (SEQ ID NO: 24);
(u) SPSGLEQELRRRLTEP (SEQ ID NO: 25);
(v) GPSRLEEELRRRLTAP (SEQ ID NO: 26);
(w) GPSRLEEELRRRLTEP (SEQ ID NO: 27);
(x) GPSRLEEELRRRLTE (SEQ ID NO: 28);
(y) MSRLEEELRRRLTEP (SEQ ID NO: 29); and
(z) MSSRLEEELRRRLTEP (SEQ ID NO: 30).

[87] The fusion protein of the invention may comprise a peptide comprising as a core structure the sequence of X1-X2-L-E-X5-E-X7-R-R-R-L-X12-X13 (SEQ ID NO: 31),

wherein X1 is G or S or P, X2 is R or G, X5 is E or Q, X7 is L or I, X12 is S or T or P or A, and X13 is P or A or S or no amino acid. Such a peptide may have a $T_{1/2}$ from about 2.5 min to about 30 min for dissociation from a single domain antibody comprising the sequence of SEQ ID NO: 133, e.g. when measured in an assay as essentially described in Example 1. The peptide may have a K_d of about 3-40 nM or less for the binding to the single domain antibody comprising the sequence of SEQ ID NO: 133.

[88] Such a peptide may comprise the sequence of G-R-L-E-E-E-L-R-R-R-L-S (SEQ ID NO: 32) or a variant thereof, wherein the variant has as compared to (SEQ ID NO: 32) 1 to 6 mutations selected from the group consisting of: G1→S, G1→P, R2→G, E5→Q, L7→I, S12→T, S12→P, and S12→A, addition of P13, addition of A13, and addition of S13. The variant may have as compared to SEQ ID NO: 32 following mutations: (a) G1→S, R2→G, E5→Q, and addition of P13; (b) R2→G, E5→Q, S12→T, and addition of A13; (c) G1→P, R2→G, E5→Q, S12→T, and addition of A13; (d) G1→S, R2→G, E5→Q, S12→T, and addition of P13; (e) G1→S, R2→G, S12→T, and addition of A13; (f) G1→S, R2→G, E5→Q, and S12→T; (g) G1→S, R2→G, E5→Q, S12→T, and addition of A13; (h) G1→S and S12→P, and addition of P13; (i) G1→S, R2→G, E5→Q, S12→T, and addition of P13; (j) E5→Q L7→I, and addition of P13; (k) addition of P13; or (l) S12→A.

[89] A peptide comprising as a core structure the sequence SEQ ID NO: 31 may comprise N-terminal of X1 the amino acids Xa-Xb, wherein Xa is M or S or P or D or G or no amino acid, and Xb is S or D or P or no amino acid. Xa-Xb may be selected from the group consisting of M-S, S-D, P-D, P-S, D-S, S-P, and G-P, preferably M-S. Such a peptide may also comprise C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or P or A or E or K or S or no amino acid, and Xz is P or S or no amino acid. Xy-Xz may be selected from the group consisting of no amino acid, G, P, A, E-P, A-S, K, and S, preferably no amino acid. The peptide may comprise a combination of Xa-Xb and Xy-Xz that is M-S and no amino acid.

[90] A peptide comprised in the fusion protein of the invention may have the core structure of SEQ ID NO: 31 and may comprise a sequence selected from the group consisting of:

(a) GRLEEELRRRLS (SEQ ID NO: 32);
(b) MSGRLEEELRRRLSP (SEQ ID NO: 33);
(c) SDSGLEQELRRRLSPG (SEQ ID NO: 34);
(d) PDGGLEQELRRRLTAP (SEQ ID NO: 35);
(e) PSGGLEQELRRRLTAP (SEQ ID NO: 36);
(f) DSPGLEQELRRRLTAP (SEQ ID NO: 37);

(g) PDSGLEQELRRRLTPA (SEQ ID NO: 38);
(h) SPSGLEEELRRRLTAEP (SEQ ID NO: 39);
(i) GPSGLEQELRRRLT (SEQ ID NO: 40);
(j) GPSGLEQELRRRLTAAS (SEQ ID NO: 41);
(k) SPSRLEEELRRRLPSK (SEQ ID NO: 42);
(l) SPSGLEQELRRRLTPS (SEQ ID NO: 43);
(m) SPGRLEQEIRRRLSPS (SEQ ID NO: 44);
(n) PSGRLEEELRRRLSPS (SEQ ID NO: 45);
(o) PSGRLEEELRRRLS (SEQ ID NO: 46);
(p) PSGRLEEELRRRLA (SEQ ID NO: 47); and
(q) PSGRLEEELRRRLSP (SEQ ID NO: 48).

[91] The fusion protein of the invention may comprise a peptide comprising as a core structure the sequence of X1-X2-L-E-X5-E-L-R-R-R-L-X12-X13 (SEQ ID NO: 49), wherein X1 is S or G or P, X2 is R or G or P, X5 is E or Q, X12 is S or T or D or E, and X13 is P or A or D or no amino acid. Such a peptide may have a T1/2 from about 20 min to about 100 min for dissociation from a single domain antibody comprising the sequence of SEQ ID NO: 133, e.g. when measured in an assay as essentially described in Example 1. The peptide may have a K_d of about 1-5 nM or less for the binding to the single domain antibody comprising the sequence of SEQ ID NO: 133.

[92] A peptide comprising as a core structure the sequence SEQ ID NO: 49 may comprise N-terminal of X1 the amino acids Xa-Xb, wherein Xa is P or D or S or G or no amino acid, and Xb is D or S or P or no amino acid. Such a peptide may also comprise C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or P or E or D or S or no amino acid, and Xz is P or no amino acid.

[93] A peptide comprised in the fusion protein of the invention may have the core structure of SEQ ID NO: 49 and may comprise a sequence selected from the group consisting of:

(a) PDSGLEQELRRRLSPG (SEQ ID NO: 50);
(b) PDSGLEQELRRRLTAP (SEQ ID NO: 51);
(c) PSSGLEQELRRRLTAP (SEQ ID NO: 52);
(d) DPSGLEQELRRRLTAP (SEQ ID NO: 53);
(e) DSGPLEQELRRRLTAP (SEQ ID NO: 54);
(f) SPSRLEEELRRRLTAEP (SEQ ID NO: 55);
(g) SPSGLEEELRRRLTAP (SEQ ID NO: 56);

(h) SPSGLEEELRRRLDAP (SEQ ID NO: 57);

(i) SPSGLEEELRRRLEAP (SEQ ID NO: 58);

(j) SPSGLEEELRRRLTDP (SEQ ID NO: 59);

(k) SPSGLEEELRRRLTADP (SEQ ID NO: 60);

(l) GPSGLEQELRRRLTA (SEQ ID NO: 169);

(m) SPSGLEQELRRRLTDP (SEQ ID NO: 170);

(n) SPSGLEQELRRRLTADP (SEQ ID NO: 171);

(o) SPSGLEQELRRRLTAEP (SEQ ID NO: 172);

(p) DSPGLEQELRRRLTAP (SEQ ID NO: 173); and

(q) SPSGLEQELRRRLSPS (SEQ ID NO: 174).

[94] The fusion protein of the invention may comprise a peptide comprising as a core structure the sequence of X1-X2-X3-X4-X5-X6-X7-X8-X9-R-L-X12-X13 (SEQ ID NO: 61), wherein X1 is G or S, X2 is R or G or A or E, X3 is L or V, X4 is E or Q, X5 is E or Q, X6 is E or Q, X7 is L or I or V, X8 is R or A or Q or E, X9 is R or A or Q or E, X12 is S or T or L or no amino acid, and X13 is K or P or S or no amino acid. Such a peptide may have a $T_{1/2}$ from about 2 min to about 10 min for dissociation from a single domain antibody comprising the sequence of SEQ ID NO: 133, e.g. when measured in an assay as essentially described in Example 1. The peptide may have a Kd of about 10-50 nM or less for the binding to the single domain antibody comprising the sequence of SEQ ID NO: 133.

[95] A peptide comprising as a core structure the sequence SEQ ID NO: 61 may comprise N-terminal of X1 the amino acids Xa-Xb, wherein Xa is D or S or G or M or no amino acid and Xb is S or D or P or M or no amino acid. Such a peptide may also comprise C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or S or P or no amino acid, Xz is S or no amino acid.

[96] A peptide comprised in the fusion protein of the invention may have the core structure of SEQ ID NO: 61 and may comprise a sequence selected from the group consisting of:

(a) GRLEEELRRRLS (SEQ ID NO: 32);

(b) MSGRLEEELRRRLSP (SEQ ID NO: 33);

(c) DSGRLEEELRRRLSKG (SEQ ID NO: 62);

(d) DSGRLEEELRRRLSPG (SEQ ID NO: 63);

(e) SDSGLEEELRRRLSPG (SEQ ID NO: 64);

(f) SDSGVEEELRRRLSPG (SEQ ID NO: 65);

(g) SDSAVEEELRRRLSPG (SEQ ID NO: 66);

(h) SDSGLQEELRRRLSPG (SEQ ID NO: 67);
(i) SDSGLEEQLRRRLSPG (SEQ ID NO: 68);
(j) SDSGLEEEIRRRLSPG (SEQ ID NO: 69);
(k) SDSGLEEEVRRRLSPG (SEQ ID NO: 70);
(l) DSGELEEELRRRLSPG (SEQ ID NO: 71);
(m) DSGRLEQELRRRLSPG (SEQ ID NO: 72);
(n) DSGRLEEEIRRRLSPG (SEQ ID NO: 73);
(o) DSGRLEQEIRRRLSPG (SEQ ID NO: 74);
(p) DSGRLEQEIARRLSPG (SEQ ID NO: 75);
(q) DSGRLEQEIQRRLSPG (SEQ ID NO: 76);
(r) DSGRLEQEIERRLSPG (SEQ ID NO: 77);
(w) DSGRLEQEIRARLSPG (SEQ ID NO: 78);
(t) DSGRLEQEIRQRLSPG (SEQ ID NO: 79);
(u) DSGRLEQEIRERLSPG (SEQ ID NO: 80);
(v) GPSRLEEELRRRL (SEQ ID NO: 81);
(w) MSGLEQELRRRLTPS (SEQ ID NO: 82);
(x) MSGRLEEELRRRLSPS (SEQ ID NO: 83);
(y) SPSAVEEELRRRLSPS (SEQ ID NO: 84);
(z) GPSAVEEELRRRLS (SEQ ID NO: 85);
(aa) MPSGLEQELRRRLTPS (SEQ ID NO: 86);
(bb) MSSGLEQELRRRLTPS (SEQ ID NO: 87);
(cc) MPSGRLEEELRRRLSPS (SEQ ID NO: 88);
(dd) MSGRLEEELRRRLSP (SEQ ID NO: 89).

[97] The fusion protein of the invention may in complex with a binding partner that specifically binds to the peptide comprised in the fusion protein. Such a specific binding partner may be an antibody disclosed herein.

[98] The fusion protein of the invention may comprise an antibody moiety. The antibody moiety may be a single domain antibody. Such antibody moiety may specifically bind to a target. Such a target may be a cell. For example, the specific target may comprise or be a structure on a cell surface, such as a cell surface receptor. A preferred target is CD62L.

[99] The present invention further provides an antibody that specifically binds to the peptide comprised in the fusion protein of the invention. Such an antibody may be a monovalent antibody. In preferred embodiments, an antibody of the invention comprises or consists of a camelid VHH domain. In preferred embodiments, an antibody of the invention is

a single domain antibody, such as a camelid single domain antibody.

[100] The term "antibody" generally refers to a proteinaceous binding molecule with immunoglobulin-like functions. Typical examples of an antibody are, but are not limited to, immunoglobulins, as well as derivatives or functional fragments thereof which still retain the binding specificity. Techniques for the production of antibodies are well known in the art. The term "antibody" also includes immunoglobulins (Ig's) of different classes (i.e. IgA, IgG, IgM, IgD, IgE, IgY etc.) and subclasses (such as IgG1, lgG2 etc.), even if recombinantly produced in foreign hosts using techniques known to those skilled in the arts. Illustrative examples of an antibody are full length immunoglobulins, F_{ab} fragments, $F(ab')_2$, F_V fragments, single-chain F_V fragments (scF_V), diabodies or domain antibodies (Holt LJ et al., *Trends Biotechnol.* 21(11), 2003, 484-490). Domain antibodies may be single domain antibodies, single variable domain antibodies or immunoglobulin single variable domain having only one variable domain, which may be VH or VL, that specifically bind an antigen or epitope independently of other V regions or domains. A particularly preferred single domain antibody is a VHH domain of a heavy chain only antibody. Such an immunoglobulin single variable domain may not only encompass an isolated antibody single variable domain polypeptide, but also a larger polypeptide that includes or consists of one or more monomers of an antibody single variable domain polypeptide sequence. It is understood that a single domain antibody may comprise a VHH domain and a fusion partner, such as a protein or peptide tag. The definition of the term "antibody" thus also includes embodiments such as chimeric, single chain and humanized antibodies. The term “antibody” may also include fragments of antibodies.

[101] Single domain antibodies are antibodies whose complementary determining regions are part of a single domain polypeptide. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived from antibodies. Single domain antibodies may be any of the art, or any future single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, goat, rabbit, or cattle. According to the invention, a single domain antibody as used herein is preferably derived from a naturally occurring antibody known as heavy chain antibody devoid of light chains. Such single domain antibodies are disclosed in WO 94/04678 for example. For clarity reasons, this variable domain derived from a heavy chain antibody naturally devoid of light chain is known herein as a VHH to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from antibodies raised in Camelidae

species, for example in camel, dromedary, llama, vicuña, alpaca and guanaco. Other species besides Camelidae may produce heavy chain antibodies naturally devoid of light chain. As an illustrative example, it is known that sharks produce heavy chain antibodies naturally devoid of light chains (commonly named IgNAR), which also comprise a VHH domain. In addition, VHHs may be obtained from synthetic libraries. All such VHHs are within the scope of the invention.

[102] VHHs, according to the present invention, and as known to the skilled addressee are preferably heavy chain variable domains derived from immunoglobulins naturally devoid of light chains such as those derived from Camelidae as described in WO 94/04678 (and referred to hereinafter as VHH domains or nanobodies). VHH molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are highly resistant to the action of proteases, which is not the case for conventional antibodies. Furthermore, *in vitro* expression of VHHs or expression in prokaryotic or eukaryotic organisms suited for recombinant protein expression produces high yield, properly folded functional VHHs. It is understood that single domain antibody according to the invention is preferably a VHH.

[103] An antibody according to the invention may carry one or more domains that have a sequence with at least about 60 %, at least about 70 %, at least about 75 %, at least about 80 %, at least about 85 %, at least about 90 %, at least about 92 %, at least about 95 %, at least about 96 %, at least about 97 %, at least about 98 % or at least about 99 % sequence identity with a corresponding naturally occurring domain of an immunoglobulin naturally devoid of light chains. It is noted in this regard, the term "about" or "approximately" as used herein means within a deviation of 20%, such as within a deviation of 10% or within 5% of a given value or range.

[104] “Percent (%) sequence identity" with respect to amino acid sequences disclosed herein is defined as the percentage of amino acid residues in a candidate sequence that are pair-wise identical with the amino acid residues in a reference sequence, i.e. an antibody molecule of the present disclosure, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publically available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve

maximum alignment over the full length of the sequences being compared. The same is true for nucleotide sequences disclosed herein.

[105] The term "variable" refers to the portions of the immunoglobulin domains that exhibit variability in their sequence and that are involved in determining the specificity and binding affinity of a particular antibody (i.e., the "variable domain(s)"). Variability is not evenly distributed throughout the variable domains of antibodies; it is concentrated in sub-domains of each of the heavy and light chain variable regions. These sub-domains are called "hypervariable regions", "HVR," or "HV," or "complementarity determining regions" (CDRs). The more conserved (i.e., non-hypervariable) portions of the variable domains are called the "framework" regions (FR). The variable domains of naturally occurring heavy and light chains each include four FR regions, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FR and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site (see Kabat et al., see below). Generally, naturally occurring immunoglobulins include six CDRs (see below); three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In naturally occurring immunoglobulins, H3 and L3 display the most diversity of the six CDRs, and H3 in particular is believed to play a unique role in conferring fine specificity to immunoglobulins. Immunoglobulins naturally devoid of light chains, however, include three CDRs that are in the VHH region. The constant domains are not directly involved in antigen binding, but exhibit various effector functions, such as, for example, antibody-dependent, cell-mediated cytotoxicity and complement activation.

[106] Each VHH, VH and VL has three CDRs and four FRs, arranged from amino-terminus (N-terminus) to carboxy-terminus (C-terminus) in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and/or light chains contain a binding domain that interacts with an epitope of an antigen. The term "immunoglobulin" may refer to protein that may two heavy chains without light chains, such as e.g. an antibody devoid of light chains, or an antigen-binding portion thereof. An immunoglobulin may also include at least two heavy (H) chains and two light (L) chains linked by disulfide bonds, or an antigen-binding portion thereof.

[107] An immunoglobulin when used herein, may be a dimeric glycosylated protein composed of two heavy chains such as a camelid heavy chain only IgG (hcIgG) or a shark IgNAR. An immunoglobulin as used herein may also be a tetrameric glycosylated protein composed of two light (L) chains of approximately 25 kDa each and two heavy (H) chains of

approximately 50 kDa each.

[108] When used in connection with a protein or peptide, the term "amino acid" or "amino acid residue" typically refers to an α-amino carboxylic acid having its art recognized definition such as an amino acid selected from the group consisting of: L-alanine (Ala or A); L-arginine (Arg or R); L-asparagine (Asn or N); L-aspartic acid (Asp or D); L-cysteine (Cys or C); L-glutamine (Gln or Q); L-glutamic acid (Glu or E); glycine (Gly or G); L-histidine (His or H); L-isoleucine (Ile or I): L-leucine (Leu or L); L-lysine (Lys or K); L-methionine (Met or M); L-phenylalanine (Phe or F); L-proline (Pro or P); L-serine (Ser or S); L-threonine (Thr or T); L-tryptophan (Trp or W); L-tyrosine (Tyr or Y); and L-valine (Val or V), although modified, synthetic, or rare amino acids such as e.g. taurine, ornithine, selenocysteine, homocystine, hydroxyproline, thioproline, iodo-tyrosine, 3-nitro-tyrosine, ornithine, citrulline, canavanine, 5-hydroxytryptophane, carnosine, cycloleucine, 3,4-dihydroxy phenylalanine, N-acetylcysteine, prolinol, allylglycine or acetidine-2-carboxylic acid may be used as desired. Generally, amino acids can be grouped as having a nonpolar side chain (e.g., Ala, Cys, Ile, Leu, Met, Phe, Pro, Val); a negatively charged side chain (e.g., Asp, Glu); a positively charged sidechain (e.g., Arg, His, Lys); or an uncharged polar side chain (e.g., Asn, Cys, Gln, Gly, His, Met, Phe, Ser, Thr, Trp, and Tyr).

[109] A target according to the invention is any substance of biological or chemical origin to which an antibody of the invention is capable to detect directly or indirectly. Targets may be, for example, proteins, peptides, nucleic acids, oligonucleic acids, saccharides, polysaccharides, glycoproteins. Examples include, but are not limited to therapeutic targets, diagnostic targets, receptors, receptor ligands, viral coat proteins, immune system proteins, hormones, enzymes, antigens, cell signaling proteins, or a fragment thereof. Targets may be native protein or a fragment thereof, a homologous sequence thereof, a functional portion thereof, or a functional portion of a homologous sequence.

[110] The term "epitope", also known as the “antigenic determinant”, refers to the portion of an antigen to which an antibody specifically binds, thereby forming a complex. Thus, the term "epitope" includes any molecule or protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. The binding site(s) (paratope) of an antibody molecule described herein may specifically bind to/interact with conformational or continuous epitopes, which are unique for the target structure. Epitopic determinants usually consist of chemically active 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. Epitope determinants may include chemically active surface

groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and/or specific charge characteristics. With regard to polypeptide antigens a conformational or discontinuous epitope is characterized by the presence of two or more discrete amino acid residues, separated in the primary sequence, but assembling to a consistent structure on the surface of the molecule when the polypeptide folds into the native protein/antigen (Sela, M., Science (1969) 166, 1365-1374; Laver, W.G., et al. Cell (1990) 61, 553-556). The two or more discrete amino acid residues contributing to the epitope may be present on separate sections of one or more polypeptide chain(s). These residues come together on the surface of the molecule when the polypeptide chain(s) fold(s) into a three-dimensional structure to constitute the epitope. In contrast, a continuous or linear epitope consists of two or more discrete amino acid residues, which are present in a single linear segment of a polypeptide chain. As an illustrative example, a "context-dependent" CD3 epitope refers to the conformation of said epitope. Such a context-dependent epitope, localized on the epsilon chain of CD3, can only develop its correct conformation if it is embedded within the rest of the epsilon chain and held in the right position by heterodimerization of the epsilon chain with either CD3 gamma or delta chain. In contrast thereto, a context-independent CD3 epitope may be an N-terminal 1-27 amino acid residue polypeptide or a functional fragment thereof of CD3 epsilon. Generally, epitopes can be linear in nature or can be a discontinuous epitope. Thus, as used herein, the term "conformational epitope" refers to a discontinuous epitope formed by a spatial relationship between amino acids of an antigen other than an unbroken series of amino acids. The term “epitope” also includes an antigenic determinant of a hapten, which is known as a small molecule that can serve as an antigen by displaying one or more immunologically recognized epitopes upon binding to larger matter such as a larger molecule e.g. a protein.

[111] An antibody or antibody molecule/fragment is said to “specifically” bind to an antigen when it recognizes its target antigen within a complex mixture of proteins and/or macromolecules. Typically, the antibody is capable of specifically interacting with and/or binding to its target but does not essentially bind to another epitope or antigen. Antibodies are said to "bind to the same epitope" if the antibodies cross-compete so that only one antibody can bind to the epitope at a given point of time, i.e. one antibody prevents the binding or modulating effect of the other.

[112] Typically, binding that is considered specific may also have a high affinity, e.g. when the binding affinity is higher than 10^{-6} M (in terms of K_d). In particular, the binding

affinity may be about 10^{-8} to 10^{-11} M (K_d), or of about 10^{-9} to 10^{-11} M or even higher. Thus, antibody molecules with an affinity in the picomolar range (with a K_d of $9.9x10^{-10}$ M to 10^{-12} M) are also encompassed in the present invention. If necessary, nonspecific binding of a binding site can be reduced without substantially affecting specific binding by varying the binding conditions.

[113] An antibody according to the invention may be an isolated antibody molecule. The term "isolated antibody molecule" as used herein refers to an antibody molecule that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are matter that would interfere with uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments the antibody molecule is purified to greater than 95% by weight of antibody as determined by the Lowry method, such as more than 99% by weight. In some embodiments the antibody molecule is purified to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator. In some embodiments the antibody is purified to homogeneity as judged by SDS-PAGE under reducing or non-reducing conditions using Coomassie blue or, preferably, silver stain. An isolated antibody molecule may in some embodiments be present within foreign host cells with one or more component(s) of the antibody's natural environment not being present. Typically an isolated antibody is prepared by at least one purification step.

[114] Unless otherwise indicated CDRs sequences of the disclosure follow the definition by AbM used by Oxford Molecular's AbM antibody modelling software. See, generally, e.g., Protein Sequence and Structure Analysis of Antibody Variable Domains. In: Antibody Engineering Lab Manual (Ed.: Duebel, S. and Kontermann, R., Springer-Verlag, Heidelberg). Other standards for defining CDRs exist as well, such as the definition according to Maass 2007 (Journal of Immunological Methods 324 (2007) 13-25). Another standard is the definition according to Kabat CDRs, as described in Sequences of Proteins of immunological Interest, US Department of Health and Human Services (1991), eds. Kabat et al. Another standard for characterizing the antigen binding site is to refer to the hypervariable loops as described by Chothia (see, e.g., Chothia, et al. (1992); J. Mol. Biol. 227:799-817; and Tomlinson et al. (1995) EMBO J. 14:4628-4638). It is understood that embodiments described with respect to the CDR definition of AbM, can alternatively be implemented using similar described relationships such as with respect to Maass, Kabat, or Chothia definition.

[115] The valence of an antibody is generally an expression of the number of antigen-binding sites for one molecule of any given antibody or the number of antibody-

binding sites for any given antigen. Most antibody molecules, and those belonging to the IgG, IgA, IgD and IgE immunoglobulin classes, have two antigen-binding sites per molecule. In general, a monovalent antibody comprises a single antigen binding site. Examples for a monovalent antibody are a single domain antibody, a VHH domain, a single Fab fragment, a Fv fragment, a scFv fragment, or a single VH or VL domain.

[116] The terms "Fab", "Fab region", "Fab portion" or “Fab fragment" are understood to define a polypeptide that includes a V_H, a C_H1, a V_L, and a C_L immunoglobulin domain. Fab may refer to this region in isolation, or this region in the context of an antibody molecule according to the invention, as well as a full-length immunoglobulin or immunoglobulin fragment. Typically a Fab region contains an entire light chain of an antibody. A Fab region can be taken to define “an arm” of an immunoglobulin molecule. It contains the epitope-binding portion of that Ig. The Fab region of a naturally occurring immunoglobulin can be obtained as a proteolytic fragment by a partial papain-digestion. A "$F(ab')_2$ portion" is the proteolytic fragment of a partially pepsin-digested immunoglobulin. A "Fab' portion" is the product resulting from reducing the disulfide bonds of an $F(ab')_2$ portion. As used herein the terms "Fab", "Fab region", "Fab portion" or “Fab fragment" may further include a hinge region that defines the C-terminal end of the antibody arm (cf. above). This hinge region corresponds to the hinge region found C-terminally of the C_H1 domain within a full length immunoglobulin at which the arms of the antibody molecule can be taken to define a Y. The term hinge region is used in the art because an immunoglobulin has some flexibility at this region.

[117] An “Fv” or “Fv fragment” consists of only the V_L and V_H domains of a “single arm” of an immunoglobulin. Thus an "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and binding site. A “two-chain” Fv fragment consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. A single-chain Fv species (scFv) includes a V_H and a V_L domain of an immunoglobulin, with these domains being present in a single polypeptide chain in which they are covalently linked to each other by a flexible peptide linker. Typically, in a scFv fragment the variable domains of the light and heavy chain associate in a dimeric structure analogous to that in a two-chain Fv species. In single chain Fv fragments, it is possible to either have the variable domain of the light chain arranged at the N-terminus of the single polypeptide chain, followed by the linker and the variable domain of the heavy chain arranged at the C-terminus of the polypeptide chain or vice versa, having the variable domain of the heavy chain arranged on the N-terminus and the variable domain of the light chain at the C-terminus with the peptide

linker arranged in between. The peptide linker can be any flexible linker known in the art, for example, made from glycine and serine residues. It is also possible to additionally stabilize the domain association between the V_H and the V_L domain by introducing disulfide bonds into conserved framework regions (see Reiter et al. Stabilization of the Fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions, Biochemistry 1994, 33, 6551-5459). Such scFv fragments are also known as disulfide-stabilized scFv fragments (ds-scFv).

[118] The term "Fc region" or “Fc fragment” is used herein to define a C-terminal region of an immunoglobulin heavy chain, including native-sequence Fc regions and variant Fc regions. The Fc part mediates the effector function of antibodies, e.g. the activation of the complement system and of Fc-receptor bearing immune effector cells, such as NK cells. In human IgG molecules, the Fc region is generated by papain cleavage N-terminal to Cys226. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy-chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody molecule, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody molecule. Accordingly, a composition of intact antibodies may include antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. Suitable native-sequence Fc regions for use in the antibodies of the invention include mammalian, e.g. human or murine, IgG1, IgG2 (IgG2A, IgG2B), IgG3 and IgG4. The Fc region contains two or three constant domains, depending on the class of the antibody. In embodiments where the immunoglobulin is an IgG the Fc region has a C_H2 and a C_H3 domain.

[119] An antibody according to the invention may be produced using any known and well-established expression system and recombinant cell culturing technology, for example, by expression in bacterial hosts (prokaryotic systems), or eukaryotic systems such as yeasts, fungi, insect cells or mammalian cells. An antibody molecule of the present invention may be produced in transgenic organisms such as a goat or a plant. An antibody may also be produced by chemical synthesis.

