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A new versatile immobilization tag based on the ultra high affinity and reversibility of the calmodulin-calmodulin binding peptide interaction

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Abstract

Reversible, high affinity immobilization tags are critical tools for myriad biological applications. However, inherent issues are associated with a number of the current methods of immobilization. Particularly, a critical element in phage display sorting is functional immobilization of target proteins. To circumvent these problems, we have used a mutant (N5A) of calmodulin binding peptide (CBP) as an immobilization tag in phage display sorting. The immobilization relies on the ultra high affinity of calmodulin to N5A mutant CBP (RWKKNFIAVSAANRFKKIS) in presence of calcium (K_D ~ 2pM), which can be reversed by EDTA allowing controlled “capture and release” of the specific binders. To evaluate the capabilities of this system, we chose eight targets, some of which were difficult to overexpress and purify with other tags and some had failed in sorting experiments. In all cases, specific binders were generated using a Fab phage display library with CBP fused constructs. K_D of the Fabs were in sub to low nanomolar (nM) ranges and were successfully used to selectively recognize antigens in cell-based experiments. Some of these targets were problematic even without any tag, so the fact that all led to successful selection endpoints means that borderline cases can be worked on with a high probability of positive outcome. Taken together with examples of successful case specific high level applications like generation of conformation, epitope and domain specific Fabs, we feel that the CBP tag embodies all the attributes of covalent immobilization tags, but does not suffer from some of their well documented drawbacks.

Graphical Abstract

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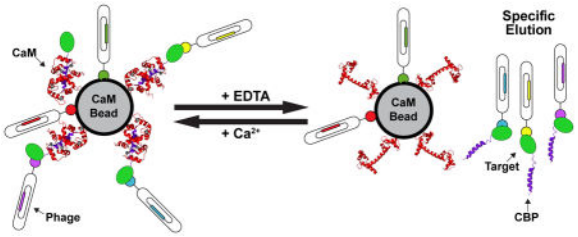

Keywords

phage display; recombinant antibodies; “capture and release”; conformation-specific; epitope binning

Introduction

High performance affinity tags play indispensable roles in almost all areas of biological research. Affinity tags are used in a variety of experiments including affinity purifications, western blots, immunoprecipitations, flow cytometry, ELISAs, directed evolution experiments, biophysical measurements, immunofluorescence and many other biological applications. There is no tag that is universal; they all have their own strengths and weaknesses depending upon the application. Some of them like His [1,2]-, FLAG [3],- cMyc [4]- and V5 [5]-tags have broader versatility, while others like Avi-tag [6], Fc-fusion tag [7] and ZZ tag [8] are designed to perform a specific function with high fidelity. They cover a spectrum of characteristics from which a researcher can tune the tag for desired properties and ease of use. Some among these, for instance the purification tags, are purposed to be reversible, while others are designed to form virtually covalent interactions with the target substrate. Thus, in most cases, the researcher is faced with trade-offs that need to be weighed before properly matching the tag with the application that it is being used for.

A case in point is tags that are used for antigen immobilization during directed evolution display experiments, particularly phage display. Phage display library sorting requires immobilization of the target antigen on a solid support in its native functional form [9] followed by vigorous washing steps to eliminate the undesired non-specific binding effects. Purification tags like His- or FLAG-tags are too weak to make them viable candidates [1, 2, 3]. For phage display the most commonly used are tags that are based on the virtually irreversible biotin-streptavidin interaction [10, 11, 12]. This requires biotinylating the protein antigen either through chemical modification of the amine and sulfydryl groups of amino acid residues or via an enzymatic biotinylation tag (Avi-tag), commonly introduced either at N- or C- terminus of the expression construct [12]. However, both these approaches have their shortcomings.

A frequently encountered issue with chemical biotinylation is that the process has to be very carefully optimized for each of the target as over-biotinylation drastically changes the surface properties of the target and often leads to partial denaturation and loss of activity of the targets. Moreover, the choice of the biotinylation reagent is especially crucial for certain classes of targets, especially for enzymes containing cysteine or lysine, which are the

primary sites for chemical biotinylation. Harsh elution conditions with either high or low pH are generally used to recover phages bound to immobilized targets chemically biotinylated using non-cleavable biotiylation reagents [13]. These non-specific elution techniques risk the enrichment of background binders, thereby reducing the chance to obtain the desired specific clones. Some of the commercially available cleavable biotinylation reagents utilize the reduction of disulfide bond necessitating the storage and handling of the biotinylated proteins in an oxidizing environment. This is detrimental for proteins that are rich in surface exposed and catalytic cysteine residues that require the presence of reducing agent in storage conditions and such non essential reactive cysteine residues must be eliminated by mutation or chemical modification before chemical biotinylation [14].

An alternate to the chemical approach is enzymatic biotinylation where the protein is selectively biotinylated at a specific lysine residue in the sequence of Avi-tag that is specifically recognized by the biotin ligase [15, 16]. Using the Avi-tag approach requires an enzymatic step for the addition of biotin, a process that can be done either *in-vivo* or *in-vitro*. But its downside is that it can lead to aggregation and solubility issues as well, which is not surprising considering the hydrophobic nature of biotin moiety. Moreover, the effectiveness of *in-vivo* (when target co-expressed with biotin ligase) biotinylation is very case specific.

Considering all the limitations associated with the current methods of immobilization of target proteins in phage display sorting experiments, there remains a need for alternate approaches that maintain the attributes of biotin associated tags, while circumventing their shortcomings. Any alternate immobilization technique should retain the structural integrity and functional properties of the targets and not affect their expression and purification. Further, the tag should be reversible, and have a high affinity to the immobilization substrate allowing enrichment of the desired clones over “background” binders and thus improve the efficiency of phage display library sorting process. Based on extensive development and testing, we propose the use of an engineered N5A mutant of a nineteen residue (RWKKNFIAVSAANRFKKIS) Calmodulin Binding Peptide (CBP) as a C-terminal fusion tag that meets the demanding criteria required for a user-friendly and versatile alternative to biotin-based tags. We present here a comprehensive evaluation of the CBP using model systems drawn from examples that, in our hands, had proven problematic in the context of biotin-based tags. We show that the CBP tag does not adversely affect expression of the target to which it is fused, it has sufficient affinity to survive vigorous washing steps required during the sorting process, can be completely released from the immobilization substrate (calmodulin) by simple addition of EDTA and it performs uniformly on virtually all targets compared to the target specific variability of the biotin-based tags.

Results

Target Set

For model systems, we selected a diverse test set of antigen targets that ranged in size, stability and chemical makeup to evaluate the capabilities of the CBP immobilization tag for use in multiple phage display applications. Maltose binding protein (MBP) was chosen as a positive control since we have extensive experience with its properties and have generated numerous MBP-specific Fabs using a variety of tags allowing direct comparisons with other

immobilization strategies. Our experience is that proteins with free cysteines are particularly challenging because they are prone to modification. Thus, we have included SETD7 (5 cysteine residues), HEF1 (4 cysteine residues), GAP1 (single catalytic cysteine) in the test set. We note that alternate immobilization methods like chemical biotinylation, which uses a reagent (NHS-PEG_4-S-S-biotin) that adds a biotin group cleavable by reducing agent is not a feasible option because the samples always need to be preserved in a reducing environment containing DTT or TCEP. Some of the chosen targets (HEF1, bromo domain from *D. melanogaster*, PGK from *S. aureus*) are prone to aggregation after chemical biotinylation. Some targets in the set are small domains of multidomain proteins that are challenging to obtain binders for. The ligand binding domain of human estrogen receptor (ESRRA) and HEF1 are targets whose expression and solubility were significantly increased as CBP fused constructs over the Avi-tagged ones. A MBP fused construct of Avi-HEF1 was more soluble than Avi-HEF1, but failed in sorting experiments enriching for non-specific binders.

CBP enhances solubility of aggregation prone proteins

To our observation, the constructs of domains of various transcription factors, the ligand binding domain of hormone receptors and zinc fingers in the context of hexa-His fusions were soluble, but the adding of an Avi-tag created significant solubility issues. This was the case irrespective of where the Avi-tag was positioned (N or C-terminal) and under all conditions of IPTG induction and temperature. Thus, it was apparent that to approach these types of targets, a different type of immobilization strategy that did not negatively impact the solubility of target antigen was required.

We speculated that the polar nature of CBP tag might actually enhance the solubility of these aggregation prone constructs. To test this, we cloned various constructs into a modified pET28a vector (Fig. 1a). The CBP was placed at the C-terminus upstream of the hexa-His tag in preference to N-terminal placement. Placement of His-tag downstream of CBP and subsequent purification by Ni-NTA chromatography precludes the chance of losing the peptide due to any proteolysis of the immobilization tag. For most of the constructs ZSCAN29 (Q8IWY8), TSC22D4 (Q9Y3Q8), ESRRA (P11474), VENTX (O95231), MAFF (Q9ULX9), LIN28A (Q9H9Z2), JARID2 (Q92833) and NEDD9 (Q14511) the amount of the overexpressed protein significantly increased in the soluble fraction compared to that of Avi-tagged ones. Some of them were quantitatively expressed in the soluble fraction with little material residing in the insoluble pellet (Fig. 1b). The high solubility of the proteins allowed us to purify the proteins in substantial yield from the soluble fraction by IMAC (Ni-NTA) followed by size exclusion chromatography (SEC) (Supplemental Fig 1.). The SEC profiles of the purified proteins showed a mono-dispersed peak lacking any soluble aggregates (Fig. 2a). Additionally, CBP did not appear to compromise the overall yield of the protein. The structural integrity of the proteins was also unaltered in contrast to chemical biotinylation as judged by thermal stability studies (Fig. 2b). This was a significant improvement over the purification of insoluble Avi-tagged constructs where the proteins have to be recovered from the inclusion bodies by denaturation and subsequent refolding to their native state.

