

# Covalently Engineered Nanobody Chimeras for Targeted Membrane Protein Degradation

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## Article

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1 **Covalently Engineered Nanobody Chimeras for Targeted Membrane Protein**  
2 **Degradation**

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12 **Abstract:**

13 Targeted degradation of membrane proteins would afford an attractive and general strategy  
14 for treating various diseases that remain difficult with the current PROTAC methodology. We  
15 herein report a covalent nanobody-based PROTAC strategy, termed GlueTAC, for targeted  
16 membrane protein degradation with high specificity and efficiency. We first designed a MS-based  
17 screening platform for rapid development of covalent nanobody (Gluebody) that allowed  
18 proximity-enabled ligation with surface antigens on cancer cells. By conjugation with the cell-  
19 penetrating peptide and lysosomal-sorting sequence, the resulting GlueTAC chimera exhibited  
20 enhanced internalization with high efficacy and sustained eradication of tumor surface antigens  
21 such as PD-L1 and EGFR both *in vitro* and *in vivo*, which has broad applications in biomedical  
22 research and therapeutics.

23

24 **Main Text:**

25 Proteolysis targeting chimeras (PROTACs) have rapidly emerged as a powerful strategy for  
26 cancer treatment by degradation of oncogenic proteins<sup>1,2</sup>. While the traditional PROTAC  
27 methodology has been focused on cytosolic proteins, the recently reported LYTACs<sup>3</sup> and  
28 AbTACs<sup>4</sup> offer an attractive approach for membrane protein elimination by targeting cation-  
29 independent mannose-6-phosphate receptor (CI-M6PR) or RNF43 with glycopolypeptides and  
30 scFv, respectively. Despite their promising results, however, the endocytosis of ternary complexes  
31 is difficult due to the large size of the IgG-based antibody scaffolds<sup>5</sup> as well as the lack of specific  
32 receptors or membrane E3 ubiquitin ligase. In addition, the potential immunogenicity of  
33 oligosaccharides used by LYTACs remains a concern for *in vivo* usage. Therefore, an all-protein

34 based membrane protein targeting and degradation platform with a smaller overall size, higher  
35 efficiency and wide-applicability without cell-type dependency is highly desirable.

36  $V_{HH}$  antibodies or nanobodies (Nbs) are heavy-chain derived antibodies that have a much  
37 smaller size than traditional IgG antibodies with remarkable stability. Although the high  
38 penetration capability of Nbs renders them particularly attractive for solid tumors<sup>6,7</sup>, they often  
39 have a relatively low binding affinity than full-length antibody, which may cause off-target effects  
40 during endocytosis. Since a stable Nb-Antigen complex is crucial for the endocytosis and  
41 degradation processes, we envisioned that covalent binding of the Nb to its antigen may fulfill this  
42 purpose, which also improves its on-target retention in a similar fashion as covalent small molecule  
43 drugs<sup>8,9</sup>. Furthermore, the conjugated cell-penetrating peptide (CPP) and lysosome-sorting  
44 sequence (LSS) may enhance the internalization and lysosome-mediated degradation of the  
45 covalent Nb-antigen complex<sup>10,11</sup>.

46 Herein, we report the covalent nanobody-based PROTAC strategy, termed GlueTAC, for  
47 targeted membrane protein degradation with high specificity and efficiency. Relying on the  
48 recently developed proximity enabled covalent binding reaction<sup>12-15</sup>, we created the covalently  
49 engineered nanobody chimeras that can irreversibly bind to membrane protein targets via  
50 proximity-enabled crosslinking, while the conjugated CPP and LSS allowed the lysosome-  
51 mediated target degradation (Fig. 1A). We first established a Mass Spectrometry-assisted  
52 screening technology (MSSP) for the rapid identification of the crosslinking site between a given  
53 antigen (Ag) and a pool of Nb variants bearing the site-specifically incorporated PrUAA at  
54 different locations. As PD-L1 is the major immune checkpoint that is highly expressed in cancer  
55 cells for suppressing immune cell responses<sup>16-18</sup>, we coupled this MSSP method with the genetic  
56 code expansion (GCE) strategy<sup>19</sup> to successfully develop the covalent Nb (Gluebody) for PD-L1,

57 and further conjugation with CPP and LSS greatly enhanced the internalization and degradation  
58 rate of PD-L1 on cancer cells (Fig. 1B). We further expanded this strategy to another tumor antigen  
59 EGFR, which together demonstrated GlueTAC as a general method for efficient and sustained  
60 membrane protein degradation in both cancer cells and mice xenograft models.

61

62 **Development of MSSP for rapid Gluebody screening.** As the incorporation sites of the  
63 PrUAA in Nbs have decisive impact on the crosslinking efficiency, we developed a MS-based  
64 screening platform (MSSP) for rapid identification of Gluebody for an antigen of interest. Our  
65 method relies on the site-specific screening of the incorporation sites and the identification of  
66 crosslinking peptides by using the tandem Mass Spectrometry (MS) and pLink software<sup>20</sup> (Fig.  
67 2A). For proof-of-concept, we applied this strategy to the Nb-PD-L1, which could restore T cell  
68 cytotoxicity by blocking this major immune checkpoint of cancer cells. According to the crystal  
69 structure of Nb-PD-L1/PD-L1 (PDB:5JDR)<sup>21</sup>, we chose the candidate sites for incorporation of  
70 PrUAA (FSY)<sup>22</sup> which are all located in the CDR3 domain, the major binding epitope of Nb-PD-  
71 L1 (Fig. 2B). The generated pool of Nb-PD-L1 variants containing FSY at selected sites were then  
72 incubated with purified PD-L1 protein, followed by SDS-PAGE analysis in which non-covalent  
73 protein-protein interactions were disrupted but not covalent Nbs-Ag complexes. To our delight, a  
74 new band with the molecular weight corresponding to the Nb-PD-L1/PD-L1 complex was  
75 observed and LC-MS/MS analysis indicated that the covalent crosslinking occurred between  
76 residues L108FSY on Nb-PD-L1 and His69 on PD-L1 (Fig. 2C, Supplementary Fig. 1).