[120] An antibody of the invention may comprise the CDR1 sequence GVTISALNAMAMG (SEQ ID NO: 115) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AVSERGNAM (SEQ ID NO: 116) or a sequence having 1

or 2 mutations relative to said sequence, and the CDR3 sequence LEDRVDSFHDY (SEQ ID NO: 117) or a sequence having 1 or 2 mutations relative to said sequence. An antibody of the invention may comprise the CDR1 sequence GVTISALNAMAMG (SEQ ID NO: 118) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AVSSRGNAM (SEQ ID NO: 119) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence. An antibody of the invention may comprise the CDR1 sequence GVTVSALNAMAMG (SEQ ID NO: 121) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AVSERGNAM (SEQ ID NO: 122) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEDRVDSFHDY (SEQ ID NO: 123) or a sequence having 1 or 2 mutations relative to said sequence. It is understood that such an antibody preferably comprises or consists of a camelid VHH domain, such as a single domain antibody, or an antibody naturally devoid of light chains. It is further understood that an antibody in which 1 or 2 mutations have been introduced to one, two, or all three of the CDR sequences is still capable of specifically binding the peptide comprised in the fusion protein of the invention, in particular a peptide having the core structure of SEQ ID NO: 3, such as a peptide of any one of SEQ ID NOs: 05-07. In such an antibody of the invention, the E or S at amino acid position 4 of CDR2 may be mutated to G, A, L, I, S, T, V, C, M, D, N, E, Q, F, Y, H, W, K, R, or P, preferably to D, N, or H. Such an antibody of the invention may comprise the FR1 sequence $EVQLX_1ESGGGLVX_2PGGSX_3RLSCTAS$, wherein X_1 is Q, V, E, or L, X_2 is Q or P, and X_3 is L or M (SEQ ID NO: 124), or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR1 sequence EVQLQESGGGLVQPGGSLRLSCTAS (SEQ ID NO: 125) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR2 sequence $WYRQX_1PGEX_2RVMVA$, wherein X_1 is A or R and X_2 is R or E (SEQ ID NO: 126) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR2 sequence WYRQAPGERRVMVA (SEQ ID NO: 127) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR2 sequence WYRQAPGEERVMVA (SEQ ID NO: 128) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR3 sequence $YRESVQGRFTVTRDFTNKMVSLQMDNLX_1PEDX_2AVYYCHV$, wherein X_1 is K or Q and X_2 is T or M (SEQ ID NO: 129) or a sequence having at least 80 %, 85 %, 90 %, or 95 %

sequence identity to said sequence. Such an antibody of the invention may comprise the FR3 sequence YRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYYCHV (SEQ ID NO: 130) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR4 sequence WGQGX$_1$QVTVSS, wherein X$_1$ is T or I (SEQ ID NO: 131) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR4 sequence WGQGTQVTVSS (SEQ ID NO: 132) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

[121] An antibody of the invention may comprise or consist of the VHH sequence selected from the group consisting of:

(a) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 133);

(b) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGEERV MVAAVSSRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYY CHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 134);

(c) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGEERV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 135);

(d) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSSRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYY CHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 136);

(e) EVQLVESGGGLVPPGGSMRLSCTASGVTVSALNAMAMGWYRQRPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLQPEDMAV YYCHVLEDRVDSFHDYWGQGIQVTVSS (SEQ ID NO: 137);

(f) EVQLVESGGGLVPPGGSMRLSCTAPGVTVSALNAMAMGWYRQRPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLQPEDMAV YYCHVLEDRVDSFHDYWGQGIQVTVSS (SEQ ID NO: 138);

(g) EVQLVESGGGVVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAV YYCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 139);

(h) EVQLVESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 140);

(i) EVQLEESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 141); and

(j) EVQLLESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 142);

(k) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSDRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 175);

(l) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSNRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 176);

(m) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSHRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 177)

or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to any one of said sequences.

[122] An antibody of the invention may comprise the CDR1 sequence GTMSAINALN (SEQ ID NO: 143) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AITDNGNAH (SEQ ID NO: 144) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEEEKLGVWVDY (SEQ ID NO: 145) or a sequence having 1 or 2 mutations relative to said sequence. An antibody of the invention may comprise the CDR1 sequence GTMSAINALN (SEQ ID NO: 146) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AITDNGNAH (SEQ ID NO: 147) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEEKLGAWVDY (SEQ ID NO: 148) or a sequence having 1 or 2 mutations relative to said sequence. An antibody of the invention may comprise the CDR1 sequence GTMSAINALN (SEQ ID NO: 149) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AITDNGNAH (SEQ ID NO: 150) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEKEKLGVWVDY (SEQ ID NO: 151) or a sequence having 1 or 2 mutations relative to said sequence. It is understood that such an antibody preferably comprises or consists of a camelid VHH domain, such as a single domain antibody, or an antibody naturally devoid of light chains. It is further understood that an antibody in which 1 or 2 mutations have been

introduced to one, two, or all three of the CDR sequences is still capable of specifically binding the peptide comprised in the fusion protein of the invention, in particular a peptide having the core structure of SEQ ID NO: 3, such as a peptide of any one of SEQ ID NOs: 05-07. Such an antibody of the invention may comprise the FR1 sequence EVQLX1ESGGGLVQPGGSLTLSCAAS, wherein X1 is V or L (SEQ ID NO: 152) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR2 sequence WYRQX1PGKERKMVA, wherein X1 is P or A (SEQ ID NO: 153) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR3 sequence YADSVKGRFTISRDNARNMVFLQMNSLX1PDDTAVYYCHY, wherein X1 is K or E (SEQ ID NO: 154) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence. Such an antibody of the invention may comprise the FR4 sequence WGQGTQVTVSS (SEQ ID NO: 155) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

[123] An antibody of the invention may comprise or consist of the VHH sequence selected from the group consisting of :

(a) EVQLVESGGGLVQPGGSLTLSCAASGTMSAINALNWYRQPPGKERKMVA AITDNGNAHYADSVKGRFTISRDNARNMVFLQMNSLKPDDTAVYYCHYL EEEKLGVWVDYWGQGTQVTVSS (SEQ ID NO: 156);

(b) EVQLLESGGGLVQPGGSLTLSCAASGTMSAINALNWYRQAPGKERKMVA AITDNGNAHYADSVKGRFTISRDNARNMVFLQMNSLEPDDTAVYYCHYL EEKLGAWVDYWGQGTQVTVSS (SEQ ID NO: 157); and

(c) EVQLVESGGGLVQPGGSLTLSCAASGTMSAINALNWYRQPPGKERKMVA AITDNGNAHYADSVKGRFTISRDNARNMVFLQMNSLKPDDTAVYYCHYL EKEKLGVWVDYWGQGTQVTVSS (SEQ ID NO: 158);

or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to any one of said sequences.

[124] The antibody of the invention may be conjugated to a detectable label. In general, such a "detectable label" may be any appropriate chemical substance or enzyme, which directly or indirectly generates a detectable compound or signal in a chemical, physical, optical, or enzymatic reaction. For example, a fluorescent or radioactive label can be conjugated to the antibody to generate fluorescence or X-rays as detectable signal. Alkaline phosphatase, horseradish peroxidase and β-galactosidase are examples of enzyme labels (and at the same time optical labels), which catalyze the formation of chromogenic reaction

products. In a preferred embodiment, the detectable label refers to detectable entities that can be used for the detection of the target of interest in microscopy, immunohistochemistry or flow cytometry. Preferably, the label does not negatively affect the characteristics of the antibody to which the label is conjugated. Examples of labels are fluorescent labels such as phycoerythrin, allophycocyanin (APC), Brilliant Violet 421, Alexa Fluor 488, coumarin or rhodamines to name only a few. There are many types of detectable labels, including a fluorescent label, a chromophore label, an isotope label, or a metal label, with a fluorescent label being preferred. The presence of a fusion protein of the invention may be detected by contacting the fusion protein with an antibody of the invention conjugated to a detectable label and detecting the signal of the detectable label. For a fluorescent label, this means detection of emitted light upon excitation of the fluorescent label. Non-exhaustive examples for suitable fluorescent labels are "green" emitters (Atto488, Alexa488, Cy2, etc.), "orange" emitters (Atto542, alexa555, Cy3, etc.), "Red-far-Red" emitters (Alexa633, Atto 647N, Cy5, etc.), infrared emitters (Atto700, LiCor IRDye700, LiCor IRDye800, etc.), ultra-violet absorbing fluorescent dyes (Atto390 or Alexa405). A fluorescent label may also be a fluorescent protein, such as GFP, eGFP, YFP, RFP, CFP, BFP, mCherry, or near-infrared fluorescent proteins. Non-exhaustive examples for a suitable chromophore label are alkaline phosphatase or peroxidase exposed to TMB (3,3',5,5' tetramethylbenzidine), DAB (3,3',4,4' diaminobenzidine), and 4CN (4-chloro-1-naphthol). ABTS (2,2'-azino-di [3-ethyl-benzthiazoline] sulfonate), OPD (o-phenylenediamine), and to BCIP/NBT (5-bromo- 4-chloro-3-indolyl-phosphate/nitroblue tetrazolium). Non-exhaustive examples for isotope labels are 13C, 15N, 19F, 27Al, 11B, 127I or different Lanthanides isotopes. Non-exhaustive examples for a metal label are Au, Pd, Pb, Pt Ag, Hg and Os. The label may be a direct label, i.e. a label that is directly detectable. Alternatively, the label may be an indirect label, i.e. a label which is an affinity tag (or epitope tag) that can be specifically bound by another specific binding partner that is conjugated to another detectable label, such as a fluorescent or chromophore label. Examples of suitable epitope tags include, but are not limited to, FLAG-tag (sequence: DYKDDDDK, SEQ ID NO: 160), Strep-tag (sequence: WSHPQFEK, SEQ ID NO: 178), Myc-tag (sequence: EQKLISEEDL, SEQ ID NO: 161), HA-tag (sequence: YPYDVPDYA, SEQ ID NO: 162), VSV-G-tag (sequence: YTDIEMNRLGK, SEQ ID NO: 163), HSV-tag (sequence: QPELAPEDPED, SEQ ID NO: 164), V5-tag (sequence: GKPIPNPLLGLDST, SEQ ID NO: 165), SPOT-tag (sequence: PDRVRAVSHWSS, SEQ ID NO: 166), BC2 tag (sequence: PDRKAAVSHWQQ, SEQ ID NO: 167), and EPEA tag (sequence: EPEA, SEQ ID NO: 168). The antigen may also be a protein, for example,

glutathione-S-transferase (GST), maltose binding protein (MBP), chitin binding protein (CBP) or thioredoxin as an antigen. The detectable label may further be a nucleic acid, such as an oligonucleotide having a recognition sequence. Such a recognition sequence may be a random sequence. This random sequence may be barcode sequence that has been incorporated into the nucleic acid molecules and can be used to identify the target molecule that has been conjugated with said nucleic acid. An "antibody of the invention may be conjugated to a detectable label" may also mean that the antibody itself is the detectable label. This may imply that the antibody is an affinity target that can be specifically recognized by another specific binding partner that specifically binds to the antibody. For example, such a specific binding partner may be an antibody that specifically recognizes camelid VHH domains. Such a specific binding partner may further be conjugated to a detectable label, such as a fluorescent label.

[125] An antibody of the invention may be conjugated to a solid support. The term "solid support" or in the context of the present invention refers to any type of carrier material that can be used for immobilization of affinity ligands such as antibodies or parts thereof and it can refer to material in particulate (e. g. beads or granules, generally used in extraction columns) or in sheet form (e. g. membranes or filters, glass or plastic slides, microtiter assay plates, dipstick, capillary fill devices or such like) which can be flat, pleated, or hollow fibers or tubes. Suitable and well-known matrices without being exhaustive: are silica (porous amorphous silica), agarose or polyacrylamide supports, or macroporous polymers. Examples include dextran, collagen, polystyrene, polypropylene, polyvinylchloride, polyacrylamide, methacrylate, celluloses, calcium alginate, controlled pore glass, aluminum, titanium and porous ceramics, synthetic polymers and co-polymers, latex, silica, agarose, metal, glass, and carbon. Alternatively, the solid surface may comprise part of a mass dependent sensor, for example, a surface plasmon resonance detector. Conveniently, an array comprising a plurality of individual affinity ligand such as antibodies or antibody fragments, which are capable of specifically binding the epitope tag of the present invention, bound or immobilized to a solid surface is provided. This array can be used to capture tagged polypeptides comprised in a solution as soon the solution is brought in contact with the immobilized affinity ligand such as antibodies or antibody fragments. A solid support can also be a magnetic bead or polymeric bead or a chromatographic stationary phase.

[126] An antibody of the invention may be in complex with an epitope it specifically binds to. Such an epitope may be a peptide that is comprised in the fusion protein of the invention. The antibody of the invention may thus be in complex with a fusion protein of the

invention. The present invention therefore encompasses a fusion protein comprising an peptide that the antibody of the invention specifically binds to.

[127] The present invention also relates to a complex comprising (a) fusion protein and (b) an antibody, wherein the fusion protein is a fusion protein of the invention and/or wherein the antibody is an antibody of the invention.

[128] The present invention also relates to a nucleic acid molecule comprising a sequence encoding a fusion protein of the invention as described herein or an antibody of the invention as described herein. The nucleic acid molecule may be a DNA or an RNA molecule. The nucleic acid molecules of the invention may be part of a vector or any other kind of cloning or expression vehicle, such as a plasmid, a phagemid, a phage, a baculovirus, a cosmid or an artificial chromosome. The nucleic acid molecule, may allow expression of the fusion protein or antibody. It may include sequence elements that contain information regarding to transcriptional and/or translational regulation, and such sequences may be "operably linked" to the nucleotide sequence encoding the protein. An operable linkage is a linkage in which the regulatory sequence elements and the sequence to be expressed are connected in a way that enables gene expression. The precise nature of the regulatory regions necessary for gene expression may vary among species, but in general these regions include a promoter, which, in prokaryotes, contains both the promoter per se, i.e., DNA elements directing the initiation of transcription, as well as DNA elements which, when transcribed into RNA, will signal the initiation of translation. Such promoter regions normally include 5' non-coding sequences involved in initiation of transcription and translation, such as the -35/-10 boxes and the Shine-Dalgarno element in prokaryotes or the TATA box, CAAT sequences, and 5'-capping elements in eukaryotes. These regions can also include enhancer or repressor elements as well as translated signal and leader sequences for targeting the native protein to a specific compartment of a host cell.

[129] Such a vehicle described herein may include, aside from the regulatory sequences described herein and a nucleic acid sequence encoding a peptide or protein described herein, replication and control sequences derived from a species compatible with a host cell that is used for expression as well as selection markers conferring a selectable phenotype on transformed or transfected cells. Large numbers of suitable cloning vectors are known in the art and are commercially available. Accordingly, the present invention also relates to a vector comprising the nucleic acid molecule of the invention.

[130] Cloning or expression of nucleic acid molecule or the vector of the invention can be conducted at least partially in vivo, using host cells transformed with the nucleic acid

or vector, or to which the nucleic acid molecule or vector has been transferred by other means including transduction or transfection. Transfer of DNA can be performed using standard techniques. Thus, the disclosure is also directed to a host cell containing a nucleic acid molecule or a vector as disclosed herein.

[131] A peptide of the invention is useful for several applications. These cellular applications may have in common that the peptide is used as an epitope tag. Non-limiting examples for applications, in which the peptide is useful include, but are not limited to, detection, immobilization, isolation, or purification of the fusion protein of the invention. Similarly also an antibody of the invention may be used for all applications that includes specific binding of an epitope tag. An antibody of the invention may be used for example for detection, immobilization, isolation, or purification of a fusion protein of the invention.

[132] The present invention also relates to a method of detecting a fusion protein of the invention. Detection can be optical detection, isotopic detection, or detection by electron microscopy. The method comprises contacting the fusion protein with an antibody of the invention, preferably under conditions allowing the formation of a complex between the peptide comprised in the fusion protein and the antibody. The antibody of the invention preferably carries a detectable label as defined herein. Where the detectable label is a fluorescent label, chromophore label, isotope label, or metal label attached to it, it is understood that the antibody preferably has a defined number of labels attached to it. The method may comprise the step of detecting the detectable label. The method may comprise expressing the fusion protein of the invention prior to contacting the fusion protein with the antibody.

[133] The term “detection” as used herein includes both, direct detection of a target (i.e. wherein the target is detected by a signal deriving from a target) and indirect detection of a target (i.e. wherein the target is detected by a signal that does not directly derive from the target, e.g. by a signal that derives from another molecule attached to the target). The term “detection” as used herein further includes both, qualitative and quantitative detection. The term “detection” may refer to determination of the presence, subcellular localization, or amount of a given molecule or structure, such as the fusion protein of the invention. The fusion protein to be detected, located and/or quantified can be detected at its intracellular location in a host cell, for example in the cell nucleus, in cell membranes or another cell compartment. The fusion protein to be detected, and/or to be quantified can also be detected in a solution comprising the tagged polypeptide or protein, for example a cell lysate obtained from a host cell or a tissue comprising the host cell.

[134] The term “optical”, as used herein, preferably refers to visible light but is generally not limited to it. The term may also refer to infrared, ultraviolet and other regions of the electromagnetic spectrum.

[135] As used herein, "isotopic detection" relates to the detection of a molecule in which one or more atoms have been replaced (i.e. "labeled") with another isotope that commonly has a detectable variation. The isotopic label can be detected by multiple means, such as their mass (e.g. by mass spectrometry, matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), or secondary-ion mass spectrometry (SIMS), vibrational mode (e.g. by infrared spectroscopy), gyromagnetic ratios (e.g. by nuclear magnetic resonance), or radioactive decay (e.g. an ionization chamber or autoradiographs of gels).

[136] Electron microscopy relates to a method of detection using an electron microscope. Types of electron microscopes include transmission electron microscope (TEM), scanning electron microscope (SEM), reflection electron microscope (REM), scanning transmission electron microscope (STEM), and Correlative Light and Electron Microscopy (CLEM).

[137] In a first step of a detection method, an antibody specifically binding the peptide comprised in the fusion protein of the present invention may be contacted with a sample comprising the fusion protein. The sample may be a host cell, a tissue, a solution comprising cell lysate of a host cell or any other sample that comprises the fusion protein, such as a supernatant, as obtained after centrifugation of a liquid comprising the host cell, wherein the host cell is capable of secreting the polypeptide of interest into the liquid or another specimen like a body fluid.

[138] This contacting step is preferably carried out at conditions that allow specific interaction of the antibody and the peptide it specifically binds to. Such conditions are well known to the person of skill in the art. Washing steps typically follow the contacting step of an antibody to its antigen, and the skilled person knows how and when to apply said washing steps. Upon contacting with the sample, the antibody will specifically interact with the fusion protein. This interaction can be detected, monitored and quantified by measuring or observing the reporter signal obtained from the detectable label. For example, if the detectable label is a fluorescent label, fluorescence can be measured and observed upon excitation.

[139] If the detectable label is an affinity tag (or epitope tag) that can be specifically bound by another specific binding partner that is conjugated to another detectable label, such

as a fluorescent or chromophore label. In such a case, detection of the detectable label attached to the antibody of the invention may be conducted by contacting the antibody with a specific binding partner that specifically binds to the detectable label conjugated to the antibody of the invention. The specific binding partner may be labeled with a further detectable label that can preferably be distinguished from the first detectable label, such as a fluorescent or chromophore label. The specific binding partner may however be a structure, which can be recognized by another specific (labeled) binding reagent. For example, the specific binding partner that binds to the detectable label conjugated to the antibody of the invention may be a (primary) antibody, which may be specifically recognized by a (secondary) antibody, which carries a detectable label that is preferably distinguishable from the first detectable label, such as a fluorescent label. The method of detection the fusion protein of the invention may thus comprise the step of contacting the fusion protein and the antibody with a specific binding partner for the detectable label comprised in the antibody. Where a second detectable label is present, the method may comprise the step of detecting the first and/ or the second detectable label.

[140] According to methods where the detectable label conjugated to the antibody of the invention is an affinity tag, the method of detecting the fusion protein may comprise, in a first step, contacting the antibody of the invention with a sample comprising or suspected to comprise the fusion protein of the invention. In a second step, a (secondary) specific binding partner can be contacted with the sample comprising the fusion protein bound to the antibody of the invention. In cases where the (secondary) specific binding partner is not conjugated to a detectable label or where the detectable label is an affinity tag, the method may further comprise the step of contacting a further specific binding partner, such as a further antibody that specifically binds to the (secondary) specific binding partner or its detectable label. The further specific binding partner may comprise a detectable label, such as a fluorescent label that can be used for detection of the fusion protein. Presence, amount and/or localization of the tagged polypeptide or protein can be detected or determined by measuring or observing a reporter signal obtained from a detectable label comprised in the (secondary) specific binding partner or further specific binding partner.

[141] An advantage of a two-step detection method using two types of binding partners is that the tag specific interaction is separated from the actual detection step. This allows that the antibody of the invention remains unchanged, as it does not need to comprise an additional detectable moiety. This may in some cases enhance its specificity or affinity compared to an antibody comprising an additional detectable label, as the detectable label

could in some cases influence the interaction of the peptide comprised in the fusion protein of the invention and the antibody of the invention. Thus, the reliability and efficiency of the detection method could be enhanced in some cases. Furthermore, using the antibody of the invention simply as a capture antibody and not as a capture and detection antibody, allows separation of the capture and the detection steps if only presence and amount of the fusion protein is to be determined. Therefore, the first step using the antibody of the invention could be followed by an isolation or enrichment step, yielding the captured fusion protein of interest. The detection step could then be carried out on the isolated and/or enriched fusion protein, leading to an enhanced reliability of the obtained quantification and an easier handling of the detection step.

[142] Suitable biophysical or biomolecular detection methods for qualitatively detecting the epitope tag/antibody interaction comprise any suitable method known in the art. Such methods include, without being limited thereto, methods as applied for qualitative or quantitative assays, e.g. for Enzyme-linked Immunosorbent Assay (ELISA), ELISPOT assay, Western Blot, or immunoassays. Such methods comprise e.g. optical, radioactive or chromatographic methods, preferably when using any of the above labels, markers or linkers, more preferably fluorescence detection methods, radioactivity detection methods, Coomassie-Blue staining, silver staining or other protein staining methods, electron microscopy methods, methods for staining tissue sections by immunohistochemistry or by direct or indirect immunofluorescence, etc. Such methods may be applied either with the antibody or may involve the use of further tools, e.g. the use of a secondary binding partner, specifically binding to a part of the fusion protein, the antibody, or the complex.

[143] In some embodiments, the subcellular localization of the tagged polypeptide or protein of interest can also be determined. For example, distinct subcellular structures such as the intermediate filamentous network or an essential part of the replication machinery can be visualized and monitored.

[144] Detection of the fusion protein can also be carried out using an antibody of the invention that is an intrabody. An "intrabody" as used herein refers to an antibody that is located within a cell to bind to an intracellular protein. Due to the lack of a reliable mechanism for bringing an antibody into a living cell from the extracellular environment, this typically requires the expression of the antibody within the target cell. After expression, the antibody may remain in the cytoplasm, or it may have a nuclear localization signal, or it may undergo co-translational translocation across the membrane into the lumen of the endoplasmic reticulum, provided that it is retained in that compartment through a KDEL sequence. A

detectable label conjugated to an intrabody may be a proteinaceous label, which can be expressed as fusion protein with the intrabody. Ideally, such a label may be optically detectable, such as by fluorescence. The detectable label may thus be a fluorescent protein.

[145] The present invention also relates to a method of isolating the fusion protein of the invention. Such a method comprises contacting the fusion protein with an antibody of the invention, preferably under conditions allowing formation of a complex between the antibody and the peptide comprised in the fusion protein. Thereby, binding of the fusion protein and the antibody is enabled. This contacting step, also referred to as capture step, may be conducted by contacting a sample, for example a solution, comprising the fusion protein with the antibody.

[146] The sample to be contacted with the tag specific antibody can be any type of sample comprising a fusion protein of the invention and can be processed to separate the polypeptide. Preferably the sample is a solution, for example a lysate of a host cell or a body fluid, comprising the fusion protein of the invention, or a supernatant, such as a supernatant obtainable by centrifugation of a liquid comprising a host cell comprising or capable of expressing fusion protein of the invention, wherein the host cell is capable of secreting or otherwise transporting the fusion protein of the invention to the liquid.

[147] The antibody used in the method of the present invention for isolation and/or purification can be used in solution or immobilized. To immobilize the antibody, the antibody can be bound to a sample carrier, solid support, or matrix. This immobilization step can occur prior to or after the binding of the antibody to the peptide comprised in the fusion protein. Methods for immobilizing antibodies and parts thereof are well-known to the person skilled in the art and any method that allows immobilization without impairing binding properties can be used.

[148] If the antibody of the present invention is not immobilized to a solid support, then the method may comprise a further step of isolating the complex, for example by using a specific binding partner for the complex, such as a secondary antibody that is specific for example for the complex or for the antibody or for a detectable label, such as an affinity tag, that is conjugated to the antibody. The secondary binding partner can be in solution or can be immobilized or immobilizable to a solid support.

[149] In an optional further step following the capture step, the solid support comprising the immobilized antibody bound to the fusion protein is washed to remove unbound and unspecifically bound constituents.

[150] Optionally, in a further step, the fusion protein can be eluted to obtain the

isolated fusion protein. Elution of the fusion protein bound to the immobilized antibody can be achieved by methods known in the art. For example, the fusion protein can be eluted by competitive elution with an epitope peptide as described herein in isolated form. This isolated epitope peptide will then be in competition with the fusion protein to bind the immobilized tag-specific antibody. If the isolated peptide is added in surplus concentration, the reaction balance of the binding will be shifted to the binding of the immobilized antibody with the isolated epitope tag. This results in the release of the fusion protein. The epitope peptide used for elution may be the same epitope peptide that is comprised in the fusion protein. The epitope peptide used for elution may also be a different peptide than the epitope peptide comprised in the fusion protein. If the epitope peptide used for elution is a different one, it is preferred that the epitope peptide used for elution has a higher binding affinity to the antibody than the epitope peptide comprised in the fusion protein. Additional steps for further purifying the released polypeptide can optionally be added, such as method steps well-known to the skilled person.

[151] The fusion protein may also remain immobilized to the solid support, such as (magnetic) beads, and processed further in downstream application such as mass spectrometry, without the elution step.

[152] The fusion protein may comprise a linker with a cleavage site that can be cleaved with an appropriate means, for example a protease, to remove the peptide. Thereby the polypeptide of the fusion protein may be released from the immobilized antibody, and the polypeptide can be obtained in its native form. For this embodiment, the nucleic acid sequence encoding the fusion protein should not only comprise a sequence encoding the epitope tag but also a sequence encoding a linker with a breakable site, for example a cleavage site recognized by a protease. The release step by enzymatic cleave can replace or follow the elution step.

[153] Where the fusion protein of the invention comprises an antibody moiety, the present invention also envisions a method of isolating the target of the antibody moiety of the invention. In principle, this method can be carried out as described above for the isolation of a fusion protein. The method may comprise the additional step of contacting the fusion protein with a specific target of the antibody moiety comprised in the fusion protein. This contacting step may be conducted prior to or after contacting the fusion protein with the antibody that binds to the peptide tag comprised in the fusion protein, with the latter alternative being preferred. In a preferred method, first the antibody specific for the peptide tag is immobilized on the solid support, and then the fusion protein is immobilized via binding to the antibody

that is specific to the peptide tag, followed by binding the target of the antibody moiety of the fusion protein to the fusion protein. Elution can be carried out as described above. The specific target may be a cell. For example, a cell surface receptor on the cell, such as CD62L. The antibody moiety of the fusion protein may be specific to a structure on the cell, such as CD62L. The antibody moiety of the fusion protein may be a single domain antibody.

[154] The present invention also envisions that detection and isolation of a fusion protein of the invention can be combined. Accordingly, the present invention envisions a method of detection and isolation of a fusion protein of the invention comprising a method of detection of the invention and a method of isolation and/or purification of the invention.

[155] Combination of both methods may thus be carried out by using one antibody conjugated to a detectable label for detection, and another antibody conjugated to a solid support for isolation of the same fusion protein. Both antibodies may be any antibody of the invention. This combination may have the advantage that only one tagged fusion protein has to be generated and detection and isolation carried out with the same transgenic construct/cell. Sometimes, it may be desired that both antibodies have an identical sequence or at least an identical antigen-binding site. For example, both antibodies may comprise CDR 1-3 sequences set forth in SEQ ID NOs: 115-117 or may comprise the sequence set forth SEQ ID NO: 133 or a sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 133. Sometimes, it may be desired that both antibodies have different sequences or different antigen-binding sites. Using different antibodies may have the advantage that tight binder, e.g, an antibody having a high affinity (e.g. about 1 pM to about 1 nM) to the peptide comprised in the fusion protein, can be used for detection, whereas a moderate binder, i.e. an antibody having moderate affinity (e.g. about 1 nM to about 500 nM) can be used for isolation of the fusion protein. For example, one of the antibodies may comprise CDR 1-3 sequences set forth in SEQ ID NOs: 115-117 or may comprise the sequence set forth SEQ ID NO: 133 or a sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 133 while the other antibody may comprise CDR 1-3 sequences set forth in SEQ ID NOs: 118-120 or may comprise the sequence set forth SEQ ID NO: 134 or a sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 134. The peptide comprised in the fusion protein may be a peptide having a core sequence of SEQ ID NO: 3 or 4 as described herein.

[156] Combination of both methods can also be carried out by using the same antibody and two different peptides for detection and purification. This has the advantage, that only one antibody has to be produced which, depending on the application, can be conjugated

to a detectable label or a solid support. When using two different peptides, it is preferred that the peptides have different binding affinity to the antibody. The peptide that is used for detection, may have a high binding affinity of e.g. about 1 pM to 1 nM, while the peptide that is used for isolation may have a moderate binding affinity of about 1 nM to 500 nM. Using peptides having different binding affinities may have the advantage that if a moderate affinity peptide is comprised in the fusion protein for isolation, a high(er) affinity peptide can be used for elution of the fusion protein. Accordingly, one peptide may be a peptide having a core sequence of SEQ ID NO: 3 or 4 as described herein, while the other peptide may be a peptide having a core sequence of SEQ ID NO: 31 or 32 as described herein. The antibody may be any antibody of the invention, e.g. the antibody may comprise CDR 1-3 sequences set forth in SEQ ID NOs: 115-117 or may comprise the sequence set forth SEQ ID NO: 133 or a sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 133.