CBP-tagged targets are effectively immobilized on CaM coated beads

A pull-down assay was employed to ascertain the level of immobilization of the CBP tagged proteins on CaM (calmodulin) coated beads. Notably, we extensively profiled the quality of the CaM coated beads from the commercially available sources and unfortunately found them generally unreliable due to severely compromised binding capacity. While this required us to fabricate our own beads for the phage sorting experiments, the procedure was straightforward as described in Materials and Methods. CBP-tagged MBP (CBP-MBP) was incubated with SA (streptavidin) beads pre-coated with Avi-tagged CaM (Avi-CaM). The supernatant was recovered to evaluate the unbound fraction and the beads were washed well to eliminate any protein adhering nonspecifically to the beads. The bound protein was eluted with 10 mM EDTA. The amount of protein effectively immobilized and eluted was analyzed on SDS-PAGE by comparing the amount of protein used (input), unbound fraction, elution and beads after elution. We observed almost quantitative capture and elution in pull-down assay (Fig. 3a).

Vigorous wash steps of the target immobilized beads after phage binding are requisite for generating binders of high quality. To ascertain whether the interaction of N5A mutant CBP tag with CaM was stable enough to withstand the washing steps, we performed a single round of mock-phage sorting. Immobilized CBP-MBP on CaM coated beads and phages displaying a Fab fragment MOS1 that was known to bind tightly to MBP were used in the experiment. As a negative control, phage displaying a Fab fragment (SETA1) for an unrelated target was used. Four sets of mock-phage sorting were performed: i) with and ii) without Avi-CaM on the beads and iii) with and iv) without CBP-MBP. MOS1 and SETA1 were, respectively, incubated with and without target, captured with Avi-CaM coated magnetic beads, washed vigorously and eluted with 10 mM EDTA. The data showed that MOS1 phages were recovered only when both CBP-MBP and CaM coated beads were used (Fig. 3b). Essentially, no phages above background level were obtained from the three other control experiments. This result demonstrated that the interaction between CBP-target and CaM was stable enough to be maintained during the sorting process in conditions under which virtually all phage sorting experiments are typically performed.

CBP tagged targets can be used in selection campaign

Having established that the affinity of CBP was suitable as an affinity tag for phage display, we created a “doped” library to demonstrate that even at very low concentration, a specific binder can be isolated from a pool containing a very high concentration of non-specific Fab phage particles. Fab phage from MOS1 was mixed with SETA1 displaying phages at different ratios ($1{:}10^2$, $1{:}10^4$ and $1{:}10^7$). Sorting experiments using such libraries allowed us to determine the level of optimal stringency required to isolate specific binders from a milieu of non-specific ones. Sorting was carried out for four successive rounds immobilizing CBP-MBP on CaM coated beads and decreasing the concentration of the target in each round (100 nM, 50 nM, 10nM, 10nM). To monitor the actual enrichment of the phage sorting, the ratio of MOS1 to the background phage SETA1 was calculated based on titer values. Randomly picked clones from the second, third and fourth rounds were analyzed by PCR via a pair of primers specifically designed to amplify only the heavy chain fragment of MOS1 displayed on phage. Results were confirmed by DNA sequencing. The enrichment

factor and results of colony PCR are summarized in Table 1. There was 20-fold enrichment of MOS1 after third round in $1:10^7$, which increased to over 100-fold after the fourth round. From the number of positive clones from colony PCR, it was determined that although the original $1:10^7$ doped library contained only 0.00001% MOS1, its presence increased to 1% (one colony positive out of ninety six) after second round, 93% (ninety colonies positive out of ninety six) after third round and 100% (all colonies were positive) after the fourth. The large enrichment obtained demonstrates that this affinity tag makes the system suitable for real selection campaigns.

Generation and characterization of Fabs from CBP tagged targets

After the promising immobilization and significant enrichment in "doping" selection experiments, we explored the general effectiveness of the CBP tag in the context of high throughput phage sorting regimes using the set of antigens described above. For successful selections, the inherent antigenicity of CaM has to be addressed; otherwise the pool of target binders gets contaminated with CaM binders. The CaM binders can be readily eliminated by pre-clearing the phage library as described in Materials and Methods.

After four rounds of sorting with increasing stringency introduced round to round, the isolated binders were screened using a single point competitive phage ELISA assay. The binders were ranked based on their apparent affinities to cognate antigens and sequenced. The CDR-H3 and L3 sequences of the binders showed high diversity in terms of length and sequences indicating they represented a large unique pool (Table 2). These unique binders were reformatted into Fab protein format in expression vector pRH2.2, expressed in *E. coli* BL21 cells, and purified to homogeneity by affinity (protein A) followed by ion exchange (Resource S) chromatography.

From this pool, the Fabs generated are all very thermally stable having melting temperatures above 70 °C and they form a tight complex with the respective target as seen in analytical SEC (Fig. 4a). It is noteworthy that the antigen-Fab complexes are more thermally stable than the individual antigens indicating that the binding of the Fabs effectively stabilizes the antigens (Fig. 4b). The binding kinetics of the antigen-Fab interactions were determined by surface plasmon resonance using a Biacore 3000 instrument (Table 2). The vast majority of the binding constants (K_D) for these Fabs are in the sub to low nanomolar (nM) ranges across all target antigen types. The high affinity is mainly attributed to their significantly low k_{off} rates (Supplemental Fig. 2). This also demonstrates that the CaM-CBP has similar capabilities as the traditional Avi-tag- streptavidin system.

Some specific cases

To further compare the capabilities of the CaM-CBP capture approach, we designed some "high performance" phage sorting experiments that had proven challenging even for the biotin-streptavidin immobilization system. These experiments included generation of conformational and regio-specific binders and binders that can be used in most common biological applications like immunoprecipitation of the target from cell lysate in native condition and immunostaining.

Conformation specific Fabs

Many proteins exist in multiple conformation states that define their function. A highly desired property of an affinity reagent is to trap a protein in a particular conformational state, either to study its structure or to induce a function. Conformational trapping usually involves some “competitive” selection steps that require highly stable immobilization of the target protein. We had previously performed a series of phage display selections where conformationally specific Fabs were generated for both the apo and ligand bound forms of MBP using biotin-streptavidin interaction [17]. For comparison, we wanted to see whether these results could be reproduced using the CaM-CBP immobilization format.

MBP consists of two domains connected by a short hinge and goes through a large conformational transition during ligand binding. Without maltose, MBP is present almost exclusively in an open (apo) conformation. Upon ligand binding, a conformational change takes place via a hinge-bending motion of ~35° bringing together the two domains to adopt a closed (ligand-bound) conformation [18]. The phage display strategy takes advantage of the structural differences between the apo and ligand-bound forms of MBP to generate Fabs that preferentially bind to either form. To generate Fabs against the specifically closed form of MBP using the CBP immobilization tag, the sorting experiments were done in presence of 1 mM maltose (Fig. 5a). After four rounds of selection, competitive phage ELISA was performed to estimate their specificity for MBP in a particular conformation.

From the intensity of ELISA signal at 450 nm, it was evident that most of the binders were specific for the closed form (Fig. 5b). The binding kinetics of each of these Fabs were determined by SPR in the presence and absence of maltose (Table 3). Representative sensograms for open conformation specific Fabs binding to either (open and closed) conformation are shown in Fig. 5c. The analyzed data indicate that the Fabs are specific for the conformation of MBP against the form from which they are generated. Fabs generated for open form (Fab2O, Fab3O, Fab4O) show no detectable binding to MBP in 1 mM maltose. This suggests that these Fabs might bind directly to the maltose binding pocket of MBP which is closed in presence of maltose. Conversely, Fabs generated for the closed ligand-bound form (Fab8M, Fab13M and Fab17M) bind to open apo-MBP, albeit with drastically reduced affinity.

Epitope specific Fabs

For many applications it is generally preferable to have a cohort of binders that associate to non-overlapping epitopes. During previous sorting experiments with SETD7, we observed that the binders were prone to be biased to a single epitope (from epitope binning experiment results, not shown). To facilitate the targeting to a set of different epitopes, we explored the use of an epitope exclusion strategy (Fig. 6a), wherein the antigen was pre-incubated with Fab23 in excess (1uM) to block the immuno-dominant epitope before sorting. The library was negatively selected against Fab23 to eliminate all phages in the pool that would bind to the masking Fab itself. Additionally, all the buffers used in the washing steps of sorting contained 1 uM Fab23. These steps basically ensured that all the isolated Fabs would bind to regions other than the masked epitope of the target protein.

Phage ELISA results confirmed that the binders obtained using this epitope masking strategy bind to other unique epitopes. There was no decrease in signal intensity even when 1 uM of Fab23 was used to completely mask the epitope to which it binds (Fig. 6b). This established the accessibility of the phage population to other epitopes even in presence of high concentration of Fab23. To minimize potential refractory artifacts often associated with phage ELISA, a protein based ELISA was preformed to bin the Fabs based on their sharing the same epitope footprint. Fig. 6c shows that that Fab1E, Fab3E, Fab4E and Fab6E bind to an epitope completely different from that recognized by the masking Fab23 and compete among each other for same binding footprint. Kinetic parameters from SPR indicate that the Fabs have very high affinity (1–10 nM), comparable to Fab23.

The ELISA binning results were further confirmed with epitope mapping experiments by SPR (Fig. 6d). In these experiments, His_{10}-SETD7 (ligand) was immobilized on a NTA chip and after saturating the surface with Fab23 (first analyte), the binding of Fab4E (second analyte) was monitored by injecting a mixture of Fab23 and Fab4E. The assumption was that signal intensity (RUs) will not increase if the two Fabs compete for the same epitope in comparison to the signal intensity when Fab23 is injected alone as the second analyte. In contrast, we observed a significant increase in response units with addition of the second analyte indicating that it is binding to a site independent of the first analyte. Similar patterns were observed after reversing the order of injection of analyte (Fab4E followed by mixture of Fab23 and Fab4E). Taken together, these results confirm that the CaM-CBP immobilization strategy is robust enough to support higher level phage display selection methods in the form of epitope masking to generate customized Fabs that can target multiple regions on a protein's surface.

Domain specific Fabs

As a final demonstration of the versatility of the immobilization method, we endeavored to generate Fabs targeting individual domains in multidomain proteins. SETD7 is composed of two domains and we were interested in obtaining binders for its N-terminal domain. While the C-terminal domain of SETD7 is stable, the N-terminal domain in isolation is much less so and has a tendency to aggregate. Therefore, to produce specific N-terminal binders, the selection had to be done in the context of the whole intact protein. One strategy for doing this would be to take all the binders to the whole protein and then to eliminate from the pool those that bound to the C-terminal domain in a second step. However, a more efficient way of achieving the same result entails just a single step. The selection strategy is depicted in Fig. 7a. During sorting, C-terminal domain was used in high excess (10–100 -fold) as a soluble competitor to eliminate all the binders targeted to the C-terminal domain. Competitive phage ELISA (Fig. 7b) of the unique binders showed that the binding was competed by the full length protein, but not at all with the C-terminal domain alone. This verified the specificity of the Fabs for the N-terminal domain.

