Directed evolution of a picomolar-affinity, high-specificity antibody targeting phosphorylated tau

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Antibodies are essential biochemical reagents for detecting protein post-translational modifications (PTMs) in complex samples. However, recent efforts in developing PTM-targeting antibodies have reported frequent nonspecific binding and limited affinity of such antibodies. To address these challenges, we investigated whether directed evolution could be applied to improve the affinity of a high-specificity antibody targeting phosphothreonine 231 (pThr-231) of the human microtubule-associated protein tau. On the basis of existing structural information, we hypothesized that improving antibody affinity may come at the cost of loss in specificity. To test this hypothesis, we developed a novel approach using yeast surface display to quantify the specificity of PTM-targeting antibodies. When we affinity-matured the single-chain variable antibody fragment through directed evolution, we found that its affinity can be improved ~20-fold over that of the WT antibody, reaching a picomolar range. We also discovered that most of the high-affinity variants exhibit cross-reactivity toward the nonphosphorylated target site but not to the phosphorylation site with a scrambled sequence. However, systematic quantification of the specificity revealed that such a tradeoff between the affinity and specificity did not apply to all variants and led to the identification of a picomolar-affinity variant that has a matching high specificity of the original phosphotau antibody. In cell- and tissue-imaging experiments, the high-affinity variant gave significantly improved signal intensity while having no detectable nonspecific binding. These results demonstrate that directed evolution is a viable approach for obtaining high-affinity PTM-specific antibodies and highlight the importance of assessing the specificity in the antibody engineering process.

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Antibodies that target post-translationally modified proteins are widely used in biology and clinical investigations. Some antibodies can bind to a single modified amino acid (e.g. phosphorylated tyrosine) within the context of diverse nearby peptide sequences (1, 2). However, most widely used are antibodies specific to a modification site in a defined target protein sequence, herein referred to as post-translational modification (PTM)2-specific antibodies. Due to a large number of experimentally identified post-translational modification sites (>87,000) (3), the demand for PTM-specific antibodies continues to grow.

PTM is a key mechanism that enables reversible regulation of protein activity, conformation, localization, turnover rate, and interactions. Abnormal PTMs result in dysregulation of these processes and are often associated with pathological conditions. In the case of the microtubule-associated protein tau, numerous abnormal PTMs regulate its biology and are associated with several neurodegenerative diseases, including Alzheimer’s disease (AD), collectively referred to as tauopathies (4). Tau contains 85 putative phosphorylation sites (5) and 14 putative acetylation sites (6). Phosphorylation sites at or near the repeat elements of the microtubule-binding domains are particularly important for the pathophysiologic of tauopathy (7). An increase in tau phosphorylation, on average from two to three phosphates to seven to eight phosphates per molecule (7), results in preferential phosphorylation at sites that decrease microtubule binding (8, 9) and increases its propensity to form oligomers and high-order aggregates (7, 10). Levels of tau oligomers showed a strong correlation with memory loss in a mouse model of tauopathy (11). Acetylation at specific lysine residues interferes with ubiquitination and subsequent degradation by the proteasome, resulting in accumulation of phosphorylated tau and associated toxicity in mouse models (12–15). Therefore, detection of tau PTMs at specific sites is valuable in identifying the cause and tracking the progression of tauopathies.

2 The abbreviations used are: PTM, post-translational modification; AD, Alzheimer’s disease; CDR, complementarity-determining region; scFv, single-chain variable fragment; V\textsubscript{L}, light variable chain fragment; V\textsubscript{H}, heavy variable chain fragment; Pphos, phosphopeptide; Pphos-b, biotinylated phosphopeptide; Pnonphos, non-phosphopeptide; Psram, scrambled phosphopeptide; Alexa, Alexa Fluor; Pphos-A488, phosphopeptide labeled with Alexa 488; EGFP, enhanced GFP; CSF, cerebrospinal fluid; 8-oxo-dGTP, 8-oxo-2’-deoxyguanosine 5’-triphosphate; dPTP, 2’-deoxy-P-nucleoside 5’-triphosphate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; DAPI, 4’6-diamidino-2-phenylindole.
High-affinity and -specificity phosphotau antibody

However, robust detection of PTMs is challenging due to their dynamic and heterogeneous nature. Only a fraction of a protein is modified at any given time, and the dynamics may vary within a tissue across different cell types (16–18). Therefore, the affinity and specificity of PTM-specific antibodies are critical for sensitive and reproducible detection of PTMs (19). PTM-specific antibodies should interact only with the modified target site with high affinity to capture potentially a small fraction of the modified form. In the case of tau PTM-specific antibodies, their affinity and specificity are particularly important because they dictate their efficacy in a wide range of applications. High-affinity antibodies are superior in detecting tau PTMs from clinical samples (20), but mouse model experiments suggest that high-specificity antibodies may be more beneficial for passive immunotherapy targeting tau (21).

Although many PTM-specific antibodies exist, high-affinity antibodies with high PTM specificity are rarely found. Rodent immunization approaches often fail or produce low-affinity antibodies. This is because the peptide epitopes containing PTMs are weak immunogens in mice (22), PTMs undergo rapid degradation in vivo regardless of the immunization route (23), and the B-cell response shows an affinity ceiling (24, 25). Screening recombinant antibody libraries in vitro is a promising alternative. However, in vitro library screening strategies tend to generate PTM-specific antibodies with a low or moderate affinity ($K_d \sim \mu M$ to $100 \text{ nM}$ range) (26–29).