77 To verify the covalent binding capacity of Nb-PD-L1 variants at identified sites, the purified  
78 FSY-bearing Nb-PD-L1 variants were incubated with PD-L1 and subjected to SDS-PAGE analysis,  
79 which showed that Nb-PD-L1-L108FSY, was the only covalent Nb of the pool that can crosslink

80 with PD-L1 (Fig. 2D, Supplementary Fig. 1D). Time-course dependent experiments showed that  
81 over 70% of the crosslinked complex formed within 5 h at a 2:1 Nb/Ag ratio under physiological  
82 conditions (Fig. 2E). In addition, we tested another PrUAA (BrC6K)<sup>23</sup> with a longer and more  
83 flexible side-chain, and found that when incorporated at residue Thr110 or Gly113, the resulting  
84 variants Nb-PD-L1-T110BrC6K and Nb-PD-L1-G113BrC6K could also crosslink with the PD-  
85 L1, which was consistent with the longer distance between the BrC6K incorporation sites and  
86 His69 of PD-L1. Of three MSSP-identified covalent Nb-PD-L1 variants with FSY or BrC6K  
87 incorporation, Nb-PD-L1-L108FSY exhibited highest crosslinking efficiency, termed as  
88 Gluebody-PD-L1 which was utilized in the following experiments.

89 To further demonstrate that our Gluebody can be covalently attached to the endogenous PD-  
90 L1 expressed on cell surface, PD-L1/MDA cells were incubated with the TAMRA-labeled  
91 Gluebody-PD-L1 followed by washing with stringent buffer that could disrupt the non-covalent  
92 nanobody-antigen interaction. Fluorescent imaging showed that only the Gluebody-PD-L1 but not  
93 its non-covalent counterpart remained bound to the PD-L1<sup>+</sup> cells under denaturing conditions (Fig.  
94 2F), indicating the formation of a covalent linkage on cell surface. Western blotting analysis also  
95 confirmed the crosslinked conjugate in PD-L1/MDA cells after incubating with Gluebody-PD-L1  
96 (Fig. 2G).

97

98 **Development of GlueTAC for membrane protein degradation.** Live-cell imaging showed  
99 that self-endocytosis rate of Gluebody-PD-L1 and its complex is relatively low (Fig. 2F), we then  
100 aimed to use the CPP-LSS peptide to accelerate the cell entry and degradation process of target  
101 proteins. Since the expression of Nbs fused with the positively charged CPP was quite difficult,  
102 we decided to conjugate the chemically synthesized functional peptide to the C terminal of

103 Gluebody via Sortase A-mediated coupling reaction<sup>24</sup>, which produced the stable and  
104 homogeneous chimera with nearly quantitative yield (Fig. 3A, Supplementary Fig. 4).  
105 Fluorescence microscopy experiments revealed that the TAMRA-labeled GlueTAC-PD-L1 co-  
106 localized with PD-L1 and lysosomes, indicating that GlueTAC-PD-L1 was able to drive membrane  
107 PD-L1 internalization and lysosome trafficking (Fig. 3B, Supplementary Fig. 5).

108

109 **GlueTAC mediated PD-L1 degradation *in vitro* and *in vivo*.** We next monitored the  
110 degradation of PD-L1 on live cells. The NSCLC cell line HCC827 cells were first treated with 100  
111 nM GlueTAC-PD-L1 for 12 h, with the Gluebody-PD-L1 alone and the non-covalent counterpart  
112 NbTAC-PD-L1 used as controls. Western blotting analysis showed that GlueTAC-PD-L1 induced  
113 the degradation of PD-L1 with maximal degradation rate of 87% when compared to NbTAC-PD-  
114 L1 with a degradation rate of 33%, indicating the essential role of the covalent linkage during the  
115 internalization and degradation process. Noteworthy, a clear crosslinked band was observed  
116 between Gluebody-PD-L1 and PD-L1, however, a much less degradation occurred, confirming  
117 that internalization of Gluebody-PD-L1 alone is not sufficient for degradation (Fig. 3C). In  
118 addition, we observed sustained degradation as PD-L1 remained at a low expression level 24 h  
119 after being treated with GlueTAC-PD-L1 for 12 h (Fig. 3D). Furthermore, to investigate the  
120 degradation mechanism, HCC827 cells were co-incubated with GlueTAC-PD-L1 or controls in  
121 the presence and absence of the lysosome inhibitor NH<sub>4</sub>Cl. Results showed that degradation of  
122 PD-L1 was significantly decreased when adding the lysosome inhibitor (Fig. 3E), demonstrating  
123 the dependency on the lysosomal pathway. Finally, to show the generality of GlueTAC mediated  
124 protein degradation on typical cancer cell lines, we demonstrated the reduction of 70-80% PD-L1

125 on H460 (lung cancer) (Fig. 3F), U87 (glioblastoma) (Fig. 3G) and PD-L1/MDA (stable cell line)  
126 (Fig. 3H) cells after the treatment of GlueTAC-PD-L1.

127 We went on to demonstrate the therapeutic potential of our GlueTAC for PD-L1<sup>+</sup> tumors *in*  
128 *vivo*. PD-L1/MDA cells were engrafted in the immuno-deficient NOG mice to form solid tumors  
129 (Fig. 3I). When the tumor size reached 100 mm<sup>3</sup>, TAMRA labeled Gluebody-PD-L1 was *i.v.*  
130 injected into the NOG mice bearing the PD-L1/MDA derived xenograft. The tumor samples were  
131 then dissected, sliced and imaged, and in parallel, digested for western blotting analysis. The  
132 results showed that our Gluebody-PD-L1 was covalently bound to tumor cells (Supplementary Fig.  
133 6) and was widely spread within the tumor (Fig. 3J), which suggested that the Gluebody-PD-L1  
134 retained the excellent permeability of Nbs. We further tested whether GlueTAC could degrade PD-  
135 L1 in tumor tissues. When the tumor size reached 60 mm<sup>3</sup>, GlueTAC-PD-L1 and the control  
136 constructs were delivered via peri-tumoral injection. After 18 h, the tumors were dissected for  
137 immunoblotting analysis which showed apparent degradation of PD-L1 by GlueTAC but not other  
138 control constructs (Fig. 3K).