Immunoprecipitation and immunofluorescence experiments

It is well established that definitive immunofluorescence and immunoprecipitation experiments require high performance antibodies. To confirm that the Fabs generated using CBP tagged antigens would be useful in cell biological applications, we performed several

immunoprecipitation and immunofluorescence experiments using the anti-SETD7 Fabs. Avi-tagged Fabs were immobilized on SA magnetic beads and the Fab coated SA beads were incubated with native HEK293 cell lysate containing overexpressed FLAG -tagged target. After overnight incubation and several washing steps, the input, unbound and bead fractions were analyzed by Western Blot to quantify the amounts of the FLAG-tagged SETD7 contained in each fraction (Fig. 8a). An unrelated anti-GFP Fab H3 and empty beads were used as negative controls and showed no detectable binding to the cell lysate. Thus, the prominent bands that are observed in the bead fractions of the samples (where the beads were coated with Anti-SETD7 Fabs) are not due to any non-specific binding, but manifest the high affinity and specificity of the generated Fabs in recognizing the cognate antigen in native form.

Immunostaining experiments on MEFs overexpressing SETD7 were performed using the anti-SETD7 Fabs as the primary antibodies. We were able to detect nuclear localization of the protein using the Fabs as primary reagents (Fig. 8b). There was no non-specific adherence of the secondary antibody AlexaFluor 488 conjugated goat anti-human IgG to the MEF cells used in the assay. −/− MEFs lacking any expression of endogenous SETD7 were used as controls to detect any level of non-specific binding. No non-specific binding was observed in the −/− MEF cells confirming again that the high specificity of the reagents for the native antigens in cellular environment.

Discussion

The current standard for high affinity immobilization is an Avi-tag, which can be biotinylated and captured irreversibly using SA coated substrates like beads or plates. Further, it is generally straightforward to introduce an enzyme-inducible cleavage site in the tag to release the antigen from the substrate during selection in a “catch and release” fashion using different proteases [19, 20]. This capability can obviate the significant effects of background binding and propagation of non-specific clones, often a pressing concern with harsh elution conditions [21]. However, there are a substantial number of antigens that behave poorly, particularly in terms of solubility when an Avi-tag is introduced as a fusion peptide. In fact, a large percentage of the affected antigens cannot be rescued no matter the effort. Use of HaloTag [22] as an immobilization tag is often associated with isolation of binders specific to the fusion tag. “Work-arounds”, such as using solubilization chaperones like GST or MBP, have their own challenges and limitations. Direct coating or passive adsorption of antigens in random orientation is associated with partial denaturation and inaccessibility of epitopes, especially in cases of membrane proteins [14, 23]. Chemical biotinylation demands optimization of reaction conditions often significantly altering surface properties of the antigen.

These shortcomings motivated us to explore for alternative solutions that would recapitulate the advantages of the Avi-tag, while minimizing the problems associated with solubility. The effects of common purification and pull down tags like His-tags and FLAG- tags are usually innocuous, but their affinities are too low to be useful in phage display experiments requiring alternate options to be used. Options based on peptides that were known to bind tightly to streptavidin were considered potential solutions. The first of these is the so-called

Nano-tag, which is a 15 amino acid peptide that is purported to bind to SA with a 4 nM affinity [24]. The second peptide tag is the streptavidin binding peptide (SBP) that is 38 amino acids long and binds with an affinity of 2.5 nM [25]. Unfortunately, we encountered myriad problems with these tags in the context of our test antigens. The first was that since these tags rather hydrophobic, they also exhibited similar solubility and expression problems that were encountered with the Avi-tag. They also proved to have insufficient affinity to SA coated beads to survive the required wash steps. The immobilized target is lost by continuous dissociation from the matrix decreasing the chance of obtaining high affinity binders. Finally, we found the efficiency of the enzyme cleavage that is necessary to release the antigen-phage complex from the bead was compromised in many cases. A third system that was considered used ^{BT}TRIS NTA in a phage display sorting format where the matrix, a high affinity capture reagent for His-tagged proteins, also has a biotin group [14, 26]. It provides a stable and reversible linkage between a His-tag and a SA coated solid support. However, ^{BT}TRIS NTA is not available commercially to date and has to be synthesized on a larger scale prior to use in a high throughput pipeline.

An alternative approach that appeared to have promising characteristics was the combination of CaM and CBP. CaM is small, highly conserved calcium binding messenger protein (148 residues) that has a key role in intracellular signal transduction, binding and activating enzymes [27]. It is also one of the few examples of a small protein capable of binding to peptides with reasonable affinity (K_D ~ 4 nM) in presence of calcium ions which can be completely disrupted by addition of calcium chelators like EDTA or EGTA due to a conformational change of CaM that is triggered on loss of calcium (Supplemental Fig. 3). The versatility of CBP as an affinity tag is evident from the successful expression and subsequent purification of recombinant protein not only from *E. coli*, but also from different eukaryotic expression system like yeast, *Drosophila* and mammalian cells [28]. Introduction of some mutations (for eg: N5A) in the 19 amino acid residue peptide (RWKKNFIAVSAANRFKKIS) derived from wild type skeletal muscle myosin light-chain kinase (MLCK), increases the affinity 1000-fold (K_D ~ 2 pM) owing to a much slower off-rate ($k_{off} < 1 \times 10^{-4}$), almost comparable to a SA-biotin interaction [29]. It had been recognized that affinity maturation of the engineered peptide makes the system an interesting candidate for biotechnological applications [30] as a useful alternative to the avidin-biotin system [31]. Due to ultra high affinity yet completely reversible interaction, CBP-CaM system thus possesses a “catch and release” capability similar to the modified Avi-tag versions, but can be manipulated in a more facile manner because it does not require an enzymatic cleavage step.

To appraise the utility of the CaM-CBP system, we devised a series of experiments that compared the ability of CBP to improve the solubility of the antigen over the Avi-tag version, and whether this tag had sufficient high binding robustness and versatility for use in challenging types of phage display sorting schemes. To address the first point, we picked a challenging set of antigen targets some of which failed to function in an Avi-tag format where they all suffered from significant solubility issues. Several also had multiple free cysteines that required the presence of reducing agents in the buffers, making them unsuitable candidates for chemical biotinylation using NHS-PEG_4-S-S-Biotin. We showed

that the CBP fusion had markedly improved solubility properties compared to the Avi-tag version. It is noteworthy that some of these targets were problematic even in the absence of any tag, so the fact that all led to successful selection endpoints means that borderline cases can be worked on with a high probability of a positive outcome.

Unfortunately, none of the commercially available CaM magnetic beads were found suitable with the Kingfisher instrument used in panning. So we decided to prepare our own CaM coated SA magnetic beads by coating Avi-CaM on SA magnetic beads. Although pretty straightforward, it adds an extra step in the process, which is undesirable for HTP pipeline. In the later stages of the project, we prepared our own beads by covalently coupling CaM to NHS-activated magnetic beads that can be stored at 4°C and thus very convenient. These beads were tested and compared with the Avi-CaM coated SA beads in a mock selection experiment using MBP. Both of them gave identical output phage titers (Supplemental Fig. 4).

To assess the robustness of all aspects of a typical phage display process, we used CBP-MBP as a model system because we could compare it directly with previous results using the Avi-tag version. Several different types of phage sorting experiments were tested. First, we tested the system in a “mock” selection where we took a known MBP binder in phage format to see at what levels of washing stringency and concentration limits we could efficiently isolate it from a large pool of non-binders. We found that even at ratios of $1:10^7$ binder vs. non-binders, extremely good enrichment was observed and after the fourth round of sorting the percentage increased to 100% effective binders. The significant enrichment of specific binders demonstrated that this CBP affinity tag exceeds the requirements for successful sorting experiments. A representative subset of the binders obtained after sorting the target set of CBP tagged antigens were reformatted into Fab protein format and their binding kinetics were determined by SPR. Kinetic parameters showed that most Fabs were both very high affinity (K_D < 10 nM) and had slow off-rates, a highly desired combination for affinity reagents.

A further demonstration of the capability of the CBP tag was the successful generation of conformationally specific Fabs using MBP as target. Here the phage sorting was done in the presence of maltose that induces the closed form and in the absence of maltose where the open form predominates. In both cases, the selections produce binders that had strong bias towards the form that were generated against. Taken together and with the other examples of successful high level applications of generating epitope and domain specific Fabs against cysteine rich antigens like SETD7 that preformed well in biological assays, provides ample evidence that the N5A mutant of CBP, which can be captured and released in a controlled manner from CaM coated beads, has virtually all the attributes that Avi-tags do, while not suffering from some of its well documented drawbacks.

Materials and Methods

Materials

All the enzymes used in molecular biology works are from Fermentas and NEB. cDNA clone of human calmodulin (Cat no: SC115829) is from OriGene. Streptavidin

MagneSphere Paramagnetic Particles (SA coated magnetic beads) are from Promega and NHS activated magnetic beads are purchased from Pierce. HRP conjugated anti-M13 mouse monoclonal antibody (Cat no: 27942101) is from GE Healthcare Life Sciences. Anti-FLAG M2 mouse monoclonal antibody (Cat no: F3165) and its HRP conjugate (Cat no: A8592) are from Sigma-Aldrich. HRP conjugated goat anti-mouse IgG (Cat no: 115-035-003) and AlexaFluor 488 conjugated goat anti-human IgG (Cat no: 109-546-097) are from Jackson ImmunoResearch. Hoechst 33342 trihydrochloride (Cat no: H3570) and ProLong Gold antifade reagent are from Invitrogen. Sypro Orange and SuperSignal West Pico chemiluminscent substrate are purchased from Life Technologies. NTA chip, Resource S 1ml column and HiTrap MabSelect SuRe 5 ml column are from GE Healthcare Life Sciences. *E. coli* DH5α and XL1-Blue cells are from Agilent Life Sciences and BL21, BL21(DE3), Tuner(DE3) are from Novagen. All the other reagents and chemicals used are of analytical grade.