Obtaining high-specificity antibodies targeting PTMs is even more challenging. In fact, many commercially available PTM-specific antibodies have not been validated for their specificity. Recent evidence using various approaches, including immunosassays using synthetic peptides and peptide arrays, have shown that indeed a significant fraction of existing PTM-targeting antibodies lack specificity (30–34). These studies have identified major species that nonspecifically bind to the antibodies, including nonmodified epitopes with the target amino acid sequence and those with modifications at nontarget sites (31, 34).

Recently, X-ray crystal structures of phosphospecific antibodies have revealed a key insight into how PTM-targeting antibodies may gain specificity (26, 27). In these antibodies, a set of complementarity-determining region (CDR) residues were found to interact with the phosphate group, whereas others make contacts with the amino acid residues nearby, leading to recognition of both the modification and target sequence. Therefore, balancing the stability of interaction of these distinct sets of CDR residues seems to be important in gaining both affinity and specificity. It is plausible that high-affinity PTM-targeting antibodies can achieve such tight binding by stabilizing the interaction toward either the modified amino acid or peptide sequence, potentially leading to unwanted cross-reactivity. Therefore, we hypothesized that a tradeoff between affinity and specificity might exist in PTM-targeting antibodies.

To test this hypothesis, we sought to assess the impact of in vitro affinity maturation on the specificity of PTM-targeting antibodies. Starting from an existing high-specificity antibody (27) targeting phosphorylated Thr-231 of human tau (pThr-231; numbering based on the 441-amino acid 2N4R isoform), we generated a single-chain variable fragment (scFv) and improved its affinity using directed evolution. To assess the specificity of PTM-targeting antibodies, we developed an experimental approach based on yeast surface display (35) to quantify scFv binding against a defined set of structurally similar epitopes. Specifically, peptides representing the phosphorylated epitope, nonphosphorylated epitope, or a phosphorylated epitope with the same amino acid composition but scrambled sequence were labeled for detection. Their interactions with yeast-displayed antibody fragments were quantified using flow cytometry. Indeed, the original high-specificity phosphotau scFv showed nearly perfect specificity in this assay in agreement with previous characterizations (27). We found that, through directed evolution, the affinity could be improved by $\geq 20$-fold, reaching picomolar monovalent dissociation constants. However, the majority of affinity-improved scFvs showed significant nonspecific binding to the nonphosphorylated epitope but not to the scrambled phosphopeptide, suggesting that high-affinity clones tend to gain affinity by stabilizing the interaction with the amino acid residues surrounding the modification site. Although the majority of clones showed a tradeoff between their affinity and specificity, this was not necessarily the case for some unique clones. By assessing the binding specificity, we identified a picomolar-affinity clone with specificity profiles that match the original high-specificity antibody. These results demonstrated that a quantitative assessment of both affinity and specificity was critical for identifying high-affinity and -specificity antibodies targeting PTMs.

Results

Yeast surface display of a phosphotau-specific scFv

To test our hypothesis, we first developed an assay to quantify antibody specificity using yeast surface display (Fig. 1). Synthetic peptides that represent the exact target epitope as well as other potentially cross-reacting species were labeled for detection. Because yeast surface display allows surface expression of 40,000–50,000 scFvs per cell (35, 36), we anticipated that binding of multiple structurally related epitopes displayed on yeast could be quantified using multicolor flow cytometry (Fig. 1). To demonstrate this concept, we used the previously identified high-specificity anti-phosphotau antibody, which targets pThr-231 (27). We cloned the antibody as a $V_L$-$V_H$ single-chain variable region fragment (pThr-231 scFv) fused to the N terminus of the yeast cell-wall protein Aga2p (Fig. 1). To detect the interaction between pThr-231 scFv and the target phosphopeptide, a synthetic phosphopeptide (KKAVVVRpTPPKpSPSSAKC where pS is phosphoserine and pT is phosphothreonine) was labeled with biotin at the C-terminal cysteine using maleimide-PEG$_{11}$-biotin. The resulting peptide was purified using high-performance LC (HPLC), and the label was validated using MS (Fig. S1). The scFv expression on the yeast cell surface and binding to the biotinylated phosphopeptide were confirmed using confocal microscopy (Fig. 2a) using streptavidin–R-phycocerythrin as a detection reagent. The affinity (dissociation constant $K_d$) of the yeast-displayed pThr-231 scFv, as determined by titrating the biotinylated phosphopeptide ($P$hos-b) was 2.2 ± 0.4 nM (Fig. 2b; mean ± S.D.), which is lower than the monovalent affinity for the full-length IgG ($K_d$ = 0.35 nM) gen-
erated using the same variable regions (27). Although scFv constructs are highly advantageous for imaging (37) and delivery (38–40), a 5–15-fold reduction in affinity upon conversion of IgG to scFv, potentially due to higher stability and functional concentration of full IgGs, has been reported (38, 41). To confirm that the yeast-displayed scFv was properly folded, we generated alanine point mutations in the CDR residues that interacted with the phosphopeptide (Fig. S2) according to the published X-ray structure of the antibody–phosphopeptide complex (27). Alanine point mutations in all phosphopeptide-interacting CDRs (single alanine substitutions in either CDR-H2, CDR-H3, CDR-L1, CDR-L2, or CDR-L3) substantially reduced phosphate binding (Fig. S2), indicating that CDR residues were correctly presented in the yeast displaying scFv.