[157] Combination of both methods can also be carried out by using two peptides and two antibodies. The fusion protein of interest may comprise a peptide having a high affinity to a given antibody. For example, the peptide may be a peptide having a core sequence of SEQ ID NO: 3 or 4 as described herein. The antibody for detection may comprise CDR 1-3 sequences set forth in SEQ ID NOs: 115-117 or may comprise the sequence set forth SEQ ID NO: 133 or a sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 133. The antibody for isolation/purification may comprise CDR 1-3 sequences set forth in SEQ ID NOs: 118-120 or may comprise the sequence set forth SEQ ID NO: 134 or a sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 134. The peptide used for elution may be a peptide having higher affinity to the antibody for elution than the peptide comprised in the fusion protein, such as another peptide described herein.

[158] The present invention further relates to a system comprising one peptide tag and two antibodies or two peptide tags and one antibody or two peptide tags and two antibodies as described herein.

[159] The present invention also relates to a kit. The kit may comprise components necessary to carry out a method of the present invention. A kit for detection or purification of a fusion protein may comprise a nucleic acid or a nucleic acid expression construct encoding a peptide/epitope tag as defined herein, which may be present in the fusion protein. The nucleic acid may comprise a site, such as a cleavage or recombination site, that facilitates genetically fusing a polypeptide to the peptide/epitope tag. A nucleic acid sequence encoding the peptide/epitope tag may be operably linked to sequence elements that contain information

regarding to transcriptional and/or translational regulation.

[160] The kit may also comprise an antibody of the invention, optionally conjugated to a detectable label described herein, preferably an optically detectable label or an affinity tag. Alternatively or additionally, the kit may comprise a detectable moiety that can be conjugated to the antibody of the invention.

[161] The kit may also comprise buffers and reagents necessary for the isolation/purification and/or detection methods of the present invention.

[162] The kit may also comprise buffers and reagents necessary to introduce the nucleic acid or the nucleic acid expression construct comprised in the kit into a host cell.

[163] The kit may also comprise at least one (secondary) specific binding partner as described herein or a (further) specific binding partner that specifically binds the (secondary) specific binding partner as described herein.

[164] The kit may also comprise a solid support comprising the antibody of the invention immobilized or attached to the solid support. The kit may also comprise an isolated peptide as described herein suitable for competitive elution of a fusion protein bound to an antibody of the invention, or other means for elution of the fusion protein, such as a proteinase.

[165] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[166] The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

[167] The term "about" or "approximately" as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. It includes, however, also the concrete number, e.g., about 20 includes 20.

[168] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or “including” or sometimes when used herein with the term “having”.

[169] When used herein “consisting of" excludes any element, step, or ingredient not

specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[170] In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms.

[171] It should be understood that this invention is not limited to the particular methodology, protocols, material, reagents, and substances, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[172] All publications cited throughout the text of this specification (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.) are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

EXAMPLES

[173] **Example 1: The system**

[174] In search for a novel epitope tag/binder system, we designed peptides SEQ ID NOs: 05-07 that form a stable α-helix in solution and are collectively referred to as "ALFAST tag". These peptides do not have a known counterpart in any eukaryotic model system, they are nearly neutral at physiological pH and do not contain any primary amines that could be a target for common fixatives. The core ALFA tag sequence (Fig 3A, amino acids written in blue) comprises an artificial peptide reported to form a stable α-helix in solution (Petukhov, M. *et al.* Design of stable alpha-helices using global sequence optimization. *J. Pept. Sci.* **15,** 359–365 (2009)). It was selected based on the following properties: The sequence i) does not have a known counterpart in any eukaryotic model system, ii) it is neutral at physiological pH and iii) does not contain any primary amines that could be a target for common amine-reactive fixatives and cross linkers.

High-affinity nanobodies against ALFAST were raised in Alpaca and selected by "Celline", a novel nanobody selection method employing antigen-specific enrichment of B-cells. To create a selective affinity resin, our favorite nanobody (NbALFAST, clone 1G5, SEQ IS NO: 133) was site-specifically coupled via a flexible linker to an agarose-based resin featuring low nonspecific protein binding. The resulting ALFA SelectorST resin was highly efficient in pulling down an ALFAST-shGFP2 fusion protein from *E.coli* extracts. Surprisingly, however,

all approaches to elute the bound protein under native conditions by competition with ALFAST peptide (Ac-PSRLEEELRRRLTEP-Amide set forth in SEQ ID NO: 179) failed. Even after 90 minutes competition with 10 column volumes of 200µM peptide, >95% of the ALFAST-shGFP2 protein remained on the resin (Fig. 1B, middle panel, grey line). We thus assumed that the off-rate of the ALFAST fusion proteins bound to the resin was too low to allow for an efficient competition by the peptide. We therefore performed systematic and rational mutagenesis in order to identify ALFAST mutants that bind sufficiently strong to NbALFAST to allow for an efficient pull-down of target proteins, but on the other side could be eluted from the nanobody using the ALFAST peptide. The peptides that were analyzed are shown in Table 2.

Table 2:

Sequence	Position	Class	$t_{1/2}$
ds**MRLEEELRRRLSK**g (SEQ ID NO: 90)	M	n.d.	n.d.
ds**MRLEEELRRRLSP**g (SEQ ID NO: 91)	M	n.d.	n.d.
sd**SG-EEELRRRLSP**g (SEQ ID NO: 92)	M	T0	< 2 min
sd**SG--EELRRRLSP**g (SEQ ID NO: 93)	M	T0	< 2 min
ds**GRLEEELRRR-SP**g (SEQ ID NO: 94)	M	T0	< 2 min
ds**GRLEEELRR--SP**g (SEQ ID NO: 95)	M	T0	< 2 min
ds**GRLEEELR---SP**g (SEQ ID NO: 96)	M	T0	< 2 min
sd**SGLEEEARRRLSP**g (SEQ ID NO: 97)	M	T0	< 2 min
sd**SGAEEELRRRLSP**g (SEQ ID NO: 98)	M	T0	< 2 min
ds**GALEEELRRRLSP**g (SEQ ID NO: 99)	M	T0	< 2 min
ds**GALEQEIRRRLSP**g (SEQ ID NO: 100)	M	T0	< 2 min
ds**GRLEQEIRRALSP**g (SEQ ID NO: 101)	M	T0	< 2 min
ds**GRLEQEIRRQLSP**g (SEQ ID NO: 102)	M	T0	< 2 min
ds**GRLEQEIRRELSP**g (SEQ ID NO: 103)	M	T0	< 2 min
pd**SGLEQELRRRLPT**a (SEQ ID NO: 104)	M	T0	< 2 min
dp**SGLEQELRRRLPT**a (SEQ ID NO: 105)	M	T0	< 2 min
ds**PGLEQELRRRLPT**a (SEQ ID NO: 106)	M	T0	< 2 min
ds**GPLEQELRRRLPT**a (SEQ ID NO: 107)	M	T0	< 2 min
gp**SGLEQELRRRL**. (SEQ ID NO: 108)	C	T0	< 2 min
M**SAVEEELRRRLSP**s (SEQ ID NO: 109)	N	T0	< 2 min
M**SGLEQELRRRLTP**s (SEQ ID NO: 110)	N	T0	< 2 min
M**SAVEEELRRRLSP**s (SEQ ID NO: 111)	N	T0	< 2 min
Mp**SAVEEELRRRLSP**s (SEQ ID NO: 112)	N	T0	< 2 min
Ms**SAVEEELRRRLSP**s (SEQ ID NO: 113)	N	T0	< 2 min
ps**GRLEEELRRRLP**. (SEQ ID NO: 114)	C	T0	< 2 min
ds**GRLEEELRRRLSK**g (SEQ ID NO: 62)	M	T1	2-30 min
ds**GRLEEELRRRLSP**g (SEQ ID NO: 63)	M	T1	2-30 min

sd**SGLEEELRRRLSP**g (SEQ ID NO: 64)	M	T1	2-30 min
sd**SGVEEELRRRLSP**g (SEQ ID NO: 65)	M	T1	2-30 min
sd**SAVEEELRRRLSP**g (SEQ ID NO: 66)	M	T1	2-30 min
sd**SGLQEELRRRLSP**g (SEQ ID NO: 67)	M	T1	2-30 min
sd**SGLEEQLRRRLSP**g (SEQ ID NO: 68)	M	T1	2-30 min
sd**SGLEEEIRRRLSP**g (SEQ ID NO: 69)	M	T1	2-30 min
sd**SGLEEEVRRRLSP**g (SEQ ID NO: 70)	M	T1	2-30 min
ds**GELEEELRRRLSP**g (SEQ ID NO: 71)	M	T1	2-30 min
ds**GRLEQELRRRLSP**g (SEQ ID NO: 72)	M	T1	2-30 min
ds**GRLEEEIRRRLSP**g (SEQ ID NO: 73)	M	T1	2-30 min
ds**GRLEQEIRRRLSP**g (SEQ ID NO: 74)	M	T1	2-30 min
ds**GRLEQEIARRLSP**g (SEQ ID NO: 75)	M	T1	2-30 min
ds**GRLEQEIQRRLSP**g (SEQ ID NO: 76)	M	T1	2-30 min
ds**GRLEQEIERRLSP**g (SEQ ID NO: 77)	M	T1	2-30 min
ds**GRLEQEIRARLSP**g (SEQ ID NO: 78)	M	T1	2-30 min
ds**GRLEQEIRQRLSP**g (SEQ ID NO: 79)	M	T1	2-30 min
ds**GRLEQEIRERLSP**g (SEQ ID NO: 80)	M	T1	2-30 min
gp**SRLEEELRRRL**. (SEQ ID NO: 81)	C	T1	2-30 min
M**SGLEQELRRRLTP**s (SEQ ID NO: 82)	N	T1	2-30 min
Ms**GRLEEELRRRLSP**s (SEQ ID NO: 83)	N	T1	2-30 min
sp**SAVEEELRRRLSP**s (SEQ ID NO: 84)	M	T1	2-30 min
gp**SAVEEELRRRLS**. (SEQ ID NO: 85)	C	T1	2-30 min
Mp**SGLEQELRRRLTP**s (SEQ ID NO: 86)	N	T1	2-30 min
Ms**SGLEQELRRRLTP**s (SEQ ID NO: 87)	N	T1	2-30 min
Mp**SGRLEEELRRRLS**ps (SEQ ID NO: 88)	N	T1	2-30 min
ms**GRLEEELRRRLSP**s (SEQ ID NO: 83)	M	T1	2-30 min
ms**GRLEEELRRRLSP** (SEQ ID NO: 89)	C	T1	2-30 min
ms**GRLEEELRRRLSP** (SEQ ID NO: 33)	N, M, C	T1	2-30 min
sd**SGLEQELRRRLSP**g (SEQ ID NO: 34)	M	T1	2-30 min
pd**GGLEQELRRRLTA**p (SEQ ID NO: 35)	M	T1	2-30 min
ps**GGLEQELRRRLTA**p (SEQ ID NO: 36)	M	T1	2-30 min
ds**PGLEQELRRRLTA**p (SEQ ID NO: 37)	M	T1	2-30 min
pd**SGLEQELRRRLTP**a (SEQ ID NO: 38)	M	T1	2-30 min
sp**SGLEEELRRRLTA**ep (SEQ ID NO: 39)	M	T1	2-30 min
gp**SGLEQELRRRLT**. (SEQ ID NO: 40)	C	T1	2-30 min
gp**SGLEQELRRRLTA**as. (SEQ ID NO: 41)	C	T1	2-30 min
sp**SRLEEELRRRLPS**k (SEQ ID NO: 42)	M	T1	2-30 min
sp**SGLEQELRRRLTP**s (SEQ ID NO: 43)	M	T1	2-30 min
sp**GRLEQEIRRRLSP**s (SEQ ID NO: 44)	M	T1	2-30 min
ps**GRLEEELRRRLSP**s (SEQ ID NO: 45)	M	T1	2-30 min
ps**GRLEEELRRRLS**. (SEQ ID NO: 46)	C	T1	2-30 min
ps**GRLEEELRRRLA**. (SEQ ID NO: 47)	C	T1	2-30 min
ps**GRLEEELRRRLSP**. (SEQ ID NO: 48)	C	T1	2-30 min

pd**SGLEQELRRRLSP**g (SEQ ID NO: 50)	M	T2	20-100 min
pd**SGLEQELRRRLTA**p (SEQ ID NO: 51)	M	T2	20-100 min
ps**SGLEQELRRRLTA**p (SEQ ID NO: 52)	M	T2	20-100 min
dp**SGLEQELRRRLTA**p (SEQ ID NO: 53)	M	T2	20-100 min
ds**GPLEQELRRRLTA**p (SEQ ID NO: 54)	M	T2	20-100 min
sp**SRLEEELRRRLTA**ep (SEQ ID NO: 55)	M	T2	20-100 min
sp**SGLEEELRRRLTA**p (SEQ ID NO: 56)	M	T2	20-100 min
sp**SGLEEELRRRLDA**p (SEQ ID NO: 57)	M	T2	20-100 min
sp**SGLEEELRRRLEA**p (SEQ ID NO: 58)	M	T2	20-100 min
sp**SGLEEELRRRLTD**p (SEQ ID NO: 59)	M	T2	20-100 min
sp**SGLEEELRRRLTA**dp (SEQ ID NO: 60)	M	T2	20-100 min
gp**SGLEQELRRRLTA**. (SEQ ID NO: 169)	C	T2	20-100 min
sp**SGLEQELRRRLTD**p (SEQ ID NO: 170)	M	T2	20-100 min
sp**SGLEQELRRRLTA**dp (SEQ ID NO: 171)	M	T2	20-100 min
sp**SGLEQELRRRLTA**ep (SEQ ID NO: 172)	M	T2	20-100 min
ds**PGLEQELRRRLTA**p (SEQ ID NO: 173)	M	T2	20-100 min
sp**SGLEQELRRRLSP**s (SEQ ID NO: 174)	M	T2	20-100 min
gr**SRLEEELRRRLTA**. (SEQ ID NO: 08)	C	T3	> 100 min
pg**SRLEEELRRRLTA**p (SEQ ID NO: 09)	M	T3	> 100 min
ps**TRLEEELRRRLTA**p (SEQ ID NO: 10)	M	T3	> 100 min
sp**SRLEEELRRRLTA**p (SEQ ID NO: 11)	M	T3	> 100 min
sp**SRLEEELRRRLDA**p (SEQ ID NO: 12)	M	T3	> 100 min
sp**SRLEEELRRRLEA**p (SEQ ID NO: 13)	M	T3	> 100 min
sp**SRLEEELRRRLTD**p (SEQ ID NO: 14)	M	T3	> 100 min
sp**SRLEEELRRRLTE**p (SEQ ID NO: 15)	M	T3	> 100 min
sp**SRLEEELRRRLTA**dp (SEQ ID NO: 16)	M	T3	> 100 min
sp**SGLEEELRRRLTE**p (SEQ ID NO: 17)	M	T3	> 100 min
gp**SRLEEELRRRLT**. (SEQ ID NO: 18)	C	T3	> 100 min
gp**SRLEEELRRRLTA**. (SEQ ID NO: 19)	C	T3	> 100 min
gp**SRLEEELRRRLTA**a. (SEQ ID NO: 20)	C	T3	> 100 min
gp**SRLEEELRRRLTA**as. (SEQ ID NO: 21)	C	T3	> 100 min
sp**SGLEQELRRRLTA**p (SEQ ID NO: 22)	M	T3	> 100 min
sp**SGLEQELRRRLDA**p (SEQ ID NO: 23)	M	T3	> 100 min
sp**SGLEQELRRRLEA**p (SEQ ID NO: 24)	M	T3	> 100 min
sp**SGLEQELRRRLTE**p (SEQ ID NO: 25)	M	T3	> 100 min
gp**SRLEEELRRRLTA**p. (SEQ ID NO: 26)	C	T3	> 100 min
gp**SRLEEELRRRLTE**p. (SEQ ID NO: 27)	C	T3	> 100 min
gp**SRLEEELRRRLTE**. (SEQ ID NO: 28)	C	T3	> 100 min
M**SRLEEELRRRLTE**p (SEQ ID NO: 29)	N	T3	> 100 min
Mp**SRLEEELRRRLTE**p (SEQ ID NO: 05)	N	T3	> 100 min
p**SRLEEELRRRLTE**p (SEQ ID NO: 06)	M	T3	> 100 min
p**SRLEEELRRRLTE** (SEQ ID NO: 07)	C	T3	> 100 min
Ms**SRLEEELRRRLTE**p (SEQ ID NO: 30)	N	T3	> 100 min

n.d: not determined
Positions: N: N-terminal; M: in between two folded domains; C: C-terminal

[175] As a rough estimation, which is based on an estimated on-rate of $2*10^5$ (1/(M*sec)), which is commonly observed for sdAbs, $t_{1/2}$ of < 2 min correspond to a K_d of > about 30 nM, $t_{1/2}$ of 2-30 min correspond to a K_d of about 2-30 nM, $t_{1/2}$ of 20-100 min correspond to a K_d of about 0.6-3 nM, and $t_{1/2}$ of more than 100 min correspond to a K_d of about 1 nM or lower.

[176] We found an ALFA mutant ($ALFA^{PE}$ for ***P***eptide ***E***lutable) (SEQ ID NO: 33) fulfilling these criteria. Proteins fused to $ALFA^{PE}$ efficiently bound to the ALFA $Selector^{ST}$. Binding was even resistant to harsh washing steps (e.g. up to 3M NaCl, 1M $MgSO_4$, 4M urea, 1% TX-100 or even 100mM DTT, Fig. 1C). Efficient elution under native conditions could, however, be accomplished within 15-20 min at room temperature by competition with 200µM of $ALFA^{ST}$ peptide (SEQ ID NO: 179) (Fig. 1B). Interestingly, both ALFA tag variants ($ALFA^{ST}$ and $ALFA^{PE}$) can be used at either terminus of the target protein or even between two protein domains with only marginal effects on the binding to $NbALFA^{ST}$ (Fig. 1B).

[177] Example 2: Application of $NbALFA^{ST}$ for pull-down of ALFA-tagged target proteins

[178] To address the specificity of ALFA $Selector^{ST}$, we performed pull-down experiments from complex lysates under physiological conditions. To this end, *E.coli* or HeLa lysates prepared in PBS were spiked with 3 µM of shGFP2 (Frey, S. *et al.* Surface Properties Determining Passage Rates of Proteins through Nuclear Pores. *Cell* **174,** 202–217.e9 (2018)) N-terminally tagged with either $ALFA^{ST}$ or $ALFA^{PE}$, that have been expressed in *E.coli* and purified via Nickel-chelate chromatography followed by size exclusion chromatography before. The purified input proteins are shown in Fig. 2A. From all lysates, both fusion proteins efficiently bound to the ALFA-$Selector^{ST}$ (Fig. 2B and Fig. 2C). As expected from our earlier experiments (Fig. 1), GFP tagged with $ALFA^{PE}$ could be efficiently eluted within 20 min under native conditions using 200µM of $ALFA^{ST}$ peptide while the $ALFA^{ST}$-tagged target protein required harsher (denaturing) conditions for efficient elution. Strikingly, pull-downs from both lysates were highly specific. Even after elution with SDS buffer, the number and strength of detectable impurities originating from lysate proteins was very low. When $ALFA^{PE}$-GFP fusion proteins were eluted using the $ALFA^{ST}$ peptide, essentially all detectable bands were identical in the eluate fractions obtained from both lysates. These bands could therefore be attributed to artifacts created by the target protein

itself (e.g. maturation bands or DTT-resistant dimers). Interestingly, especially after peptide elution, the eluate fractions contained significantly less contaminating proteins than the substrates used as input material (which had been purified using two consecutive chromatographic steps; Figure 2A).

[179] Example 3: Co-immunoprecipitation using ALFA^{PE}-tagged target protein and ALFA SelectorST

[180] To see if the ALFA system can also be applied for more delicate co-immunoprecipitation experiments, we tried to pull down the binary *E.coli* YfgM-PpiD inner membrane protein complex (Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)) under native conditions (Fig. 2D). To this end, either wild-type YfgM or YfgM-ALFA^{PE} was expressed in a *yfgMΔ* strain. To ensure nearly physiological expression levels, both YfgM variants were expressed from a low-copy plasmid under the control of the endogenous promoter. When using the YfgM-ALFA^{PE}-containing total lysate prepared in the presence of the mild non-ionic detergent DDM as input, ALFA SelectorST was able to pull down the YfgM-PpiD complex in a specific and detergent-resistant manner. This indicated that the ALFA^{PE} tag was compatible with the formation of this labile membrane complex. Importantly, the native and non-modified membrane protein complex could be recovered from ALFA SelectorST resin within 20min under physiological conditions using 200µM of ALFAST peptide. YfgM and its interaction partner PpiD specifically associated with ALFA SelectorST via the ALFA^{PE} tag present on the (periplasmic) C-terminus of YfgM, as the complex could not be purified from a control lysate expressing non-tagged YfgM. The ALFA^{PE} tag together with the ALFA SelectorST resin can thus not only be used for purification of proteins from various sources, it is also suited for native pull-downs of challenging (membrane) protein complexes.

[181] Example 4: Detection of ALFAST-tagged proteins by direct immunofluorescence

[182] We first tested if fluorescently labeled NbALFAST could be applied for immuno-detection of ALFA-tagged proteins in PFA-fixed samples. Indeed, using the NbALFAST (SEQ ID NO: 133) coupled to two fluorophores (FluoTag-X2 anti-ALFA AbberiorStar635P), a specific staining pattern could be obtained irrespective of the localization of the ALFAST tag or ALFA^{PE} tag within the target proteins in mammalian cells. More specifically, we successfully tested target proteins with ALFA tags placed at the C-

terminus (Tom70-EGFP-ALFAST, Fig. 3C; Tom70-EGFP-ALFA^{PE}, Fig. 3C), the N-terminus (ALFAST-FLAG-Vimentin, Fig. 3D and Fig. 4A; ALFA^{PE}-FLAG-Vimentin, Fig. 4A), or between a folded domain and a transmembrane domain (EGFP-ALFAST-TM, Fig. 4B).

[183] Example 5: ALFAST-tagged proteins show normal folding, targeting and multimerization status

[184] Importantly, all assayed target proteins showed their characteristic localization (Tom70-EGFP-ALFA: mitochondrial outer membrane; ALFA-Vimentin: characteristic filamentous structures; EGFP-ALFAST-TM: plasma membrane), indicating that the ALFA-tags did not interfere with general folding or proper targeting of the tagged proteins. Proper incorporation of ALFAST-Vimentin and ALFA^{PE}-Vimentin into characteristic intermediate filament structures (Fig. 3D and Fig. 4A) furthermore suggests that the ALFA-tags does not interfere with proper filament assembly.

[185] To more sensitively address if the ALFAST tag influence the intracellular localization of a fused protein of interest, we statistically analyzed the localization of cytosolic EGFP harboring N- or C-terminal ALFAST tags transfected into mammalian cells (Fig. 5). In this assay, the nucleocytoplasmic distribution of EGFP tagged with ALFAST at either terminus was indistinguishable from non-tagged EGFP. Importantly, we did not observe any signs for an atypical association of the analyzed ALFAST fusion proteins to cellular compartments (e.g. membranes or organelles). Furthermore, gelfiltration of recombinant ALFAST-tagged EGFP variants confirmed their monomeric state indicating that the ALFAST tag does not induce multimerization. We therefore conclude that the ALFAST tag does not generally impair the behavior of target proteins. We, however, note that (as for any other tag), specific effects on given target proteins have to be analyzed on a protein-to-protein basis.

[186] Example 6: ALFA tags are compatible with common fixation conditions

[187] Immunofluorescence (IF) applications often require optimization of fixation conditions. This may be complicated, especially if proteins requiring different fixation conditions need to be localized in the same specimen. In addition, established epitope tags often contain lysines that render them potentially sensitive towards modification by amine-reactive fixatives (Table 1). The ALFA tags, in contrast, do not contain lysines. In line with these considerations, the ALFAST tag and the ALFA^{PE} tag could be detected after standard fixation with 4% paraformaldehyde (PFA) or precipitative fixation with 100% methanol and

was even resistant to fixation with 2% glutaraldehyde (Fig. 3D). Both ALFA tags are thus compatible with most standard fixation methods and may even prove to be useful in electron microscopic applications, where glutaraldehyde is preferred due to its ability to preserve structures at the nanoscale.

[188] Example 7: Detecting ALFAST-tagged proteins *in vivo*

[189] We next wanted to know if our NbALFAST can also be used as an intrabody. Such nanobodies expressed *in situ* in the cytoplasm (or other compartments) of a target cell are often used to localize or manipulate target proteins in life cells (Caussinus, E., Kanca, O. & Affolter, M. Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat Struct Mol Biol* **19,** 117–121 (2012); Kirchhofer, A. *et al.* Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol* **17,** 133–138 (2010)). Such applications depend on the stability and functionality of a given nanobody in the reducing environment of the cytoplasm of a eukaryotic host cell. To test our nanobody under such conditions, we co-expressed ALFAST-tagged target proteins (ALFAST-Vimentin or Tom70-EGFP-ALFAST) with NbALFAST fused to mScarlet-I (Bindels, D. S. *et al.* mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nat Methods* **11,** 121–122 (2016)). Indeed, in cells co-transfected with both constructs, the mScarlet-I signal robustly co-localized with the respective ALFAST-tagged target protein (Fig. 3E).

[190] Example 8: Western-Blot

[191] In order to test if the ALFA-tag can be detected with a Western-blot using fluorescently labeled NbALFAST, we analyzed lysates from COS-7 cells transfected with ALFAST-tagged Vimentin (Fig. 6A and Fig. 7A) or ALFA^{PE}-tagged Vimentin (Fig. 6A). Lysates from cells transfected with a non-related plasmid served as control. After SDS-PAGE and Western-blotting, ALFA-tagged vimentins could specifically be detected using NbALFAST labeled with IRDye800CW (FluoTag-X2 anti-ALFA IRDye800CW). Only a limited number of minor non-specific bands could be detected in the control lysate lacking ALFA-tagged proteins (Fig. 7A).

[192] To directly compare the performance of NbALFAST with commonly used monoclonal tools recognizing epitope tags, we produced maltose-binding protein (MBP) fused to multiple epitope tags (HA, myc, FLAG and ALFAST, Fig. 6B). Individual detection of each epitope tag with identical concentrations of primary antibody (or nanobody) showed significant differences in signal strength and sensitivity (Fig. 6C, Fig. 7B). The detected signal

obtained for the ALFAST tag employing fluorescently labeled NbALFAST was overall 3-10-fold stronger as compared to signals obtained for all other epitope tags. This result was especially striking as the detection with the monoclonal antibodies (anti-FLAG M2, anti-HA F-7, anti-myc 9E10) involved signal amplification due to the use of a polyclonal secondary antibody, whilst detection of the ALFAST tag exclusively relied on directly labeled NbALFAST. Without further optimizing the detection conditions, NbALFAST yielded a remarkably linear signal over at least three orders of magnitude (Fig. 6D) and was able to detect target protein amounts as low as 100pg. The detection limit was thus ~10-times lower than observed for all other epitope tags.

[193] Example 9: Capture of ALFAST-tagged target proteins using ALFA Selector resins

[194] Next, we site-specifically immobilized NbALFAST on an agarose-based resin with ultra-low background via a hydrophilic and flexible linker. Binding to the nanobody-coupled resin was analyzed using an ALFAST-tagged GFP variant (shGFP2; Frey, S. *et al.* Surface Properties Determining Passage Rates of Proteins through Nuclear Pores. *Cell* **174,** 202–217.e9 (2018); Fig. 8A, B). As expected, shGFP2-ALFAST efficiently and tightly bound to the resulting resin. Binding was, however, too strong to allow for a competitive peptide elution from the resin even when a significant excess of free ALFAST peptide was used (Fig. 8A, black solid line). Even after 60 minutes competition with 10 column volumes of 200µM peptide, >95% of the shGFP2-ALFAST protein remained on the resin. We therefore called the NbALFAST-charged resin "ALFA SelectorST" (for ***S***uper-***T***ight). Based on the structure of the NbALFAST-ALFAST complex, we followed a rational mutagenesis approach to identify weaker NbALFAST mutants that would allow for an efficient peptide elution while stably associating with ALFAST-tagged proteins in the absence of free ALFAST peptide. We found an NbALFA mutant, NbALFA^{PE} (for ***P***eptide ***E***lution), fulfilling these criteria: An agarose-resin with immobilized NbALFA^{PE} (ALFA Selector^{PE}) tightly bound shGFP2-ALFAST. Even upon washing for >1h, the target protein remained stably bound to the resin. It was, however, efficiently released under native conditions within ~15-20min ($t_{1/2}$ ~3min) at room temperature by competition with free ALFAST peptide (Fig. 8B and Fig. 10A, black solid line). Similar elution kinetics were found when the ALFAST tag was placed between two folded domains (Fig. 10B), while an N-terminally ALFAST-tagged shGFP2 eluted slightly quicker from ALFA Selector^{PE} ($t_{1/2}$~50sec; Fig. 10C). Remarkably, in the absence of competing peptide, spontaneous elution of all target proteins from both ALFA SelectorST and

ALFA Selector^{PE} was insignificant (Fig. 8B and Fig. 10 dotted grey lines).