Construction of CBP-tagged vector

CBP-tagged vector (pCBPH6) was prepared by modifying pET28a by Kunkel mutagenesis [32]. ssDNA from pET28a was used as template with the primers: (a) pET28a_ΔNHis (5′ GCACCAGGCCGCTGCTCCCGCCGCTGCTGCCCATGG3′) and (b) pET28a_CBP_Cterm(5′GGTGGTGGTGGTGGTGTTAATGATGATGATGATGATGGCT GCCGCTGCCGCTAATTTTTTTAAAGCGGTTCGCCGCGCTCACCGCAATAAACGCT TTTTT CCAGCGGCTGCCGCTGCCTCGAGTGCGGCC 3′). pET28a_ΔNHis replaces the N-terminal His_6 tag of pET28a with a pair of glycine residues. pET28a_CBP_Cterm adds –$(GlySer)_2$-CBP-$(GlySer)_2$-His_6 followed by a stop codon at the C-terminus of the vector. The product from Kunkel reaction was purified using PCR purification kit (Qiagen). Chemically competent *E. coli* DH5α cells were transformed with the purified DNA and selected on LB-Agar/Kan plates. Colonies obtained were verified by sequencing for the proper construct.

Cloning and overexpression of the target proteins

The genes of all the target proteins were amplified by PCR and cloned into the BamHI/XhoI sites of PCBPH6. MBP (Uniprot: P0AEX9), SETD7 (Uniprot: Q8WTS6), GAP1 (Uniprot: Q6GIL8), PGK (Uniprot: Q6GB57) and bromo domain (Uniprot: B4NS38) were amplified from pHFT2/MBP [33, 34], pNICBio2/SETD7, pQE30/GAP1 [35], pQE30/PGK [36] and pET21/bromo respectively. ESRRA (Uniprot: P11474), NEDD9 (Uniprot: Q14511), ZSCAN29 (Uniprot: Q8IWY8), TSC22D4 (Uniprot: Q9Y3Q8), VENTX (Uniprot: O95231), MAFF (Uniprot: Q9ULX9), LIN28A (Uniprot: Q9H9Z2) and JARID2 (Uniprot: Q92833) were amplified from the plasmids HR7097C, HR5554A, HR8429A, HR7683A, HR7703A, HR8265A, HR7525A and HR8400C respectively. The HR- plasmids have the N-terminal Avi-tagged constructs of the target proteins. Open reading frame of CaM was amplified by PCR from cDNA of human CAM and cloned into the BamHI/XhoI sites of pHBT [37] that adds an N-terminal Avi-tag to the overexpressed protein. SEDT7 was cloned in pHFT2 to produce FLAG-tagged SETD7.

MBP, SETD7, GAP1, PGK, bromo domain were overexpressed in *E. coli* BL21(DE3) cells while Tuner (DE3) cells were used to overexpress ESRRA, NEDD9, ZSCAN29, TSC22D4,

VENTX, MAFF, LIN28A and JARID2. Avi-tagged proteins were expressed in Tuner (DE3)/pBirA cells that co-express *E. coli* BirA (biotin ligase) [38] for *in-vivo* biotinylation in presence of 50 uM D-biotin.

For expression and solubility studies, cells were grown in 10 ml cultures and induced with varying concentrations of IPTG at different temperatures (37, 22 and 18°C). Harvested cells were resuspended in phosphate buffer saline (PBS) supplemented with DNase I and protease inhibitors. Cells were lysed by sonication, spun down at 22,000g and supernatant fraction recovered. Total protein was estimated by Bradford's method [39]. Total protein, supernatant and the pellet fractions were analyzed by SDS-PAGE electrophoresis.

All proteins were purified by Ni-NTA followed by size exclusion chromatography.

Pull-down Assay

The pull down assay was performed at room temperature and all incubation steps were for 15 minutes. 100 ul of SA magnetic beads (Streptavidin MagneSphere Paramagnetic Particles) was washed twice with binding buffer (Tris buffer saline (TBS) + 1mM $CaCl_2$). 5ug of biotinylated Avi-CaM in binding buffer was incubated with the beads. Unbound fraction (FT: flow-through) was collected and the beads were washed well with binding buffer to eliminate any non specific binding. Input, unbound fractions and the beads were subjected to SDS-PAGE electrophoresis to analyze the extent of capture of Avi-CaM by the SA beads. These CaM coated beads were then used for pull-down assay of CBP-tagged targets. 100 uL of CaM coated beads were incubated with CBP-tagged targets in 1:1 molar ratio (considering entire 5ug biotinylated CaM has been captured by 100 ul SA beads as observed from SDS PAGE analysis) in binding buffer. The unbound fractions were collected and the beads washed twice in binding buffer. Finally the bound CBP-tagged targets were eluted after incubating with 10 mM EDTA in TBS. After elution, the beads were washed once in TBS. Input (TP: total protein), unbound (FT: flow-through), elution (E) fractions and the washed beads after elution were analyzed on SDS-PAGE.

Mock selection

To ascertain whether the interaction of mutant CBP tag with CaM was strong enough to withstand the vigorous washing steps in sorting, a single round of mock-selection was performed. 50 nM of CBP-MBP was immobilized on 20 ul of SA magnetic beads coated with 50 nM Avi-CaM. Virions displaying a MBP-specific Fab fragment [17] were used as the input. As a negative control, equal number of phage particles (based on cfu) displaying a Fab fragment (SETA1) specific for an unrelated epigenetic target were used. TBST/ 0.5%BSA (TBS buffer containing 0.05 %(v/v) Tween-20 and 0.5% (w/v) BSA) with 1mM $CaCl_2$ was used as buffer for sorting. Four sets of sorting were performed: i) with and ii) without Avi-CaM on beads and iii) with and iv) without CBP-MBP. Phages bound after binding and rigorous washing steps in Kingfisher [40] were eluted with 10 mM EDTA. Eluted binders were used to infect log phase *E. coli* XL1-Blue cells. The infected cultures were plated on LB-Agar/Amp plate and grown overnight at 37°C. Titer of recovered phages (cfu) was calculated from the colonies the next day.

Doping selection

After a single round of "mock" selection with MBP specific Fab MOS1, we created a "doped" library to demonstrate that a specific binder can be isolated from a vast excess of non-specific phage particles. Phages displaying MOS1 and SETA1 Fabs were amplified in *E. coli* XL1-Blue cells, precipitated using PEG-NaCl and their titer determined. They were then mixed in ratios of $1:10^2$, $1: 10^5$ and $1:10^7$ with the non-specific SETA1 in excess. These phage mixtures were then used separately as inputs for panning against immobilized CBP-MBP on CaM coated beads. Four rounds of selection were done with decreasing the target concentration from 100 nM from the 1st round to 50 nM in the 2nd and 3rd rounds and finally to 10 nM in the 4th round using the amplified virions from the preceding round as inputs. TBST/0.5% BSA with 1mM $CaCl_2$ was used as buffer for sorting. The enrichment ratio, defined as -fold enrichment of MOS1 over background SETA1, was calculated from the titer values of phages eluted from 2nd round onwards with 10 mM EDTA. Ninety-six randomly picked clones from each of the 2nd, 3rd and 4th rounds were amplified by PCR by a pair of specific primers MOS1_HC_FP: 5′CGCGTGTACGTTCCATACATCAGCCAATATTATTCCTGGTCGTACCGCGGTATT 3′ and MOS1_HC_RP:5′GCCGCCAGCATTGACAGGAGGTTGAGGCAGGT 3′. These primers were designed to only anneal and amplify a part of the HC fragment (535 bp) of MOS1 displayed on p3 of phage. Results were also confirmed by DNA sequencing.

Library sorting

Library sorting was performed at room temperature following published procedures [14, 40, 41]. SA coated magnetic beads (Streptavidin MagneSphere Paramagnetic Particles) were pre-coated with Avi-CaM to prepare the CaM coated magnetic beads. These CaM coated beads were used in sorting to capture the CBP-tagged targets. The concentration of Avi-CAM used to coat SA beads was identical to the concentration of the targets used in each successive round of selection. All the buffers used in sorting, except the elution buffer, were supplemented with 1 mM $CaCl_2$ to keep CaM in the desired conformation. To get rid of the potential CaM binders from the pool of desired virions, the phage library was negatively selected against 250 uL of SA magnetic beads coated with 200 nM Avi-CaM from the very first round before the phages were added to the target. This pre-clearing step is mandatory in every round to make sure that most of the CaM binders are eliminated from the amplified pool. The preclearing step helped to eliminate the potential straptavidin binders as well. In the first round, 100 nM target was immobilized on 250 uL of CaM coated magnetic beads, followed by addition of 1uM D-biotin solution to block any unoccupied SA sites. After the blocking step, the beads were extensively washed to get rid of the unbound target and excess biotin. Six of such targets bound to CaM coated beads were combined. 1ml of phage library E [42] containing 10^{12}–10^{13} virions were added to the combined beads and incubated for 30 minutes. After the incubation step, the beads were washed and resuspended in the sorting buffer. The resuspended beads containing bound virions were used to infect freshly-grown log phase *E. coli* XL1-Blue cells. Phages were amplified overnight in 2YT media with 50 ug/ml Amp and 10^9 pfu/ml of M13 KO7 helper phage. To increase the stringency of selection, three additional rounds of sorting were performed with decreasing the target concentration in each round (2nd round: 50 nM, 3rd

round: 50 nM and 4th round: 10 nM) using the amplified pool of virions of the preceding round as the input. Sorting from 2nd to 4th rounds were done on Kingfisher instrument. From 2nd to 4th round, the targets were premixed with the amplified phage pool and then CaM coated beads were added to the mixture. 2nd round onwards, 10 mM EDTA was used to elute the bound phage by disrupting the interaction of CBP-tagged targets with CaM on the beads.

To obtain conformational specific (closed) Fabs for MBP, sorting buffer was supplemented with 1 mM maltose in all rounds. Fabs specific for the open conformation of MBP were obtained without any maltose in the selection conditions.

Epitope-specific Fabs were obtained by “epitope exclusion strategy”. Before sorting, CBP-SETD7 was pre-incubated with 1uM Fab23 to block the immunodominant epitope which binders are prone to be biased towards (unpublished data). Before each round, the phage pool was incubated with 250 ul of SA beads coated with 100 nM biotinylated Fab23 at room temperature for 15 minutes. This pre-clearing step is performed to eliminate all virions that will bind to the masking Fab23 itself. Additionally 1 uM Fab23 was added in all the buffers during each of the four rounds. This ensures that the epitope is masked and all binders specific for the masking Fab are washed away.