139  
140 **GlueTAC mediated EGFR degradation.** To demonstrate the generality of our GlueTAC  
141 strategy, we further applied it for the degradation of EGFR, a well-known target antigen for  
142 treating cancers<sup>25</sup>. By site-specific incorporation of FSY into Nb-EGFR<sup>26</sup> followed by MSSP, we  
143 found apparent crosslinking between FSY incorporated at Gln116 on Nb-EGFR and His409 of  
144 EGFR. The resultant Nb-EGFR-Q116FSY, termed Gluebody-EGFR, was purified, TAMRA-  
145 labeled and incubated with the purified EGFR extracellular domain before SDS-PAGE analysis  
146 (Supplementary Fig. 7). A covalent complex of Gluebody-EGFR and EGFR was detected, but not  
147 for Nb-EGFR, confirming FSY-mediated covalent targeting of EGFR (Supplementary Fig. 8).

148 Next, to test if Gluebody-based GlueTAC strategy could degrade EGFR on cancer cells, GlueTAC-  
149 EGFR was constructed and incubated with HeLa cells highly expressing EGFR. Western blotting  
150 analysis showed that GlueTAC-EGFR demonstrated higher efficiency in decreasing EGFR on  
151 HeLa cells over its non-covalent counterpart as well as the non-conjugated Gluebody-EGFR alone.  
152 (Supplementary Fig. 9).

153 In summary, conventional therapeutic antibodies inhibit oncogenic signaling from cell-  
154 surface receptors by antagonizing extracellular receptor-ligand interactions, which cannot  
155 completely prevent intracellular signaling as many receptors including PD-L1 exhibited ligand-  
156 independent activity. Our covalent nanobody-based GlueTAC strategy thus provides an alternative  
157 approach for eliminating surface receptor signaling.

158 In compare with the recently reported LYTAC and AbTAC technologies, GlueTAC has three  
159 unique advantages. Firstly, the irreversible attachment of covalent nanobody (Gluebody) to surface  
160 antigen can largely avoid off-target effects during the endocytosis process. This covalent feature  
161 also enables Gluebody to improve its half-life through on-target retention. Secondly, the  
162 endocytosis is driven by enzymatically conjugated polyarginine peptide which is independent of  
163 specific receptor or E3 ligase. This is particularly attractive as the tumors are highly heterogeneous  
164 and PD-L1 level in both cancer cells and other immunosuppressive cell types will be increased  
165 during the immunotherapy due to the adaptive resistance mechanism of immune escaping<sup>27</sup>. Our  
166 cell-type independent degradation strategy would thus allow elimination of PD-L1 regardless of  
167 cell types within the tumor microenvironment. Finally, the straightforward protocol for  
168 constructing GlueTAC chimeras, in conjunction with its outstanding tissue permeability due to the  
169 small overall size, makes it an excellent choice for solid tumor treatment.

170 Notably, the MSSP platform offers us a high-throughput strategy capable of simultaneous  
171 screening of multiple PrUAAs and incorporation sites, allowing rapid identification of proper  
172 incorporation site in nanobodies for generating proximal reactive Gluebody. We envision that, by  
173 combining the MSSP strategy and mature nanobody discovery technology, the GlueTAC strategy  
174 may offer a general platform for targeted cell-surface protein degradation.

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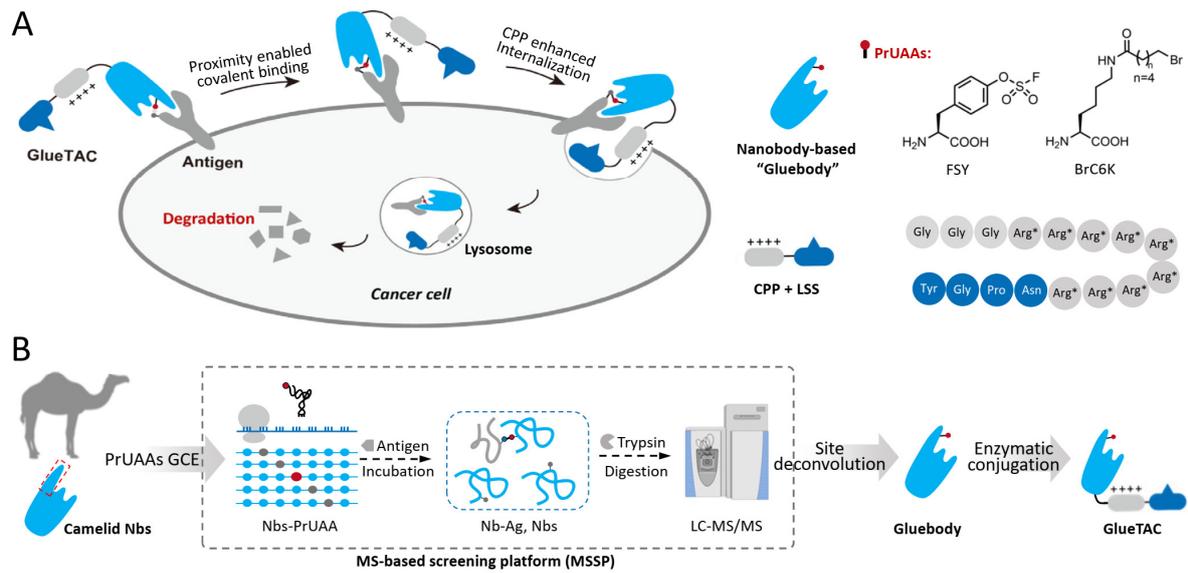
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245 **Author contributions:** H. Zhang, Y. Han, J. Lin and P. R. Chen designed the research and  
246 conducted most of the experiments. Y. Yang synthesized the FSY. F. Lin conducted some *in vitro*  
247 assays. H. Zhang, Y. Han, J. Lin and P. R. Chen wrote the paper with input from all authors. The  
248 experimental data sets used in this study are available as supplementary materials.

249 **Competing interests:** The authors declare no competing interests.