**Assessment of antibody phosphospecificity using yeast surface display**

Having verified the binding activity of the pThr-231 scFv, we quantified the specificity of the scFv to structurally similar epitopes, including the nonphosphopeptide and a phosphopeptide with scrambled amino acid sequence. We labeled peptides representing the nonphosphopeptide (Pnonphos; KKVAVRTPPKSPSSAKC) and a scrambled phosphopeptide (Psram; KAVpSASVPSRKpTKPPKC) with biotin at the C-terminal cysteine (Fig. S1 and data not shown). To enable quantitation of binding specificity, we generated a phosphopeptide labeled with a second tag, Alexa 488, using Alexa Fluor 488 C5-maleimide (Fig. S1). When we assessed the binding of pThr-231 to the phosphopeptide labeled with Alexa 488 (Pphos-A488) using flow cytometry, we found that the signal intensity of scFv-phosphopeptide was lower compared with the signal obtained from Pnonphos (Fig. S3). Such a difference could be attributed to the fact that the brightness of R-phycocerythrin (molar absorption coefficient × quantum yield = 19.6 × 10^5 M^-1 cm^-1 × 0.82 = 16.1 × 10^5 M^-1 cm^-1) (42) is 24-fold higher than that of Alexa 488 (0.73 × 10^5 M^-1 cm^-1 × 0.92 = 0.67 × 10^5 M^-1 cm^-1; manufacturer specifications). Therefore, we amplified the signal from Alexa 488 using an anti-Alexa 488 antibody (rabbit polyclonal) followed by an anti-rabbit IgG conjugated with Alexa 488, which resulted in 10-fold increase in the scFv-Pphos-A488 signal (Fig. S3). When yeast cells displaying the pThr-231 scFv were incubated with an equimolar mixture of Pphos-b and Pphos-A488 (both at 0.5 μM), signals from both labels were detected using flow cytometry (Fig. 2C) as anticipated in the multicolor labeling scheme (Fig. 1). To assess the specificity, yeast cells displaying pThr-231 scFv were incubated with a mixture of either Pphos-A488 and Pnonphos-b (both at 0.5 μM) or Pphos-A488 and Psram-b (both at 0.5 μM). In these experiments, the yeast cells showed labeling almost exclusively by the target phosphopeptide (Pphos-A488) and no detectable binding to the nonphosphopeptide (Pnonphos-b) or scrambled phosphopeptide (Psram-b) (Fig. 2C). These results confirmed the previous report of high specificity of the pThr-231 antibody (27) and demonstrated the effectiveness of our specificity quantification assay.

**Directed evolution of pThr-231 scFv for improved affinity**

We performed directed evolution of the pThr-231 scFv by applying selection pressure to enhance the affinity (Fig. 3a). We generated a random mutation library of the pThr-231 scFv using error-prone polymerase chain reaction (PCR) (43) followed by electroporation in yeast (44), yielding ~3 × 10^7 transformants. Three clones displaying the scFv variants were subjected to three rounds of fluorescence-activated cell sorting (FACS) (Fig. 3a). After rounds 1 and 2, the sorted scFv sequences were further diversified using DNA shuffling followed by random mutagenesis (Fig. 3a). FACS process, selection pressure for higher affinity was applied by incubating yeast cells displaying pThr-231 mutants with progressively lower concentration of Pphos-b (Fig. 3a) followed by incubation with 1 μM unlabeled phosphopeptide (Pphos) for dissociation (45) and sorting for high Pphos-b binding versus scFv expression (using anti-c-Myc antibody) (Pphos) for dissociation (45) and sorting for high Pphos-b binding versus scFv expression (using anti-c-Myc antibody) (Pphos) for dissociation (45) and sorting for high Pphos-b binding versus scFv expression (using anti-c-Myc antibody) (Pphos) for dissociation (45) and sorting for high Pphos-b binding versus scFv expression (using anti-c-Myc antibody) (Pphos) for dissociation (45) and sorting for high Pphos-b binding versus scFv expression (using anti-c-Myc antibody). After three rounds of FACS, a population of high Pphos-b was enriched (Fig. 3c). We sequenced individual clones from this pool and identified 27 unique scFv mutant sequences (Fig. 4). Although all 27 clones had distinct sequences with mutations throughout the scFv sequence, several high-occurrence mutations were found (Fig. 4). The majority of frequent mutations (residues with occurrence labeled in bold in Fig. 4) were found in or near the CDRs (Kabat numbering): V_{H}W50L and V_{L}S66C (L2), V_{H}A95T, V_{L}G100,D (or S), V_{H}D101G, and V_{L}A102T.
Characterization of affinity and specificity of scFv mutants