Fabs specific for the N-terminal domain of SETD7 were obtained by “domain exclusion” strategy. Full length CBP-SETD7 was used as a target with the C-terminal domain as a soluble competitor. The competitor was added starting from the 2nd round. The phage pool was incubated with 5uM C-terminal domain of SETD7 for 15 minutes at room temperature before adding the target. All the washing steps in panning had 5uM C-terminal domain of SETD7 to remove binders targeting the C-terminal domain.

Single point competitive phage ELISA

A single-point competitive phage ELISA was used to rapidly estimate the affinities of the obtained Fabs in phage format. Colonies of *E. coli* XL1-Blue harboring phagemids were inoculated directly into 500 μl of 2YT broth supplemented with 100 ug/ml Ampicillin and M13-KO7 helper phage. The cultures were grown at 37 °C for 16–20 hours at 280 rpm in a 96 deep-well block plate. Culture supernatants containing Fab-phage were diluted ten-fold in PBST buffer (PBS buffer containing 0.05 %(v/v) Tween-20) with or without 50 nM target proteins as soluble competitor. After 15 minutes incubation at room temperature, the mixtures were transferred to ELISA plates that were coated with target proteins and blocked with 0.5% BSA in PBS. The ELISA plates were incubated with the phage-competitor mixture for another 15 minutes and then washed with PBST. The washed ELISA plates were incubated with HRP conjugated anti-M13 mouse monoclonal antibody (1:5000 dilution in PBST buffer) for 30 minutes. The plates were again washed with PBST, developed with TMB substrate, quenched with 1.0 M H_3PO_4, and absorbance (A_{450}) determined spectrophotometrically at 450 nm. For each clone, the ratio of A_{450} in the presence of 50 nM competitor to that in absence of competitor gives the fraction of Fab-phage uncomplexed with soluble competitor. A plot of the signal intensities (Y-axis) without any competitor as a function of this ratio (X-axis) gives an estimate about the affinities of the binders. For further characterization, clones with high Y-values and low X-values are chosen. To

determine conformational specificity of the Fabs against the closed form of MBP, all the buffers used in ELISA were supplemented with 1mM maltose.

Cloning, overexpression and purification of Fabs

Unique clones of Fabs were sub-cloned into the HindIII/SalI sites of expression vector pRH2.2. Avi-tagged Fabs were obtained by cloning them into a modified version of pRH2.2 that adds an Avi-tag at the C-terminus of the heavy chain. *E. coli* BL21 cells were transformed with sequence verified clones of Fabs in pRH2.2. Fabs were grown in 2YT media with 100 ug/ml Ampicillin at 37 °C for 2.5–3 hours during which A_{600} reached 0.6–0.8, induced with 1mM IPTG and further grown for 4 hours at 37 °C to maximize the overexpression. Harvested cells were resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, pH 7.5 containing 0.1 mg/ml DNase I, 0.1 mM each of leupeptin, pepstatin, aprotinin and 0.02 mM PMSF). The suspension was lysed by ultrasonication. The cell lysate was incubated at 65°C for 30 minutes to get rid of any undesired proteolyzed fragment of the Fabs produced during overexpression. Heat-treated lysate was then cleared by centrifugation, filtered and loaded onto a HiTrap MabSelect SuRe 5 ml column, equilibrated with buffer containing 50 mM Tris, 500 mM NaCl; pH 7.5. The column was washed with 10 column volumes of equilibration buffer followed by elution of Fabs with 0.1 M acetic acid. Fractions containing protein were directly loaded onto an ion exchange Resource S 1 ml column. Column was washed with buffer containing 50 mM sodium acetate pH 5.0 at 5 ml/min. Fabs were eluted with a linear gradient 0–50% of buffer containing 50 mM sodium acetate, 2 M NaCl, pH 5.0. Affinity and ion exchange chromatography were performed using an automated program on ÄKTA explorer (GE Life Sciences). Purified Fabs were dialyzed overnight against 20 mM Tris, 150 mM NaCl, pH 7.5. Avi tagged Fabs were coexpressed with *E. coli* BirA (biotin ligase) in BL21/pBirA cells for *in-vivo* biotinylation in presence of 50 uM D-biotin. Avi-tagged Fabs were purified in a similar manner.

Surface plasmon resonance measurements

Interaction analyses between targets and Fabs were performed at 20 °C using a BIACORE 3000 (GE Healthcare). SEC purified, CBP-targets were immobilized onto an NTA sensor chip via the C-terminal His-tag. All dilutions of ligand (target) and analytes (Fabs) were prepared in running buffer that contained 10 mM HEPES pH 7.4, 150 mM NaCl, 50 uM EDTA supplemented with 0.05% Tween 20. The targets were captured on the chip at a flow rate of 5 μl/min. Running buffer without analyte was injected at least three times to ensure stability of the surface before analyte injections were started. For each assay, three fold dilution series of Fabs were injected at a flow rate of 30 μl/min. All conditions were tested for five different Fab concentrations, each in triplicate. Sensograms were double referenced using blank channel (channel 1) and buffer injections. Data processing and kinetic analysis were performed using the Scrubber 2 program (BioLogic software). To determine kinetic rate constants, all data sets were fit to a simple 1:1 Langmuir interaction model. To measure the affinity of the Fabs for the closed form of MBP, the running buffer was supplemented with 1 mM maltose and all the dilutions of sample were prepared in the running buffer with maltose.

For the epitope mapping experiments of SETD7 by SPR, 5ul of 5 nM CBP-SETD7 was immobilized (~ 40 RUs) on NTA chip at a flow rate of 5ul/min via the C-terminal His_6 tag at 20°C. 100 nM Fab23 was injected for 100 sec at a flow rate of 30 ul/min to saturate the epitope. After saturating SETD7 on the chip with Fab23 and reaching equilibrium, a mixture of 100 nM Fab23 and 100 Fab4E was injected. 100 nM Fab23 was added in the second injection as well to make sure that the epitope recognized by the first Fab remains fully saturated during the interaction of SETD7 with the second Fab. Control experiment was done using Fab23 alone in the second injection. A similar experiment was performed by reversing the order of analyte injection, ie: injection of 100 nM Fab4E followed by injection of 100 nM Fab23 + 100 nM Fab4E. 2^{nd} injection of Fab4E was used as a control in this case.

Epitope binning by protein ELISA

50 ul of 12.5 nM Avi-tagged Fab23, Fab6E, Fab4E, Fab3E, Fab1E were added to six consecutive wells along five rows in an ELISA plate that was pre coated with neutravidin and blocked with 0.5 % BSA in PBS, ie: Fab23, Fab6E, Fab4E, Fab3E and Fab1E were added in rows A1-A6, B1-B6, C1-C6, D1-D6 and E1-E6 respectively. After incubating with the Fabs for 15 minutes at room temperature, the plate was washed with PBST. 50 ul of 100 nM FLAG-tagged SETD7 was mixed with 50 ul of 5uM each of non Avi-tagged Fab23, Fab6E, Fab4E, Fab3E and Fab1E in a separate plate. The competing Fabs were used in excess to mask the epitope completely in the complex. SETD7/Fab complexes were then added to five consecutive wells along each column of the ELISA plate coated with Avi-tagged Fabs ie SETD7/Fab23, SETD7/Fab6E, SETD7/Fab4E, SETD7/Fab3E and SETD7/Fab1E were added in columns A2–E2, A3–E3, A4–E4, A5–E5 and A5–A6 respectively. Only SETD7 without any Fab was added along the A1–E1 as controls. The ELISA plates were incubated for 30 minutes with these complexes and then washed with PBST. The washed ELISA plates were incubated with HRP conjugated anti-FLAG M2 mouse monoclonal antibody (1:10,000 dilution in PBST buffer) for 30 minutes. The plates were again washed with PBST, developed with TMB substrate, quenched with 1.0 M H_3PO_4, and absorbance (A_{450}) determined spectrophotometrically at 450 nm.

Differential Scanning Fluorimetry (DSF)

Thermal stabilities of samples were measured by DSF [43]. DSF measurements were performed on a BioRad-CFX384 real-time PCR instrument. Samples were prepared in triplicates in 50 mM sodium phosphate buffer (pH 7.4) with 150 mM sodium chloride and 4×(250-fold dilution of stock) Sypro Orange dye to a total sample volume of 25µl. Concentration of protein was 4 uM in samples containing either Fab or target. For target-Fab complexes, 4 uM target was mixed with 6 uM Fab in 1:1.5 molar ratio. Thermal melts were performed by heating the samples 25 °C to 95 °C, increasing the temperature in steps of 0.5 °C/30 s. Wavelengths of 490 and 575 nm were used for excitation and emission, respectively. Obtained data were processed with the CFX software provided by the manufacturer. The first derivative of the curve is used to determine the melting temperature (T_m).

Immunoprecipitation

Human Embryonic Kidney (HEK) cells overexpressing FLAG-tagged SETD7 were grown on 15 cm round dish for 24 – 48 hours. Cells at 80%–90% confluence were washed with ice cold PBS, lyzed with 1.5 ml IP buffer (25 mM TRIS, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v)Triton X-100 and 5% (v/v) glycerol supplemented with EDTA free Roche protease inhibitor tablets). DNase I (final concentration of 0.1 mg/ml) was added to the lysate and further incubated on ice for 30 minutes to complete lysis. The lysed cells were harvested by centrifugation at 13000g at 4 °C for 10 minutes. Pellets containing cellular debris were discarded and supernatant added to 100 ul of pre-blocked SA beads (Streptavidin MagneSphere Paramagnetic Particles) coated with biotinylated Avi-tagged anti-SETD7 Fabs. The supernatant was incubated overnight with the beads at 4°C. The unbound fraction (also called flow-through, FT) was collected and the beads were washed five times with ice cold IP buffer to remove the non-specifically adhered proteins. The samples (input, flow-through and the beads fraction) were subjected to SDS-PAGE electrophoresis and then transferred onto PVDF membrane for immunoblotting. After the membrane was transferred, it was blocked with PBS-T containing 4% non-fat milk. Blocked membrane was incubated with primary anti-FLAG M2 mouse monoclonal antibody (1:2000 dilution), washed, and subsequently incubated with secondary HRP conjugated goat anti-mouse IgG (1:10,000 dilution). Immunoprecipitated FLAG-tagged SETD7 was detected in a ChemiDoc MP Imaging System from Biorad after adding SuperSignal West Pico chemiluminescent substrate.