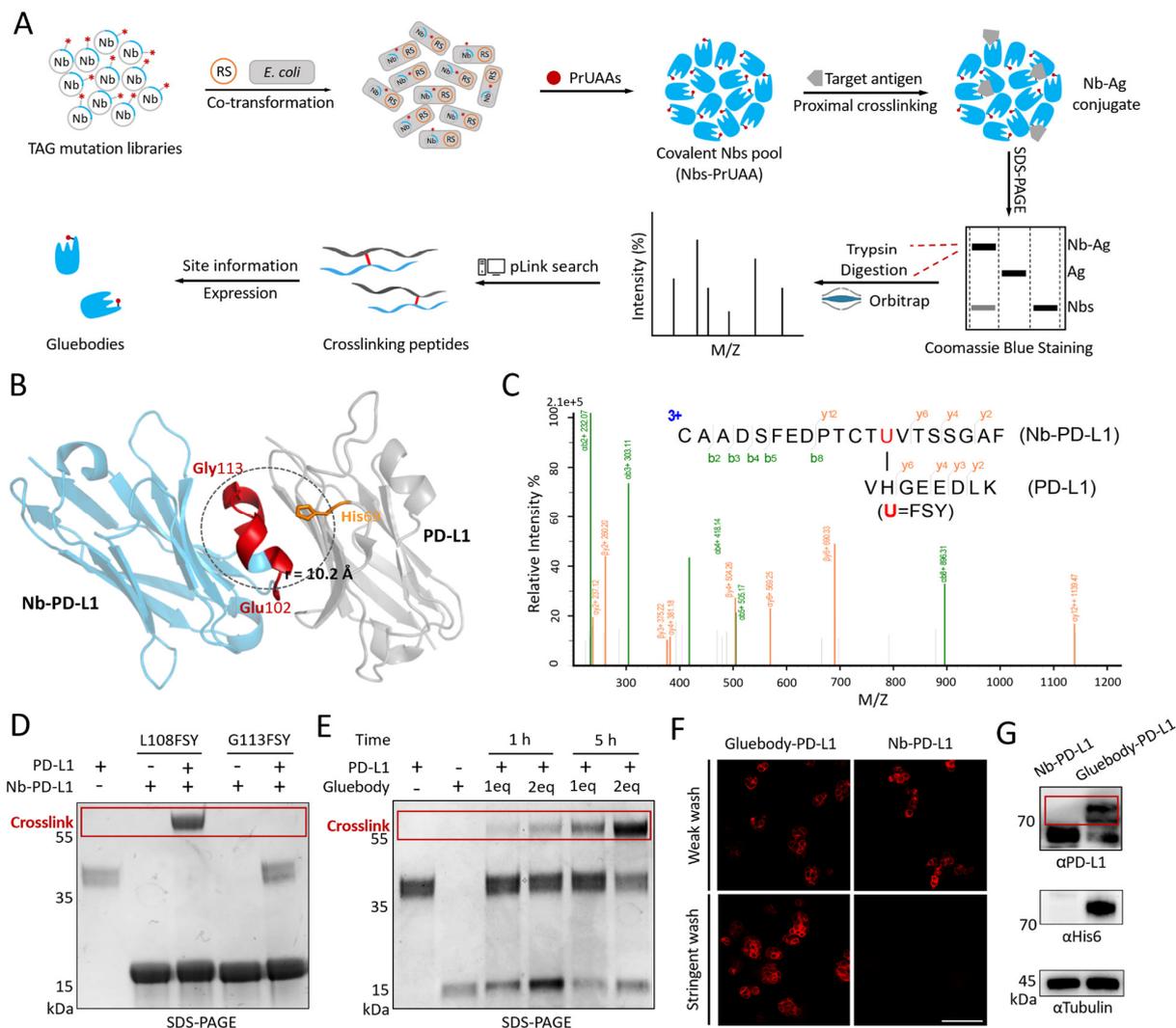
250 **Correspondence and requests for materials** should be addressed to P. R. Chen and J. Lin.

251 **Data and materials availability:** All data are available in the manuscript or the Supplementary  
252 Information. More details are available from the corresponding author upon request.



253  
254

255 **Fig. 1. Design and development of the Gluebody chimera-based GlueTAC strategy for**  
 256 **targeted membrane protein degradation.** (A) Schematic illustration of the GlueTAC strategy.  
 257 The nanobody variant containing a site-specifically incorporated proximal reactive uncanonical  
 258 amino acid (PrUAA, e.g., FSY, BrC6K) can covalently bind to the membrane antigen on tumor  
 259 cells via proximity-enabled crosslinking. The resulting Gluebody can be further conjugated with  
 260 the cell-penetrating peptide (CPP) consisting of nine *D*-Arginine and lysosomal-sorting sequence  
 261 (LSS) consisting of NPGY to generate the final GlueTAC construct with enhanced internalization  
 262 and degradation of the bound membrane antigen. \* means *D* conformation. (B) Schematic  
 263 illustration of the pipeline for preparation of GlueTAC. High affinity camelid nanobodies (Nbs)  
 264 are genetically incorporated with PrUAAs at various sites in the CDR3 region (red frame),  
 265 resulting a pool of Nbs-PrUAAs. The MS-based screening platform (MSSP) can then be applied  
 266 to this Nbs pool to identify the covalent variant that exhibited the highest crosslinking efficiency  
 267 with the antigen. Further enzymatic conjugation of CPP and LSS to this covalent Nb (Gluebody)  
 268 will produce the desired GlueTAC construct for targeted membrane protein degradation.



