Supplementary data for

Covalently Engineered Nanobody Chimeras for Targeted Membrane Protein Degradation

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- **Materials and methods**
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- **Reference**

Cell culture

The human cancer MDA-MB-231, HCC827, H460, U87 and HeLa cells were purchased from Procell Life Science&Technology Co., Ltd.. All cell lines were tested and verified to be free of mycoplasma. HCC827 and H460 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Invitrogen). MDA-MB-231, U87 and HeLa cells were maintained in DMEM medium (Gibco) supplemented with 10% FBS (Invitrogen), and all of them were cultured in a humidified incubator at 37 °C under 5% CO_2. MDA-MB-231-PD-L1-GFP (PD-L1/MDA) cells stably expressing PD-L1-GFP were constructed by infecting MDA cells with lentivirus containing the full-length human PD-L1-GFP gene, and sorted on the basis of GFP fluorescence.

Antibodies and reagents

The primary antibodies for PD-L1 (Cell Signaling Technologies, Cat# 13684), GAPDH (Abcam, Cat# ab128915), His tag (Cell Signaling Technologies, Cat# 12698S), alpha-Tubulin (Cell Signaling Technologies, Cat# 2125S) and EGFR (Abcam, Cat# ab52894) were commercially available. Secondary antibodies for Western blots were: goat anti-rabbit IgG, HRP-linked (Cell Signaling Technologies, Cat# 7074S). Recombinant proteins such as PD-L1 (Cat# 10084-HNAH) and EGFR (Cat# 10001-H08H) were purchased from Sino Biological Inc.. Synthetic peptides were purchased from Hangzhou Allpeptide Co., Ltd.. Uncanonical amino acids FSY and BrC6K were synthesized according to the previous literature[1,2]. Small molecular compounds such as MTSET (Biorigin, Cat# BN15003), LysoTracker™ Deep Red (ThermoFisher, Cat# L12492), NH_4Cl (Acros Organics, Cat# 199975000), TAMRA-PEG_3-maleimide (TMR-PEG_3-Mal, Confluo, Cat# BDR-15) were also purchased from the indicated suppliers.

Western blotting

40 μg of protein samples were loaded in 4-20% or 8-16% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE) for electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). After protein transfer, the membrane was blocked with 5% bovine serum albumin in TBST buffer (20 mM Tris, pH 8.0,150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature with gentle shaking. The membranes were respectively incubated with primary antibodies (PD-L1 antibody, rabbit, 1:500; GAPDH antibody, rabbit, 1:1,000; His tag antibody, Rabbit, 1:1,000; Tubulin antibody, Rabbit, 1:1,000; EGFR antibody, Rabbit, 1:1,000) overnight at 4 °C. After incubation with the primary antibody, the membrane was washed three times (each for 5 min) with TBST buffer. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:5,000 dilution) for 1 h at room temperature. Finally, the membrane was washed three times (each for 5 min) with TBST buffer. The western blot bands were detected by using an electro-chemiluminescence (ECL) western blotting substrate (Millipore, Cat# WBKLS0500).

Plasmids construction

The expression plasmid pET20b-Nb-PD-L1-His or pET20b-Nb-EGFR-His was constructed by inserting synthesized DNA encoding Nb-PD-L1/ Nb-EGFR and His tag (protein sequence shown below) into the pET20b vector, which contains a pelB signal sequence, using the NdeI/NotI multiple cloning sites (MCS). The plasmid pBAD-Nb-PD-L1-His and pBAD-Nb-EGFR-His were constructed by a similar way.

The plasmids pBAD-Nb-PD-L1-His(TAGs) for UAA incorporation were generated by introducing the TAG codon at different sites and the corresponding primers were used to perform site-directed mutagenesis on plasmid pBAD-Nb-PD-L1-His.

The plasmid pSupAR-chFSYRS encoding chimeric pyrrolysyl-tRNA synthetase (PylRS) for FSY incorporation was constructed by combining MbPylRS(1-149) with four beneficial mutants (IPYE)[3] and the previously reported MmFSYRS(185-454)[1]. The plasmid for BrC6K incorporation (pSupAR-BrC6KRS) was previously reported to encode the PylRS for DiZASeC incorporation[4].

Sequence of Nb-PD-L1 (WO2018/133873A1):

MA QVQLQESGGG LVQPGGSLRL SCAASGKMSS RRCMAWFRQA PGKERERVAK LLTTSGSTYL ADSVKGRFTI SQNNAKSTVY LQMNSLKPED TAMYYCAADS F***EDPT***C***TLVT SSGAFQY***WGQ GTQVTVSS GGGGSGGGGSLPETGGHHHHHH

The ***italic and bold residues*** were used for the incorporation of FSY while the underlined residues were used for the incorporation of BrC6K.

Sequence of $\alpha EGFR_{VHH}$[5]:

MD QVKLEESGGG SVQTGGSLRL TCAASGRTSR SYGMGWFRQA PGKEREFVSG ISWRGDSTGY ADSVKGRFTI SRDNAKNTVD LQMNSLKPED TAIYYCAAAA GSAWYGTLYE YDYWGQGTQV TVSS AGQGTSGLPETGGHHHHHH

Expression and purification of Nb-PD-L1

Plasmid pET20b-Nb-PD-L1-His was transformed into *E. coli* Shuffle T7-B competent cells. For expression of Nb-PD-L1-His, transformed bacteria were cultured at 37℃ in 2×YT media with 100 μg/mL ampicillin and induced with 1 mM IPTG at 30℃ when OD_{600} reached 0.6-0.8. After induction of expression at 30℃ for 12 h, bacteria were collected by centrifugation at 4500 rpm for 20 min at 4℃. The cell pellets were re-suspended in 10 mL lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl) per gram of bacteria wet weight. The cell suspension was lysed in an ice-water bath for 20 min by sonication. The cell lysate was centrifuged at 20,000 g for 30 min at 4℃ and the supernatant was loaded in a Ni-NTA column (HisTrap HP, 5mL, GE Healthcare) and washed with wash buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 50 mM imidazole) in 10 column volume. The protein was eluted with elution buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 250 mM imidazole). The eluted protein solution was concentrated by ultrafiltration centrifugation (Millipore, Cat# UFC900324, MWCO = 3 kDa) and then further

purified by size exclusion chromatography with Superdex 75 10/300 GL (GE Healthcare) equilibrated with PBS.