[195] Example 10: The interaction of ALFA-tagged proteins with ALFA Selector is compatible with stringent washing.

[196] We decided to further analyze the biochemical properties of both ALFA Selector resins. To this end, both resins were charged with either ALFAST-shGFP2 or shGFP2-ALFAST and subjected to stringent washing steps (Fig. 8D). For all combinations of resin and substrate, the interaction was resistant even to harsh washing steps including up to 3M NaCl, 1M $MgSO_4$, 2M Guanidinium-HCl or 1% non-denaturing detergents like TX-100, DDM or Sodium-Desoxycholate. No dissociation was observed even after incubation with 100mM DTT at room temperature. Slight differences between the different resins/substrate combinations were observed under denaturing conditions: A partial release of ALFAST-shGFP2 and, to an even lesser extend shGFP2-ALFAST from ALFA Selector^{PE} was observed upon washing with 4M or 6M urea, while both target proteins remained tightly bound to ALFA SelectorST under the same conditions. Surprising differences were observed after incubation with 0.1% SDS, as ALFAST-shGFP2 attached to ALFA Selector^{PE} partially lost its fluorescence while staying bound to the resin. This effect was not observed with any other combination of resin and substrate.

[197] Example 11: pH resistance

[198] In a similar assay, the loaded ALFA Selector resins were washed with buffers adjusted to different pH (Fig. 8E). The interaction was resistant at pH7.5 to 9.5 and only slightly affected at pH4.5. However, even after neutralization, both ALFA Selector resins remained completely non-fluorescent when washed with 100mM Glycin at pH2.2. The eluted material, in contrast, successfully recovered its fluorescence at neutral pH (not shown), indicating that acidic elution with Glycin at pH2.2 is possible even from the tightly binding ALFA SelectorST.

[199] Example 12: Affinity estimation

[200] The affinity of shGFP2-ALFAST to both NbALFAST and NbALFA^{PE} was determined in solution by microscale thermophoresis (MST). This technique provided evidence that NbALFA^{PE} has a dissociation constant of ~15nM, while NbALFAST binds shGFP2-ALFAST with a K_d of ~10pM, which is the lower detection limit of the device. These values are well in line with the dissociation kinetics observed during peptide elution (Fig. 8B)

when assuming an on-rate of ~$2x10^5$/M*sec, which is commonly observed for nanobody-target interactions.

[201] Example 13: Pull-down of ALFAST-tagged target proteins from complex lysates

[202] To address the specificity of our ALFA Selector resins, we performed pull-down experiments from complex lysates under physiological conditions (Fig. 9 A-C). To this end, *E.coli* or HeLa lysates prepared in PBS were spiked with 3µM of recombinant purified ALFAST-shGFP2 (Fig. 9A). The fusion protein specifically bound to both ALFA Selectors but not to a control resin without coupled nanobody ("Selector control"). As expected from our earlier experiments, ALFAST-shGFP2 efficiently eluted from ALFA Selector^{PE} under native conditions using 200µM of ALFAST peptide. In contrast, successful elution from ALFA SelectorST was observed only after treatment with SDS sample buffer. Strikingly, pull-downs from both, *E.coli* and HeLa lysates were highly specific (Fig. 9B and C). After peptide elution from ALFA Selector^{PE} essentially all visible bands could be attributed to the input protein, and even in the SDS eluate, the number and strength of detectable impurities originating from lysate proteins was very low. In fact, ALFAST-shGFP2 obtained by peptide elution from ALFA Selector^{PE} in a single step contained significantly less contaminations than the protein used for spiking the input lysates (Fig. 9A). This observation was especially striking as the input protein had been purified using two consecutive chromatographic steps.

[203] Example 14: Co-immunoprecipitation using ALFA Selector^{PE} resin

[204] To see if the ALFA system can also be applied for more delicate co-immunoprecipitation experiments, we tried to pull down the binary *E.coli* YfgM-PpiD inner membrane protein complex (Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)) under native conditions (Fig. 9D). To this end, either wild-type YfgM or YfgM-ALFAST was expressed in a *yfgMΔ* strain. To ensure nearly physiological expression levels, both YfgM variants were expressed from a low-copy plasmid under the control of the endogenous promoter. When using the YfgM-ALFAST-containing total lysate prepared in the presence of the mild non-ionic detergent DDM as input, ALFA Selector^{PE} was able to pull down the YfgM-PpiD complex in a specific and detergent-resistant manner. This indicated that the ALFAST tag was compatible with the formation of this labile membrane complex. Importantly, the native and non-modified membrane protein complex could be recovered from ALFA Selector^{PE} resin

within 20min under physiological conditions using 200µM of ALFAST peptide. YfgM and its interaction partner PpiD specifically associated with ALFA Selector^{PE} via the ALFAST tag present on the (periplasmic) C-terminus of YfgM, as the complex could not be purified from a control lysate expressing non-tagged YfgM. The ALFAST tag together with the ALFA Selector^{PE} resin can thus not only be used for purification of proteins from various sources, it is also suited for native pull-downs of challenging (membrane) protein complexes.

[205] Example 15: Isolation of live lymphocytes

[206] An envisioned application for the ALFA Selector^{PE} is the specific enrichment of cells under physiological conditions. This may be particularly interesting e.g. for the generation of chimeric antigen receptor-modified T (CAR-T) cells, the precursors of which are usually obtained from blood (Tokarew, N. et al. Teaching an old dog new tricks: next-generation CAR T cells. Br. J. Cancer (2018). doi:10.1038/s41416-018-0325-1). To investigate if the ALFA system can be applied to enrich live blood cells, human peripheral blood mononuclear cells (PBMCs) were passed through an ALFA Selector^{PE} column pre-charged with an ALFA-tagged nanobody recognizing CD62L, a surface marker typically present on naïve T cells (Lefrançois, L. Development, trafficking, and function of memory T-cell subsets. Immunological Reviews (2006). doi:10.1111/j.0105-2896.2006.00393.x) (Fig.12a). After washing, bound cells were eluted using ALFAST peptide, stained with antibodies recognizing CD62L, the pan T cell marker CD3 and the pan B cell marker CD19, and analyzed by FACS (Fig.12). Total PBMCs served as a control. Using this strategy, CD62L+ lymphocytes were enriched from 71.8 to 97.7% (Fig.12b). In addition, we confirmed that the vast majority of ALFA peptide-eluted cells were CD3-positive T cells, while B cells represented a minor population of the isolated cells (Fig.12c).

[207] Discussion

[208] We reported the development and initial characterization of the ALFA system. This system comprises the ALFAST tag, a novel and highly versatile epitope tag, a mutant variant thereof (ALFA^{PE} tag) and a set of related single-domain antibodies (nanobodies) recognizing the ALFAST tag with extraordinarily high or moderate affinity, respectively. Importantly, the rational approach chosen allowed us to equip the ALFA system with features that are crucial for its generic applicability. When selecting the ALFA tag sequences, it was preferred that the tag was small, devoid of lysines, hydrophilic without carrying any net charge and absent within the proteome of relevant model organisms, but also that it would adopt a stable fold in solution. As a result, the ALFA tags are preferably *by design* highly specific, insensitive to amine-reactive fixatives, generally well tolerated by the tagged target

proteins and can easily refold after denaturation.

[209] As binders we preferred nanobodies, because in contrast to conventional antibodies they are small, monovalent and robust probes that can easily be modified by genetic means and recombinantly produced in various expression systems. It is therefore possible to site-specifically immobilize nanobodies or to quantitatively introduce fluorescent labels (Pleiner, T. *et al.* Nanobodies: site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation. *Elife* **4,** (2015)). NbALFAST, our preferred high affinity nanobody recognizing the ALFAST tag, can thus readily be used e.g. for direct immunofluorescence. Due to the small size of the nanobodies (~3-4nm diameter) and the defined number and location of the attached dyes, fluorescently labeled NbALFAST is an ideal tool for high-resolution or quantitative imaging. We could show that NbALFAST faithfully interacts with various ALFAST-tagged target proteins expressed in mammalian cells. Importantly, NbALFAST can even fold in the cytoplasm of eukaryotic cells and can thus be used as an "intrabody" for detecting or manipulating ALFA-tagged target proteins *in vivo* (Rothbauer, U. *et al.* Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat Methods* **3,** 887–889 (2006); Kirchhofer, A. *et al.* Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol* **17,** 133–138 (2010); Röder, R. *et al.* Intracellular Delivery of Nanobodies for Imaging of Target Proteins in Live Cells. *Pharm. Res.* (2016). doi:10.1007/s11095-016-2052-8). This finding is in line with our biochemical evidence showing that NbALFAST is resistant to at least 100mM DTT at room temperature and suggests that the conserved internal disulfide bridge common to all nanobodies is largely dispensable for a faithful interaction with the ALFAST tag. For intrabody applications, we found that expression of both, NbALFAST fused to a fluorescent reporter and target proteins under the control of a CMV or PGK promoter, led to good results with low background. For optimal results or detection of low-abundant ALFAST-tagged target proteins, more sophisticated titrations of the relative expression levels may be required.

[210] Generally, most nanobodies recognize three-dimensional epitopes on the surface of their target proteins and thus do not recognize denatured proteins (e.g. in Western-Blots). We could show that NbALFAST is an exception from this rule as it can also be used for highly sensitive target protein detection in Western-Blot applications. This fact suggests that the ALFAST and ALFA^{PE} tags can efficiently refold after transfer to the membrane and removal of SDS. A direct comparison showed that – despite its monovalent binding mode – NbALFAST significantly outperformed established monoclonal anti-epitope tag tools with

respect to absolute signal intensities and detection limit. We envision similar advantages in other applications like ELISA or microarray assays that require high sensitivity. Due to the resistance to amine-reactive fixatives, we believe it will be possible to also adapt the ALFA system to immuno-EM applications in the future.

[211] The affinity of NbALFAST for the ALFAST tag is extraordinarily high. While this is ideal for high profile imaging applications and highly sensitive detection, it prevents an elution under physiological conditions within a reasonable time frame and thus sets limits for biochemical applications. We therefore aimed at lowering the affinity of the nanobody for its substrate without affecting its specificity. We approached this on two separate ways: 1) We screened a large selection of ALFA peptides for reduced binding strength. This approach led to the ALFA^{PE} tag, which binds efficiently to NbALFAST but can efficiently be eluted by competition with free ALFAST peptide. 2) Based on the crystal structure of NbALFAST in complex with the ALFAST peptide, we introduced specific mutations in NbALFAST that successfully increased the off-rate to a level allowing for an efficient peptide elution under physiological conditions. When immobilized to an agarose resin with low background binding, the mutant nanobody (NbALFA^{PE}) proved to be ideally suited for native purifications of proteins and protein complexes from various lysates under physiological conditions. ALFA SelectorST displaying the wild-type high affinity nanobody (NbALFAST), in turn, might have advantages in special applications requiring harsh washing with up to 6M urea or up to 0,1% SDS, or when extremely low-abundant proteins need to be depleted from dilute lysates. Elution from ALFA SelectorST, however, requires strongly denaturing or acidic elution (e.g. 1% SDS or Glycin pH2.2), which is in general incompatible with a native target protein conformation.

[212] The structure of NbALFAST bound to the ALFAST peptide shows that NbALFAST recognizes the ALFAST peptide in its alpha-helical conformation. In order to minimize the potential influences of neighboring secondary structures on the conformation of the ALFAST tag, we placed the core ALFAST sequence (SRLEEELRRRLTE, SEQ ID NO: 04)) between two prolines acting as "insulators". Using this approach, the interaction of NbALFAST with the ALFAST tag is largely independent of the tag's localization within the protein, i.e. both NbALFAST and NbALFA^{PE} will recognize ALFAST tags placed both at the N- and C-terminus of a target protein or even within two protein domains.

[213] Taken together, we here introduced a novel epitope tag system with exceptionally broad applicability. Using the ALFA system, a single transgenic cell line or organism harboring an ALFAST-tagged target protein is sufficient for a wealth of different

applications including (super-resolution) imaging, *in-vivo* manipulation of proteins, *in-vitro* detection by Western-blot or even native pull-down applications aiming at detecting specific interaction partners. ALFA Selector^{PE} could even be applied for the selective enrichment of CD62L-positive lymphocytes from PBMC preparations (Fig.12). We believe that this technique can easily be transferred to the highly validated recombinant Fab and scFv fragments that are currently used for cell isolation approaches and similar purposes (Mohr, F. et al. Minimally manipulated murine regulatory T cells purified by reversible Fab Multimers are potent suppressors for adoptive T-cell therapy. Eur. J. Immunol. (2017). doi:10.1002/eji.201747137), or to novel nanobodies recognizing surface markers that can easily be equipped with an ALFA tag. Our new technology can therefore contribute to current advances in biomedical research and therapy including the CAR-T technology (Tokarew, N. et al. Teaching an old dog new tricks: next-generation CAR T cells. Br. J. Cancer (2018). doi:10.1038/s41416-018-0325-1). We strongly believe that due to the wide range of applications the ALFA system is an important contribution that will significantly stimulate the scientific community.

[214] Material and Methods

[215] Transfection of 3T3 and COS-7 cells

[216] For immunofluorescence experiments, 3T3 or COS-7 cells were transiently transfected with appropriate plasmids listed in **Table 3**, using the PolyJet transfection kit (SignaGen) according to the manufacturers recommendations. In short, for each experiment cells were seeded on 12- well plates. Volumes were adjusted according to the size of the well. 1µg of each plasmid was premixed with 38µl of serum-free medium and subsequently supplemented with PolyJet transfection reagent diluted in 38µl of serum-free medium. The suspension was incubated at room temperature for 15min and afterwards added drop-wise to the cells. Cells were incubated for 24h at 37°C with 5% CO_2. For co-expression experiments, plasmid DNA was premixed in a 1:1 ratio and further processed as described above.

[217] Fixation and staining of COS-7 cells

[218] Transiently transfected cells were fixed 24h post transfection in either 4% paraformaldehyde (PFA) (*w/v*) or 2% glutaraldehyde (GA) (*v/v*) for 30min at room-temperature. Alternatively, fixation was performed in ice cold methanol for 15min at -20°C. Cells were blocked and permeabilized in PBS containing 10% normal goat serum (*v/v*) and 0.1% Triton-X 10 (*v/v*) for 15min at room temperature. Fluorescently labeled NbALFAST (FluoTag-X2 anti-ALFA AbberiorStar635P, NanoTag Biotechnologies N1502-Ab635P-L) was diluted 1:500 in PBS containing 3% normal goat serum and 0.1% Triton-X 100 (*v/v*). The

cells were incubated in this staining solution for 1h at room temperature and subsequently washed 3 times for 5min with PBS. To stain the nucleus, DAPI (0.4 µg/ml) was included in one of the PBS washing steps. Coverslips were mounted on cover-slides using MOWIOL solution, dried at 37°C and imaged using an epifluorescence microscope (Axio, Zeiss) equipped with a 20x lens. Constructs expressed at the cell-surface were co-stained with anti-FLAG M2 (primary antibody, Sigma, F1804) and FluoTag-X2 anti-mouse IgG Atto488 (secondary nanobody, NanoTag, N1202-At488-L) diluted 1:1000 and 1:500 respectively, in PBS containing 3% normal goat serum and 0.1% Triton-X 100 (*v/v*).

[219] Impact of ALFA tags on the localization of EGFP

[220] Transiently transfected 3T3 cells were imaged using an epifluorescence microscope (Axio, Zeiss) equipped with a 40x 1.3 oil lens. For cells transfected with either pCMV ALFAST-EGFP, pCMV EGFP-ALFAST, or pEGFP-N1, 107-133 cells were imaged on a total of six to seven individual images. For each individual image, cells were grouped and counted according to the localization of EGFP ("slightly nuclear", "equally distributed", "other"). The fraction of cells in each group was statistically analyzed using Student's t-test.

[221] Western Blots with COS-7 lysates

[222] Transfected cells from a confluent 10 cm petri dish were washed with PBS and lysed in 2 mL SDS sample buffer. Lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% milk powder in TBS-T, membranes were incubated with mouse anti -tubulin (SYSY #302 211; 1:1000 dilution) followed by a FluoTag-X2 anti-Mouse IgG IRDye680 (NanoTag Biotechnologies #N1202; 1:1000 dilution) and FluoTag-X2 anti-ALFA IRDye800 (NanoTag Biotechnologies #1502; 1:1000 dilution). Membranes were scanned using Odyssey CLx (Li-COR).

[223] Sensitivity assay

[224] A serial dilution of MBP fused to FLAG, HA, myc and ALFAST tags was prepared in PBS pH7.4, 0.1µg/mL BSA. 1µl of each dilution was spotted on nitrocellulose membranes.

[225] The membrane was blocked and washed with 5 % milk powder in TBS-T. Established monoclonal antibodies (anti-FLAG M2 – Sigma #F1804, anti-myc 9E10 – SynapticSystems #343 011, anti-HA F-7 – SantaCruz #sc-7392) were used in combination with a secondary goat anti-mouse IgG IRDye800CW (Li-COR #925-32210, dilution 1:500) to detect FLAG, myc and HA-tag, respectively. The ALFAST tag was detected using a FluoTag-X2 anti-ALFA (NanoTag Biotechnologies #N1502) directly coupled to IRDye800CW. All primary antibodies and the nanobody were used at 2.7nM final concentration. Detection of

MBP by a rabbit polyclonal serum recognizing MBP (SynapticSystems) and an anti-rabbit IgG IRDye680RD (Li-COR #925-68071) served as an internal loading control. Membranes were scanned using Odyssey CLx (Li-COR). Quantifications were performed using ImageStudioLight (Li-COR).

[226] Off-rate assays

[227] 20µl ALFA SelectorST or ALFA Selector^{PE} (NanoTag Biotechnologies) was saturated with the respective recombinant target protein. After washing 4x with PBS, the beads were suspended in a 10-fold excess of PBS containing 200µM free ALFAST peptide and mixed at 25°C. Control reactions were carried out without peptide. At indicated time points, specific elution from the beads was quantified using the GFP fluorescence released into the supernatant (Q-Bit 3.0; Thermo-Fischer Scientific). Three independent experiments were performed in parallel. Mean values, standard deviations and exponential fits were calculated using GraphPad Prism 5.0. Photographic pictures were taken upon UV illumination using a Nikon D700 equipped with a 105mm macro lens (Nikon).

[228] Resistance towards stringent washing and pH

[229] Depending on the experiment, 10-15µl of ALFA SelectorST or ALFA Selector^{PE} saturated with indicated ALFA-tagged shGFP2 fusion proteins were washed with PBS and incubated with 100µl of the indicated substances for 60min at room temperature. Photos were taken after sedimentation of the beads upon UV illumination. To assay for pH resistance, the same beads were incubated with 150mM NaCl buffered to various pH (100mM Glycin-HCl, pH2.2; 100mM Na-Acetate pH4.5, 100mM Tris-HCl pH 7.5, 100mM Carbonate pH9.5) for 30min at RT. The resin was washed twice with the same buffer. Photos were taken after equilibrating several times with PBS.

[230] One-step affinity purifications using the ALFA Selector Resins.

[231] To obtain defined input materials for pull-down experiments from *E.coli* or HeLa lysates, respective mock lysates were blended with 3µM of the indicated purified ALFA-tagged shGFP2 variant. 1mL of each lysate/substrate mixture was incubated with 25µl of ALFA SelectorST or ALFA Selector^{PE} for 1h at 4°C. Depending on the experimental setup, either an analogous resin without immobilized sdAb (Selector Control) or a mock lysate without target protein served as a specificity control. After washing 3 times with 600µL of PBS, the resins were transferred into MiniSpin columns (NanoTag Biotechnologies). Excess buffer was removed by centrifugation (3000x g, 30sek) before incubating twice for 10min at room temperature with 50µl each of 200µM ALFAST peptide in PBS. Proteins remaining on the beads were afterwards eluted with SDS sample buffer. 0.5µL (*E.coli*) or 1.5µL (HeLa) of

input and non-bound fractions were resolved by SDS-PAGE (12%) and Coomassie staining. Shown eluate fractions correspond to the material eluted from 1µl of the respective resins.

[232] YfgM pull-downs using ALFA Selector^{PE}

[233] A *yfgM* deletion strain was complemented with either C-terminally ALFAST-tagged or untagged YfgM expressed from a pSC-based low-copy vector under control of the endogenous promoter. Membrane protein complexes were solubilized from total lysates prepared in buffer LS (50mM Tris pH7.5, 300mM NaCl, 5mM $MgCl_2$) using 1% DDM within 1h on ice (Maddalo, G. *et al.* Systematic analysis of native membrane protein complexes in Escherichia coli. *J Proteome Res* **10,** 1848–1859 (2011)). Both lysates were incubated with 20µl of ALFA Selector^{PE} resin for 1h at 4°C on a roller drum. After washing in PBS + 0.3% DDM, bound proteins were eluted under native conditions by sequentially incubating twice with 50µl PBS containing 200µM ALFAST peptide. Samples corresponding to 1/800 of the input and non-bound material or 1/80 of eluate fractions were resolved by SDS-PAGE. Analysis was performed by Western-blotting using a polyclonal rabbit antiserum raised against the YfgM-PpiD complex (Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)) followed by an HRP-conjugated goat anti-rabbit IgG (Dianova). Blots were developed using the Western Lightning Plus-ECL Kit (Perkin Elmer) and imaged using a LAS 4000 mini luminescence imager (Fuji Film).

[234] YfgM pull-down using ALFA SelectorST

[235] A *yfgM* deletion strain was complemented with either C-terminally ALFA^{PE}-tagged or untagged YfgM expressed from a pSC-based low-copy vector under control of the endogenous promoter. Membrane protein complexes were solubilized from total lysates prepared in buffer LS (50mM Tris pH7.5, 300mM NaCl, 5mM $MgCl_2$) using 1% DDM within 1h on ice (Maddalo, G. *et al.* Systematic analysis of native membrane protein complexes in Escherichia coli. *J Proteome Res* **10,** 1848–1859 (2011)). Both lysates were incubated with 20µl of ALFA SelectorST resin for 1h at 4°C on a roller drum. After washing in PBS + 0.3% DDM, bound proteins were eluted under native conditions by sequentially incubating twice with 50µl PBS containing 200µM ALFAST peptide. Samples corresponding to 1/800 of the input and non-bound material or 1/80 of eluate fractions were resolved by SDS-PAGE. Analysis was performed by Western-blotting using a polyclonal rabbit antiserum raised against the YfgM-PpiD complex (Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)) followed by an HRP-conjugated goat anti-rabbit IgG (Dianova). Blots were developed using the

Western Lightning Plus-ECL Kit (Perkin Elmer) and imaged using a LAS 4000 mini luminescence imager (Fuji Film).

[236] Table 3: Plasmids

Transfection			
Identifier	**Promoter**	**Encoded protein**	**Origin/Citation**
pNT1112	pCMV	$ALFA^{ST}$-FLAG-Vimentin	This application
pNT1077	pCMV	$ALFA^{PE}$-FLAG-Vimentin	This application
pNT1067	pPGK	Tom70-EGFP-$ALFA^{ST}$	This application
pNT1178	pPGK	Tom70-EGFP-$ALFA^{PE}$	This application
pNT1066	pCMV	EGFP-$ALFA^{ST}$-myc-TM	This application
pNT1004	pCMV	Nb$ALFA^{ST}$-mScarlet-I	This application
pEGFP-N1	pCMV	EGFP	Clontech
pNT1137	pCMV	EGFP-$ALFA^{ST}$	This application
pNT1135	pCMV	$ALFA^{ST}$-EGFP	This application
Bacterial expression			
Identifier	**Encoded protein**		**Origin/Citation**
pNT1208	His_{14}-bdSUMO-FLAG-HA-MBP-myc-$ALFA^{ST}$		This application
pNT1177	$ALFA^{ST}$-shGFP2-His_6		This application
pNT1176	$ALFA^{PE}$-shGFP2-His_6		This application
pNT1050	His_{14}-bdSUMO-shGFP2-$ALFA^{ST}$		This application
pNT1116	His_{14}-bdSUMO-shGFP2-$ALFA^{PE}$		This application
pNT1063	His_{14}-bdSUMO-$ALFA^{ST}$-shsfGFP		This application
pNT1115	His_{14}-bdSUMO-$ALFA^{PE}$-shsfGFP		This application
pNT1209	pSC YfgM-$ALFA^{ST}$		This application
pNT1123	pSC YfgM-$ALFA^{PE}$		This application
pSC-yfgM	pSC *YfgM* (native promoter in pUA66)		[a]
pNT0076	His_{14}-bdSUMO-TwinStrepTag-bdNEDD8-$ALFA_{min}$		This application

[a] Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)

[237] E.coli strains

[238] *E. coli* MC4100 Δ*yfgM* Δ*ppiD* (Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014))

[239] **Table 4 Antibodies**

Antibody	Supplier	Order No
FluoTag-X2 anti-ALFA AbberiorSTAR635P	NanoTag Biotechnologies	N1502-Ab635P
FluoTag-X2 anti-ALFA IRDye800CW	NanoTag Biotechnologies	N1502 (Custom)
FluoTag-X2 anti-Mouse IRDye680RD	NanoTag Biotechnologies	N1202 (Custom)
anti-FLAG M2	Sigma	F1804
anti-myc 9E10	SynapticSystems	#343 011
anti-HA F-7	SantaCruz	#sc-7392
Goat anti-rabbit IRDye680RD	Li-COR	#925-68071
Goat anti-mouse IRDye800CW	Li-COR	#925-32210
anti-Tubulin	SynapticSystems	#302 211
polyclonal serum recognizing MBP	SynapticSystems	
goat anti-rabbit IgG HRP-conjugate	Dianova	#GAR/IgG(H+L)/PO
anti-YfgM/PpiD	[a]	

[a] Götzke, H. *et al.* YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli. *J Biol Chem* **289,** 19089–19097 (2014)

[240] **Table 5 Fusion proteins**

Transfection		
Identifier	**Encoded protein**	**Sequence of encoded protein**
pNT1112	**ALFAST**-FLAG-*Vimentin* (SEQ ID NO: 180)	M**PSRLEEELRRRLTEP**DYKDDDDKG*STRSVSSSSYRRMFGGSGTSSRPSSNRSYVTTSTRTYSLGSALRPSTSRSLYSSSPGGAYVTRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQVDQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAESTLQSFRQDVDNASLARLDLERKVESLQEEIAFLKKLH*

		DEEIQELQAQIQEQHVQIDVDVSKPDLTAALR DVRQQYESVAAKNLQEAEEWYKSKFADLSEAA NRNNDALRQAKQESNEYRRQVQSLTCEVDALK GTNESLERQMREMEENFALEAANYQDTIGRLQ DEIQNMKEEMARHLREYQDLLNVKMALDIEIA TYRKLLEGEESRISLPLPTFSSLNLRETNLESLPL VDTHSKRTLLIKTVETRDGQVINETSQHHDDLE
pNT1077	**ALFA^{PE}**-FLAG-*Vimentin* (SEQ ID NO: 181)	**MSGRLEEELRRRLSP**DYKDDDDKG*STRSVS SSSYRRMFGGSGTSSRPSSNRSYVTTSTRTYSLGS ALRPSTSRSLYSSSPGGAYVTRSSAVRLRSSVPGV RLLQDSVDFSLADAINTEFKNTRTNEKVELQEL NDRFANYIDKVRFLEQQNKILLAELEQLKGQG KSRLGDLYEEEMRELRRQVDQLTNDKARVEVE RDNLAEDIMRLREKLQEEMLQREEAESTLQSF RQDVDNASLARLDLERKVESLQEEIAFLKKLHD EEIQELQAQIQEQHVQIDVDVSKPDLTAALRDV RQQYESVAAKNLQEAEEWYKSKFADLSEAANR NNDALRQAKQESNEYRRQVQSLTCEVDALKGT NESLERQMREMEENFALEAANYQDTIGRLQDE IQNMKEEMARHLREYQDLLNVKMALDIEIATY RKLLEGEESRISLPLPTFSSLNLRETNLESLPLVD THSKRTLLIKTVETRDGQVINETSQHHDDLE*
pNT1067	Tom70-*EGFP*-**ALFAST** (SEQ ID NO: 182)	MKSFITRNKTAILATVAATGTAIGAYYYYGN SPVAT*MVSKGEELFTGVVPILVELDGDVNGHK FSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT LVTTLTYGVQCFSRYPDHMKQHDFFKSAMPE GYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI ELKGIDFKEDGNILGHKLEYNYNSHNVYIMAD KQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPI GDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVL LEFVTAAGITLGMDELYK*GS**PSRLEEELRRRL TE**
pNT1178	Tom70-*EGFP*-**ALFA^{PE}** (SEQ ID NO: 183)	MKSFITRNKTAILATVAATGTAIGAYYYYGN SPVAT*MVSKGEELFTGVVPILVELDGDVNGHK*