The beads used in IP were prepared as follows: 100 ul of SA magnetic beads (Streptavidin MagneSphere Paramagnetic Particles) were washed twice with PBS followed by incubation with 5ug biotinylated Fab in PBS for at least 20 minutes at 4°C. Beads were washed once with PBS and blocked with blocking buffer (PBS/4%BSA) for 30 minutes at 4°C. Unoccupied SA sites were then blocked with 5 uM D-biotin in blocking buffer for 30 minutes at 4°C and washed once with PBS. These beads were ready for use with IP.

Immunofluorescence

Wild type and −/− SETD7 mouse embryonic fibroblasts (MEFs) were grown in high glucose Dublecco’s modified Eagle’s medium on 18 mm coverslips in 12 well plates for approximately 24 hours until they reached 70%–80% confluence. Cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Fixed cells were washed with PBS, permeabilized with 0.1% Triton X-100, washed three times with PBS and blocked in blocking buffer (PBS-T with 2% BSA) for 30 minutes. Fabs generated against CBP-SETD7 (final concentration of 15 ug/ml in the blocking buffer) were used as primary antibodies and incubated with permeabilized MEF cells overnight at 4°C. Next day, cells were washed three times with PBS-T. Secondary AlexaFluor 488 conjugated goat anti-human IgG (final dilution 1:1000 in blocking buffer) was incubated with cells in the presence of DNA staining dye Hoechst 33342 (1:1000 dilution) for 2 hours at room temperature. Cells were then washed three times with PBS-T and dehydrated, and the coverslips were mounted on slides in ProLong Gold antifade reagent. Images were captured using Andor DL-6584-TIL camera connected to the inverted Zeiss Axio Observer A1 epi-fluorescence microscope with X63

oil-immersion objective and the acquisition software Andor Solis. Deconvoluted images were processed as TIFF files using the Image J software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CaM	Calmodulin
CBP	Calmodulin binding peptide
Fab	Fragment antigen binding
SA	Streptavidin
MBP	Maltose binding protein
PBS	phosphate buffered saline
TBS	TRIS buffered saline
HRP	Horseradish peroxidase

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Highlights

- Reversible high affinity immobilization tags have myriad research applications.
- N5A mutant of CBP tag offers controlled “capture and release” of specific binders.
- CBP improves solubility over Avi-tagged constructs.
- High affinity domain, epitope, conformation specific Fabs for challenging targets.
- Significant improvement over current techniques to obtain affinity reagents.

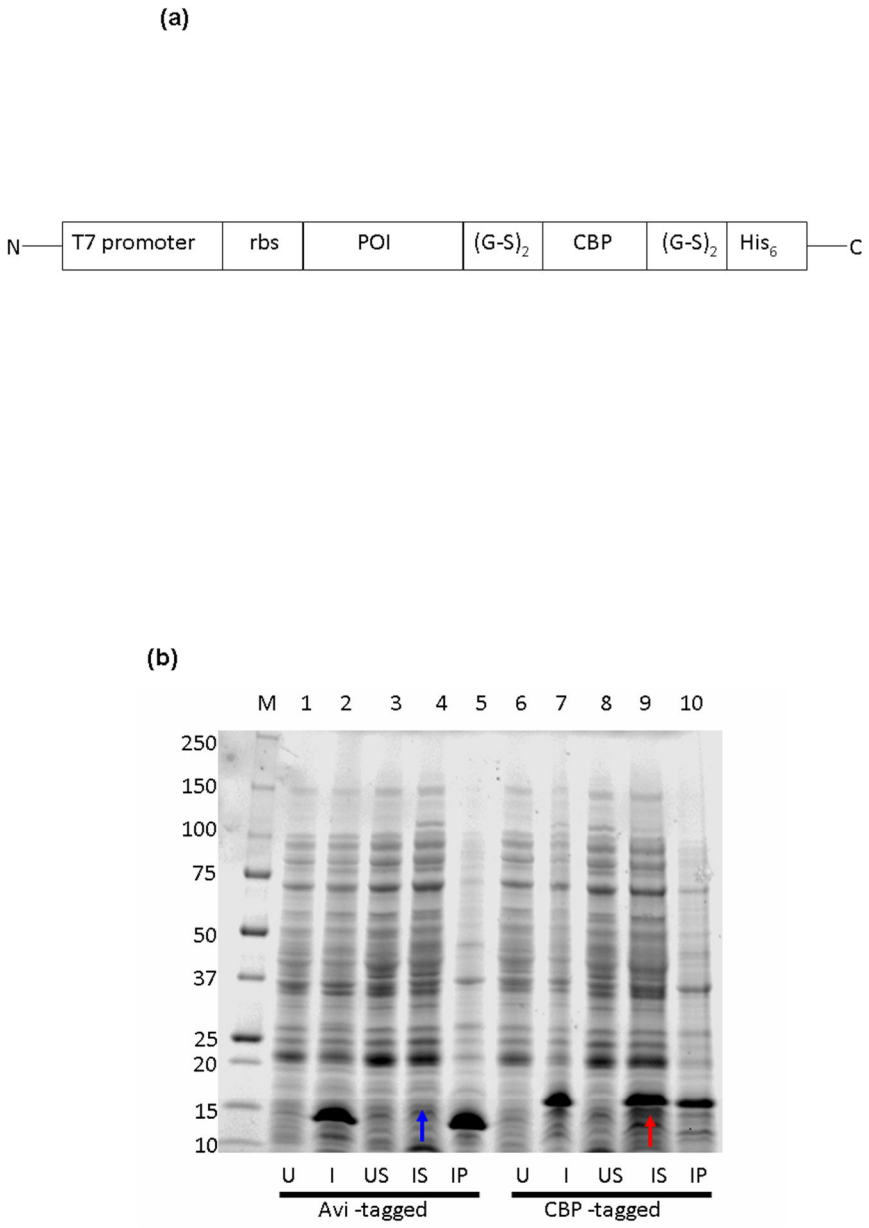


Fig. 1.

Construct design and solubility of CBP-tagged targets. (a) Representation of the expression vector, pCBPH6, used to express CBP-tagged proteins. The vector is an engineered pET28a vector containing T7 promoter. RBS, POI, CBP, Gly-Ser and His_6 stand for ribosome binding site, protein of interest, N5A mutant of calmodulin binding peptide tag (RWKK**A**FIAVSAANRFKKIS), linker of glycine and serine residues and hexahistidine tag respectively. (b) Solubility comparison of Avi-tagged vs CBP-tagged VENTX. CBP-tagged construct was fairly soluble (red arrow) while there was hardly any protein in soluble fraction from the Avi-tagged construct (blue arrow). U: uninduced cell lysate, I: Induced cell lysate, US: supernatant fraction from uninduced culture, IS: supernatant fraction from

induced culture, IP: pellet fraction from induced culture. CBP-tagged target is localized more in IS fraction than Avi-tagged ones. Equal amount of protein was loaded in each well.

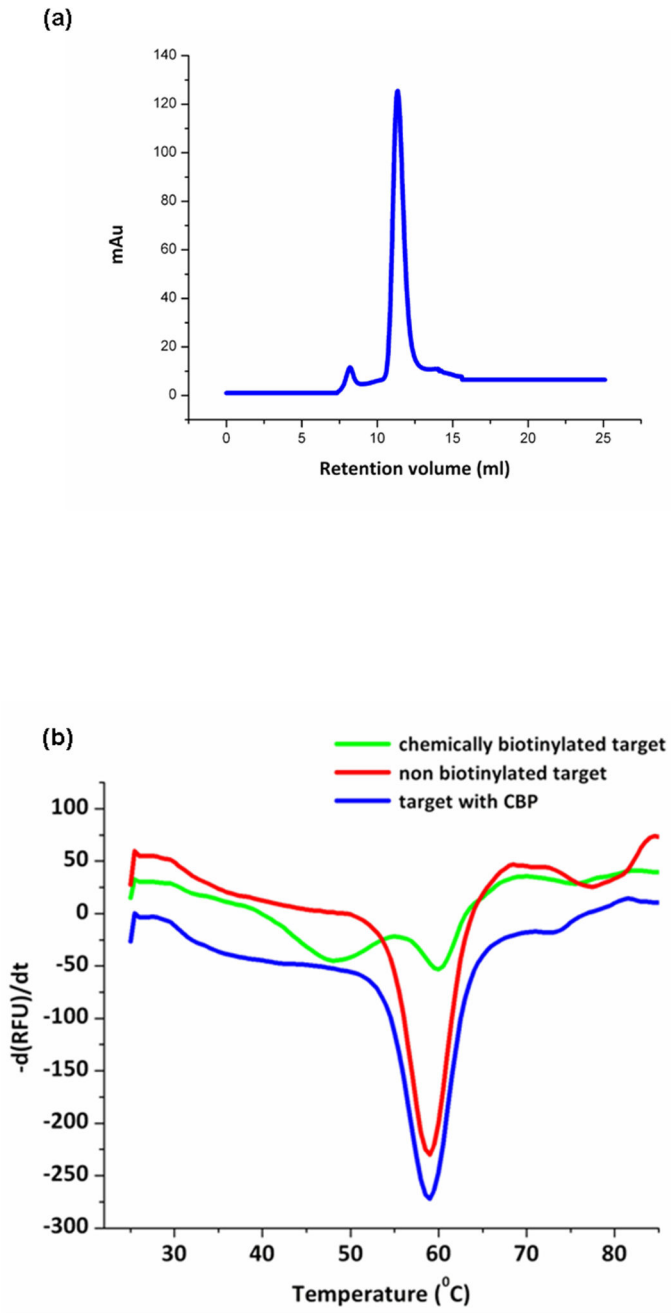


Fig. 2.

Purification and Characterization of CBP-tagged targets. (a) Analytical size exclusion (aSEC) profile of purified CBP-target (CBP-SETD7) by Ni-NTA chromatography shows a mono dispersed peak lacking any soluble aggregates. (b) Comparison of thermal stability of CBP-tagged proteins (blue) with His_6-tagged (red) and chemically biotinylated (green) protein samples by DSF. Stability is significantly compromised in some cases with chemical biotinylation while no such effect is observed in CBP-tagged constructs.