To assess the effectiveness of the directed evolution, we characterized the clones isolated after three rounds of sorting for their affinity and specificity. For these analyses, we chose the first 14 unique clones identified from sequencing (clones 3.1–3.14; Fig. 4). To rapidly compare the affinity with that of the wildtype (WT) pThr-231 scFv, we incubated yeast cells displaying mutant scFvs with 2 nM Pphos-b, which was near the $K_d$ of the WT pThr-231 scFv. At this peptide concentration, seven of 14 clones showed significantly higher binding (normalized to the scFv expression level) compared with that of the WT scFv (Fig. 6a; $p < 0.05$, Dunnett’s multiple comparisons test). Based on the binding/expression level, we chose scFv mutant 3.05 (containing six mutations; Fig. 4) and measured its affinity by displaying it on yeast and generating a titration curve for the Pphos-b binding (Fig. 6b). scFv 3.05 showed a 21-fold improvement in affinity over that of the WT pThr-231 scFv, reaching picomolar monovalent binding affinity ($K_d = 92 \pm 1$ pM) (Fig. 6b). To characterize the phosphospecificity of the 14 mutant clones, we first measured the individual binding to Pnonphos-b and Pphos-A488 (second column), Psram-b and Pphos-A488, or Pphos-b and Pphos-A488, all at 0.5 μM. When we assessed the specificity of the high-affinity scFv 3.05 using competitive binding between Pphos-A488 and Pnonphos-b, significant binding to Pnonphos-b was detected (Fig. S4). Taken together, these results suggested that some of the clones gained affinity by stabilizing...
the interaction to the target peptide sequence, whereas none of the clones gained affinity to the phosphate group alone to cause cross-reactivity toward Pscram-b. Conversely, the fact that some of the clones did not show significant binding to Pnon-phos-b (Fig. 6c) suggested that improving affinity did not necessarily lead to enhanced cross-reactivity toward the nonphosphorylated epitope. To identify an optimal clone with improved affinity and high specificity, we selected additional clones based on the sequence homology and occurrence of mutations (Fig. 4) and assessed their binding to Pphos-b, Pnonphos-b, and Pscram-b. From this characterization, we identified scFv 3.24 (containing five mutations) that showed 11-fold improvement in binding affinity over the WT pThr-231 (Kd = 50 pM) and no significant change in binding to Pnonphos-b compared with that of WT pThr-231 (Fig. 6f). These results indicate that, in phosphospecific antibodies, applying selection pressure toward improved affinity does result in cross-reactivity in a significant fraction of clones. Therefore, high-affinity clones need to be checked for phosphospecificity to improve the binding affinity through directed evolution.

Cell and tissue section labeling using purified scFvs

High-affinity and -specificity reagents are expected to enable sensitive detection of tau phosphorylation in complex samples. We tested the performance of the scFvs in immunocytochemistry and immunohistochemistry experiments. To perform the labeling experiments, we cloned the WT scFv and mutant 3.24 into a vector containing a synthetic secretion signal to secrete them using yeast Saccharomyces cerevisiae. The secreted scFvs were purified using a hexahistidine motif at the C terminus (Fig. S5). When we resolved the purified scFvs using SDS-PAGE followed by Coomassie staining, the gel loaded with purified scFvs showed a single protein band with a molecular mass of 28 kDa, which is typical for scFvs, with ~92% purity as quantified by densitometry of the gel (Fig. S5). To enable direct imaging of the scFvs without the use of secondary reagents, the purified scFvs were fluorescently labeled with Alexa 594 through modification of lysine residues and purified. To assess the specificity, the fluorescent scFvs were used to label human embryonic kidney cells (HEK293FT) transiently expressing human tau (microtubule-associated protein tau 0N4R) as a GFP fusion (EGFP-tau) (48). When high concentrations (100 nM) of the WT pThr-231 scFv or scFv 3.24 were used for staining, clear scFv signal was detected in HEK293FT cells expressing WT tau (Fig. 7a, top row) but not to the same cells treated with A-phosphatase (Fig. 7a, second row). These results indicate that the binding of WT pThr-231 scFv and mutant 3.24 requires phosphorylation of tau, and as reported previously, the tau expressed in HEK293 cells is phosphorylated (49, 50). To further assess the specificity of the scFvs, we generated point mutants of human tau at the target phosphorylation site (Thr-231) either incapable of phosphorilation (T231A) or pseudophosphorylated (T231E). In HEK293FT cells expressing EGFP-tau T231A, both the WT pThr-231 scFv and mutant 3.24 showed no detectable binding (Fig. 7a, third row), whereas in the same cells expressing EGFP-tau T231E, both scFvs showed clear binding (Fig. 7a, bottom row). These results indicate that the scFv binding requires tau phosphorylation at Thr-231. When we compared the labeling of WT and scFv 3.24 at lower concentrations, scFv 3.24 showed a significantly higher binding signal above background compared with that of WT at scFv concentrations ranging from 80 pM to 50 nM (Fig. 7b; p < 0.05, Student’s t test).

We proceeded to test the fluorescently labeled scFvs in the staining of tissue sections from both PS19 mouse tissue slices and human AD tissue slices; PS19 mice express the aggregation-prone mutant P301S of human tau (1N4R) under the...
mouse prion protein (Prnp) promoter (51). In brain slices of 8-month-old PS19 mice, WT pThr-231 scFv and mutant 3.24 demonstrated staining patterns characteristic of neurofibrillary tangles (Fig. 8a), suggesting specific labeling of tau. This staining was dose-dependent for both scFvs (Fig. 8b). Neither scFv showed detectable staining in either tau knockout or WT C57Bl/6 mice (Fig. 8c). Staining of human AD tissue also revealed characteristic tangle staining (Fig. 8d) with no detectable staining in aged control brain tissues. However, in the human tissues, the mutant scFv 3.24 showed significantly higher signal than the WT scFv at the 50 nM working concentration (Fig. 8e), suggesting higher affinity and more robust staining for the mutant scFv. These results further demonstrate that the high-affinity antibody generated in this study has exceptionally high specificity for phosphotau.