		*FSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT LVTTLTYGVQCFSRYPDHMKQHDFFKSAMPE GYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI ELKGIDFKEDGNILGHKLEYNYNSHNVYIMAD KQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPI GDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVL LEFVTAAGITLGMDELYK*GS**PSRLEEELRRRL TE**
pNT1066	*SS*-**HA**-*EGFP*-**ALFAST-myc**-*TM* (SEQ ID NO: 184)	*METDTLLLWVLLLWVPGSTG*D**YPYDVPDYA**S NGT*SKGEELFTGVVPILVELDGDVNGHKFSVS GEGEGDATYGKLTLKFICTTGKLPVPWPTLVTT LTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ ERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKG IDFKEDGNILGHKLEYNYNSHNVYIMADKQKN GIKVNFKIRHNIEDGSVQLADHYQQNTPIGDG PVLLPDNHYLSTQSALSKDPNEKRDHMVLKEF VTAAGITLGMDELYK*GS**PSRLEEELRRRLTE PGD**EQKLISEEDL**N*AVGQDTQEVIVVPHSLP FKVVVISAILALVVLTIISLIILIMLWQKKPR*
pNT1004	**NbALFAST-***mScarlet-I* (SEQ ID NO: 185)	MGSGDASDSE**VQLQESGGGLVQPGGSLRL SCTASGVTISALNAMAMGWYRQAPGERR VMVAAVSERGNAMYRESVQGRFTVTRDF TNKMVSLQMDNLKPEDTAVYYCHVLEDR VDSFHDYWGQGTQVTVSS**EPKTPKPQTSGS TGENVAT*MVSKGEAVIKEFMRFKVHMEGSMN GHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLP FSWDILSPQFMYGSRAFIKHPADIPDYYKQSFP EGFKWERVMNFEDGGAVTVTQDTSLEDGTLIY KVKLRGTNFPPDGPVMQKKTMGWEASTERLY PEDGVLKGDIKMALRLKDGGRYLADFKTTYKA KKPVQMPGAYNVDRKLDITSHNEDYTVVEQYE RSEGRHSTGGMDELYK*
pEGFP-N1	EGFP (SEQ ID NO: 186)	MVSKGEELFTGVVPILVELDGDVNGHKFSV SGEGEGDATYGKLTLKFICTTGKLPVPWPTL

		VTTLTYGVQCFSRYPDHMKQHDFFKSAMPE GYVQERTIFFKDDGNYKTRAEVKFEGDTLV NRIELKGIDFKEDGNILGHKLEYNYNSHNVY IMADKQKNGIKVNFKIRHNIEDGSVQLADH YQQNTPIGDGPVLLPDNHYLSTQSALSKDPN EKRDHMVLLEFVTAAGITLGMDELYK
pNT1137	*EGFP*-ALFAST (SEQ ID NO: 187)	*MVSKGEELFTGVVPILVELDGDVNGHKFSVSG* *EGEGDATYGKLTLKFICTTGKLPVPWPTLVTTL* *TYGVQCFSRYPDHMKQHDFFKSAMPEGYVQE* *RTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGI* *DFKEDGNILGHKLEYNYNSHNVYIMADKQKNG* *IKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPV* *LLPDNHYLSTQSALSKDPNEKRDHMVLLEFVT* *AAGITLGMDELYK*GS**PSRLEEELRRRLTE**
pNT1135	**ALFAST-***EGFP* (SEQ ID NO: 188)	M**PSRLEEELRRRLTEP***MVSKGEELFTGVVPI* *LVELDGDVNGHKFSVSGEGEGDATYGKLTLKF* *ICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHM* *KQHDFFKSAMPEGYVQERTIFFKDDGNYKTRA* *EVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY* *NYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSV* *QLADHYQQNTPIGDGPVLLPDNHYLSTQSALS* *KDPNEKRDHMVLLEFVTAAGITLGMDELYK*
Bacterial expression		
Identifier	**Encoded protein**	**Sequence of encoded protein**
pNT1208	His_{14}-bdSUMO-**FLAG-HA-***MBP*-**myc-ALFAST** (SEQ ID NO: 189)	MSKHHHHSNHHRHNHHHHSGNHHHSGSAA GGEEDKKPAGGEGGGAHINLKVKGQDGNE VFFRIKRSTQLKKLMNAYCDRQSVDMTAIA FLFDGRRLRAEQTPDELEMEDGDEIDAMLH QTGGAS**DYKDDDDK**GSTGD**YPYDVPDYA**S NGT*KTEEGKLVIWINGDKGYNGLAEVGKKFEK* *DTGIKVTVEHPDKLEEKFPQVAATGDGPDIIF* *WAHDRFGGYAQSGLLAEITPDKAFQDKLYPFT* *WDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPK* *TWEEIPALDKELKAKGKSALMFNLQEPYFTWP*

		*LIAADGGYAFKYENGKYDIKDVGVDNAGAKAG LTFLVDLIKNKHMNADTDYSIAEAAFNKGETA MTINGPWAWSNIDTSKVNYGVTVLPTFKGQPS KPFVGVLSAGINAASPNKELAKEFLENYLLTDE GLEAVNKDKPLGAVALKSYEEELAKDPRIAAT MENAQKGEIMPNIPQMSAFWYAVRTAVINAAS GRQTVDEALKDAQTN*GSVSAGD**EQKLISEED** **L**NAVGQDTAST**PSRLEEELRRRLTE**
pNT1177	**ALFA**ST-*shGFP2*-His_6 (SEQ ID NO: 190)	M**PSRLEEELRRRLTEP***SKGEELFTGTVPIKV ELDGDVNGHKFSVRGEGEGDATEGKLTLKFIC TTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKR HDFFKSAMPEGYVQERTIEFKDDGTYKTRAEV KFEGDTLVNRIELKGNDFKEDGNILGHKLEYN HNSHNVRIEADKQKNGIKANFKIRHNVEDGSQ QEADHKQQNTPIGDGPVRLPDNHYLSTQTTLS KDPNEKRDHMVLKEFVTAAGITKGEDERDK*HHHHHH
pNT1176	**ALFA**^{PE}-*shGFP2*-His_6 (SEQ ID NO: 191)	**MSGRLEEELRRRLSP***SKGEELFTGTVPIKVE LDGDVNGHKFSVRGEGEGDATEGKLTLKFICT TGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRH DFFKSAMPEGYVQERTIEFKDDGTYKTRAEVK FEGDTLVNRIELKGNDFKEDGNILGHKLEYNH NSHNVRIEADKQKNGIKANFKIRHNVEDGSQQ EADHKQQNTPIGDGPVRLPDNHYLSTQTTLSK DPNEKRDHMVLKEFVTAAGITKGEDERDKHH HHHH
pNT1050	His_{14}-bdSUMO-*shGFP2*-**ALFA**ST (SEQ ID NO: 192)	MSKHHHHSNHHRHNHHHHSGNHHHSGSAA GGEEDKKPAGGEGGGAHINLKVKGQDGNE VFFRIKRSTQLKKLMNAYCDRQSVDMTAIA FLFDGRRLRAEQTPDELEMEDGDEIDAMLH QTGG*GSKGEELFTGTVPIKVELDGDVNGHKFS VRGEGEGDATEGKLTLKFICTTGKLPVPWPTL VTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGY VQERTIEFKDDGTYKTRAEVKFEGDTLVNRIEL*

		*KGNDFKEDGNILGHKLEYNHNSHNVRIEADKQ KNGIKANFKIRHNVEDGSQQEADHKQQNTPIG DGPVRLPDNHYLSTQTTLSKDPNEKRDHMVLK EFVTAAGITKGEDERDK*GSGNSDG**PSRLEEE LRRRLTE**
pNT1116	His_{14}-bdSUMO-*shGFP2*-**ALFA^{PE}** (SEQ ID NO: 193)	MSKHHHHSNHHRHNHHHHSGNHHHSGSAA GGEEDKKPAGGEGGGAHINLKVKGQDGNE VFFRIKRSTQLKKLMNAYCDRQSVDMTAIA FLFDGRRLRAEQTPDELEMEDGDEIDAMLH QTGG*SKGEELFTGTVPIKVELDGDVNGHKFS VRGEGEGDATEGKLTLKFICTTGKLPVPWPTL VTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGY VQERTIEFKDDGTYKTRAEVKFEGDTLVNRIEL KGNDFKEDGNILGHKLEYNHNSHNVRIEADKQ KNGIKANFKIRHNVEDGSQQEADHKQQNTPIG DGPVRLPDNHYLSTQTTLSKDPNEKRDHMVLK EFVTAAGITKGEDERDK*GSGNSDG**MSGRLEE ELRRRLSP**
pNT1063	His_{14}-bdSUMO-**ALFAST**-*shsfGFP* (SEQ ID NO: 194)	MSKHHHHSNHHRHNHHHHSGNHHHSGSAA GGEEDKKPAGGEGGGAHINLKVKGQDGNE VFFRIKRSTQLKKLMNAYCDRQSVDMTAIA FLFDGRRLRAEQTPDELEMEDGDEIDAMLH QTGGSGDASDS**PSRLEEELRRRLTEP***SKGE ELFTGTVPIKVELDGDVNGHKFSVRGEGEGDA TEGKLTLKFICTTGKLPVPWPTLVTTLTYGVQC FSRYPDHMKRHDFFKSAMPEGYVQERTIEFKD DGTYKTRAEVKFEGDTLVNRIELKGNDFKEDG NILGHKLEYNHNSHNVRIEADKQKNGIKANFKI RHNVEDGSQQEADHKQQNTPIGDGPVRLPDN HYLSTQTTLSKDPNEKRDHMVLKEFVTAAGITK GEDERDKA*
pNT1115	His_{14}-bdSUMO-**ALFA^{PE}**-*shsfGFP* (SEQ ID NO: 195)	MSKHHHHSNHHRHNHHHHSGNHHHSGSAA GGEEDKKPAGGEGGGAHINLKVKGQDGNE VFFRIKRSTQLKKLMNAYCDRQSVDMTAIA

		FLFDGRRLRAEQTPDELEMEDGDEIDAMLH QTGGSGDASDS**MSGRLEEELRRRLSP***SKGE ELFTGTVPIKVELDGDVNGHKFSVRGEGEGDA TEGKLTLKFICTTGKLPVPWPTLVTTLTYGVQC FSRYPDHMKRHDFFKSAMPEGYVQERTIEFKD DGTYKTRAEVKFEGDTLVNRIELKGNDFKEDG NILGHKLEYNHNSHNVRIEADKQKNGIKANFKI RHNVEDGSQQEADHKQQNTPIGDGPVRLPDN HYLSTQTTLSKDPNEKRDHMVLKEFVTAAGITK GEDERDKA*
pNT1209	*YfgM*-**ALFA**ST (SEQ ID NO: 196)	*MEIYENENDQVEAVKRFFAENGKALAVGVILG VGALIGWRYWNSHQVDSARSASLAYQNAVTAVS EGKPDSIPAAEKFAAENKNTYGALASLELAQQF VDKNELEKAAAQLQQGLADTSDENLKAVINLR LARVQVQLKQADAALKTLDTIKGEGWAAIVAD LRGEALLSKGDKQGARSAWEAGVKSDVTPALS EMMQMKINNLSI*GS**PSRLEEELRRRLTE**
pNT1123	*YfgM*-**ALFA**^{PE} (SEQ ID NO: 197)	*MEIYENENDQVEAVKRFFAENGKALAVGVILG VGALIGWRYWNSHQVDSARSASLAYQNAVTAVS EGKPDSIPAAEKFAAENKNTYGALASLELAQQF VDKNELEKAAAQLQQGLADTSDENLKAVINLR LARVQVQLKQADAALKTLDTIKGEGWAAIVAD LRGEALLSKGDKQGARSAWEAGVKSDVTPALS EMMQMKINNLSI*GS**MSGRLEEELRRRLS**
pSC-yfgM	*YfgM* (SEQ ID NO: 198)	*MEIYENENDQVEAVKRFFAENGKALAVGVILG VGALIGWRYWNSHQVDSARSASLAYQNAVTAVS EGKPDSIPAAEKFAAENKNTYGALASLELAQQF VDKNELEKAAAQLQQGLADTSDENLKAVINLR LARVQVQLKQADAALKTLDTIKGEGWAAIVAD LRGEALLSKGDKQGARSAWEAGVKSDVTPALS EMMQMKINNLSI*
pNT0076	His_{14}-bdSUMO-*TwinStrepTag*-bdNEDD8-**ALFA$_{min}$**	MSKHHHHSNHHRHNHHHHSGNHHHSGSAA GGEEDKKPAGGEGGGAHINLKVKGQDGNE VFFRIKRSTQLKKLMNAYCDRQSVDMTAIA

	(SEQ ID NO: 199)	FLFDGRRLRAEQTPDELEMEDGDEIDAMLH QTGGACA*WSHPQFEKGGGSGGSSGGSAWSH PQFEK*GSGSAESEAASSTMIKVKTLTGKEIEI DIEPTDTIDRIKERVEEKEGIPPVQQRLIYAGK QLADDKTAKDYNIEGGSVLHLVLALRGGAT GTAST**RLEEELRRRL**AS

[241] Protein expression and purification

[242] All recombinant proteins were expressed under the control of the Tac-promoter from expression vectors with ColE1 origin that confer resistance to Kanamycin.

[243] The MBP fusion protein harboring multiple epitope tags, $ALFA^{ST}$-shGFP2, $ALFA^{PE}$-shGFP2 and TwinStrepTag-bdNEDD8-$ALFA_{min}$ were expressed as N-terminal His_{14}-bdSUMO fusions. For protein expression, *E.coli* was cultured in Terrific broth (TB) supplemented with 0.3mM IPTG for 14-16h at 23°C. After harvest, *E. coli* cells were lysed in LS buffer (50 mM Tris/HCl pH 7.5, 300 mM NaCl) supplemented with 15 mM imidazole/HCl pH 7.5 and 10 mM DTT, and purified by binding to Ni(II)-chelate beads. After extensive washing, proteins were eluted by on-column-cleavage with bdSENP1 as described before (Frey, S. & Görlich, D. A new set of highly efficient, tag-cleaving proteases for purifying recombinant proteins. J Chromatogr A 1337, 95–105 (2014); Frey, S. & Görlich, D. Purification of protein complexes of defined subunit stoichiometry using a set of orthogonal, tag-cleaving proteases. J Chromatogr A 1337, 106–115 (2014)).

[244] $ALFA^{ST}$-shGFP2-His_6, $ALFA^{PE}$-shGFP2-His_6, His_{14}-bdSUMO-$ALFA^{ST}$-shsfGFP and His_{14}-bdSUMO-$ALFA^{PE}$-shsfGFP were expressed and purified in a similar fashion; Elution was, however, performed using 250mM Imidazole in buffer LS.

[245] For affinity determinations and binding studies from complex lysates, substrate proteins were in addition purified via size exclusion chromatography on a Superdex200 10/30 column (GE Healthcare).

[246] Selection of specific sdAb clones by affinity purification of B-cells "Celline"

[247] 1mL of T-Catch resin (IBA Lifesciences) was washed with B cell isolation buffer (PBS pH7.4, 1% BSA, 1mM EDTA) and incubated with saturating amounts of a TwinStrepTag-bdNEDD8-$ALFA_{min}$ fusion protein for 30min rolling at RT. The resins were cleared form excess bait protein by extensively washing with B cell isolation buffer. 100mL of blood sample was taken from alpaca immunized with ALFA peptide fusions and

immediately incubated with 5000 IU/mL heparin (Sigma) to prevent clotting. From the fresh blood (less than 4h past sampling) PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare). To remove residual serum, PBMCs were washed three times consecutively with B cell isolation buffer. PBMCs were passed over the loaded T-Catch resin for three times before washing the resins with 10 column volumes B cell isolation buffer. Bound B cells were eluted from the resins by incubating 2 μM NEDP1 (Frey, S. & Görlich, D. A new set of highly efficient, tag-cleaving proteases for purifying recombinant proteins. J Chromatogr A 1337, 95–105 (2014); Frey, S. & Görlich, D. Purification of protein complexes of defined subunit stoichiometry using a set of orthogonal, tag-cleaving proteases. J Chromatogr A 1337, 106–115 (2014)) for 30min at RT. From the eluted B cells an sdAb-specific cDNA library was amplified by a multistep nested RT-PCR and cloned into a bacterial expression vector. 96 single clones were tested by ELISA for expression of ALFA-reactive sdAbs.

[248] Preparation of human PBMCs

[249] Human peripheral blood mononuclear cells (PBMCs) were obtained from fresh blood using standard density gradient centrifugation. Briefly, 60 mL of fresh blood were diluted with 40 mL of phosphate-buffered saline (PBS) supplemented with 1 mM EDTA and placed on top of a layer of CELLPURE Roti-Sep 1077 (Carl Roth) in 50 mL LEUCOSEP tubes (Greiner Bio- One) and centrifuged at 800 x g for 20 minutes at room temperature. Subsequently, the PBMC-containing layer was collected and washed five times in cold PBS + EDTA to remove platelets.

[250] Isolation of CD62L-positive lymphocytes

[251] Approximately 2 x 10^7 PBMCs were passed by gravity flow through an ALFA Selector^{PE} resin loaded with an ALFA-tagged anti-human CD62L nanobody, followed by extensive washing with PBS supplemented with 1 mM EDTA 1 and 1 % (w/v) bovine serum albumin. Subsequently, bound cells were eluted in the same buffer containing 200 μM ALFA peptide.

[252] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by exemplary embodiments and optional features, modification and

variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[253] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[254] Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

CLAIMS

1. A fusion protein comprising:
   (a) a peptide comprising the sequence of X1-X2-X3-X4-X5-X6-X7-X8-X9-R-L-X12-X13 (SEQ ID NO: 01), wherein X1 is G or S or T or P, X2 is R or G or A or E or P, X3 is L or V, X4 is E or Q, X5 is E or Q, X6 is E or Q, X7 is L or I or V, X8 is R or A or Q or E, X9 is R or A or Q or E, X12 is S or T or D or E or P or A or no amino acid, and wherein X13 is E or K or P or S or A or D or no amino acid;
   (b) and a polypeptide.

2. The fusion protein of claim 1, wherein the peptide is fused to the N-terminus or the C-terminus or at a position between the N-terminus and the C-terminus of the polypeptide.

3. The fusion protein of claim 1 and 2, wherein the peptide is an epitope tag.

4. The fusion protein of any one of the preceding claims, wherein the polypeptide has a stable fold that is independent from the peptide.

5. The fusion protein of any one of the preceding claims, wherein the peptide has an α-helical secondary structure.

6. The fusion protein of any one of the preceding claims, wherein the peptide specifically binds to a camelid VHH domain comprising the CDR sequences GVTISALNAMAMG (SEQ ID NO: 115), AVSERGNAM (SEQ ID NO: 116), and LEDRVDSFHDY (SEQ ID NO: 117).

7. The fusion protein of any one of the preceding claims, further comprising one or more linker linking the peptide and the polypeptide.

8. The fusion protein of any one of the preceding claims, wherein the polypeptide comprises at least one protein domain.

9. The fusion protein of claim 8, wherein the peptide is fused to the polypeptide at a position that is located outside the at least one protein domain.

10. The fusion protein of any one of the preceding claims, wherein the polypeptide is a globular protein, a membrane protein, a fibrous protein, or natively unfolded protein, or is a subunit of a globular protein, a membrane protein, a fibrous protein, or natively unfolded protein.

11. The fusion protein of any one of the preceding claims, wherein the peptide has a length of 8 to 25 amino acids.

12. The fusion protein of any one of the preceding claims, wherein the polypeptide has a length of at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids.

13. The fusion protein of any one of the preceding claims, wherein the peptide comprises the sequence of X1-X2-L-E-X5-E-X7-R-R-R-L-X12-X13 (SEQ ID NO: 02), wherein X1 is G or S or P or T, X2 is R or G or P, X5 is E or Q, X7 is L or I, X12 is S or T or P or A or D or E, and wherein X13 is P or A or S or A or D or E or no amino acid.

14. The fusion protein of any one of the preceding claims, wherein the peptide comprises N-terminal of X1 the amino acids Xa-Xb, wherein Xa is D or S or G or M or P or no amino acid and Xb is S or D or P or M or R or G no amino acid.

15. The fusion protein of any one of the preceding claims, wherein the peptide comprises C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or S or P or D or A or E or K or no amino acid, and Xz is S or P or no amino acid.

16. The fusion protein of claim 13 or 14, wherein the peptide comprises C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or P or A or E or K or S or D or no amino acid, and Xz is P or S or no amino acid.

17. The fusion protein of any one of the preceding claims, wherein the peptide comprises the sequence of X1-X2-L-E-X5-E-L-R-R-R-L-X12-X13 (SEQ ID NO: 03), wherein X1

is S or T, X2 is R or G, X5 is E or Q, X12 is T or D or E, and wherein X13 is A or D or E or no amino acid.

18. The fusion protein of any one of the preceding claims, wherein the peptide comprises the sequence of S-R-L-E-E-E-L-R-R-R-L-T-E (SEQ ID NO: 04) or a variant thereof, wherein the variant has as compared to (SEQ ID NO: 04) 1 to 5 mutations selected from the group consisting of: S1→T, R2→G, E5→Q, T12→D, T12→E, E13→A, E13→D, and deletion of E13.

19. The fusion protein of claim 18, wherein the variant has as compared to SEQ ID NO: 04 following mutations:
    (a) S1→T and E13→A;
    (b) R2→G;
    (c) R2→G and E5→Q;
    (d) R2→G, E5→Q and E13→A;
    (e) R2→G, E5→Q, T12→D, and E13→A;
    (f) R2→G, E5→Q, T12→E, and E13→A;
    (g) T12→D and E13→A;
    (h) T12→E and E13→A;
    (i) and E13→A;
    (j) and E13→D; or
    (k) deletion of E13.

20. The fusion protein of any one of claims 17-19, wherein the peptide comprises N-terminal of X1 the amino acids Xa-Xb, wherein Xa is S or G or M or P or no amino acid, and Xb is R or G or S or P or M or no amino acid.

21. The fusion protein of claim 20, wherein Xa-Xb is selected from the group consisting of P, M-P, G-R, P-G, P-S, S-P, G-P, S-P, M, and M-S, preferably P or M-P.

22. The fusion protein of any one of claims 17-21, wherein the peptide comprises C-terminal of X13 the amino acids Xy-Xz, wherein Xy is P or D or A or no amino acid, and Xz is P or S or no amino acid.

23. The fusion protein of claim 22, wherein Xy-Xz is selected from the group consisting of no amino acid, P, D-P, A, and A-S, preferably no amino acid or P.

24. The fusion protein of any one of claims 17-23, wherein the peptide comprises a combination of Xa-Xb and Xy-Xz selected from the group consisting of:
    (a) M-P and P;
    (b) P and P; and
    (c) P and no amino acid.

25. The fusion protein of any one of the preceding claims, wherein the peptide comprises a sequence selected from the group consisting of:
    (a) MPSRLEEELRRRLTEP (SEQ ID NO: 05);
    (b) PSRLEEELRRRLTEP (SEQ ID NO: 06);
    (c) PSRLEEELRRRLTE (SEQ ID NO: 07);
    (d) GRSRLEEELRRRLTA (SEQ ID NO: 08);
    (e) PGSRLEEELRRRLTAP (SEQ ID NO: 09);
    (f) PSTRLEEELRRRLTAP (SEQ ID NO: 10);
    (g) SPSRLEEELRRRLTAP (SEQ ID NO: 11);
    (h) SPSRLEEELRRRLDAP (SEQ ID NO: 12);
    (i) SPSRLEEELRRRLEAP (SEQ ID NO: 13);
    (j) SPSRLEEELRRRLTDP (SEQ ID NO: 14);
    (k) SPSRLEEELRRRLTEP (SEQ ID NO: 15);
    (l) SPSRLEEELRRRLTADP (SEQ ID NO: 16);
    (m) SPSGLEEELRRRLTEP (SEQ ID NO: 17);
    (n) GPSRLEEELRRRLT (SEQ ID NO: 18);
    (o) GPSRLEEELRRRLTA (SEQ ID NO: 19);
    (p) GPSRLEEELRRRLTAA (SEQ ID NO: 20);
    (q) GPSRLEEELRRRLTAAS (SEQ ID NO: 21);
    (r) SPSGLEQELRRRLTAP (SEQ ID NO: 22);
    (s) SPSGLEQELRRRLDAP (SEQ ID NO: 23);
    (t) SPSGLEQELRRRLEAP (SEQ ID NO: 24);
    (u) SPSGLEQELRRRLTEP (SEQ ID NO: 25);
    (v) GPSRLEEELRRRLTAP (SEQ ID NO: 26);
    (w) GPSRLEEELRRRLTEP (SEQ ID NO: 27);

(x) GPSRLEEELRRRLTE (SEQ ID NO: 28);

(y) MSRLEEELRRRLTEP (SEQ ID NO: 29); and

(z) MSSRLEEELRRRLTEP (SEQ ID NO: 30).

26. The fusion protein of any one of claims 1-16, wherein the peptide comprises the sequence of X1-X2-L-E-X5-E-X7-R-R-R-L-X12-X13 (SEQ ID NO: 31), wherein X1 is G or S or P, X2 is R or G, X5 is E or Q, X7 is L or I, X12 is S or T or P or A, and X13 is P or A or S or no amino acid.

27. The fusion protein of any one of the preceding claims, wherein the peptide comprises the sequence of G-R-L-E-E-E-L-R-R-R-L-S (SEQ ID NO: 32) or a variant thereof, wherein the variant has as compared to (SEQ ID NO: 32) 1 to 6 mutations selected from the group consisting of: G1→S, G1→P, R2→G, E5→Q, L7→I, S12→T, S12→P, S12→A, addition of P13, addition of A13, and addition of S13.

28. The fusion protein of claim 27, wherein the variant has as compared to SEQ ID NO: 32 following mutations:

(a) G1→S, R2→G, E5→Q, and addition of P13;

(b) R2→G, E5→Q, S12→T, and addition of A13;

(c) G1→P, R2→G, E5→Q, S12→T, and addition of A13;

(d) G1→S, R2→G, E5→Q, S12→T, and addition of P13;

(e) G1→S, R2→G, S12→T, and addition of A13;

(f) G1→S, R2→G, E5→Q, and S12→T;

(g) G1→S, R2→G, E5→Q, S12→T, and addition of A13;

(h) G1→S and S12→P, and addition of P13;

(i) G1→S, R2→G, E5→Q, S12→T, and addition of P13;

(j) E5→Q L7→I, and addition of P13;

(k) addition of P13; or

(l) S12→A.

29. The fusion protein of any one of claims 26-28, wherein the peptide comprises N-terminal of X1 the amino acids Xa-Xb, wherein Xa is M or S or P or D or G or no amino acid, and Xb is S or D or P or no amino acid.

30. The fusion protein of claim 29, wherein Xa-Xb is selected from the group consisting of M-S, S-D, P-D, P-S, D-S, S-P, and G-P, preferably M-S.

31. The fusion protein of claim any one of claims 26-30, wherein the peptide comprises C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or P or A or E or K or S or no amino acid, and Xz is P or S or no amino acid.

32. The fusion protein of claim 31, wherein Xy-Xz is selected from the group consisting of no amino acid, G, P, A, E-P, A-S, K, and S, preferably no amino acid.

33. The fusion protein of claim any one of claims 26-32, wherein the peptide comprises a combination of Xa-Xb and Xy-Xz that is M-S and no amino acid.

34. The fusion protein of any one of the preceding claims, wherein the peptide comprises a sequence selected from the group consisting of:
    (a) GRLEEELRRRLS (SEQ ID NO: 32);
    (b) MSGRLEEELRRRLSP (SEQ ID NO: 33);
    (c) SDSGLEQELRRRLSPG (SEQ ID NO: 34);
    (d) PDGGLEQELRRRLTAP (SEQ ID NO: 35);
    (e) PSGGLEQELRRRLTAP (SEQ ID NO: 36);
    (f) DSPGLEQELRRRLTAP (SEQ ID NO: 37);
    (g) PDSGLEQELRRRLTPA (SEQ ID NO: 38);
    (h) SPSGLEEELRRRLTAEP (SEQ ID NO: 39);
    (i) GPSGLEQELRRRLT (SEQ ID NO: 40);
    (j) GPSGLEQELRRRLTAAS (SEQ ID NO: 41);
    (k) SPSRLEEELRRRLPSK (SEQ ID NO: 42);
    (l) SPSGLEQELRRRLTPS (SEQ ID NO: 43);
    (m) SPGRLEQEIRRRLSPS (SEQ ID NO: 44);
    (n) PSGRLEEELRRRLSPS (SEQ ID NO: 45);
    (o) PSGRLEEELRRRLS (SEQ ID NO: 46);
    (p) PSGRLEEELRRRLA (SEQ ID NO: 47); and
    (q) PSGRLEEELRRRLSP (SEQ ID NO: 48).

35. The fusion protein of any one of claims 1-16, wherein the peptide comprises the sequence of X1-X2-L-E-X5-E-L-R-R-R-L-X12-X13 (SEQ ID NO: 49), wherein X1 is S or G or P, X2 is R or G or P, X5 is E or Q, X12 is S or T or D or E, and X13 is P or A or D or no amino acid.

36. The fusion protein of claim 35, wherein the peptide comprises N-terminal of X1 the amino acids Xa-Xb, wherein Xa is P or D or S or G or no amino acid, and Xb is D or S or P or no amino acid.