Mass spectrometric analysis of intact proteins

Wild type and UAA-incorporated intact proteins were analyzed by a Waters ACQUITY UPLC I-Class SQD 2 MS spectrometer with electrospray ionization (ESI). LC separation for sortase-mediated protein conjugation was carried out with a BEH300 C4 Acquity column (1.7 μm, 2.1 × 100 mm) and positive mode was chosen for ESI-MS to analyze all samples. The total mass of proteins was calculated using MassLynx V4.1 software (Waters).

Mass-based screening platform (MSSP) for identification of Gluebodies

1. Constructing a pool of Nb-PD-L1(PrUAA)

Plasmids pBAD-Nb-PD-L1-His(TAGs) were separately co-transform with plasmid pSupAR-chFSYRS or pSupAR-BrC6KRS into DH10B competent cells. For incorporation of proximal reactive uncanonical amino acids (PrUAA, FSY or BrC6K) into proteins, transformed bacteria were mixed together and cultured at 37℃ in 2×YT media with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The mixed bacteria were incubated with 1mM FSY when OD_{600} reached 0.6 and induced with 0.2% arabinose when OD_{600} reached 1.0. After induction of expression at 30℃ for 12 h, bacteria were harvested and lysed to prepare the library of Nb-PD-L1(PrUAA) proteins following the purification steps of Nb-PD-L1 described above. Protein concentration was measured by NanoDrop 2000 (ThermoFisher) based on its molecular weight and extinction coefficient.

2. *In vitro* crosslinking of Gluebodies with PD-L1

The purified pool of Nb-PD-L1(PrUAA) was incubated with PD-L1 at the molar ratio of 10:1 in PBS buffer at 37℃ for 24 h or 72 h. The amount of PD-L1 was 2 μg. 5× reduced loading buffer (CWBio, Cat# CW0027) was added into the tube and heated at 95℃ for 15 min. These samples were then separated by 8-16% SDS-PAGE gel followed by staining with coomassie brilliant blue.

3. Deconvolution of "Glue" sites for PrUAA incorporation

The Gluebody-PD-L1/PD-L1 crosslinking proteins were excised from the SDS-PAGE gel according to the mass shift. The desired bands were cut into pieces and discolored in discoloring buffer (50% acetonitrile, 50% 100mM ammonium bicarbonate) until they all turned transparent. After dehydrated in pure acetonitrile at 37℃, the gel pieces were then incubated in reduction buffer (10 mM DTT, 100 mM ammonium bicarbonate) at 56 °C for 30 min and further incubated in alkylation buffer (55 mM iodoacetamide, 100 mM ammonium bicarbonate) at room temperature for 30 min in the dark. Then the gel pieces were dehydrated in pure acetonitrile twice and then digestion buffer (20 ng/μL trypsin, 20 ng/μL chymotrypsin, 100 mM ammonium bicarbonate, 1mM calcium chloride) was added. The gel pieces were

incubated at 25 °C for 12 h and then further digested at 37°C for 6 h. The resulting peptides were extracted three times with extraction buffer (50% acetonitrile, 45% water, 5% formic acid) and then concentrated to dryness with vacuum. The samples were desalted by Pierce™ C18 tips (ThermoFisher, Cat# 87784) and dried with vacuum centrifugation. All samples were resuspended in 0.1% formic acid in water and analyzed on a Thermo Scientific Q Exactive Orbitrap mass spectrometer in conjunction with an Easy-nLC II HPLC (ThermoFisher). 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile-20% water as mobile phase B. The MS/MS analysis was performed under the positive-ion mode with a full-scan m/z range from 350 to 1,800 and a mass resolution of 70,000. Peptides were eluted using a 70 min gradient (0 min 3% B; 10 min 10% B; 58 min 37% B; 59 min 100% B; 64 min 100% B; 65 min 3% B and 70min 3% B) with a flow rate of 300 nL/min. MS/MS fragmentation was performed in a data-dependent mode, of which TOP 20 most intense ions are selected for MS2 analysis with a resolution of 17,500 using HCD (high-energy collision dissociation). Other important parameters: isolation window, 2.0 m/z units; default charge, +2, charge exclusion, unassigned, 8, >8 ; normalized collision energy, 28%; maximum IT, 50 ms; dynamic exclusion, 20.0 s. Mass spectrometry raw data was searched by pLink to identify the proper "Glue" sites for PrUAA incorporation and the corresponding reactive residues of PD-L1.

Cell labeling and confocal imaging

The TAMRA-labeled Nb-PD-L1 or Gluebody-PD-L1 were separately added to PD-L1/MDA cells (~70% confluent) at the concentration of 100 nM. Cells were cultured at 37 ℃ and 5% CO_2 in the dark for 4 h, after which the cells were washed with weak wash buffer (Hank's Balanced Salt Solution, pH 7.4, room temperature, 10 min, three times) or stringent wash buffer (500 mM NaCl, 3% tween 20, 100 mM glycine, pH 3.0, room temperature, 10 min, three times). The TAMRA and GFP fluorescence was visualized at room temperature on Zeiss LSM 710 Confocal Laser Scanning Microscope using a 20x objective. The images from each filter were combined to produce an overlay image.