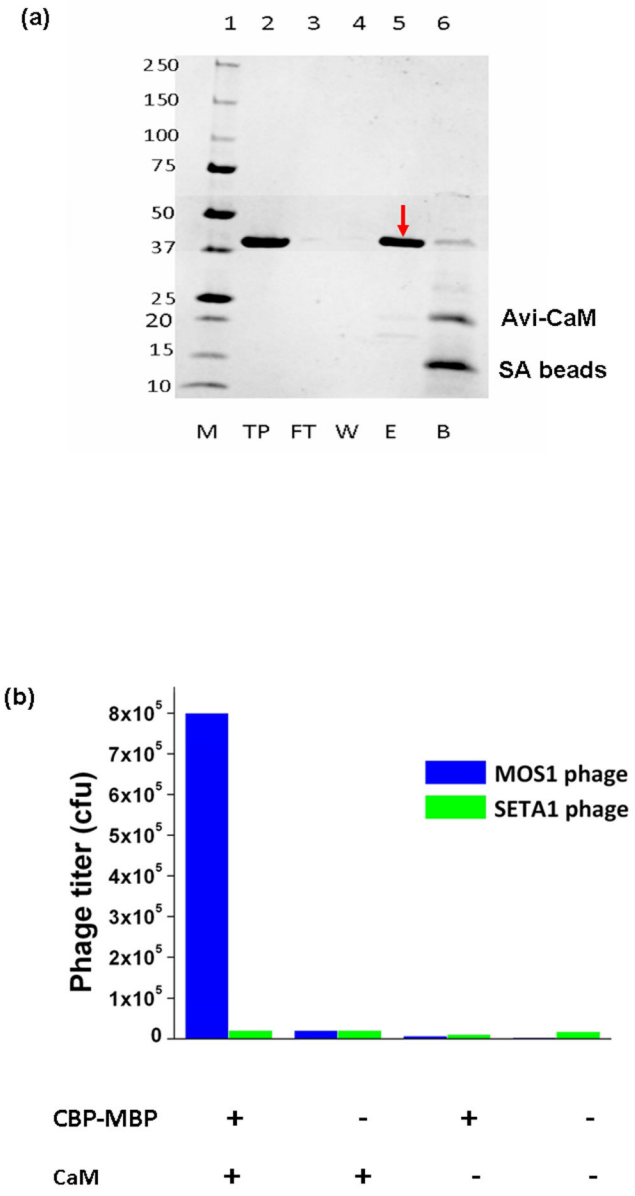


Fig. 3.

CBP-tagged targets are effectively immobilized on CaM coated beads. (a) Pull-down assay: Lane 1: Molecular weight marker (Precision Plus Protein Unstained Standard from Bio-Rad), Lane 2: Input or total protein used (TP), Lane 3: Unbound fraction or flowthrough (FT), Lane 4: Wash (W) fraction, Lane 5: Elution (E) fraction with 10 mM EDTA, Lane 6: Avi-CaM coated SA beads treated with SDS loading buffer. Avi-CaM is highlighted (red asterisk). Almost quantitative capture and elution of the CBP-MBP is observed (red arrow). (b) Recovery of phage displaying MBP binding Fab (MOS1) is maximized (1st panel) only when both CaM and CBP-MBP are used in the mock sorting. The other panels are negative controls where either one of the two or both are missing. SETA1 is phage displaying Fab specific for an unrelated target. Titer values of MOS1 and SETA1 used as inputs were similar.

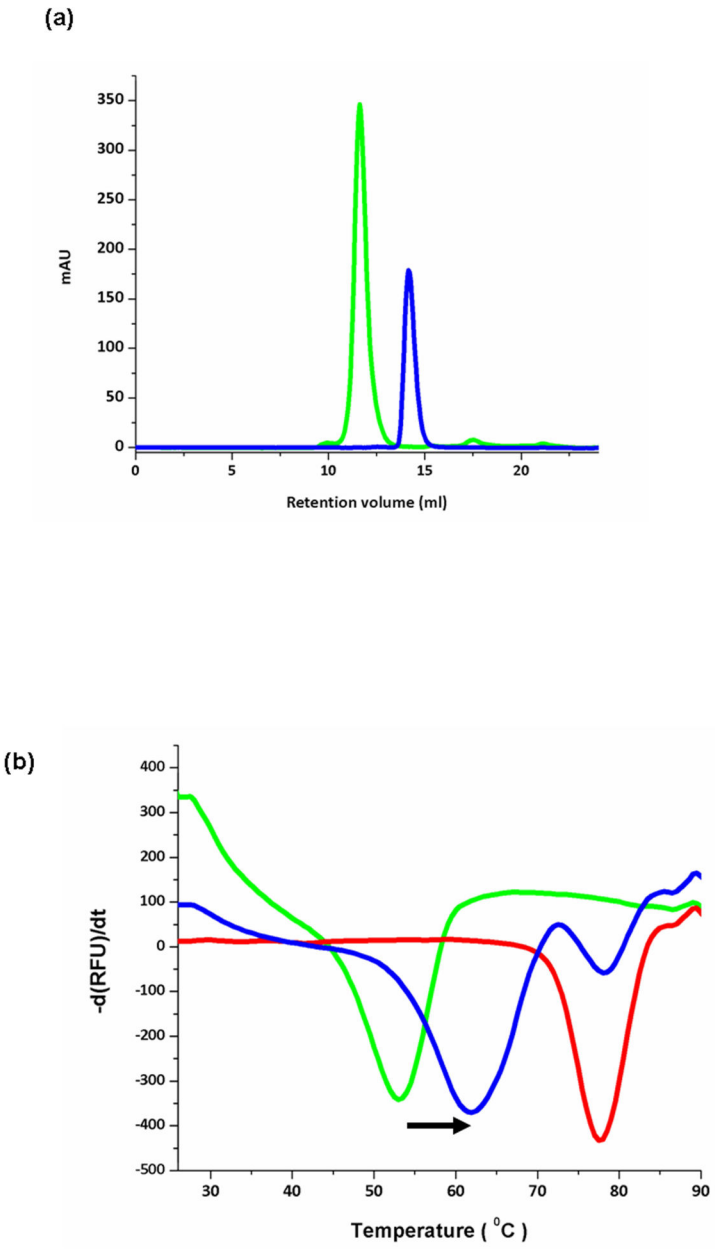


Fig. 4.

Characterization of target-Fab complexes. (a) aSEC profile of individual target (blue) and target-Fab (green) complex. Fabs form high affinity complexes with target proteins that can be separated by SEC. (b) Melting curves of individual targets (green), Fabs (red) and target-Fab (blue) complex. Fabs are thermally very stable (T_m> 70°C) and stabilize the targets in the complex to an appreciable extent (marked by black arrow). Target-Fab complex was prepared by mixing target: Fab in 1:1.5 molar ratio. The melting peak of the excess Fab in the complex at higher temperature is also observed.

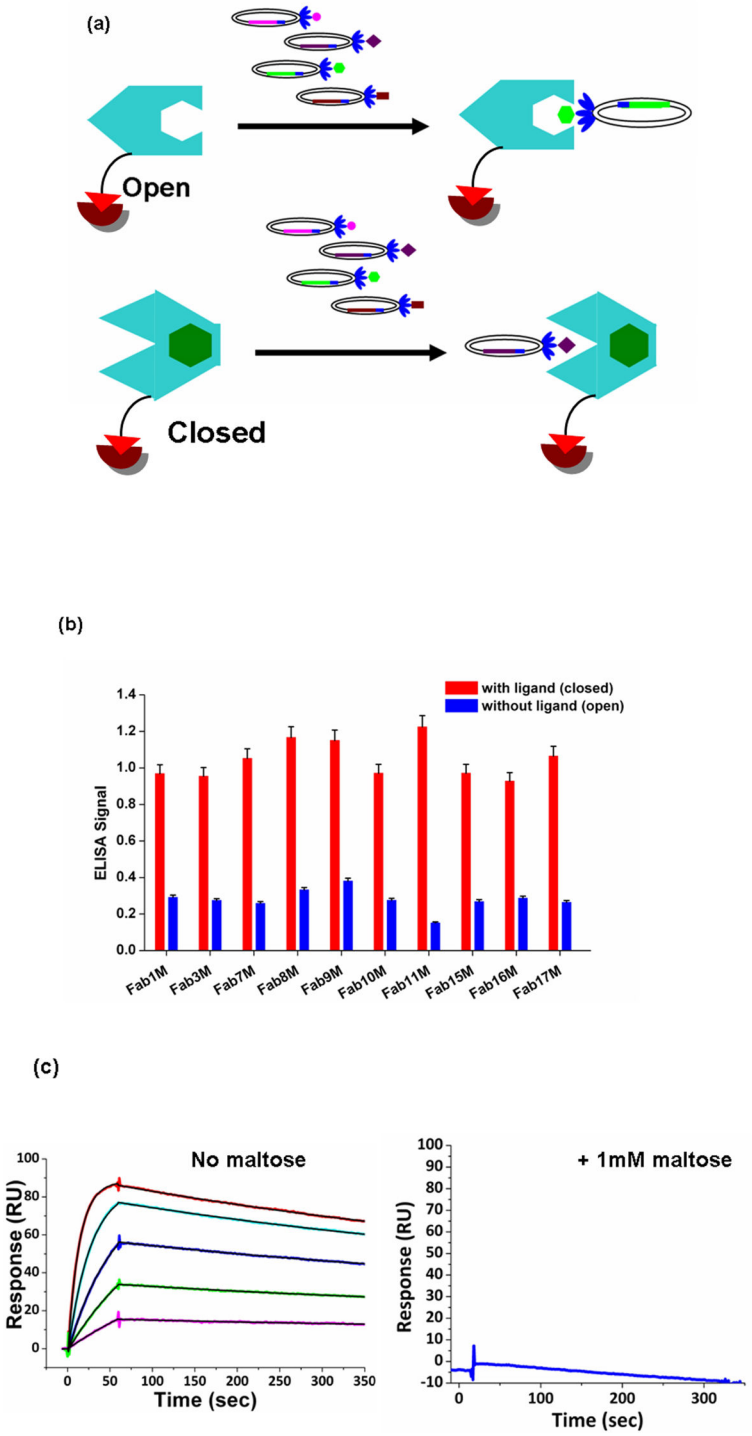
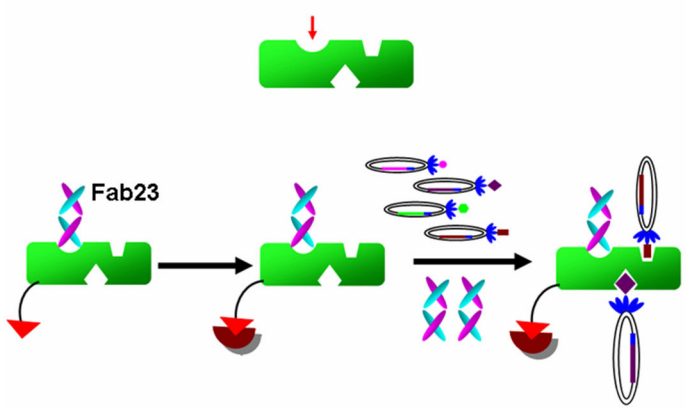


Fig. 5.