269

270 **Fig. 2. The MS-based screening platform (MSSP) for Gluebody development and verification.**  
 271 (A) Diagram of the MSSP selection strategy for identifying the covalent nanobody (Gluebody) for  
 272 an antigen of interest. The selected residues in the CDR3 region of a native nanobody were mutated  
 273 to amber suppression codon (TAG) to generate the covalent Nbs pool in the presence of both  
 274 PrUAA and the corresponding aaRS/tRNA pair. Candidate covalent Nbs were incubated with the  
 275 antigen to initiate *in situ*, proximity-induced crosslinking, followed by SDS-PAGE analysis and  
 276 trypsin digestion of the crosslinked bands. The crosslinking peptides can be identified by searching  
 277 the resulting tandem mass spectrum by pLink software, with the PrUAA incorporation site  
 278 deconvoluted and the corresponding Nb variant named as the Gluebody. (B) Structure of PD-L1  
 279 in complex with the Nb-PD-L1. The side-chain of His69 on the PD-L1 antigen and the  $\alpha$ -helix  
 280 between Glu102 and Gly113 on Nb-PD-L1 are highlighted, which are within 10.2 Å distance. PDB:  
 281 5jdr. (C) Tandem mass spectrum of the crosslinked Gluebody-PD-L1/PD-L1 complex indicated  
 282 that L108FSY of Nb-PD-L1 reacted with His69 on PD-L1. (D) Verification of the covalent binding  
 283 of Gluebody-PD-L1 to PD-L1 by SDS-PAGE. Purified proteins were incubated with PD-L1 in  
 284 PBS buffer at 37 °C for 5 h before SDS-PAGE and coomassie staining analysis. Covalent complex

285 of Gluebody-PD-L1/PD-L1 was indicated by the red frame. (E) Time-course and dosage study of  
286 the reaction between Gluebody-PD-L1 and PD-L1 as verified by SDS-PAGE. (F) Irreversible  
287 binding of Gluebody-PD-L1 to the endogenous PD-L1 on PD-L1/MDA cells indicated by  
288 fluorescence imaging and (G) western blotting analysis. PD-L1/MDA cells were incubated with  
289 TAMRA labeled Gluebody-PD-L1 or Nb-PD-L1 in medium at 37 °C for 5 h, after which the cells  
290 were washed with weak washing buffer or stringent washing buffer (denaturing condition). The  
291 crosslinked band was indicated by the red frame. Scale bar = 100 μm.

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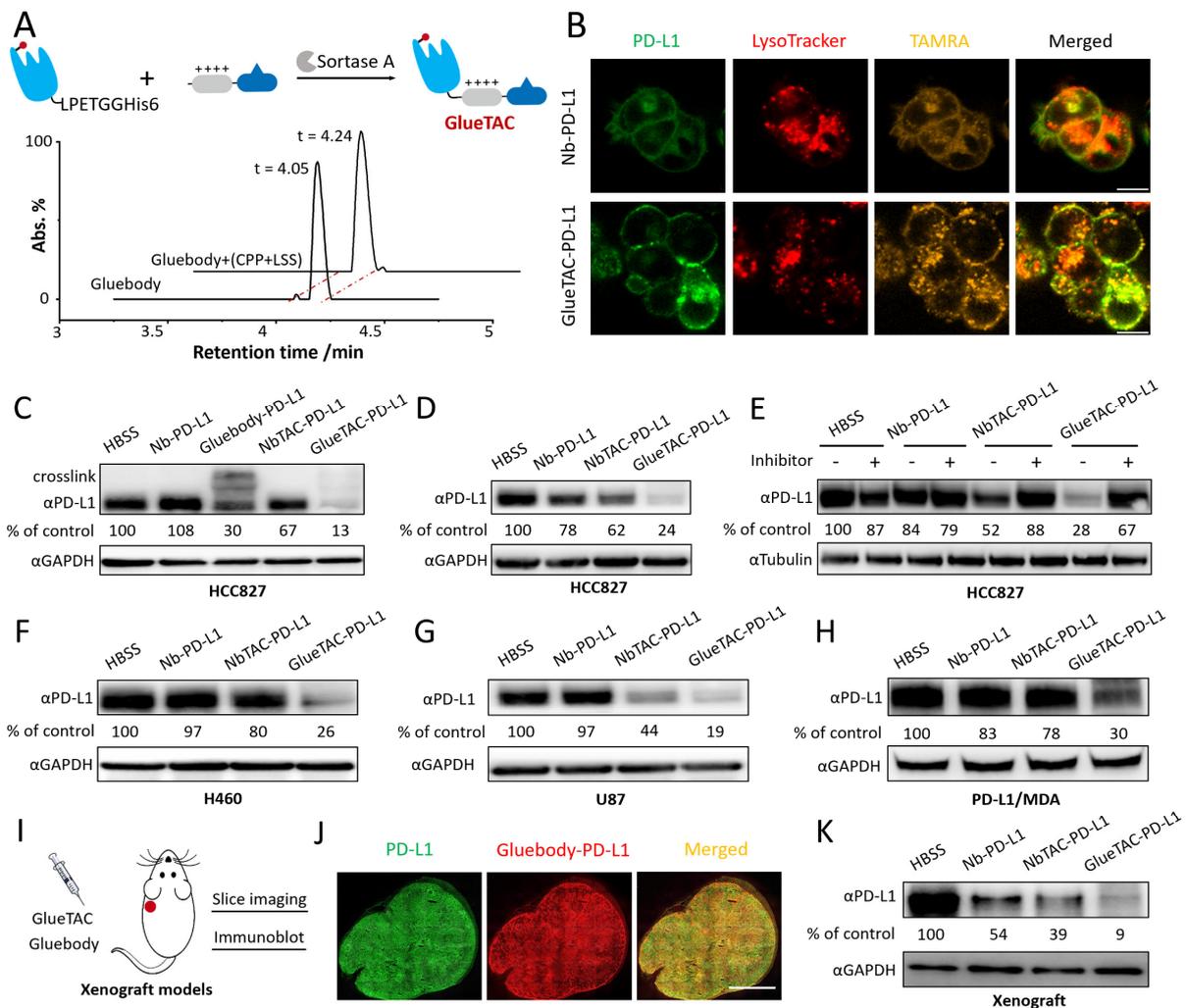
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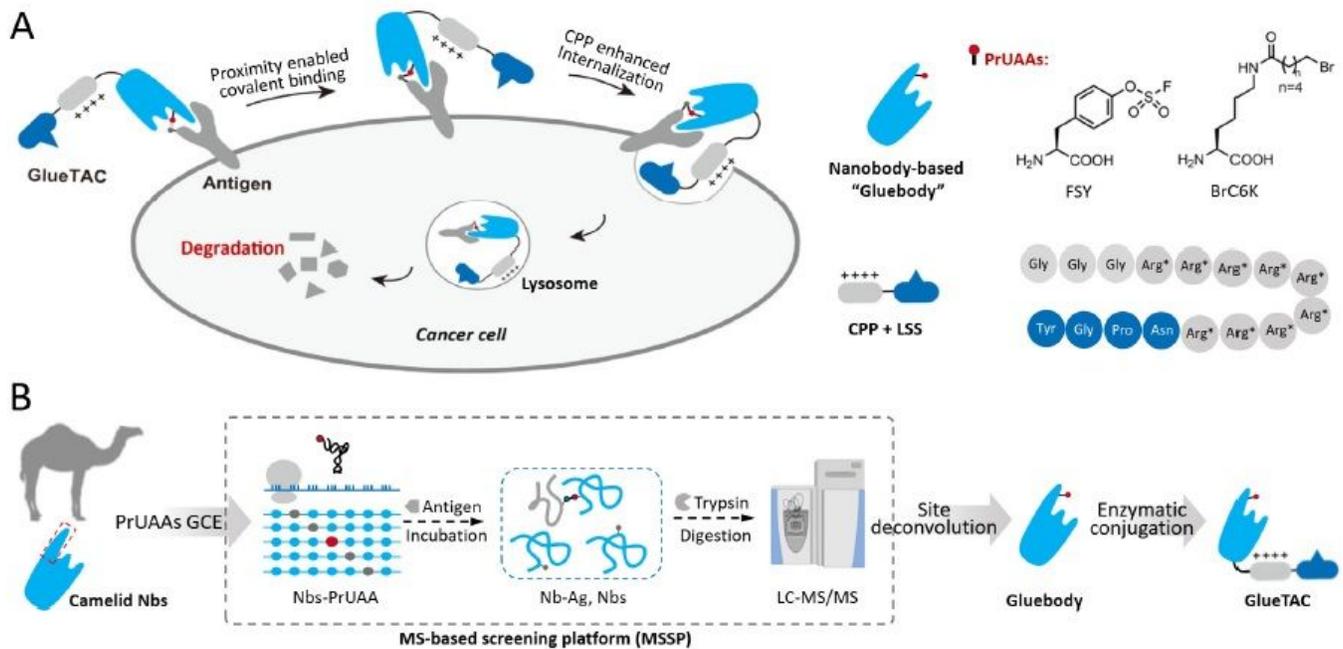