Crosslinking of Gluebody-PD-L1 with PD-L1 on cells

PD-L1/MDA cells (1×10^5) were seeded in 24-well plates and cultured with DMEM (+10% FBS and 1:100 penicillin-streptomycin). After 12 h, Nb-PD-L1 or Gluebody-PD-L1 (Nb-PD-L1-L108FSY) was added into culture media at a final concentration of 100 nM. After incubation at 37℃ for 12 h, the culture media was removed and the cells were lysed on ice by adding cold 60 μL RIPA buffer (ThermoFisher, Cat# 89901) with 1× protease inhibitor cocktail (Cell Signaling Technology, Cat# 5872). Protein concentration was quantified using the BCA protein assay kit (ThermoFisher, Cat# 23222). The samples were then heated at 95℃ for 20 min after adding 5× reduced loading buffer (CWBio, Cat# CW0027). The denatured samples were analyzed by electrophoresis in 4-20% SDS-PAGE followed by western blot.

Crosslinking of Gluebody-PD-L1 with PD-L1 in tumor

All animal studies were approved by the Institutional Animal Care and Use Committee of Peking University. PD-L1/MDA cells (2×10^6) were resuspended with 200 μL HBSS (Hank's Balanced Salt Solution) and respectively injected into the flank of 4-6 week old female NOG mice (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/JicCrl, Beijing Charles River Laboratory Animal Technology Co. Ltd., China, Cat# 408). After 10 days, tumor size was about 100 mm^3. 20 μg or 100 μg Nb-PD-L1 or Gluebody-PD-L1 was injected into the skin site near the tumor. After 4 h, mice were sacrificed and tumors were harvested. The tumors were added with 10 μL RIPA buffer (ThermoFisher, Cat# 89901) with 1× protease inhibitor cocktail (Cell Signaling Technology, Cat# 5872) per milligram of tumor wet weight. Then the tumors were split by a homogenizer and lysed by ultrasonication. Western blot was then performed using the same procedures as described in the section above.

Sortase-mediated conjugation

Recombinant mgSrtA was expressed and purified as described in the literature[6]. The conjugation reactions between proteins having a C-terminal LPETGG-His_6 sequence and substrate peptides having a N-terminal GGG(C) sequence were conducted by incubating 10-100 μM proteins (1 eq), 0.1-1 mM substrate peptides (10 eq) and 1-10 μM mgSrtA (0.1 eq) in reaction buffer (1mM $CaCl_2$ in PBS, pH 7.4) for 30 min at room temperature. Terminator MTSET (2 mM) was added into the reaction mixture for 5 min at room temperature followed by desalting with Micro Bio-Spin P-6 Gel Columns (Bio-rad, Cat# 7326221). The unreacted proteins and mgSrtA were removed from the desired products by adding magnetic Ni-NTA beads (Promega, Cat# V8565).

Cell surface degradation experiment

Adherent cells (70% confluency for 12 h experiments, 50% confluency for 24h experiments) were treated with HBSS or 100 nM of Nb-PD-L1, NbTAC-PD-L1, GlueTAC-PD-L1 in complete growth medium. At the indicated time, the growth medium was removed and the cells were lysed on ice by adding cold 60 μL RIPA buffer (ThermoFisher, Cat# 89901) with 1× protease inhibitor cocktail (Cell Signaling Technology, Cat# 5872). Protein concentration was quantified using the BCA protein assay kit (ThermoFisher, Cat# 23222). The samples were then heated at 95℃ for 20 min after adding 5× reduced loading buffer (CWBio, Cat# CW0027). The denatured samples were analyzed by electrophoresis in 4-20% SDS-PAGE followed by western blot.

Tumor tissue imaging

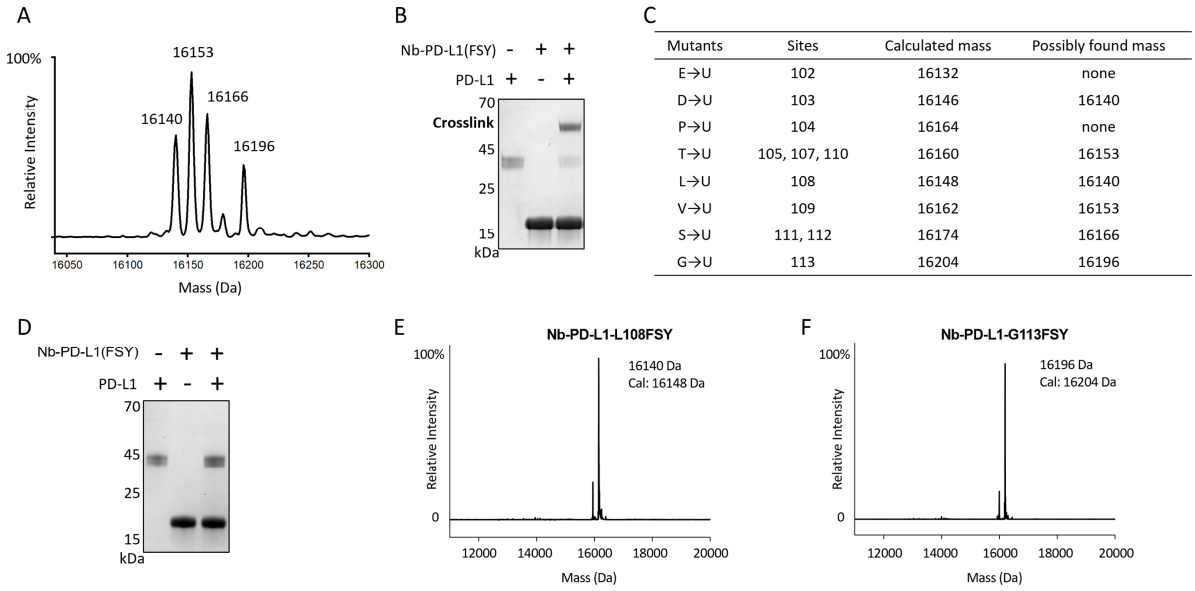
PD-L1/MDA cells (1×10^5) in 50% matrigel (100 μL) was implanted subcutaneously in the flank of 4-6 week old female NOG mice (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/JicCrl, Beijing Vital River Laboratory Animal Technology Co. Ltd., China, Cat# 408). At day 10, mice were assigned randomly to two groups (n= 3 mice per group) and were given 2 mg/kg of Nb-PD-L1-TAMRA or Gluebody-TAMRA (200 μL) with intravenous injection. After 4 h, tumors