37. The fusion protein of claim 35 or 36, wherein the peptide comprises C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or P or E or D or S or no amino acid, and Xz is P or no amino acid.

38. The fusion protein of any one of the preceding claims, wherein the peptide comprises a sequence selected from the group consisting of:
    (a) PDSGLEQELRRRLSPG (SEQ ID NO: 50);
    (b) PDSGLEQELRRRLTAP (SEQ ID NO: 51);
    (c) PSSGLEQELRRRLTAP (SEQ ID NO: 52);
    (d) DPSGLEQELRRRLTAP (SEQ ID NO: 53);
    (e) DSGPLEQELRRRLTAP (SEQ ID NO: 54);
    (f) SPSRLEEELRRRLTAEP (SEQ ID NO: 55);
    (g) SPSGLEEELRRRLTAP (SEQ ID NO: 56);
    (h) SPSGLEEELRRRLDAP (SEQ ID NO: 57);
    (i) SPSGLEEELRRRLEAP (SEQ ID NO: 58);
    (j) SPSGLEEELRRRLTDP (SEQ ID NO: 59);
    (k) SPSGLEEELRRRLTADP (SEQ ID NO: 60);
    (l) GPSGLEQELRRRLTA (SEQ ID NO: 169);
    (m) SPSGLEQELRRRLTDP (SEQ ID NO: 170);
    (n) SPSGLEQELRRRLTADP (SEQ ID NO: 171);
    (o) SPSGLEQELRRRLTAEP (SEQ ID NO: 172);
    (p) DSPGLEQELRRRLTAP (SEQ ID NO: 173); and
    (q) SPSGLEQELRRRLSPS (SEQ ID NO: 174).

39. The fusion protein of any one claims 1-16, wherein the peptide comprises the sequence of X1-X2-X3-X4-X5-X6-X7-X8-X9-R-L-X12-X13 (SEQ ID NO: 61), wherein X1 is G or S, X2 is R or G or A or E, X3 is L or V, X4 is E or Q, X5 is E or Q, X6 is E or Q, X7 is L or I or V, X8 is R or A or Q or E, X9 is R or A or Q or E, X12 is S or T or L or no amino acid, and X13 is K or P or S or no amino acid.

40. The fusion protein of claim 39, wherein the peptide comprises N-terminal of X1 the amino acids Xa-Xb, wherein Xa is D or S or G or M or no amino acid and Xb is S or D or P or M or no amino acid.

41. The fusion protein of claim 39 or 40, wherein the peptide comprises C-terminal of X13 the amino acids Xy-Xz, wherein Xy is G or S or P or no amino acid, Xz is S or no amino acid.

42. The fusion protein of any one of the preceding claims, wherein the peptide comprises a sequence selected from the group consisting of:
    (a) GRLEEELRRRLS (SEQ ID NO: 32);
    (b) MSGRLEEELRRRLSP (SEQ ID NO: 33);
    (c) DSGRLEEELRRRLSKG (SEQ ID NO: 62);
    (d) DSGRLEEELRRRLSPG (SEQ ID NO: 63);
    (e) SDSGLEEELRRRLSPG (SEQ ID NO: 64);
    (f) SDSGVEEELRRRLSPG (SEQ ID NO: 65);
    (g) SDSAVEEELRRRLSPG (SEQ ID NO: 66);
    (h) SDSGLQEELRRRLSPG (SEQ ID NO: 67);
    (i) SDSGLEEQLRRRLSPG (SEQ ID NO: 68);
    (j) SDSGLEEEIRRRLSPG (SEQ ID NO: 69);
    (k) SDSGLEEEVRRRLSPG (SEQ ID NO: 70);
    (l) DSGELEEELRRRLSPG (SEQ ID NO: 71);
    (m) DSGRLEQELRRRLSPG (SEQ ID NO: 72);
    (n) DSGRLEEEIRRRLSPG (SEQ ID NO: 73);
    (o) DSGRLEQEIRRRLSPG (SEQ ID NO: 74);
    (p) DSGRLEQEIARRLSPG (SEQ ID NO: 75);
    (q) DSGRLEQEIQRRLSPG (SEQ ID NO: 76);
    (r) DSGRLEQEIERRLSPG (SEQ ID NO: 77);

(s) DSGRLEQEIRARLSPG (SEQ ID NO: 78);
(t) DSGRLEQEIRQRLSPG (SEQ ID NO: 79);
(u) DSGRLEQEIRERLSPG (SEQ ID NO: 80);
(v) GPSRLEEELRRRL (SEQ ID NO: 81);
(w) MSGLEQELRRRLTPS (SEQ ID NO: 82);
(x) MSGRLEEELRRRLSPS (SEQ ID NO: 83);
(y) SPSAVEEELRRRLSPS (SEQ ID NO: 84);
(z) GPSAVEEELRRRLS (SEQ ID NO: 85);
(aa) MPSGLEQELRRRLTPS (SEQ ID NO: 86);
(bb) MSSGLEQELRRRLTPS (SEQ ID NO: 87);
(cc) MPSGRLEEELRRRLSPS (SEQ ID NO: 88);
(dd) MSGRLEEELRRRLSP (SEQ ID NO: 89).

43. The fusion protein of any one of the preceding claims, wherein the fusion protein is in complex with a binding partner that specifically binds to the peptide comprised in the fusion protein.

44. The fusion protein of any one of the preceding claims, wherein the fusion protein comprises an antibody.

45. An antibody that specifically binds to the peptide comprised in the fusion protein of any one of claims 1-44.

46. The antibody of claim 45, wherein the antibody is a monovalent antibody.

47. The antibody of claim 45 or 46, wherein the antibody is a single domain antibody.

48. The antibody of any one of claims 45-47, wherein the antibody comprises a camelid VHH domain.

49. The antibody of any one of claims 45-48, comprising the CDR1 sequence GVTISALNAMAMG (SEQ ID NO: 115) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AVSERGNAM (SEQ ID NO: 116) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence

LEDRVDSFHDY (SEQ ID NO: 117) or a sequence having 1 or 2 mutations relative to said sequence.

50. The antibody of any one of claims 45-48, comprising the CDR1 sequence GVTISALNAMAMG (SEQ ID NO: 118) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AVSSRGNAM (SEQ ID NO: 119) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEDRVDSFHDY (SEQ ID NO: 120) or a sequence having 1 or 2 mutations relative to said sequence.

51. The antibody of any one of claims 45-48, comprising the CDR1 sequence GVTVSALNAMAMG (SEQ ID NO: 121) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AVSERGNAM (SEQ ID NO: 122) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEDRVDSFHDY (SEQ ID NO: 123) or a sequence having 1 or 2 mutations relative to said sequence.

52. The antibody of any one of claims 49-51, wherein the E or S at amino acid position 4 of CDR2 is mutated to G, A, L, I, S, T, V, C, M, D, N, E, Q, F, Y, H, W, K, R, or P, preferably to D, N, or H.

53. The antibody of any one of claims 49-52, comprising the FR1 sequence EVQLX$_1$ESGGGLVX$_2$PGGSX$_3$RLSCTAS, wherein X$_1$ is Q, V, E, or L, X$_2$ is Q or P, and X$_3$ is L or M (SEQ ID NO: 124), or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

54. The antibody of any one of claims 49-52, comprising the FR1 sequence EVQLQESGGGLVQPGGSLRLSCTAS (SEQ ID NO: 125) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

55. The antibody of any one of claims 49-54, comprising the FR2 sequence WYRQX$_1$PGEX$_2$RVMVA, wherein X$_1$ is A or R and X$_2$ is R or E (SEQ ID NO: 126) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

56. The antibody of any one of claims 49-54, comprising the FR2 sequence WYRQAPGERRVMVA (SEQ ID NO: 127) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

57. The antibody of any one of claims 49-54, comprising the FR2 sequence WYRQAPGEERVMVA (SEQ ID NO: 128) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

58. The antibody of any one of claims 49-57, comprising the FR3 sequence YRESVQGRFTVTRDFTNKMVSLQMDNLX$_1$PEDX$_2$AVYYCHV, wherein X$_1$ is K or Q and X$_2$ is T or M (SEQ ID NO: 129) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

59. The antibody of any one of claims 49-57, comprising the FR3 sequence YRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYYCHV (SEQ ID NO: 130) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

60. The antibody of any one of claims 49-59, comprising the FR4 sequence WGQGX$_1$QVTVSS, wherein X$_1$ is T or I (SEQ ID NO: 131) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

61. The antibody of any one of claims 49-59, comprising the FR4 sequence WGQGTQVTVSS (SEQ ID NO: 132) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

62. The antibody of any one of claims 49-61, comprising the VHH sequence
    (a) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 133);
    (b) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGEERV MVAAVSSRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYY CHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 134);

(c) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGEERV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 135);

(d) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSSRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYY CHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 136);

(e) EVQLVESGGGLVPPGGSMRLSCTASGVTVSALNAMAMGWYRQRPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLQPEDMAV YYCHVLEDRVDSFHDYWGQGIQVTVSS (SEQ ID NO: 137);

(f) EVQLVESGGGLVPPGGSMRLSCTAPGVTVSALNAMAMGWYRQRPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLQPEDMAV YYCHVLEDRVDSFHDYWGQGIQVTVSS (SEQ ID NO: 138);

(g) EVQLVESGGGVVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAV YYCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 139);

(h) EVQLVESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 140);

(i) EVQLEESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 141);

(j) EVQLLESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 142);

(k) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSDRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 175);

(l) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSNRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 176); or

(m) EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRV MVAAVSHRGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS (SEQ ID NO: 177);

or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to any one of

said sequences.

63. The antibody of any one of claims 45-48, comprising the CDR1 sequence GTMSAINALN (SEQ ID NO: 143) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AITDNGNAH (SEQ ID NO: 144) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEEEKLGVWVDY (SEQ ID NO: 145) or a sequence having 1 or 2 mutations relative to said sequence.

64. The antibody of any one of claims 45-48, comprising the CDR1 sequence GTMSAINALN (SEQ ID NO: 146) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AITDNGNAH (SEQ ID NO: 147) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEEKLGAWVDY (SEQ ID NO: 148) or a sequence having 1 or 2 mutations relative to said sequence.

65. The antibody of any one of claims 45-48, comprising the CDR1 sequence GTMSAINALN (SEQ ID NO: 149) or a sequence having 1 or 2 mutations relative to said sequence, the CDR2 sequence AITDNGNAH (SEQ ID NO: 150) or a sequence having 1 or 2 mutations relative to said sequence, and the CDR3 sequence LEKEKLGVWVDY (SEQ ID NO: 151) or a sequence having 1 or 2 mutations relative to said sequence.

66. The antibody of any one of claims 63-65, comprising the FR1 sequence EVQLX$_1$ESGGGLVQPGGSLTLSCAAS, wherein X$_1$ is V or L (SEQ ID NO: 152) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

67. The antibody of any one of claims 63-66, comprising the FR2 sequence WYRQX$_1$PGKERKMVA, wherein X$_1$ is P or A (SEQ ID NO: 153) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

68. The antibody of any one of claims 63-67, comprising the FR3 sequence YADSVKGRFTISRDNARNMVFLQMNSLX$_1$PDDTAVYYCHY, wherein X$_1$ is K or

E (SEQ ID NO: 154) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

69. The antibody of any one of claims 63-68, comprising the FR4 sequence WGQGTQVTVSS (SEQ ID NO: 155) or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to said sequence.

70. The antibody of any one of claims 63-69, comprising the VHH sequence
    (a) EVQLVESGGGLVQPGGSLTLSCAASGTMSAINALNWYRQPPGKERKMVA AITDNGNAHYADSVKGRFTISRDNARNMVFLQMNSLKPDDTAVYYCHYL EEEKLGVWVDYWGQGTQVTVSS (SEQ ID NO: 156);
    (b) EVQLLESGGGLVQPGGSLTLSCAASGTMSAINALNWYRQAPGKERKMVA AITDNGNAHYADSVKGRFTISRDNARNMVFLQMNSLEPDDTAVYYCHYL EEKLGAWVDYWGQGTQVTVSS (SEQ ID NO: 157); or
    (c) EVQLVESGGGLVQPGGSLTLSCAASGTMSAINALNWYRQPPGKERKMVA AITDNGNAHYADSVKGRFTISRDNARNMVFLQMNSLKPDDTAVYYCHYL EKEKLGVWVDYWGQGTQVTVSS (SEQ ID NO: 158);
    or a sequence having at least 80 %, 85 %, 90 %, or 95 % sequence identity to any one of said sequences.

71. The antibody of any one of claims 45-70, wherein the antibody is conjugated to a detectable label.

72. The antibody of claim 71, wherein the detectable label is a fluorescent label.

73. The antibody of claim 71, wherein the detectable label is an affinity tag.

74. The antibody of claim 71 or 73, wherein the detectable label is selected from the group consisting of FLAG-tag, Strep-tag, Myc-tag, His-tag, HA-tag, VSV-G-tag, HSV-tag, V5-tag, GST-tag, Spot-tag, BC2-tag, EPEA-tag, maltose binding protein (MBP), chitin binding protein (CBP), thioredoxin, and biotin.

75. The antibody of any one of claims 45-74, wherein the antibody is conjugated to a solid support.

76. The antibody of any one of claims 45-75, wherein the antibody is in complex with an epitope it specifically binds to.

77. A fusion protein comprising a peptide that the antibody of any one of claims 45-76 binds to.

78. A complex comprising
(a) a fusion protein; and
(b) an antibody;
wherein the fusion protein is the fusion protein of any one of claims 1-44 and 77 and/or wherein the antibody is the antibody of any one of claims 45-76.

79. A nucleic acid encoding a fusion protein of any one of claims 1-44 and 77 or an antibody of any one of claims 45-76.

80. A vector comprising the nucleic acid of claim 79.

81. A host cell comprising the nucleic acid of claim 79 or the vector of claim 80 or expressing the fusion protein of any one of claims 1-44 and 77 or the antibody of any one of claims 45-76.

82. Use of a peptide as defined in any one of claims 1-44 and 77 as an epitope tag or for the detection, immobilization, isolation, or purification of the fusion protein of any one of claims 1-45 and 76.

83. Use of the antibody of any one of claims 45-76 for the detection, immobilization, isolation, or purification of the fusion protein of any one of claims 1-44 and 77.

84. A method of detecting the fusion protein of any one of claims 1-44 and 77, comprising contacting the fusion protein with an antibody of any one of claims 71-74.

85. The method of claim 84, further comprising contacting the fusion protein and the antibody with a specific binding partner for the detectable label comprised in the antibody.

86. The method of claim 85, wherein the specific binding partner is conjugated to a second detectable label.

87. The method of claim 86, wherein the second detectable label is a fluorescent label.

88. The method of any one of claims 84 to 87, further comprising the step of detecting the detectable label or the second detectable label.

89. The method of any one of claims 84-88, further comprising expressing the fusion protein prior to contacting the fusion protein with the antibody.

90. The method of any one of claims 84-89, wherein detecting the fusion protein comprises determining the presence, subcellular localization, or amount of the fusion protein.

91. A method of isolating the fusion protein of any one of claims 1-44 and 77 or a specifc target of an antibody moiety comprised in the fusion protein of claim 44, comprising contacting the fusion protein with an antibody of any one of claims 45-76.

92. The method of claim 91, wherein the antibody is prior to, during, or after binding to the fusion protein attached to a solid support.

93. The method of claim 91 or 92, further comprising eluting the fusion protein.

94. The method of claim 93, wherein the elution is conducted by contacting the complex of the fusion protein and the antibody with an agent that competes with the fusion protein for binding to the antibody.

95. The method of claim 94, wherein the agent is a peptide as defined in any one of claims 1-43 or 76.

96. The method of any one of claims 91-95, further comprising contacting the fusion protein of claim 44 with a specific target of the antibody moiety comprised in the fusion protein.

97. The method of claim 96, wherein the specific target comprises a cell surface receptor.

98. The method of any one of claims 91 to 97, further comprising a wash step.

99. A kit comprising a nucleic acid or a nucleic acid expression construct encoding a peptide as defined in any one of claims 1-44 or 77 and optionally an antibody of any one of claims 45-76.

Fig. 1

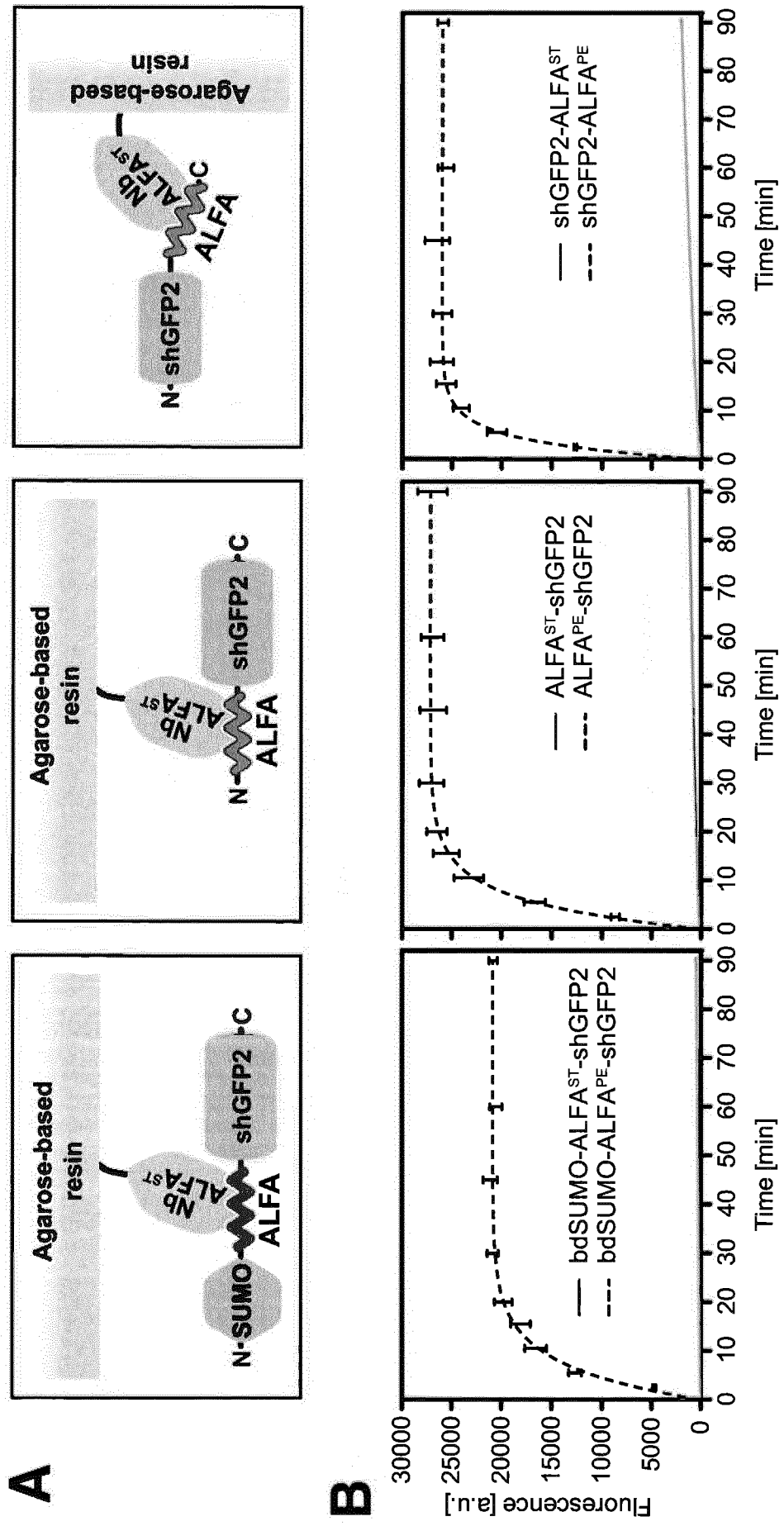

A
Agarose-based resin
N-SUMO
Nb ALFAST
ALFA
shGFP2
C
N
N- shGFP2
ALFA
Nb ALFAST
C
B
Fluorescence [a.u.]
Time [min]
bdSUMO-ALFAST-shGFP2
bdSUMO-ALFA^{PE}-shGFP2
ALFAST-shGFP2
ALFA^{PE}-shGFP2
shGFP2-ALFAST
shGFP2-ALFA^{PE}

Fig. 1 (continued)

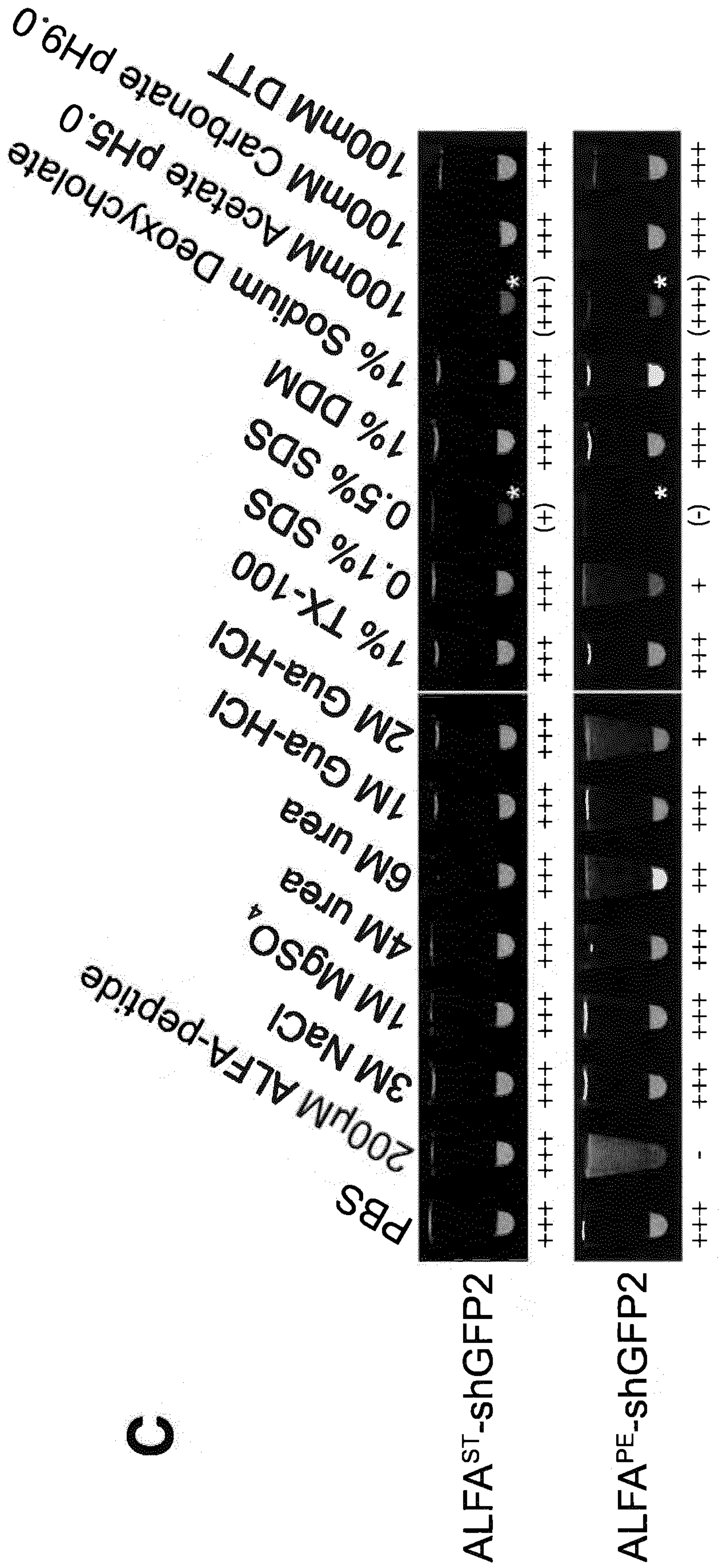


Fig. 2

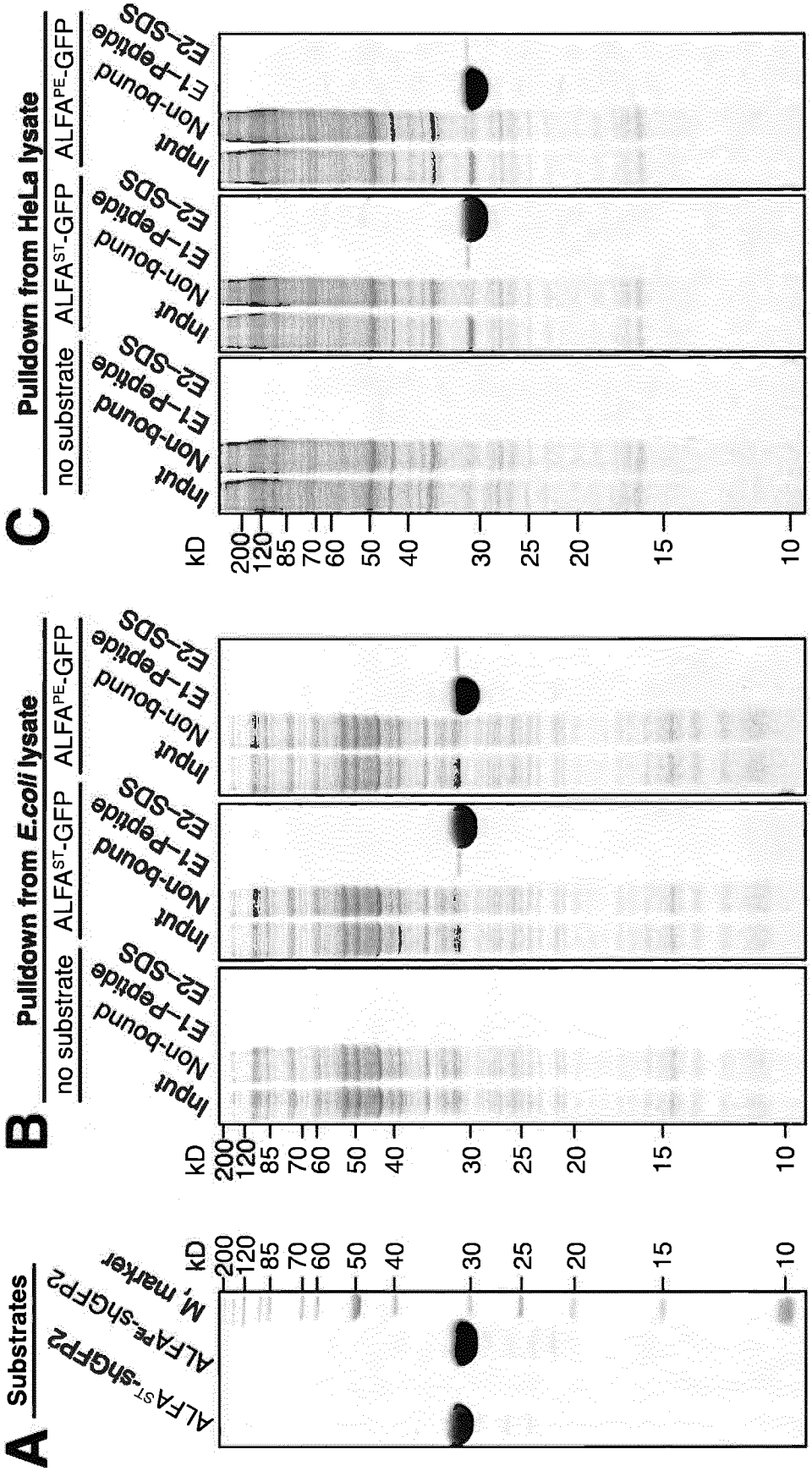

A
Substrates
ALFA^ST-sfGFP2
ALFA^PE-sfGFP2
M, marker
kD
200
120
85
70
60
50
40
30
25
20
15
10
B
Pulldown from E.coli lysate
no substrate
ALFA^ST-GFP
ALFA^PE-GFP
Input
Non-bound
E1-Peptide
E2-SDS
C
Pulldown from HeLa lysate

Fig. 2 (continued)