**Discussion**

In this study, we applied directed evolution to enhance the affinity of a phosphospecific antibody and quantified the impact of *in vitro* affinity maturation on specificity. High-affinity antibody fragments with dissociation constants reaching the picomolar range were successfully generated. Based on the crystal structure of the WT pThr-231 antibody bound to the phosphopeptide (27), only one mutation occurred in a residue...
that directly contacts the phosphoepitope (Fig. 5: V1W50L). Five of nine high-occurrence mutations were located near the direct contact sites (V1S56C, V1A95T, V1G100H, D (or S), V1D101G, and V1A102T). The pattern of mutations occurring in amino acid positions near but not at the direct contact site has been frequently observed in high-affinity antibody variants (52, 53). Such mutations may contribute to enhanced affinity through preorganization and rigidification of the paratope (54–56). Mutations V1G100H, D, V1D101G, and V1A102T are also located at the V1-V2 interface, suggesting that stability of interaction between the two domains may contribute to affinity improvement. Interestingly, high-occurrence mutations found in the optimal variant 3.24 (V1D57G, V1G74C, and V1A93V) were exclusively located in the framework region. This suggests that this variant may have gained affinity through optimization of structural plasticity (57, 58). Further investigation of the impact of individual mutations on affinity and specificity may provide new insights into designing PTM-specific antibodies.

High-affinity reagents would be highly advantageous in detecting PTMs from complex samples. Our results show that the high-affinity variant scFv generated significantly higher signal compared with that of the WT scFv in detecting tau from human tissue but not in mouse tissue. The discrepancy in staining intensities may be explained by differences in the expression level of human tau and level of phosphorylation. In the transgenic PS19 mice, the expression of human tau P301S is driven by the prion protein promoter, which is strongly induced in neuronal cells (59), leading to 3–5-fold higher levels of tau expression than endogenous levels (51). Moreover, in PS19 mice, the solubility of tau decreases by 3 months of age, and it becomes hyperphosphorylated (51, 60). Therefore, the level of insoluble aggregates of phosphorylated tau in mouse tissue may be significantly higher than in human AD patient tissue. Additionally, in the mouse tissue, the labeling intensity was significantly diminished at a scFv concentration of 10 nM, suggesting a possibility for mass transfer limitation of the labeling reagent. Very slow or limited antibody penetration has been reported for fixed samples (61). This shows the need for high antibody concentrations in labeling fixed tissue and highlights the importance of antibody specificity.

Although the scFv identified in this study can be converted to full-length IgGs, the scFv construct provides unique advantages for detecting tau due to its small size. Compared with full-length IgGs (150 kDa), scFvs are approximately one-sixth the molecular mass (25 kDa). This has led to significant enhancement in processes that rely on diffusion, such as tissue penetration. For example, when the blood–brain barrier was transiently opened through focused ultrasound, scFvs showed 5–57-fold higher uptake into the central nervous system compared with IgGs (40). In a mouse model of tauopathy, intravenously injected scFvs were detected in the brain tissue in significantly higher levels compared with that of IgGs (37). Moreover, scFv constructs enable wider modes of delivery because they are encoded by a short single gene that can be packaged in viruses. For example, intracerebroventricular injection of adeno-associated viruses carrying an anti-tau scFv resulted in reduced tau accumulation in a mouse model of tauopathy (39). Finally, we demonstrated cell and tissue staining using scFvs directly labeled with a fluorophore. These reagents have the potential to enable subdiffraction-limited imaging of phosphotau due to greatly reduced linkage error, which limits image resolution (62).

Phosphorylation of tau at Thr-231 is a widely recognized modification for its association with AD. In vitro and in cultured cells, pThr-231 decreases the ability of tau to bind and stabilize microtubules (63, 64). pThr-231 seems to occur early in AD progression and precedes paired helical filament formation (65). pThr-231 was found in the cerebrospinal fluid (CSF) of patients with mild cognitive impairments who eventually developed AD (66). In addition, levels of pThr-231 in the CSF were correlated with rates of hippocampal atrophy in AD patients (67). An immunobassay of pThr-231 in the CSF demonstrated an 85% sensitivity (detected in 23 of 27 AD samples) and a 97% specificity (detected in one of 31 non-AD samples) in AD brain extracts (68). The high-affinity and specificity pThr-231 tau antibody developed in this study is expected to enhance the sensitivity and enable earlier detection of pThr-231.

A major finding of this study is that in vitro affinity maturation of the phosphospecific antibody led to an increase in unwanted cross-reactivity toward the nonphosphorylated target sequence in a majority of high-affinity variants. Considering the fact that the area of interaction available from a modified amino acid is limited, stabilization of the interaction with the nearby amino acid sequence may be a common phenomenon in high-affinity PTM-specific antibodies. In a recent study, monoclonal antibodies generated in guinea pig against pThr-18 of p53 showed picomolar affinity (Kd = 0.20 nM) but also significant binding to the nonphosphorylated p53 (Kd = 130 nM) (69). If such an antibody is used at concentration ranges commonly

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**Figure 5. High-occurrence mutations identified from the screen mapped on the WT pThr-231 structure.** Heavy chain is shown in green, and light chain is shown in red and labeled with the WT amino acid. Boxed amino acids indicate those mutated in scFv 3.24. The structure was generated using CCP4MG, based on Protein Data Bank code 4GLR.
used for cell or tissue staining (10–50 nM), false-positive signal from cross-reactivity toward the nonmodified target may be detected. This illustrates an important point that antibody specificity is a function of antibody concentration. Therefore, existing PTM-specific antibodies should be validated for their specificity under varying concentrations. We suggest setting an upper concentration limit for each PTM-specific antibody to prevent false-positive detection. Until now, efforts to validate and enhance specificity have rarely been made. Nonspecific antibodies would be a major factor in irreproducibility in biological experiments. Ongoing efforts to standardize ways to quantify and document specificity of binding reagents (70) need to consider these aspects of antibody specificity.