were dissected and frozen in -80 ℃ immediately. Frozen slicing were conducted by Beijing Biosource Co., Ltd. and the GFP and TAMRA fluorescence was scanned at room temperature on Zeiss LSM 710 Confocal Laser Scanning Microscope using a 10x objective. The images from each filter were combined to produce an overlay image.

PD-L1 degradation experiment on tumor xenograft NOG-mice

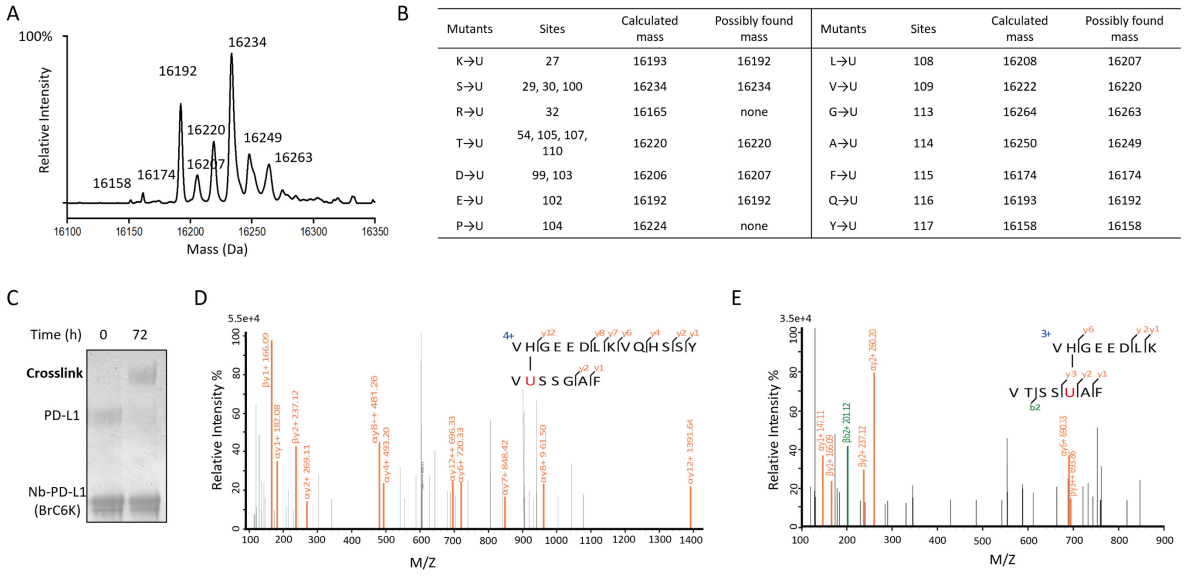
PD-L1/MDA cells (2×10^6) were resuspended with 200 μL HBSS and respectively injected into the flank of 4-6 week old female NOG mice (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/JicCrl, Beijing Vital River Laboratory Animal Technology Co. Ltd., China, Cat# 408). After 10 days, tumor size was about 60 mm^3. 100 μg Nb-PD-L1, NbTAC-PD-L1 or GlueTAC-PD-L1 was peri-tumoral injected. After 18 h, mice were sacrificed and tumors were harvested. The tumors were added with 10 uL RIPA buffer (ThermoFisher, Cat# 89901) with 1× protease inhibitor cocktail (Cell Signaling Technology, Cat# 5872) per milligram of tumor wet weight. Then the tumors were split by a homogenizer and lysed by ultrasonication. Western blot was then performed using the same procedures as described in the section above.