300

301 **Fig. 3 Targeted degradation of PD-L1 on cancer cells by GlueTAC *in vitro* and *in vivo*.** (A)  
 302 Verification of GlueTAC-PD-L1 construction through enzymatic conjugation between the  
 303 Gluebody-PD-L1 and the CPP-LSS peptide. The functional peptide was added to the C-terminal  
 304 of Gluebody-PD-L1 through the LPETG tag via Sortase A mediated conjugation and validated by  
 305 LC-MS analysis. (B) Live-cell confocal microscopy imaging confirms the co-localization of the  
 306 PD-L1-GFP and TAMRA labeled Nb-PD-L1 or GlueTAC-PD-L1 on tumor cells. The enhanced  
 307 internalization and lysosomal trafficking of GlueTAC-PD-L1 bound PD-L1-GFP was indicated by  
 308 LysoTracker Deep Red. Scale bar = 10  $\mu$ m. (C) Western blotting analysis confirms PD-L1  
 309 degradation mediated by GlueTAC on tumor cells. PD-L1 levels in HCC827 cells after the  
 310 treatment of GlueTAC-PD-L1 or control constructs in RPMI 1640 supplemented with 10% FBS  
 311 at 37  $^{\circ}$ C for 12 h. (D) GlueTAC exhibits a sustained degradation effect. Recovery of PD-L1 levels  
 312 in HCC827 cells after being cultured with GlueTAC-PD-L1 or controls for 12 h and a further  
 313 incubation in fresh medium for another 12 h. (E) PD-L1 levels in HCC827 cells after treatment  
 314 with GlueTAC-PD-L1 or controls for 12 h in the presence and absence of lysosome inhibitor (10  
 315 mM  $\text{NH}_4\text{Cl}$ ). (F) GlueTAC mediates PD-L1 degradation in H460 cells, (G) U87 cells or (H) PD-  
 316 L1/MDA cells for 12 h, respectively. (I) NOG mice bearing PD-L1/MDA derived xenograft tumor