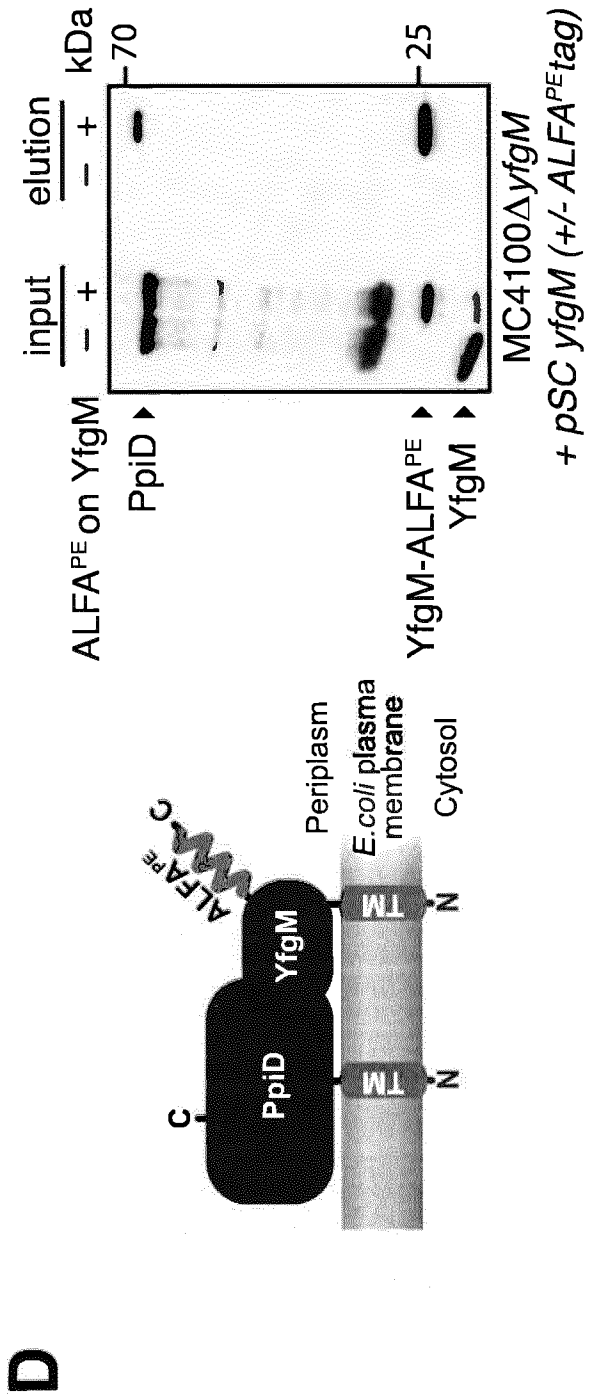


Fig. 3

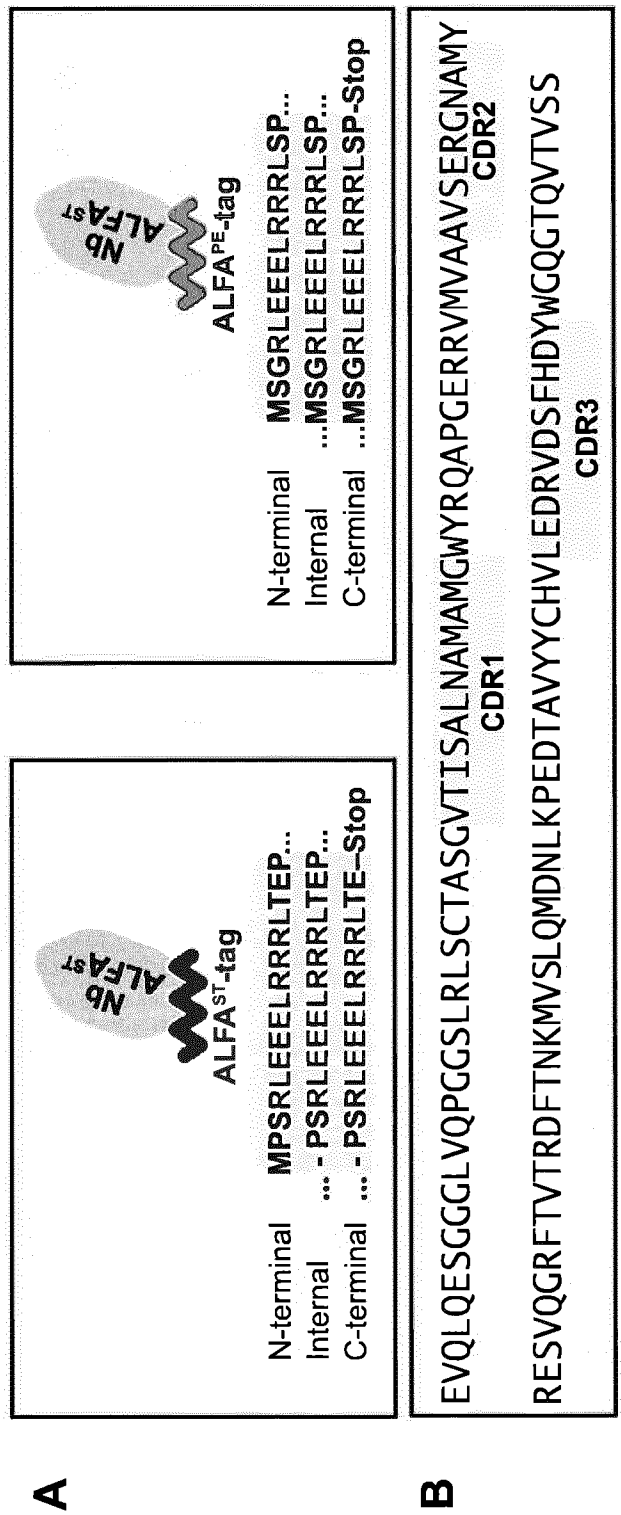

A
Nb ALFA^ST
ALFA^ST-tag
N-terminal MPSRLEEELRRRRLTEP...
Internal ...-PSRLEELRRRRLTEP...
C-terminal ...-PSRLEEELRRRRLTE–Stop
Nb ALFA^ST
ALFA^PE-tag
N-terminal MSGRLEEELRRRRLSP...
Internal ...MSGRLEEELRRRRLSP...
C-terminal ...MSGRLEEELRRRRLSP-Stop
B
EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRVMVAAVSERGNAMY
CDR1
CDR2
RESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYYCHVLEDRVDSFHDYWGQGTQVTVSS
CDR3

Fig. 3 (continued)

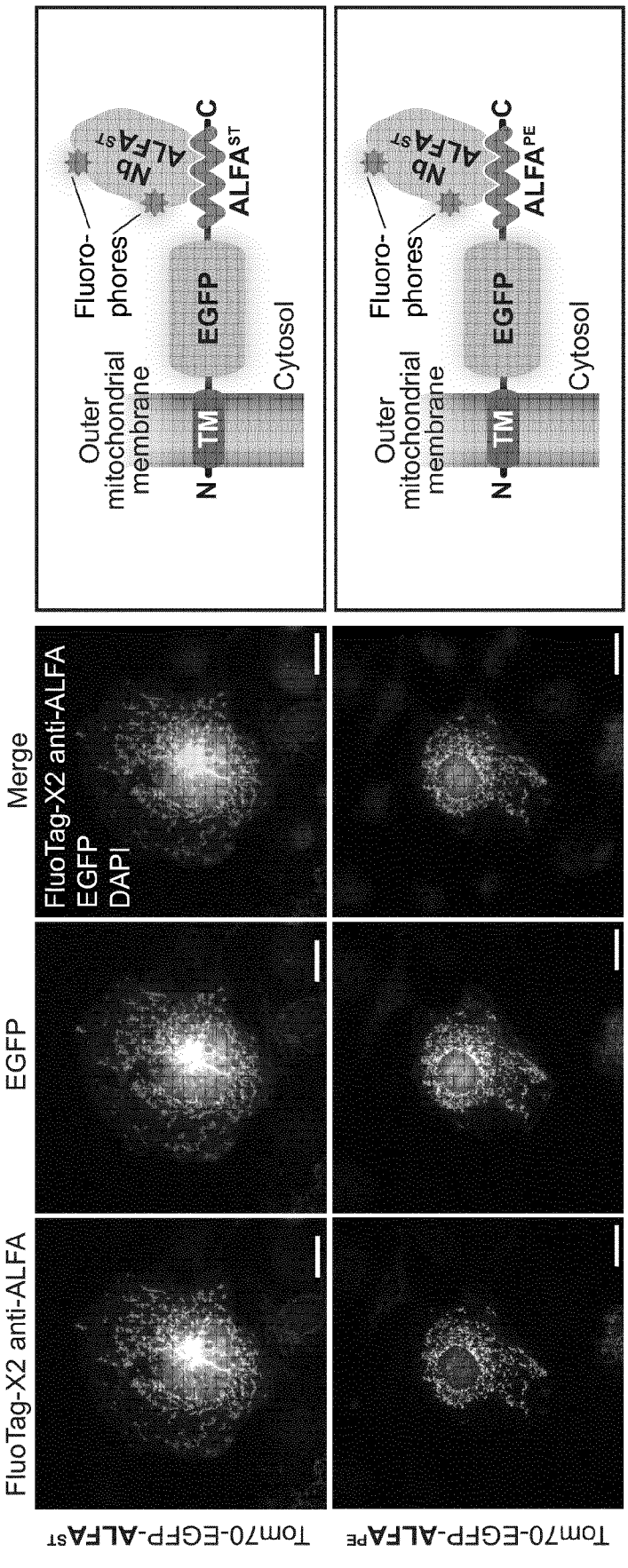


Fig. 3 (continued)

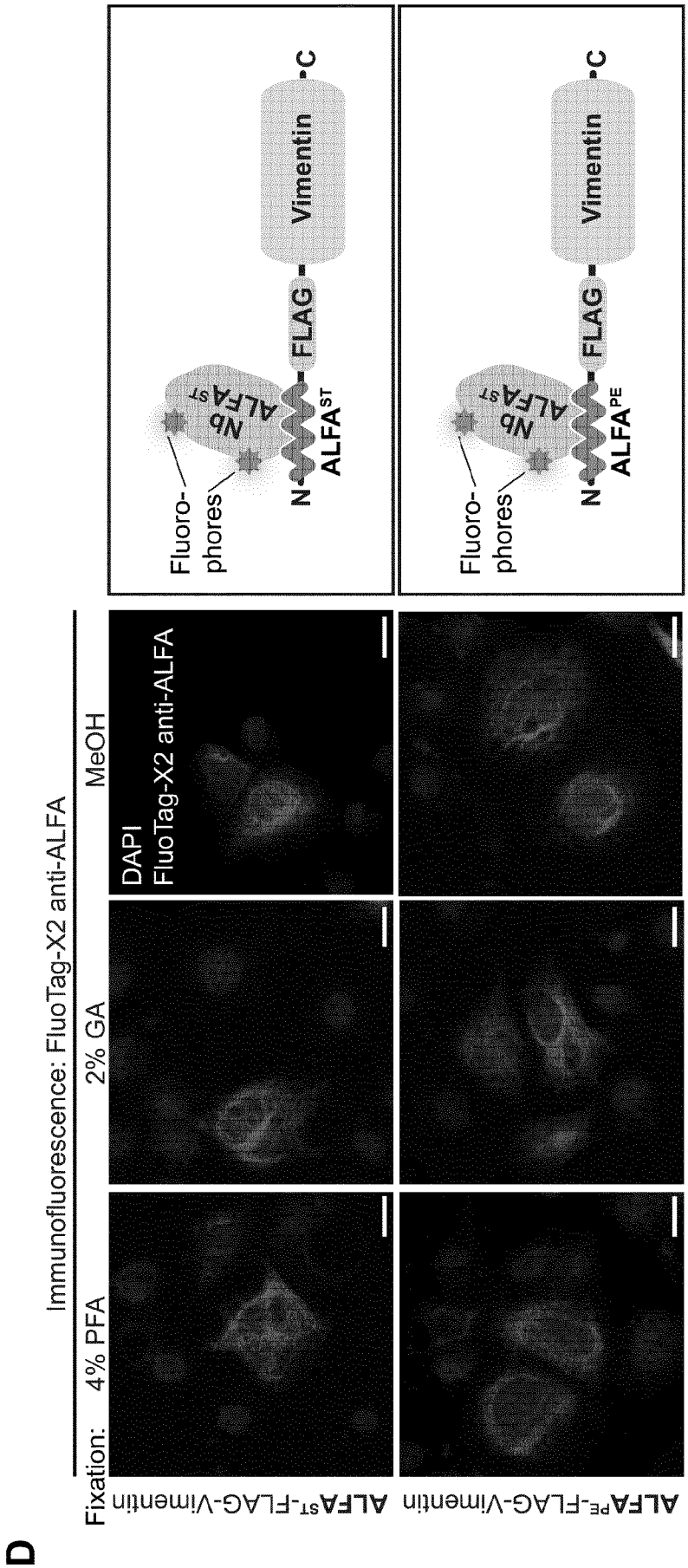


Fig. 3 (continued)

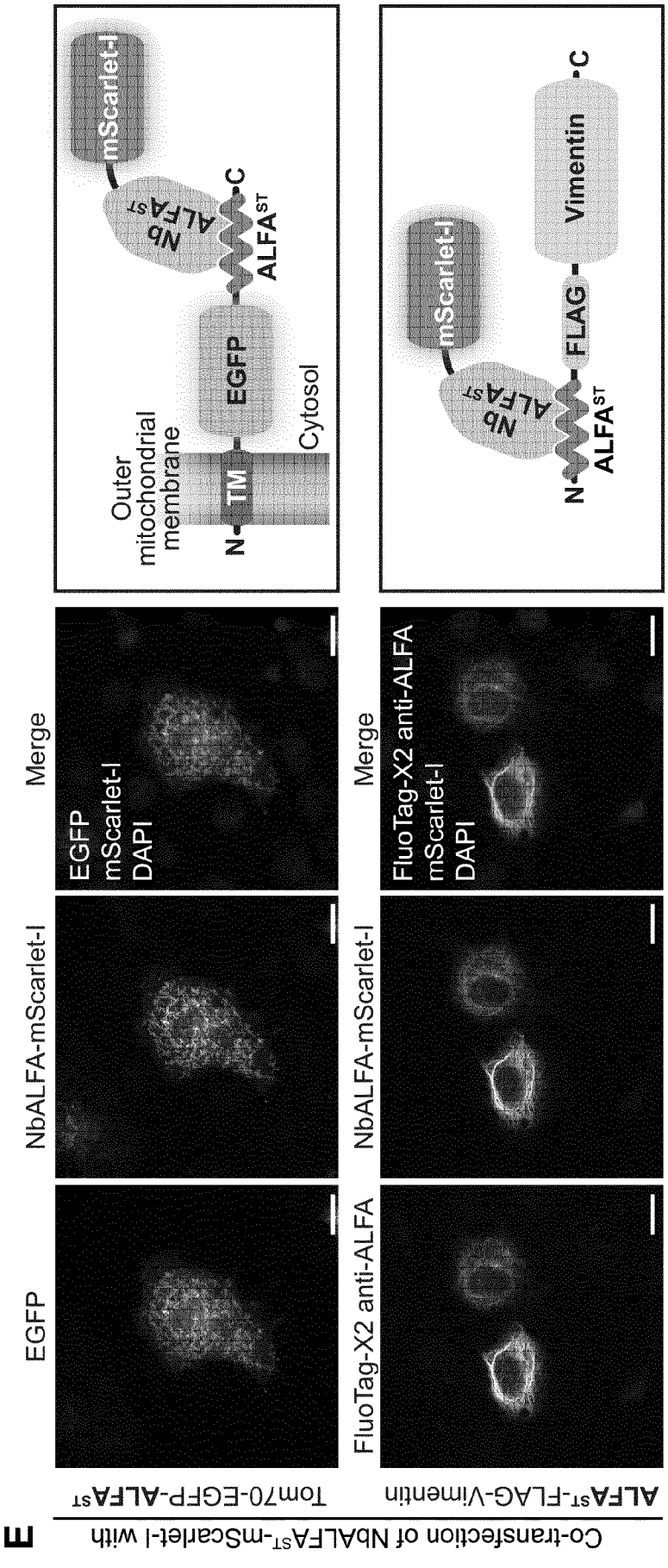


Fig. 4

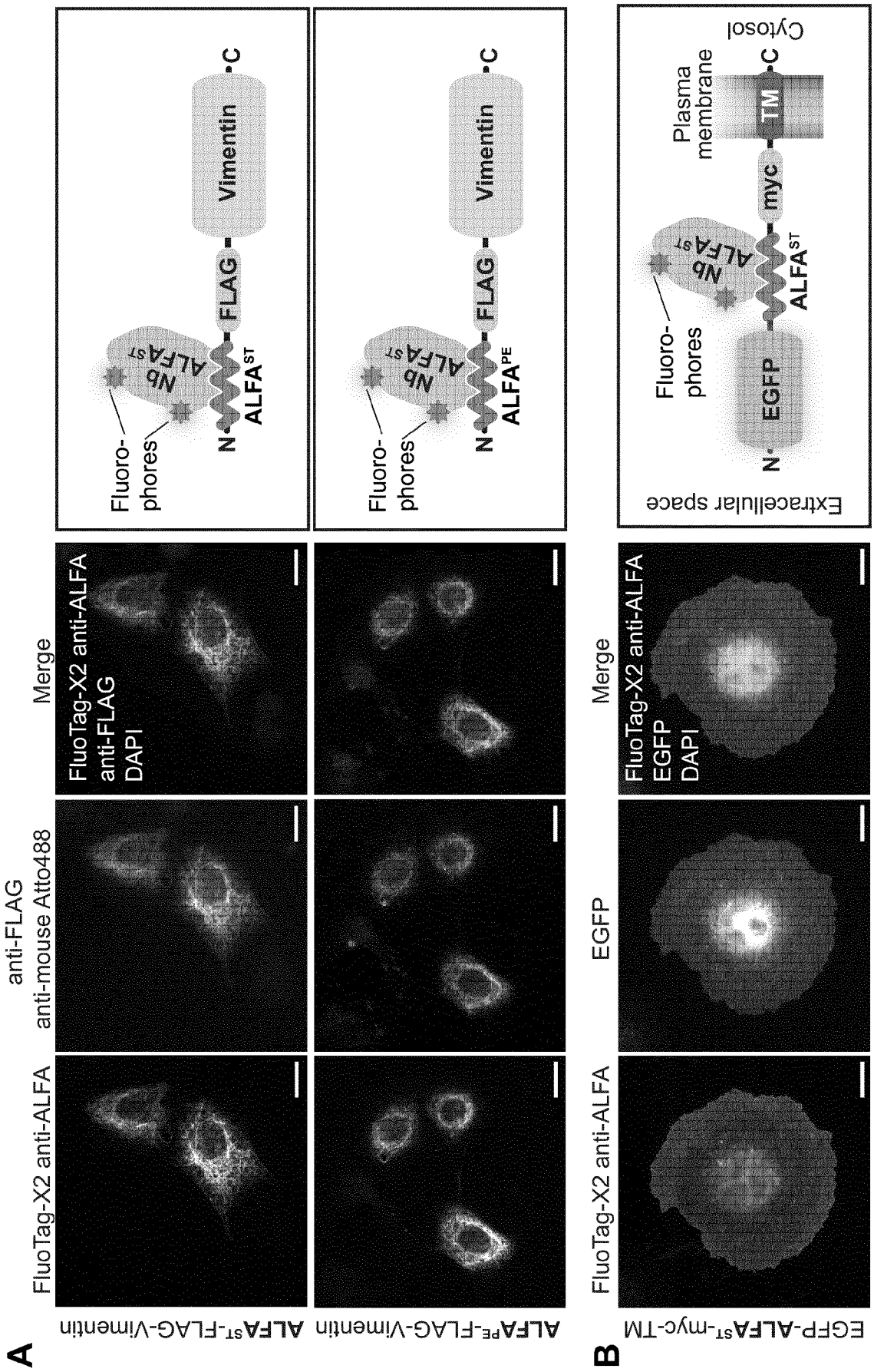

A
B
anti-FLAG
anti-mouse Atto488
FluoTag-X2 anti-ALFA
Merge
FluoTag-X2 anti-ALFA
anti-FLAG
DAPI
ALFA^ST-FLAG-Vimentin
ALFA^PE-FLAG-Vimentin
Nb ALFA^ST
Fluoro-phores
N
ALFA^ST
FLAG
Vimentin
C
ALFA^PE
EGFP
EGFP-ALFA^ST-myc-TM
Extracellular space
Plasma membrane
Cytosol
myc
TM

Fig. 5

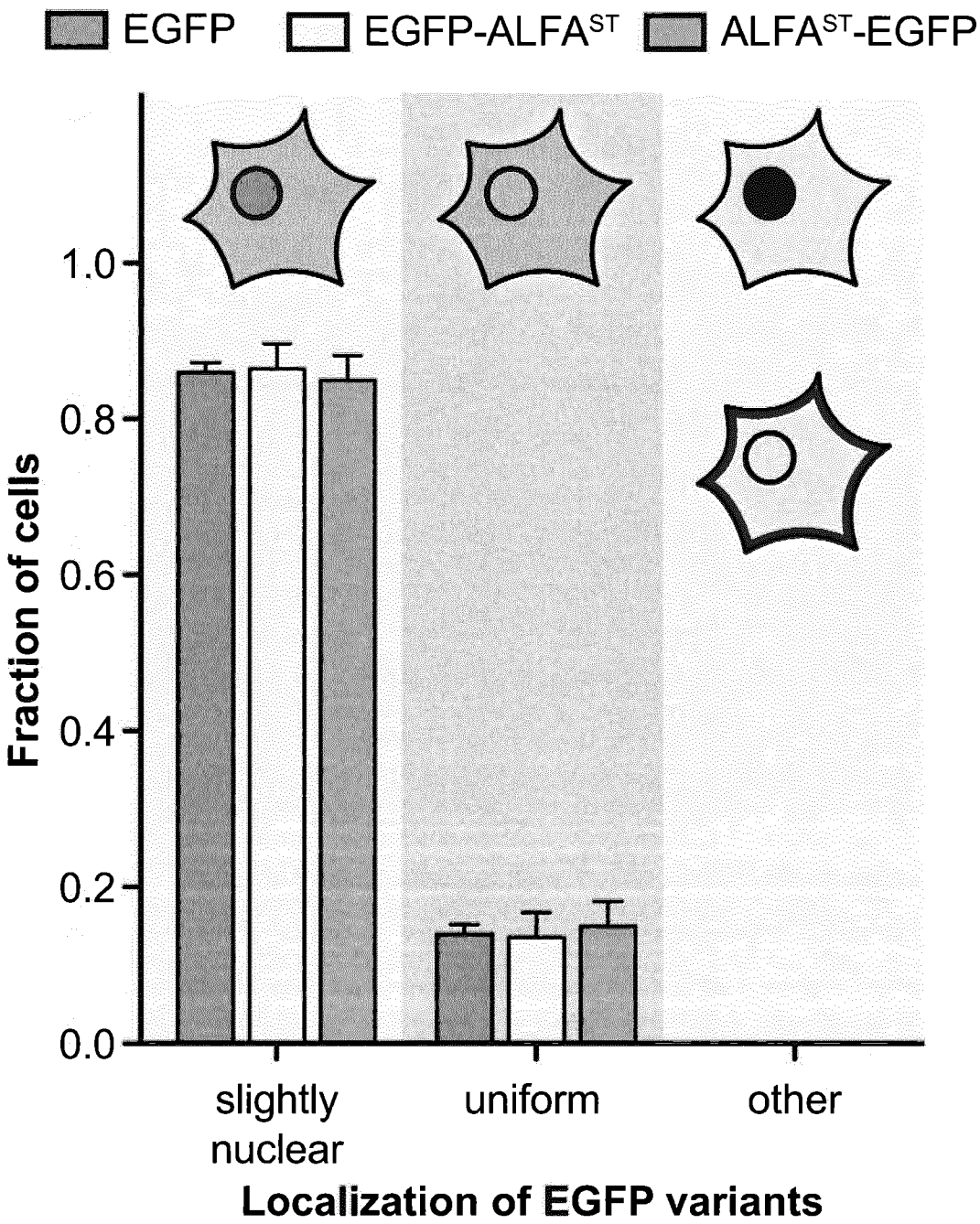

EGFP
EGFP-ALFAST
ALFAST-EGFP
Fraction of cells
1.0
0.8
0.6
0.4
0.2
0.0
slightly nuclear
uniform
other
Localization of EGFP variants

Fig. 6

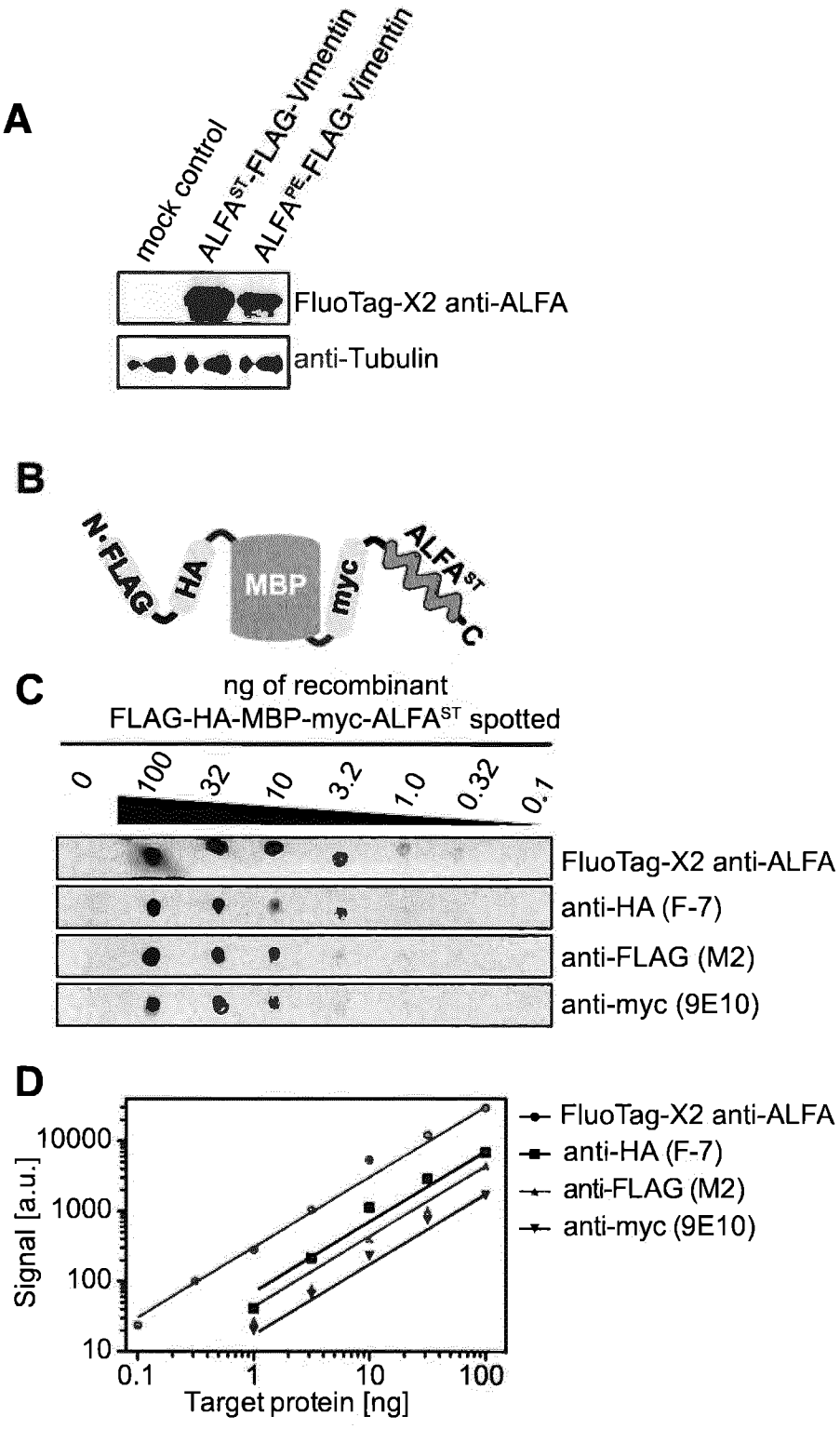

A
mock control
ALFA^ST-FLAG-Vimentin
ALFA^PE-FLAG-Vimentin
FluoTag-X2 anti-ALFA
anti-Tubulin
B
N-FLAG
HA
MBP
myc
ALFA^ST
C
C
ng of recombinant
FLAG-HA-MBP-myc-ALFA^ST spotted
0
100
32
10
3.2
1.0
0.32
0.1
FluoTag-X2 anti-ALFA
anti-HA (F-7)
anti-FLAG (M2)
anti-myc (9E10)
D
Signal [a.u.]
10000
1000
100
10
0.1
1
10
100
Target protein [ng]
FluoTag-X2 anti-ALFA
anti-HA (F-7)
anti-FLAG (M2)
anti-myc (9E10)