Supplementary Figures



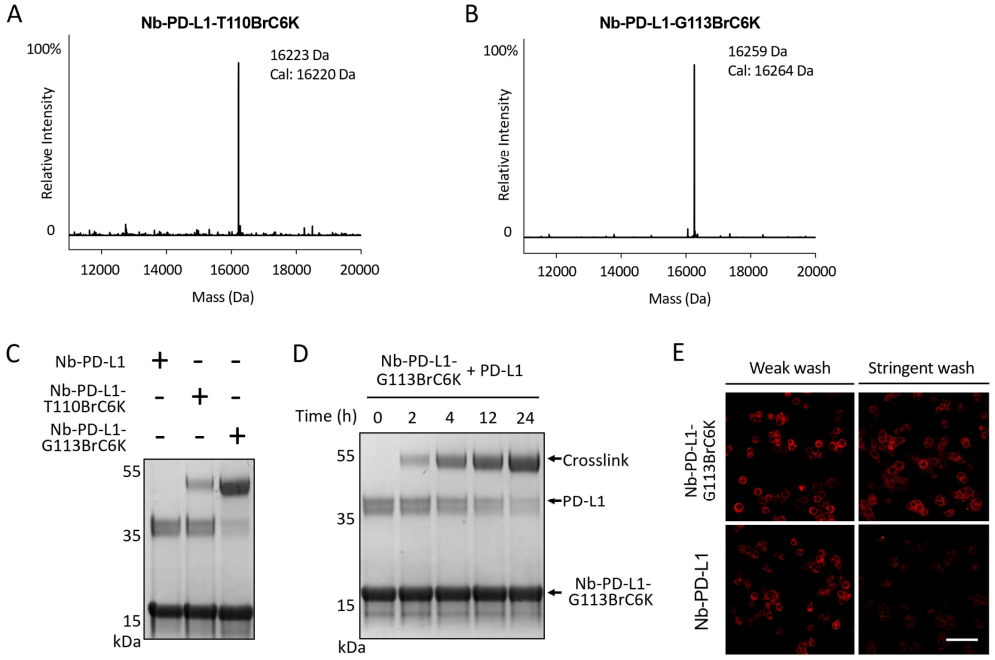
Mutants	Sites	Calculated mass	Possibly found mass
E→U	102	16132	none
D→U	103	16146	16140
P→U	104	16164	none
T→U	105, 107, 110	16160	16153
L→U	108	16148	16140
V→U	109	16162	16153
S→U	111, 112	16174	16166
G→U	113	16204	16196

Supplementary Fig. 1. Gluebody identified from the pool of covalent nanobody variants covalently binds to PD-L1 *in vitro*. (A) A pool of covalent nanobody variants were generated from the mixed bacteria expressing Nb-PD-L1 with FSY incorporation at different sites in CDR3 region. Four deconvoluted mass of mixed Nb-PD-L1(FSY) proteins might be contributed to nine possible FSY-incorporated sites of Nb-PD-L1 (details shown in right table). (B) Verification of the covalent binding of Nb-PD-L1(FSY) to PD-L1 by SDS-PAGE. The pool of Nb-PD-L1(FSY) proteins was incubated with PD-L1 in PBS buffer at 37 °C for 5 h before SDS-PAGE and coomassie staining analysis. Covalent complex of Nb-PD-L1(FSY)/ PD-L1 was indicated in the figure. (C) Mass of Nb-PD-L1(FSY) proteins. Eleven sites (E102-T105, T107-G113) were mutated to TAG for FSY incorporation. (D) Verification of the covalent binding of Nb-PD-L1-L108FSY to PD-L1 by SDS-PAGE. Nb-PD-L1-L108FSY was solely excluded from the pool of Nb-PD-L1(FSY) proteins when incubated with PD-L1 in PBS buffer at 37 °C for 5 h before SDS-PAGE and coomassie staining analysis. Covalent complex of Nb-PD-L1(FSY)/PD-L1 was not observed. (E) The Gluebody-PD-L1 (Nb-PD-L1-L108FSY) and (F) Nb-PD-L1-G113FSY were separately purified and characterized before verification of the covalent binding to PD-L1.