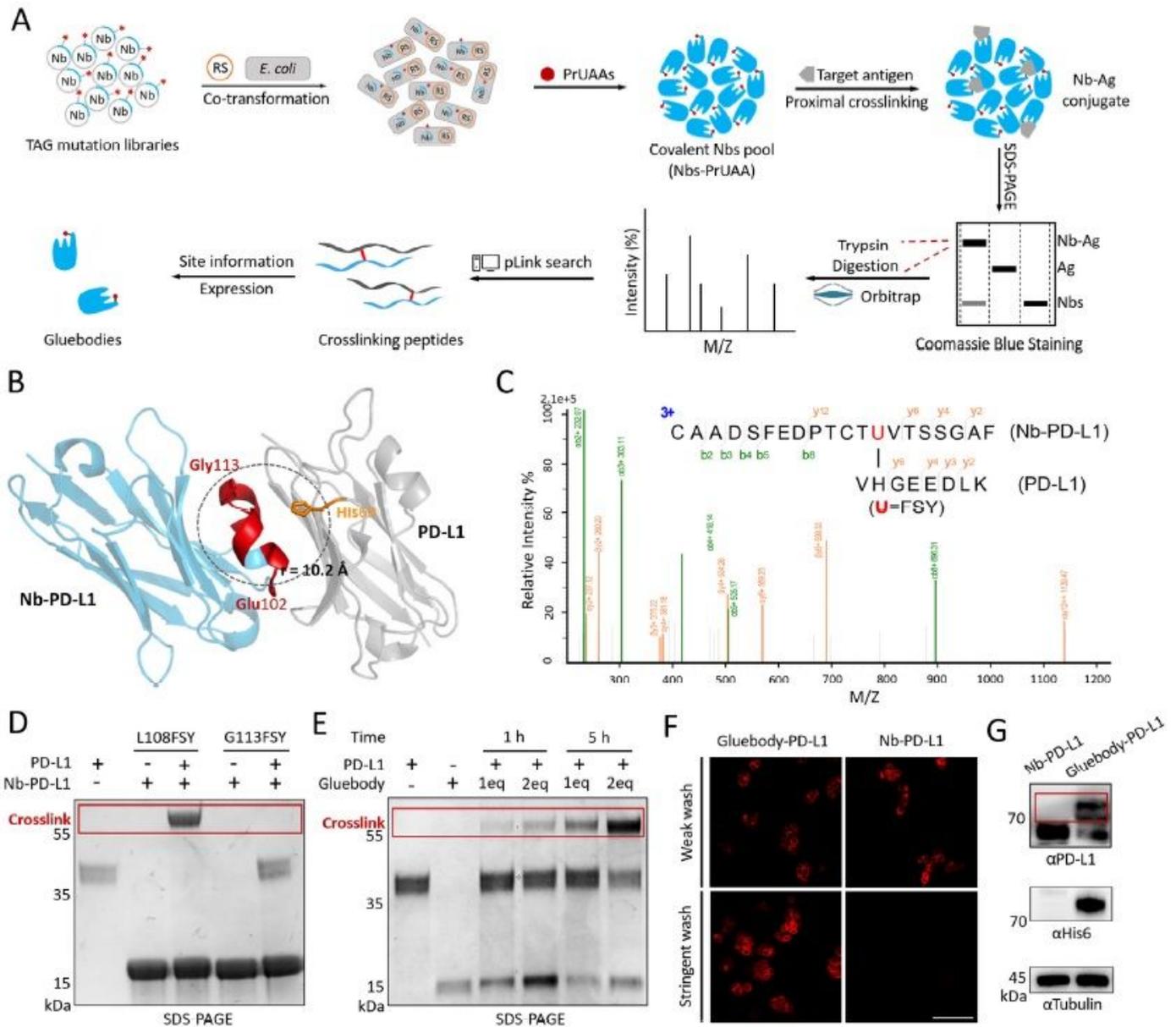
317 underarm. (J) Fluorescent images of tissue slice of separated PD-L1<sup>+</sup> tumors from the xenograft  
318 models after *i.v.* injection with TAMRA-labeled Gluebody-PD-L1 for 4 h. Scale bar= 2 mm. (K)  
319 PD-L1 levels in tumors after peri-tumoral injection with GlueTAC-PD-L1 or control constructs  
320 for 18 h. The presented data are representative of three independent replicates.

# Figures



**Figure 1**

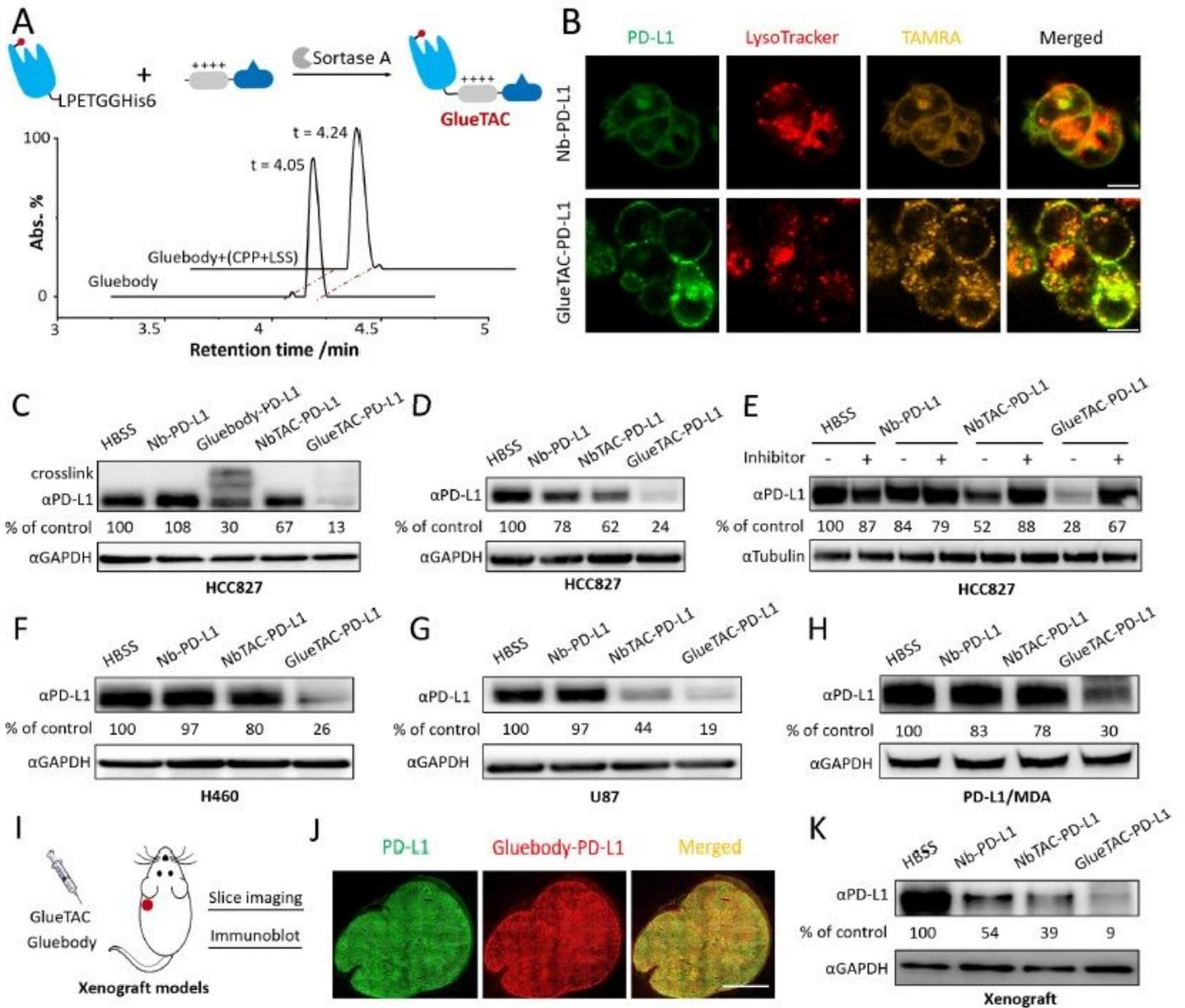
Design and development of the Gluebody chimera-based GlueTAC strategy for targeted membrane protein degradation. (A) Schematic illustration of the GlueTAC strategy. The nanobody variant containing a site-specifically incorporated proximal reactive uncanonical amino acid (PrUAA, e.g., FSY, BrC6K) can covalently bind to the membrane antigen on tumor cells via proximity-enabled crosslinking. The resulting Gluebody can be further conjugated with the cell-penetrating peptide (CPP) consisting of nine D-Arginine and lysosomal-sorting sequence (LSS) consisting of NPGY to generate the final GlueTAC construct with enhanced internalization and degradation of the bound membrane antigen. \* means D conformation. (B) Schematic illustration of the pipeline for preparation of GlueTAC. High affinity camelid nanobodies (Nbs) are genetically incorporated with PrUAAs at various sites in the CDR3 region (red frame), resulting a pool of Nbs-PrUAAs. The MS-based screening platform (MSSP) can then be applied to this Nbs pool to identify the covalent variant that exhibited the highest crosslinking efficiency with the antigen. Further enzymatic conjugation of CPP and LSS to this covalent Nb (Gluebody) will produce the desired GlueTAC construct for targeted membrane protein degradation.