We introduced an approach using yeast surface display to quantify the specificity of PTM-targeting antibodies. This enabled us to find that, even among antibody variants selected only for improved affinity, a fraction of clones maintained high specificity. This specificity quantification approach may be widely adopted due to the general applicability of yeast surface display and its compatibility with flow cytometry. We demonstrated the use of this approach for a phosphospecific antibody, but it is readily applicable to other PTM-specific antibodies. In principle, this approach would be suitable for assessing the specificity of affinity reagents in general. The identified high-affinity variant showed exceptional specificity as validated by cell- and tissue-staining experiments. Because of the compatibility with FACS, this approach has potential to enable directed evolution of antibody specificity. In particular, it would be useful in engineering the specificity of antibodies with known cross-reactivity. For example, a recent study showed that a significant fraction of commercially available PTM-specific tau antibodies has cross-reactivity (34). Directed evolution may be applied to reduce the cross-reactivity of these antibodies.

The results from this study demonstrate that in vitro affinity maturation is a viable option for improving the affinity of PTM-specific antibodies. In this process, it is essential to quantify the specificity, particularly to the nonmodified target sequence. By
quantifying the specificity, it is possible to apply selection pressures for improved affinity and specificity simultaneously.

Experimental procedures

Yeast surface display of pThr-231 scFv

The amino acid sequence of VL and VH from an anti-tau pThr-231 antibody (27) was back-translated to the DNA sequence, yeast-codon optimized, and synthesized (Integrated DNA Technologies). A (G4S)3 linker was inserted between the VL and VH sequences (5′/H11032-VL-linker-VH) to create a fusion construct encoding an scFv sequence. The synthesized scFv gene was cloned using PCR to add a 5′/H11032-NheI and a 3′/H11032-BsrGI (New England Biolabs) restriction site. The PCR products were ligated into the vector pCT4RE (35) (a gift from Dr. Eric Shusta). This plasmid included an N-terminal c-Myc epitope tag and a C-terminal FLAG epitope tag. The pCT4RE plasmid was transformed into S. cerevisiae EBY100 (35) (a gift from Dr. Dane Wittrup).

Figure 7. Cell labeling using purified pThr-231 scFvs. a, confocal microscope images of purified, fluorescently labeled WT pThr-231 scFv and mutant 3.24 binding to HEK293FT cells expressing WT tau (top row), WT tau treated with lambda phosphatase (Lambda PP) (second row), tau T231A (third row), and tau T231E (bottom row). All human tau constructs were fused to EGFP at the N terminus (EGFP-tau). The first column shows DAPI staining in blue. The second column shows tau expression in green. The third column shows scFv binding in red. Scale bars, 50 µm. b, quantification of the fluorescence intensity of WT pThr-231 scFv and mutant 3.24 binding to HEK293FT cells expressing WT tau at scFv concentrations from 16 pM to 50 nM. Error bars indicate S.D. from three independent experiments. Statistics for b, Student’s t test; *, p < 0.05. MAPT, microtubule-associated protein tau.

Peptide modification and purification

All peptides used were purchased from a commercial source (Peptide 2.0). Peptides contained a C-terminal cysteine for chemical modification. Peptides to be modified were dissolved at 1 mM in phosphate-buffered saline (PBS; 8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter Na2HPO4, 0.24 g/liter KH2PO4, pH 7.4) at room temperature. A 10 mM stock solution of Alexa Fluor 488 C5-maleimide (Thermo Fisher Scientific) was prepared in deionized H2O, and a 100 mM stock solution of EZ-Link maleimide-PEG11-biotin (Thermo Fisher Scientific) was prepared in dimethyl sulfoxide (DMSO). The Alexa Fluor 488 C5-maleimide stock solution was protected from light by wrapping the container with aluminum foil. For peptide modification, either Alexa Fluor 488 C5-maleimide or EZ-Link maleimide-PEG11-biotin stock solution was added to the peptide PBS solution at a final reagent concentration of 2 mM. The reactions proceeded at 4 °C overnight in the dark.
Modified peptides were purified using an HPLC system (Shimadzu SCL-10Avp) equipped with a preparative column (Waters, Atlantis T3, 100 Å, 10 μm, 10 × 250 mm). For the mobile phase, solvent A consisted of 99.9% H2O and 0.1% TFA, and solvent B consisted of 99.9% methanol and 0.1% TFA. The solvent flow rate was 3.5 ml/min. The column temperature was set at 50 °C. For each HPLC run, 100 μl of the peptide reaction mixture was injected. The gradient for peptides was as following: 0% solvent B at 0 min, 20% solvent B at 1 min, 40% solvent B at 26 min, 75% solvent B at 31 min, and 100% solvent B at 36 min. Purified peptide fractions, which were detected at 214 nm, were collected, lyophilized, and stored at 20 °C.