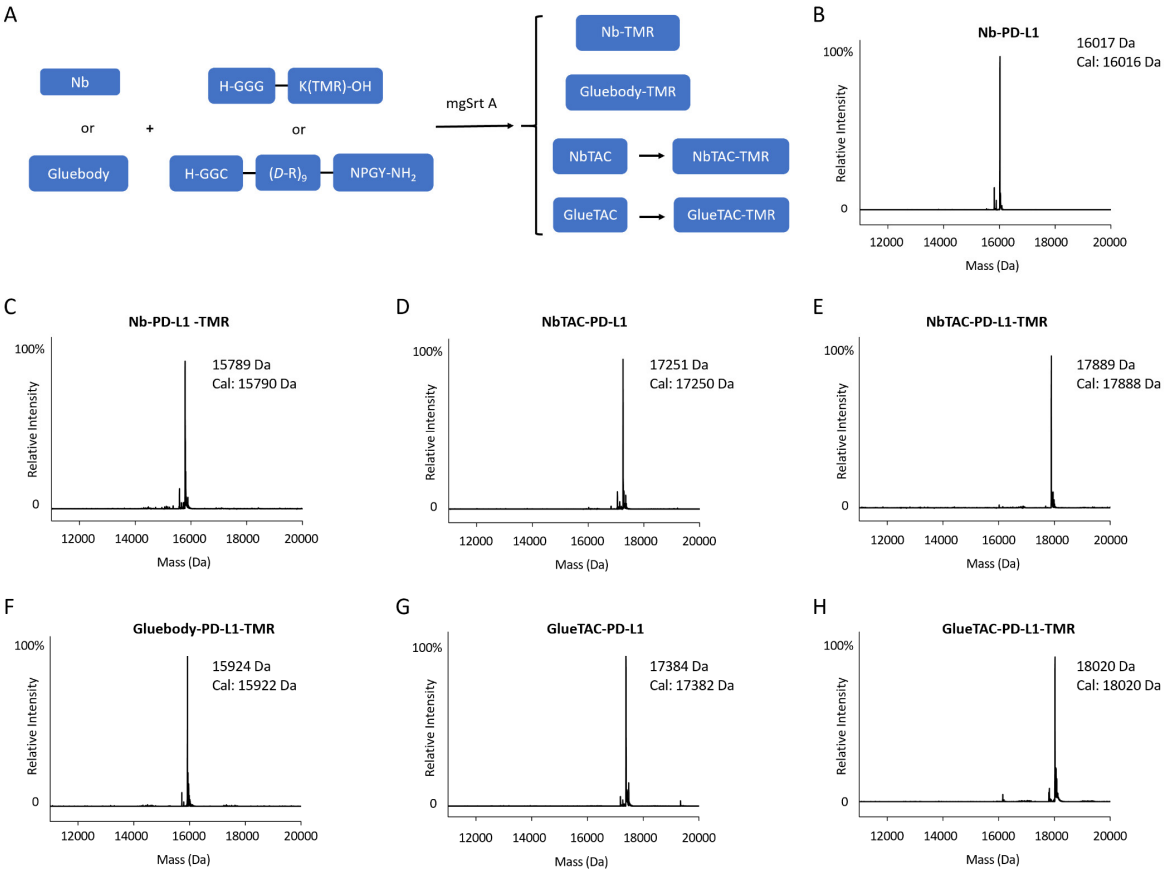


Mutants	Sites	Calculated mass	Possibly found mass	Mutants	Sites	Calculated mass	Possibly found mass
K→U	27	16193	16192	L→U	108	16208	16207
S→U	29, 30, 100	16234	16234	V→U	109	16222	16220
R→U	32	16165	none	G→U	113	16264	16263
T→U	54, 105, 107, 110	16220	16220	A→U	114	16250	16249
D→U	99, 103	16206	16207	F→U	115	16174	16174
E→U	102	16192	16192	Q→U	116	16193	16192
P→U	104	16224	none	Y→U	117	16158	16158

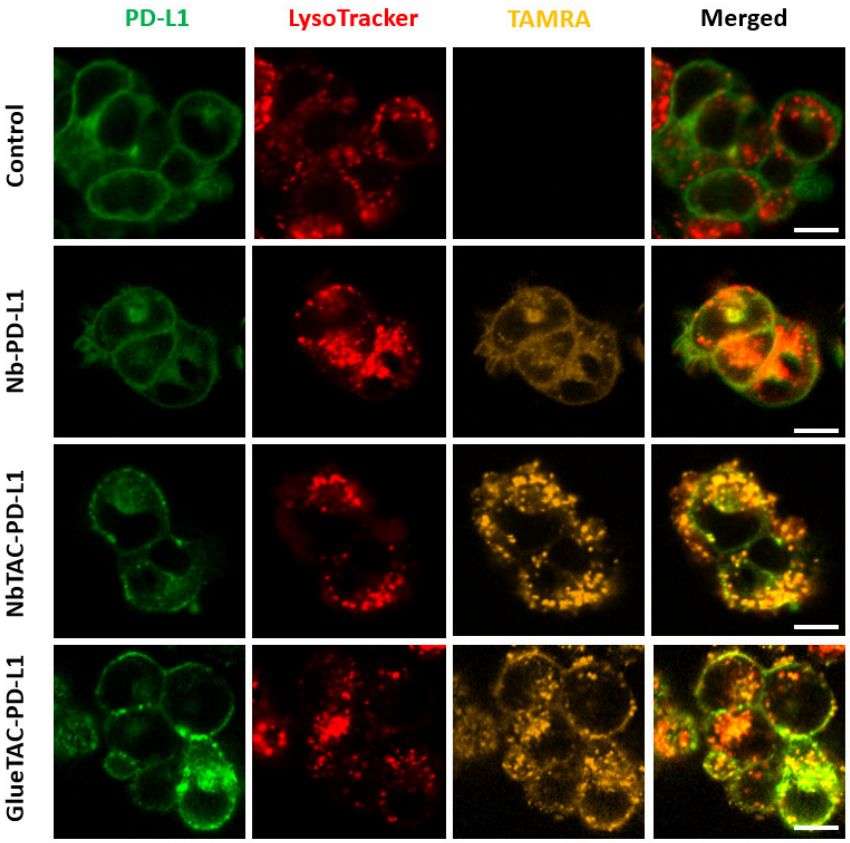
Supplementary Fig. 2. BrC6K is an alternative PrUAA served to be incorporated in Nb-PD-L1 for covalent binding to PD-L1 *in vitro*. (A) A pool of covalent nanobody variants were generated from the mixed bacteria expressing Nb-PD-L1 with BrC6K incorporation at different sites around the Nb-PD-L1/PD-L1 interface. Eight deconvoluted mass of mixed Nb-PD-L1(BrC6K) proteins might be contributed to nineteen possible BrC6K-incorporated sites of Nb-PD-L1 (details shown in right table). (B) Mass of Nb-PD-L1(BrC6K) proteins. Twenty one sites were mutated to TAG for BrC6K incorporation. (C) Verification of the covalent binding of Nb-PD-L1(BrC6K) to PD-L1 by SDS-PAGE. The pool of Nb-PD-L1(BrC6K) proteins was incubated with PD-L1 in PBS buffer at 37 °C for 72 h before SDS-PAGE and coomassie staining analysis. Covalent complex of Nb-PD-L1(BrC6K)/PD-L1 was indicated in the figure. The crosslinked Nb-PD-L1(BrC6K)/PD-L1 complex were excised from the SDS-PAGE gel and cut into pieces, then subjected to in-gel digestion for identification of desired incorporation sites of BrC6K. (D) Tandem mass spectrum of crosslinked Nb-PD-L1(BrC6K)/PD-L1 complex indicated that Nb-PD-L1-T110BrC6K and (E) Nb-PD-L1-G113BrC6K reacted with His69 on PD-L1.