Fig. 7

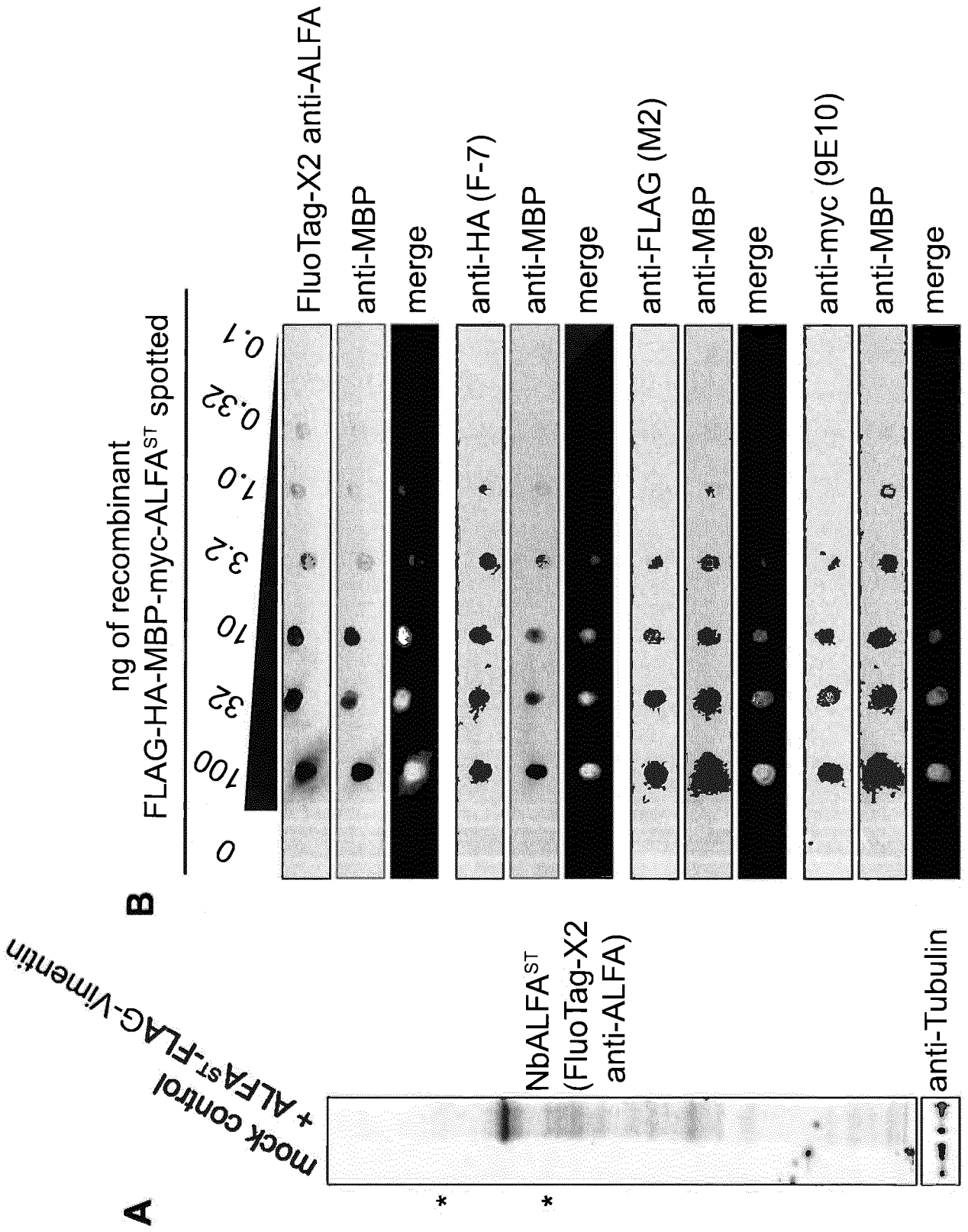

A
mock control
+ ALFAST-FLAG-Vimentin
NbALFAST
(FluoTag-X2
anti-ALFA)
anti-Tubulin
*
*
B
ng of recombinant
FLAG-HA-MBP-myc-ALFAST spotted
0
100
32
10
3.2
1.0
0.32
0.1
FluoTag-X2 anti-ALFA
anti-MBP
merge
anti-HA (F-7)
anti-MBP
merge
anti-FLAG (M2)
anti-MBP
merge
anti-myc (9E10)
anti-MBP
merge

Fig. 8

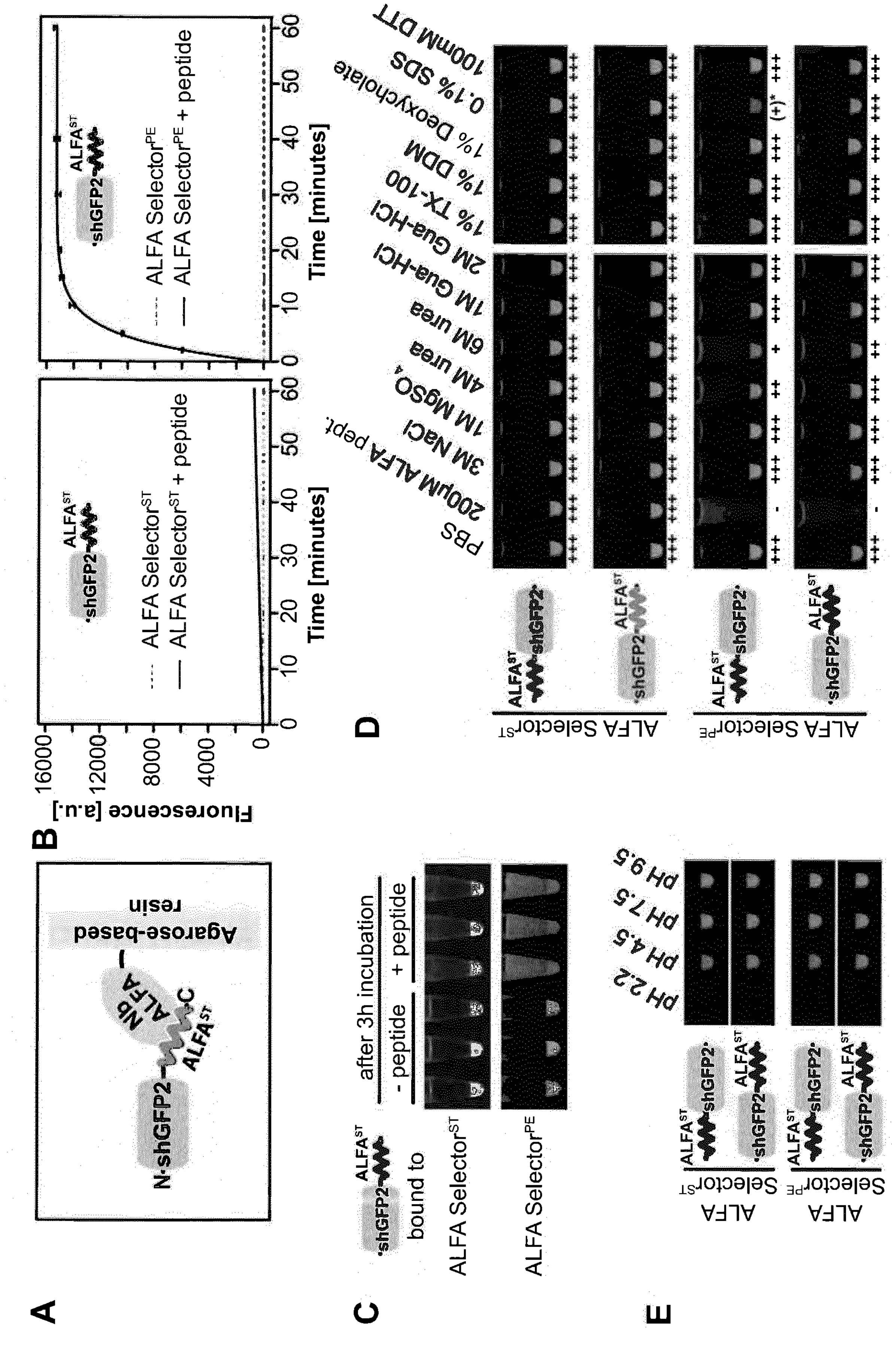

A
N·shGFP2
Nb ALFA
C
ALFA^ST
Agarose-based resin
B
Fluorescence [a.u.]
16000
12000
8000
4000
0
Time [minutes]
0 10 20 30 40 50 60
ALFA Selector^ST
ALFA Selector^ST + peptide
ALFA Selector^PE
ALFA Selector^PE + peptide
C
·shGFP2 ALFA^ST bound to
after 3h incubation
- peptide
+ peptide
ALFA Selector^ST
ALFA Selector^PE
D
PBS
200µM ALFA pept.
3M NaCl
1M MgSO4
4M urea
6M urea
1M Gua-HCl
2M Gua-HCl
1% TX-100
1% DDM
1% Deoxycholate
0.1% SDS
100mM DTT
ALFA Selector^ST
ALFA Selector^PE
E
pH 2.2
pH 4.5
pH 7.5
pH 9.5
ALFA Selector^ST
ALFA Selector^PE

Fig. 9

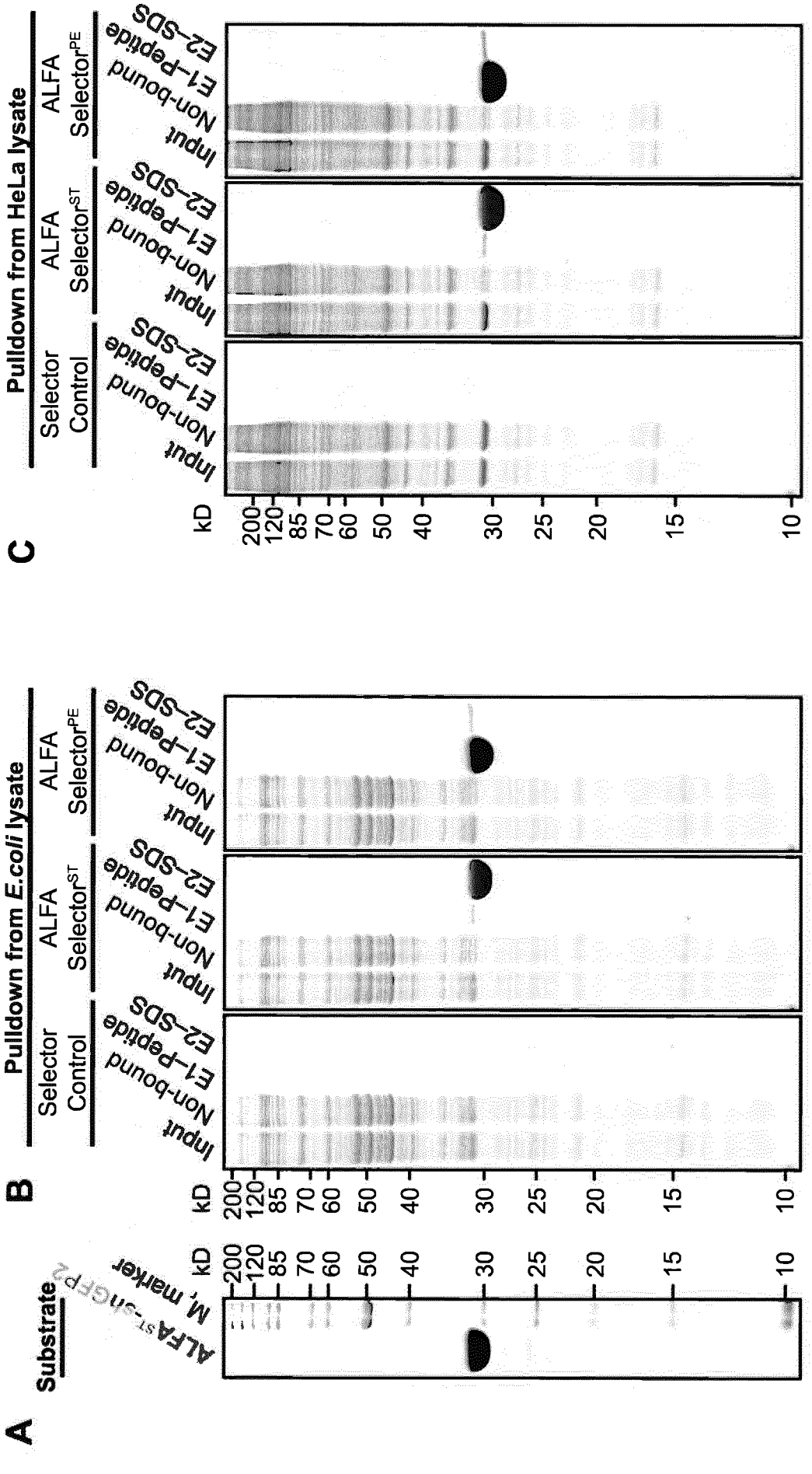
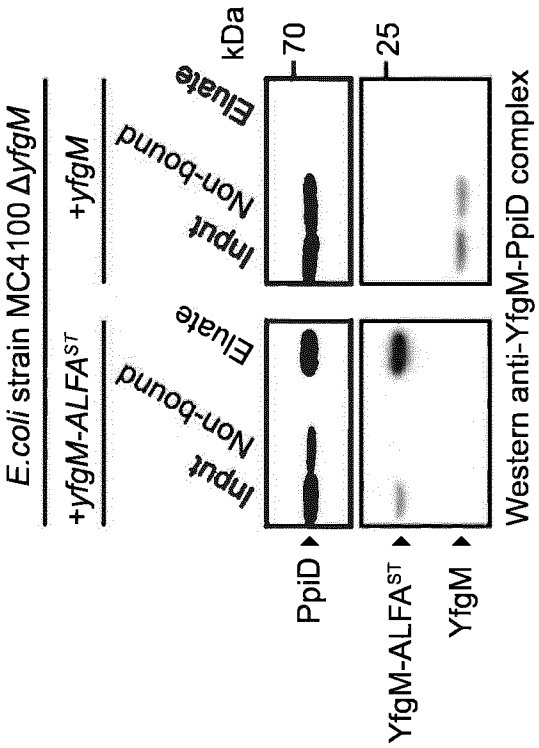

A
Substrate
ALFAST-sfGFP2
M, marker
kD
200
120
85
70
60
50
40
30
25
20
15
10
B
Pulldown from *E.coli* lysate
Selector
Control
ALFA
SelectorST
ALFA
Selector^{PE}
Input
Non-bound
E1-Peptide
E2-SDS
C
Pulldown from HeLa lysate
Selector
Control
ALFA
SelectorST
ALFA
Selector^{PE}
Input
Non-bound
E1-Peptide
E2-SDS

Fig. 9 (continued)



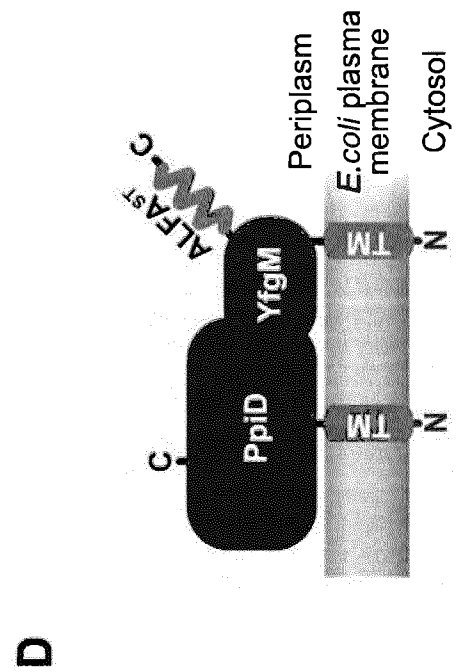


Fig. 10

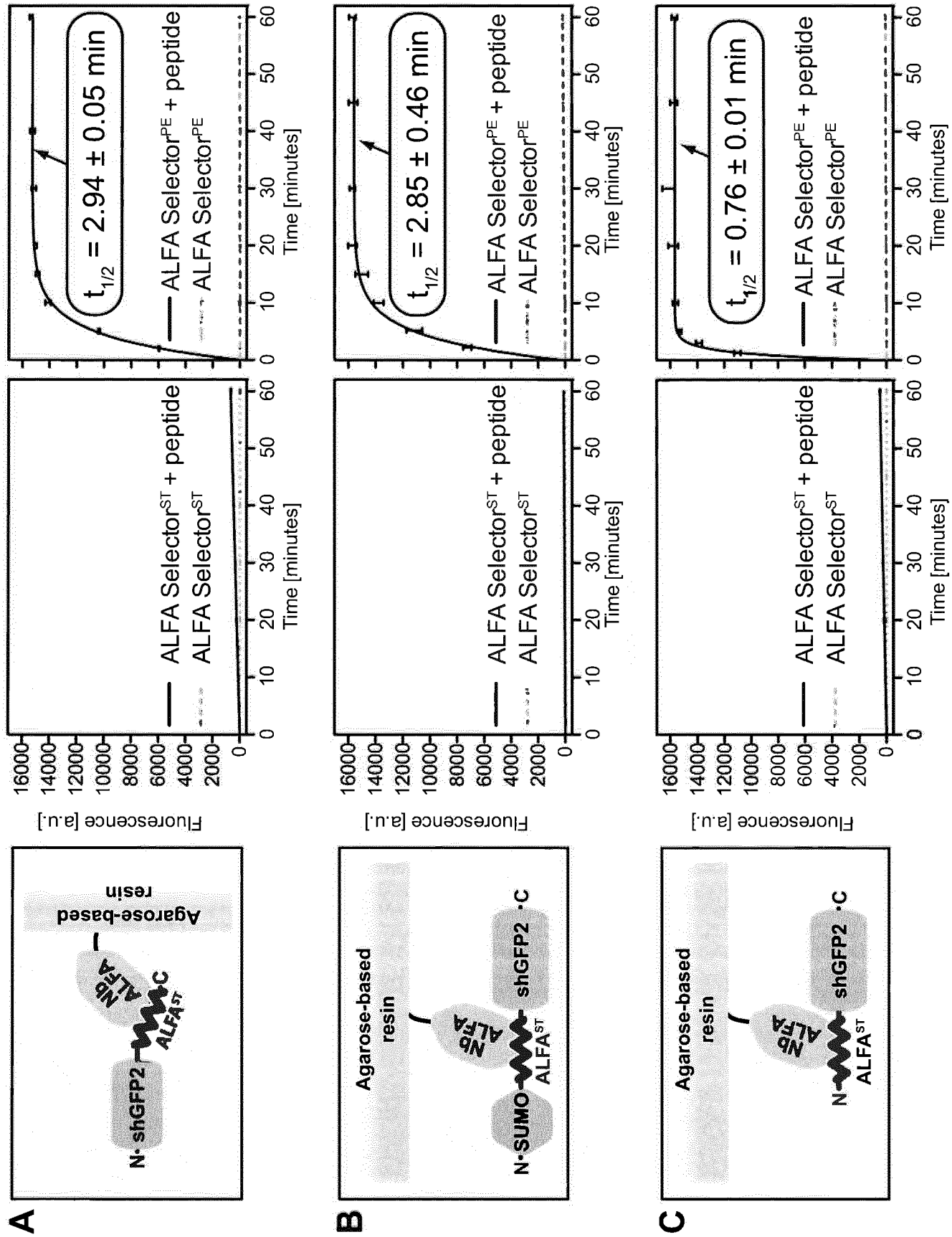

A
B
C
Agarose-based resin
N· shGFP2
ALFA^NB
ALFA^ST ·C
N·SUMO
shGFP2 ·C
N
Fluorescence [a.u.]
Time [minutes]
ALFA Selector^ST + peptide
ALFA Selector^ST
ALFA Selector^PE + peptide
ALFA Selector^PE
t1/2 = 2.94 ± 0.05 min
t1/2 = 2.85 ± 0.46 min
t1/2 = 0.76 ± 0.01 min

Fig.11

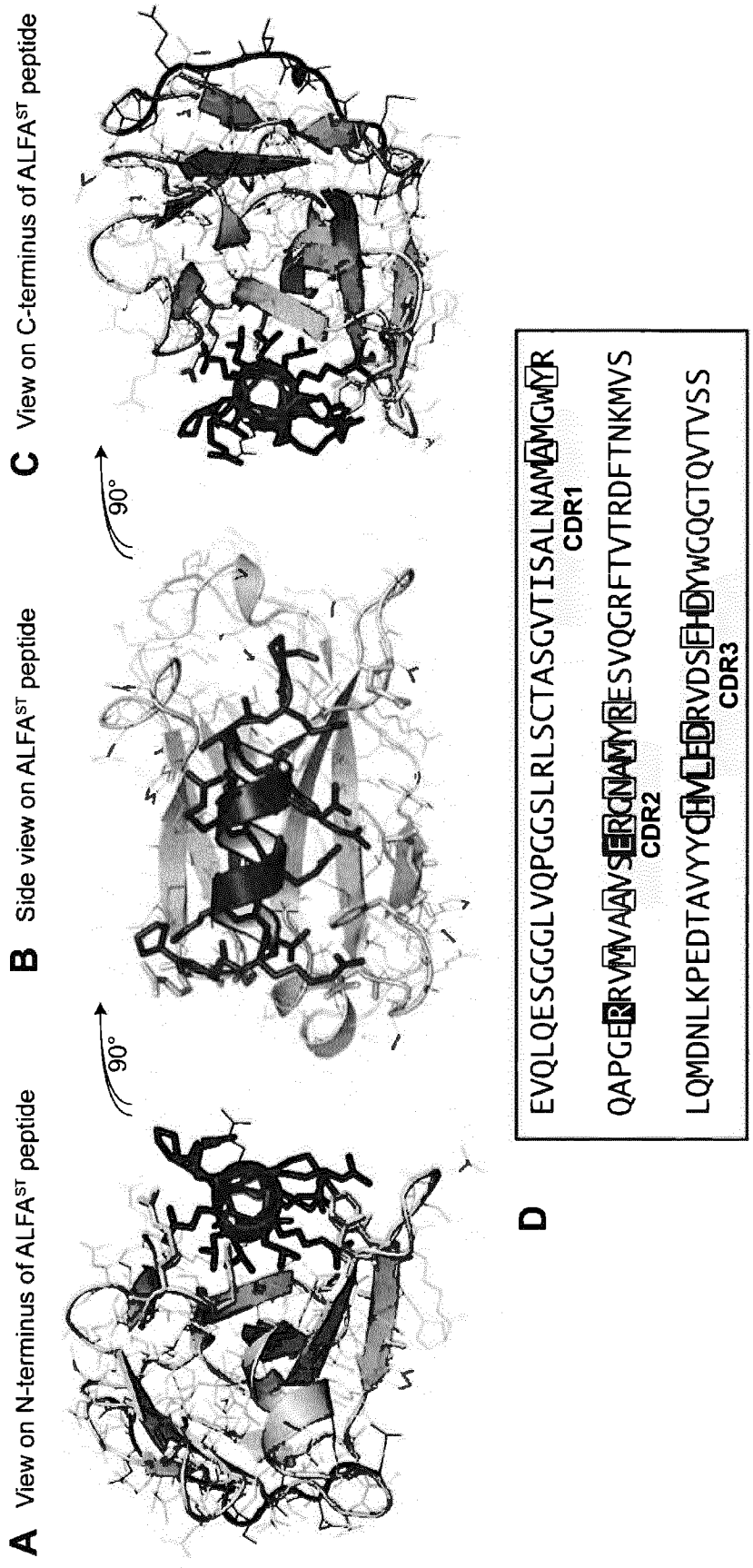
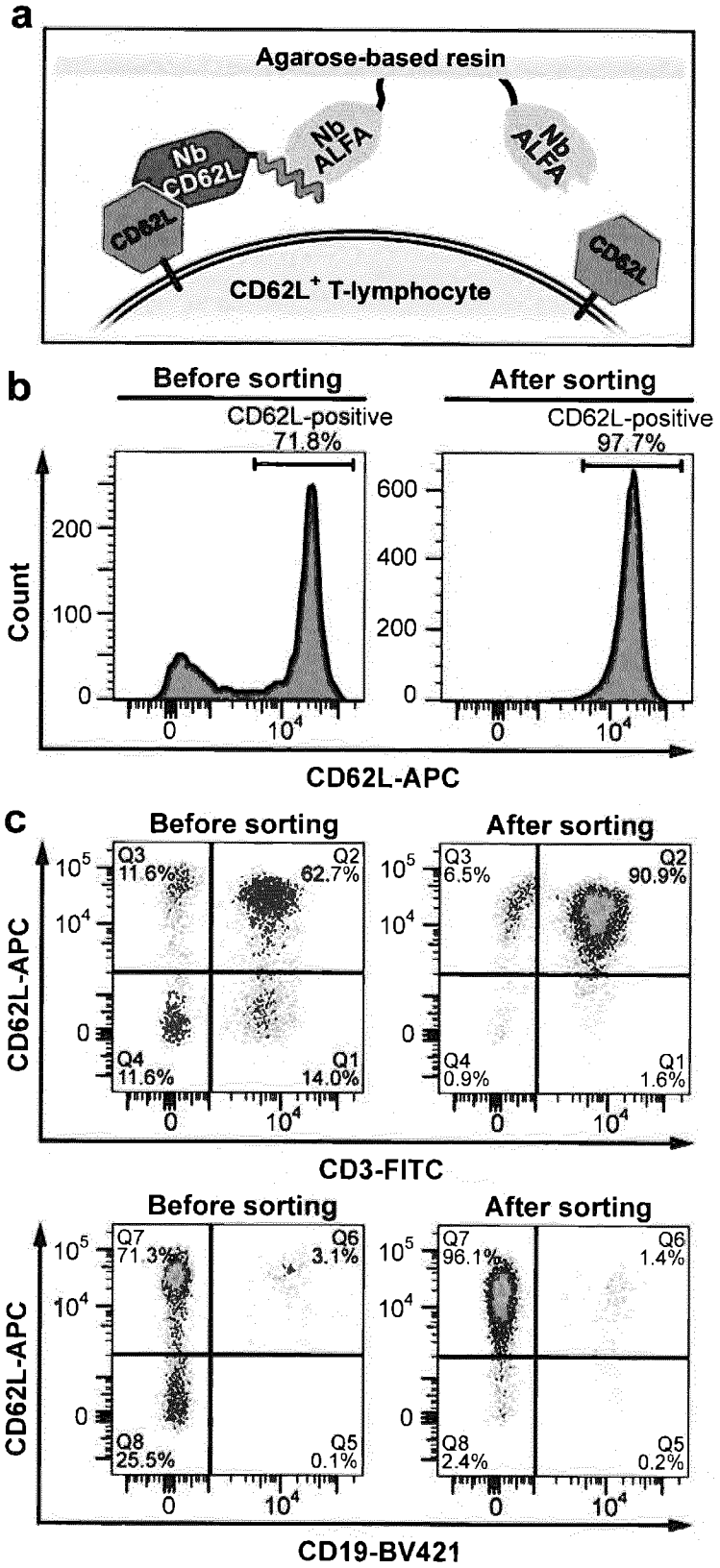

A View on N-terminus of ALFA^ST peptide
B Side view on ALFA^ST peptide
C View on C-terminus of ALFA^ST peptide
90°
90°
D
EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAWYR
CDR1
QAPGERRVMVAAVSSERGNAMYRESVQGRFTVTRDFTNKMVS
CDR2
LQMDNLKPEDTAVYYCHMLEDRVDSFHDYWGQGTQVTVSS
CDR3

Fig. 12


a
Agarose-based resin
Nb CD62L
Nb ALFA
Nb ALFA
CD62L
CD62L
CD62L+ T-lymphocyte
b
Before sorting
After sorting
CD62L-positive
71.8%
CD62L-positive
97.7%
Count
CD62L-APC
c
Before sorting
After sorting
Q3 11.6%
Q2 62.7%
Q4 11.6%
Q1 14.0%
Q3 6.5%
Q2 90.9%
Q4 0.9%
Q1 1.6%
CD62L-APC
CD3-FITC
Before sorting
After sorting
Q7 71.3%
Q6 3.1%
Q8 25.5%
Q5 0.1%
Q7 96.1%
Q6 1.4%
Q8 2.4%
Q5 0.2%
CD62L-APC
CD19-BV421

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/074153

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/04 C07K14/435 C07K14/705
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/04678 A1 (CASTERMAN CECILE [BE]; HAMERS RAYMOND [BE]) 3 March 1994 (1994-03-03) cited in the application claims 1-20	45-48
X	PETUKHOV MICHAEL; TATSU YOSHIRO; TAMAKI KAZUYO; MURASE SACHIKO; UEKAWA HIROKO; YOSHIKAWA SUSUMU; SERRANO LUIS; YUMOTO NOBORU: "Design of stable alpha-helices using global sequence optimization.", JOURNAL OF PEPTIDE SCIENCE : AN OFFICIAL PUBLICATION OF THE EUROPEAN PEPTIDE SOCIETY, vol. 15, no. 5, 1 May 2009 (2009-05-01), pages 359-365, XP002795930, cited in the application the whole document	1-44, 49-99

-/--

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

[X] See patent family annex.

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2019	16/12/2019

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

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/074153

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MICHAEL B. BRAUN ET AL: "Peptides in headlock - a novel high-affinity and versatile peptide-binding nanobody for proteomics and microscopy", SCIENTIFIC REPORTS, vol. 6, 21 January 2016 (2016-01-21), page 19211, XP055267206, DOI: 10.1038/srep19211 the whole document	1-44, 49-99
T	H. GÖTZKE ET AL.: "The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications", NATURE COMMUNICATIONS, vol. 10, 27 September 2019 (2019-09-27), pages 1-12, XP002795929, the whole document	1-99

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/074153

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9404678 A1	03-03-1994	AT 203767 T	15-08-2001
		AT 420178 T	15-01-2009
		AT 427968 T	15-04-2009
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		DK 1498427 T3	26-04-2010
		DK 1589107 T3	26-04-2010
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		EP 1087013 A2	28-03-2001
		EP 1498427 A1	19-01-2005
		EP 1589107 A1	26-10-2005
		EP 1621554 A1	01-02-2006
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		ES 2162823 T3	16-01-2002
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		ES 2325541 T3	08-09-2009
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		FI 950782 A	20-04-1995
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		JP 3444885 B2	08-09-2003
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		JP 4414900 B2	10-02-2010
		JP H08500487 A	23-01-1996
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		JP 2005170953 A	30-06-2005
		JP 2009280608 A	03-12-2009
		PT 656946 E	28-12-2001
		PT 1087013 E	15-04-2009
		PT 1498427 E	22-03-2010
		PT 1589107 E	29-03-2010
		PT 1621554 E	13-07-2009
		US 5759808 A	02-06-1998
		US 5800988 A	01-09-1998
		US 5840526 A	24-11-1998
		US 5874541 A	23-02-1999
		US 6015695 A	18-01-2000
		WO 9404678 A1	03-03-1994