Mass spectrometry of the modified peptides was conducted using a QStar Elite Hybrid Q-TOF mass spectrometer (AB Sciex). TOF spectra (300–2000 m/z) were acquired using Analyst QS 2.0 software.

Directed evolution of the pThr-231 scFv

pThr-231 scFv was randomly mutagenized as described previously (71) with the following modifications. Error-prone PCR was performed using a mixture of triphosphates of nucleoside analogs. Each PCR consisted of ~250 ng of plasmid template, 0.5 μM forward and reverse primers, 200 μM dNTP mixture (Thermo Fisher Scientific), Taq PCR buffer (without MgCl2), 2 mM MgCl2, 2.5 units of Platinum Taq DNA polymerase (Thermo Fisher Scientific), 2 μM 8-oxo-dGTP (Tri-Link Biotechnologies) and 2 μM dPTP (TriLink BioTechnologies) in a 50-μl reaction volume. The mixture was denatured at 94 °C for 3 min followed by 19 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 60 s and a final extension of 72 °C for 10 min on a thermocycler. The PCR product was gel-purified using a gel extraction kit (Zymo Research). 2 μl of purified mutagenesis product was added to a 100 μl of PCR solution without using nucleoside analogs to amplify the mutated fragments. About 6.4 μg of mutated pThr-231 scFv gene and 1 μg of vector pCT4RE (a gift from Dr. Eric Shusta) were combined with 200 μl of electrocompetent EBY100 (a gift from Dr. Dane Wittrup) and electroporated at 2.5 kV and 25-microfarad capacitance using a GenePulser (Bio-Rad; 0.2-cm electrode gap cuvette). The electroporated cells were grown in SD-CAA medium (0.1
m sodium phosphate, pH 6.0, 6.7 g/liter yeast nitrogen base, 5 g/liter casamino acids, 20 g/liter glucose) at 30 °C and 250 rpm. Frozen aliquots of the library were prepared by freezing cells (10× library diversity) in yeast freezing medium (2% glycerol, 6.7 g/liter yeast nitrogen base) at −80 °C for long-term storage.

The following rounds of gene diversification were realized by shuffling the library screened in the previous round followed by the above random mutagenesis procedure to incorporate additional mutations. The plasmid DNA from the library pool was the above random mutagenesis procedure to incorporate additions.

**Purification of pThr-231 scFvs**

The pThr-231 scFv gene was inserted into the vector pRS316 (a gift from Dr. Eric Shusta), which had been restriction-digested with Nhel and BsrGI (New England Biolabs). The pRS316 plasmid was transformed into S. cerevisiae YVH10 cells (72) (a gift from Dr. Eric Shusta) for protein secretion and purification.

Cells were grown in 500 ml of SD-CAA plus tryptophan medium at 30 °C and 250 rpm for 3 days, pelleted, and resuspended in 500 ml of SG-CAA (0.1 m sodium phosphate, pH 6.0, 6.7 g/liter casamino acids, 20 g/lITER galactose) plus tryptophan with 0.1% BSA (Sigma-Aldrich) at 20 °C and 250 rpm for 3 days to induce and secrete the scFvs. The cells were pelleted, and supernatant containing scFv was collected and filtered using a bottle-top filter (Corning, polyethersulfone membrane, 0.22 μm) to remove the yeast cells and cell lysate. The scFv supernatant was first concentrated to 50 ml by ultrafiltration using a selective permeable membrane (MilliporeSigma, 10 kDa) under nitrogen pressure. The concentrated supernatant was then dialyzed to exchange buffer in 5 litters of PBS (0.01 m sodium phosphate, 0.15 m NaCl, pH 8.0) at 4 °C overnight. The dialyzed scFvs solution was purified as described previously (46) using SuperFlow nickel-nitrotriacetic acid beads (Qiagen). The purified scFvs were resolved using SDS-PAGE in Tris-glycine running buffer (14.4 g/liter glycine, 1 g/liter SDS, 3.03 g/liter Tris base) with 12% Bis-Tris gels. SeeBlue® prestained protein standard (Thermo Fisher Scientific) was used to determine the molecular weights.

**Fluorescent tagging of purified pThr-231 scFvs**

The scFv elution fractions were pooled and concentrated using a centrifugal filter unit (MilliporeSigma, Amicon Ultra, 10-kDa molecular mass cutoff). The concentrated scFvs were diluted to 3 mg/ml in 0.1 m sodium bicarbonate buffer, pH 8.3. The amine-reactive dye Alexa Fluor 594 NHS ester (Thermo Fisher Scientific) was prepared in dimethylformamide at 10 mg/ml immediately before use. 35 μg of Alexa Fluor 594 NHS ester from the stock solution was added to 100 μg of purified scFv. The reaction mixture was incubated for 1 h at room temperature in the dark. Unreacted dye was removed using Zeba desalting columns (Thermo Fisher Scientific, 7-kDa molecular mass cutoff), and the concentrations of modified scFvs were determined using a Bradford assay (Thermo Fisher Scientific).