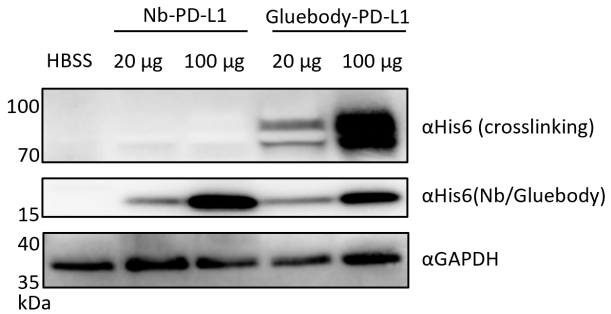
Supplementary Fig. 3. Gluebodies(BrC6K) can covalently bind to PD-L1. (A) The Nb-PD-L1-T110BrC6K and (B) Nb-PD-L1-G113BrC6K were separately purified and characterized before verification of the covalent binding to PD-L1. (C) Verification and comparison of the covalent binding of Nb-PD-L1-T110BrC6K and Nb-PD-L1-G113BrC6K to PD-L1 by SDS-PAGE. The crosslinking efficiency of Nb-PD-L1-G113BrC6K was better than Nb-PD-L1-T110BrC6K. Gluebody(BrC6K) Nb-PD-L1-T110BrC6K or Nb-PD-L1-G113BrC6K was incubated with PD-L1 in PBS buffer at 37 °C for 24 h before SDS-PAGE and coomassie staining analysis. Covalent complex of Nb-PD-L1(BrC6K)/PD-L1 was indicated in the figure. (D) Time-course study of the covalent binding between Nb-PD-L1-G113BrC6K and PD-L1 as verified by SDS-PAGE. (E) Irreversible binding of Nb-PD-L1-G113BrC6K to the endogenous PD-L1 on PD-L1/MDA cells was confirmed by fluorescence imaging. PD-L1/MDA cells were incubated with TAMRA labeled Nb-PD-L1-G113BrC6K or Nb-PD-L1 in medium at 37 °C for 24 h, after which the cells were washed with weak wash buffer or stringent wash buffer (denaturing condition). Scale bar = 50 μm. The cells washed with stringent wash buffer (denaturing condition) were harvested and then subjected to western blotting.



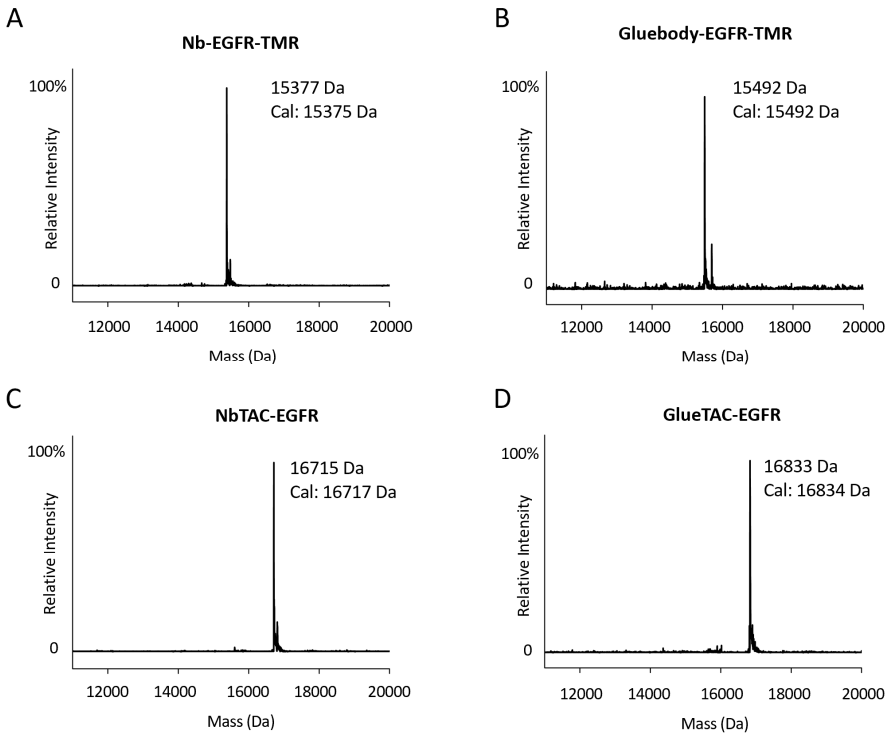
Supplementary Fig. 4. Construction of Nb-PD-L1-derived formats by sortase-mediated conjugation. (A) Schematic illustration showed the general procedure of sortase-mediated conjugation between Nb-PD-L1 or Gluebody-PD-L1 and functional peptides. TAMRA (TMR) modified peptides: H-GGGK(TMR)-OH; cell-penetrating peptide and lysosome-sorting sequence complex: H-GGC-(*D*-R)$_9$-NPGY-NH_2, the free Cys could be further modified by TMR-PEG_3-Mal. The conjugation protocol was described in the above method. (B) All of the Nb-PD-L1-derived formats were purified and characterized before imaging and degradation assays. The deconvoluted mass spectra of purified Nb-PD-L1, expected 16016 Da; observed 16017 Da; (C) Nb-PD-L1-TMR, expected 15790 Da; observed 15789 Da; (D) NbTAC-PD-L1, expected 17250 Da; observed 17251 Da; (E) NbTAC-PD-L1-TMR, expected 17888 Da; observed 17889 Da; (F) Gluebody-PD-L1-TMR, expected 15922 Da; observed 15924 Da; (G) GlueTAC-PD-L1, expected 17382 Da; observed 17384 Da; (H) GlueTAC-PD-L1-TMR, expected 18020 Da; observed 18020 Da.