**Figure 2**

The MS-based screening platform (MSSP) for Gluebody development and verification. (A) Diagram of the MSSP selection strategy for identifying the covalent nanobody (Gluebody) for an antigen of interest. The selected residues in the CDR3 region of a native nanobodies were mutated to amber suppression codon (TAG) to generate the covalent Nbs pool in the presence of both PrUAA and the corresponding aaRS/tRNA pair. Candidate covalent Nbs were incubated with the antigen to initiate in situ, proximity-induced crosslinking, followed by SDS-PAGE analysis and tryptic digestion of the crosslinked bands. The crosslinking peptides can be identified by searching the resulting tandem mass spectrum by pLink software, with the PrUAA incorporation site deconvoluted and the corresponding Nb variant named as the Gluebody. (B) Structure of PD-L1 in complex with the Nb-PD-L1. The side-chain of His69 on the PD-L1 antigen and the  $\alpha$ -helix between Glu102 and Gly113 on Nb-PD-L1 are highlighted, which are within 10.2 Å distance. PDB: 5jdr. (C) Tandem mass spectrum of the crosslinked Gluebody-PD-L1/PD-L1 complex

indicated that L108FSYof Nb-PD-L1 reacted with His69 on PD-L1. (D) Verification of the covalent binding of Gluebody-PD-L1 to PD-L1 by SDS-PAGE. Purified proteins were incubated with PD-L1 in PBS buffer at 37 °C for 5 h before SDS-PAGE and coomassie staining analysis. Covalent complex of Gluebody-PD-L1/PD-L1 was indicated by the red frame. (E) Time-course and dosage study of the reaction between Gluebody-PD-L1 and PD-L1 as verified by SDS-PAGE. (F) Irreversible binding of Gluebody-PD-L1 to the endogenous PD-L1 on PD-L1/MDA cells indicated by fluorescence imaging and (G) western blotting analysis. PD-L1/MDA cells were incubated with TAMRA labeled Gluebody-PD-L1 or Nb-PD-L1 in medium at 37 °C for 5 h, after which the cells were washed with weak washing buffer or stringent washing buffer (denaturing condition). The crosslinked band was indicated by the red frame. Scale bar = 100  $\mu$ m.



**Figure 3**

Targeted degradation of PD-L1 on cancer cells by GlueTAC in vitro and in vivo. (A) Verification of GlueTAC-PD-L1 construction through enzymatic conjugation between the Gluebody-PD-L1 and the CPP-

LSS peptide. The functional peptide was added to the C-terminal of Gluebody-PD-L1 through the LPETG tag via Sortase A mediated conjugation and validated by LC-MS analysis. (B) Live-cell confocal microscopy imaging confirms the co-localization of the PD-L1-GFP and TAMRA labeled Nb-PD-L1 or GlueTAC-PD-L1 on tumor cells. The enhanced internalization and lysosomal trafficking of GlueTAC-PD-L1 bound PD-L1-GFP was indicated by LysoTracker Deep Red. Scale bar = 10  $\mu$ m. (C) Western blotting analysis confirms PD-L1 degradation mediated by GlueTAC on tumor cells. PD-L1 levels in HCC827 cells after the treatment of GlueTAC-PD-L1 or control constructs in RPMI 1640 supplemented with 10% FBS at 37 °C for 12 h. (D) GlueTAC exhibits a sustained degradation effect. Recovery of PD-L1 levels in HCC827 cells after being cultured with GlueTAC-PD-L1 or controls for 12 h and a further incubation in fresh medium for another 12 h. (E) PD-L1 levels in HCC827 cells after treatment with GlueTAC-PD-L1 or controls for 12 h in the presence and absence of lysosome inhibitor (10 mM NH<sub>4</sub>Cl). (F) GlueTAC mediates PD-L1 degradation in H460 cells, (G) U87 cells or (H) PD L1/MDA cells for 12 h, respectively. (I) NOG mice bearing PD-L1/MDA derived xenograft tumor underarm. (J) Fluorescent images of tissue slice of separated PD-L1+ tumors from the xenograft models after i.v. injection with TAMRA-labeled Gluebody-PD-L1 for 4 h. Scale bar= 2 mm. (K) PD-L1 levels in tumors after peri-tumoral injection with GlueTAC-PD-L1 or control constructs for 18 h. The presented data are representative of three independent replicates.

## Supplementary Files

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