**Immunocytochemistry in HEK293 cells**

The HEK293 cells were transfected with pRK5-EGFP-tau (Addgene 46904), which contained human tau (microtubule-associated protein tau 0N4R) fused to EGFP at the N terminus. Cells were rinsed once with 0.5 ml of ice-cold PBSCM (0.15 m NaCl, 1.9 mM Na2HPO4, 8.1 mM NaH2PO4, 1 mM CaCl2, 0.5 mM MgSO4, pH 7.4), fixed in 4% paraformaldehyde (Sigma) in PBSCM at room temperature for 15 min, and then permeabilized using 0.1% Triton X-100 (Acros Organics) in PBSCM at room temperature for 15 min. After washing three times with PBSCM, the cells were incubated with 0.1 μM Alexa Fluor 594–conjugated scFv in 0.5 ml of PBSCM with 40% goat serum at 4 °C overnight in the dark. The cells were washed three times with 0.5 ml of ice-cold PBSCM, stained with 0.3 μM DAPI (Thermo Fisher Scientific) at room temperature for 5 min, and washed once with 0.5 ml of ice-cold PBSCM. The cells were imaged using a Nikon A1R confocal microscope (University of Connecticut Center for Open Research Resources and Equipment).

**Immunohistochemistry in brain tissue sections**

All mouse (n = 3 PS19, n = 3 WT C57BL6/J), and n = 3 tau knockout) and human (n = 2 AD cases and n = 2 aged control) tissues were sectioned at 30 μm and stained as free-floating sections using Netwell baskets (VWR catalog number 29442-132) in 12-well Falcon plates (VWR catalog number 353043).

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**High-affinity and -specificity phosphotau antibody**

The HEK293 cells were transfected with pRK5-EGFP-tau (Addgene 46904), which contained human tau (microtubule-associated protein tau 0N4R) fused to EGFP at the N terminus. Cells were rinsed once with 0.5 ml of ice-cold PBSCM (0.15 m NaCl, 1.9 mM Na2HPO4, 8.1 mM NaH2PO4, 1 mM CaCl2, 0.5 mM MgSO4, pH 7.4), fixed in 4% paraformaldehyde (Sigma) in PBSCM at room temperature for 15 min, and then permeabilized using 0.1% Triton X-100 (Acros Organics) in PBSCM at room temperature for 15 min. After washing three times with PBSCM, the cells were incubated with 0.1 μM Alexa Fluor 594–conjugated scFv in 0.5 ml of PBSCM with 40% goat serum at 4 °C overnight in the dark. The cells were washed three times with 0.5 ml of ice-cold PBSCM, stained with 0.3 μM DAPI (Thermo Fisher Scientific) at room temperature for 5 min, and washed once with 0.5 ml of ice-cold PBSCM. The cells were imaged using a Nikon A1R confocal microscope (University of Connecticut Center for Open Research Resources and Equipment).

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**High-affinity and -specificity phosphotau antibody**

Extracted mouse tissues were flash frozen on dry ice, sectioned onto slides (Millenia 2.0 Adhesion Slides, catalog number 318), and fixed in cold 4% paraformaldehyde for 3 min prior to staining. Human tissues were fixed and stored in periodate-lysine-paraformaldehyde until sectioned using a Leica VT1200S vibrotome. To quench lipofuscin autofluorescence, human tissue sections were photobleached under a 1500-lumen white light-emitting diode bulb for 72 h at 4 °C while suspended in PBS with no lid or covering (73). Following mouse tissue fixation and human tissue bleaching, all sections were then washed three times in PBS for 30 s per wash followed by a 5-min incubation in detergent medium (Tris-buffered saline with 0.25% Triton X-100). Tissue was then incubated for 30 min in citrate-based antigen unmasking solution (Vector Laboratories, catalog number H-3300) at 95 °C. Tissue was blocked with gentle rotation for 2 h in detergent medium with 5% donkey serum (Sigma-Aldrich, D9663, 10 ml). After blocking, free-floating human tissue sections were moved into separate wells of a 24-well plate with 300 µl of the appropriate scFv dilution and no basket; mouse tissue was stained directly on the slides with 200 µl of the appropriate scFv dilution. Tissues were incubated with the scFVs for 3 h at room temperature with gentle rotation.

Following scFv incubation, tissue sections were incubated with 1 µg/ml DAPI in the dark for 5 min at room temperature. Tissues were then washed four times in PBS for 5 min each and carefully placed on Millenia 2.0 slides using a histology brush if free floating (Fisher Scientific, catalog number NC0344756). Excess water was allowed to evaporate for 15 min, and then each slide was mounted with 100 µl of Prolong Gold Antifade Reagent (Thermo Fisher, catalog number P36930) and a 1-mm coverslip. Imaging was done using a Zeiss LSM 710 confocal microscope, ensuring equal parameters for digital gain (790), pinhole (30 µm), and laser power (4.4%) for all images. Following imaging, images were processed using the Fiji distribution of ImageJ. The magic wand tool was used to select individual tangles as a region of interest, which was then measured for digital gain (790).

**Author contributions**—D. L. and Y. K. C. conceptualization; D. L. and B. F. M. data curation; D. L. and B. F. M. formal analysis; D. L., L. W., B. F. M., and Y. K. C. investigation; D. L., L. W., and Y. K. C. methodology; D. L., L. W., B. F. M., X. Y., B. W., and Y. K. C. writing—original draft; D. L., L. W., B. F. M., X. Y., B. W., and Y. K. C. writing—review and editing; L. W., B. F. M., B. W., and Y. K. C. validation; B. F. M. visualization; B. W. and Y. K. C. supervision; Y. K. C. funding acquisition; Y. K. C. project administration.

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