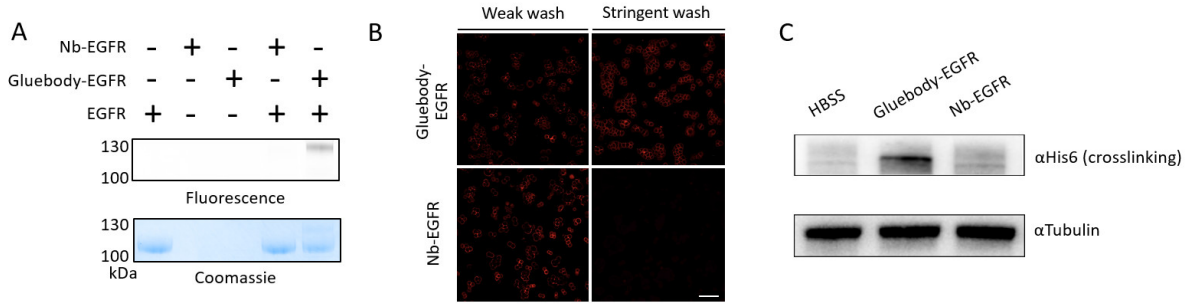
Supplementary Fig. 5. Live-cell confocal microscopy images of PD-L1-GFP/MDA cells treated by 100 nM Nb-PD-L1-TMR, NbTAC-PD-L1-TMR or GlueTAC-PD-L1-TMR for 4 h, then labelled with LysoTracker Deep Red for 30 min. The CPP-LSS peptide enhanced internalization and lysosomal trafficking of Nb-PD-L1/PD-L1 complex. Scale bar = 10 μm.



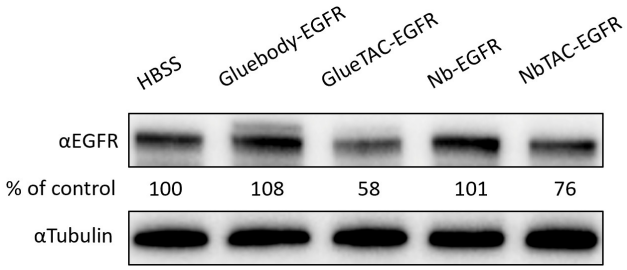
Supplementary Fig. 6. Western blotting analysis confirms the irreversible binding of Gluebody-PD-L1 to the endogenous PD-L1 on tumors engrafted in mice. Gluebody-PD-L1 and Nb-PD-L1 were both retained in tumor while only Gluebody covalently bound to PD-L1 *in vivo*.



Supplementary Fig. 7. Construction of Nb-EGFR-derived formats by sortase-mediated conjugation. (A) All of the Nb-EGFR-derived formats were purified and characterized before imaging and degradation assays. The deconvoluted mass spectra of purified Nb-EGFR-TMR, expected 15375 Da; observed 15377 Da; (B) Gluebody-EGFR-TMR, expected 15492 Da; observed 15492 Da; (C) NbTAC-EGFR, expected 16717 Da; observed 16715 Da; (D) GlueTAC-EGFR, expected 16834 Da; observed 16833 Da.



Supplementary Fig. 8. Gluebody-EGFR can covalently bind to EGFR. (A) Fluorescence-gel imaging and coomassie staining analysis confirm the irreversible binding of Gluebody-EGFR (Nb-EGFR-Q116FSY) to EGFR *in vitro*. TAMRA (TMR) labelled Nb-EGFR or Gluebody-EGFR was incubated with EGFR in PBS buffer at 37 °C for 12 h before SDS-PAGE analysis. The crosslinking band with TAMRA fluorescence was consistent with the covalent complex of Gluebody-EGFR-TMR/EGFR. (B) Irreversible binding of Gluebody-EGFR to the endogenous EGFR on HeLa cells was confirmed by fluorescence imaging and (C) western blotting analysis. HeLa cells were incubated with TAMRA labeled Nb-EGFR or Gluebody-EGFR in medium at 37 °C for 5 h, after which the cells were washed with weak wash buffer or stringent wash buffer (denaturing condition). Scale bar = 100 μm.



Supplementary Fig. 9. Western blotting analysis of EGFR levels in HeLa cells after the treatment of 100 nM Gluebody-EGFR, GlueTAC-EGFR, Nb-EGFR or NbTAC-EGFR in RPMI 1640 supplemented with 10% FBS at 37°C for 18 h.

Reference

1. Wang, N. *et al.* Genetically Encoding Fluorosulfate-l-tyrosine To React with Lysine, Histidine, and Tyrosine via SuFEx in Proteins in Vivo. *J. Am. Chem. Soc.* **140**, 4995-4999 (2018).
2. Chen, X. H. *et al.* Genetically encoding an electrophilic amino acid for protein stapling and covalent binding to native receptors. *ACS Chem. Biol.* **9**, 1956-1961 (2014).
3. Bryson, D. I. *et al.* Continuous directed evolution of aminoacyl-tRNA synthetases. *Nat. Chem. Biol.* **13**, 1253-1260 (2017).
4. He, D. *et al.* Quantitative and Comparative Profiling of Protease Substrates through a Genetically Encoded Multifunctional Photocrosslinker. *Angew. Chem. Int. Ed. Engl.* **56**, 14521-14525 (2017).
5. Schmitz, K. R., Bagchi, A., Roovers, R. C., van Bergen en Henegouwen, P. M. & Ferguson, K. M. Structural evaluation of EGFR inhibition mechanisms for nanobodies/VHH domains. *Structure* **21**, 1214-1224 (2013).
6. Ge, Y. *et al.* Enzyme-Mediated Intercellular Proximity Labeling for Detecting Cell-Cell Interactions. *J. Am. Chem. Soc.* **141**, 1833-1837 (2019).