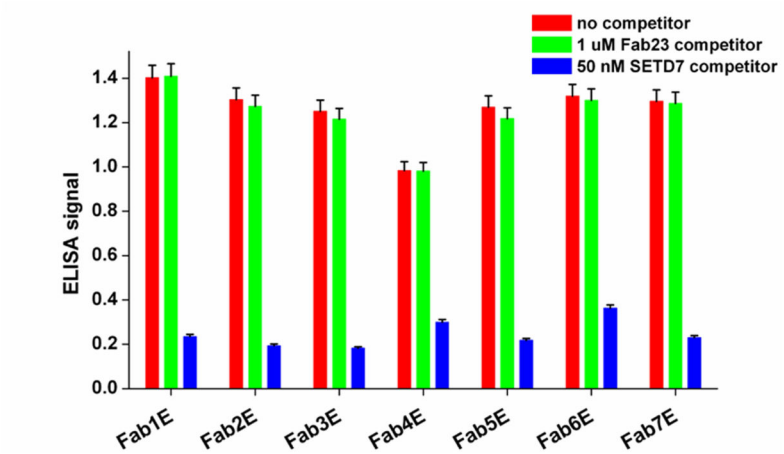
Generation of conformation specific Fabs. (a) Selection strategy to obtain conformation specific Fabs from CBP-tagged targets. CBP-MBP is in open conformation without maltose (green hexagon). In presence of maltose, it adopts the closed conformation. Separate sorting experiments with immobilized targets in specific conformations generate Fabs that are

conformation specific. (b) Phage ELISA results show that Fabs generated against closed form of MBP in presence of maltose have a drastically compromised binding to open form of MBP in absence of maltose. (c) SPR sensograms showing that Fabs recognize specific conformation of MBP. Fab 7O binds to open form of MBP immobilized on NTA chip without maltose. No significant binding was observed in 1mM maltose.

(a)



(b)



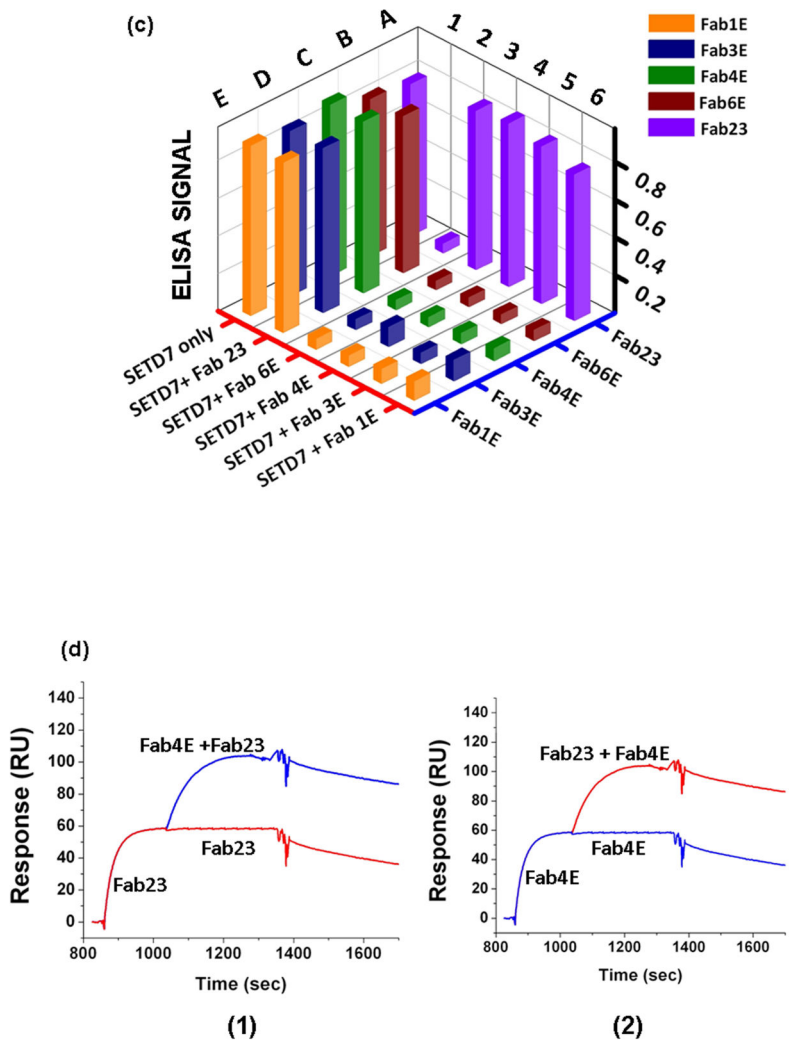


Fig. 6.

Generation of epitope specific Fabs. (a) “Epitope exclusion” sorting strategy to generate Fabs binding to epitope other than the immunodominant one (shown in red arrow). The antigen was pre-incubated with excess of Fab23 so that the immunodominant epitope is masked, driving the phage pool to bind to other epitopes during selection. Excess of Fab23 in the solution blocks the epitope and also helps to wash away the binders specific for the masking Fab. (b) Competitive phage ELISA results show that Fabs generated by “epitope masking” bind to SETD7 even in presence of 1uM Fab23 (green bars) but successfully competed off by 50nM SETD7 (blue bars). This proves that these Fabs are very specific and bind to epitopes different from Fab23 which are available even in presence of 1 uM Fab23. (c) Epitope binning experiment by protein ELISA proves that the Fabs bind to an epitope distinct from Fab23 as they are not competed by Fab23. Fab1E, Fab3E, Fab4E and Fab6E compete among themselves for the same epitope. (d) Epitope binning experiment using SPR. His_{10}-SETD7 is immobilized on NTA sensor chip. (1) A mixture of Fab4E + Fab23 (blue sensogram) is injected after saturating the ligand surface with first analyte (Fab23). 2nd injection with Fab23 alone is used as control (red sensogram). Overlay of the two

sensograms shows a net increase in signal intensity when Fab4E + Fab23 is injected indicating that Fab4E is binding to an epitope that is non overlapping with the epitope to which Fab23 binds. (2) Same pattern is observed on reversing the order of analyte injection. Here the ligand is first saturated with Fab4E followed by injecting a mixture of Fab23 +Fab4E (red sensogram). Control experiment was done using Fab4E alone in the second injection (blue sensogram). Similar increase in net signal intensity in comparison to control further confirms sequential binding of the Fabs to different epitopes on SETD7.

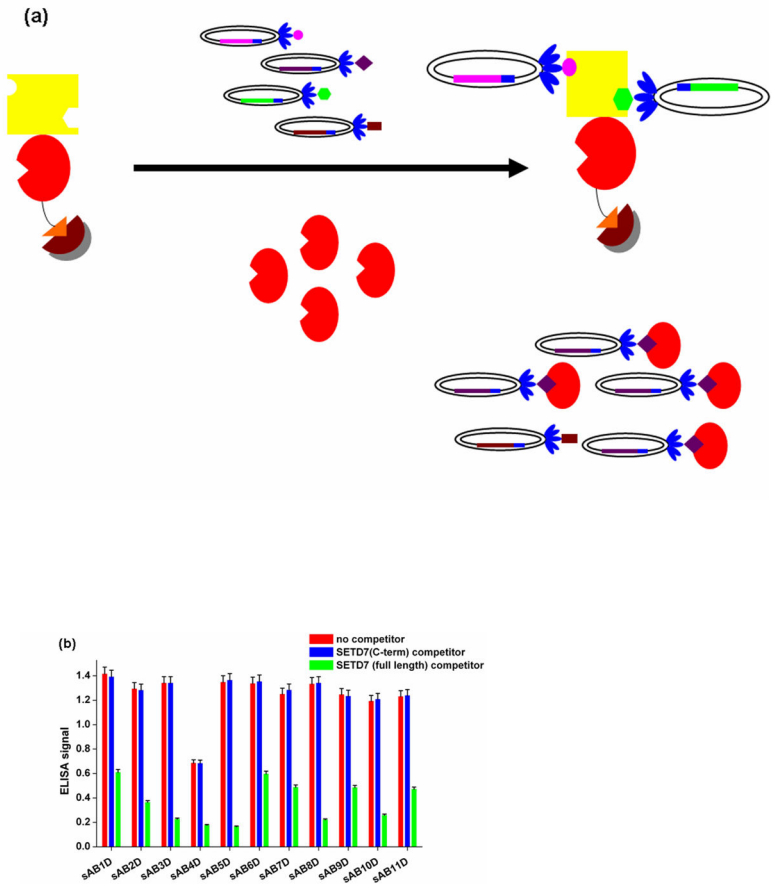


Fig. 7.

Generation of domain specific Fabs. (a) “Domain exclusion” sorting strategy to generate Fabs exclusively to N-terminal domain (yellow). The C-terminal domain (red) is used in huge excess as soluble competitor to enrich for binders specific for N-terminal domain. Phages binding to the C-terminal domain are washed away. (b) Competitive phage ELISA results show that Fabs are not competed by C-terminal domain of SETD7 (blue bars) but by full length construct of SETD7 (green bars). This proves that the sABs bind to N- terminal domain of SETD7.

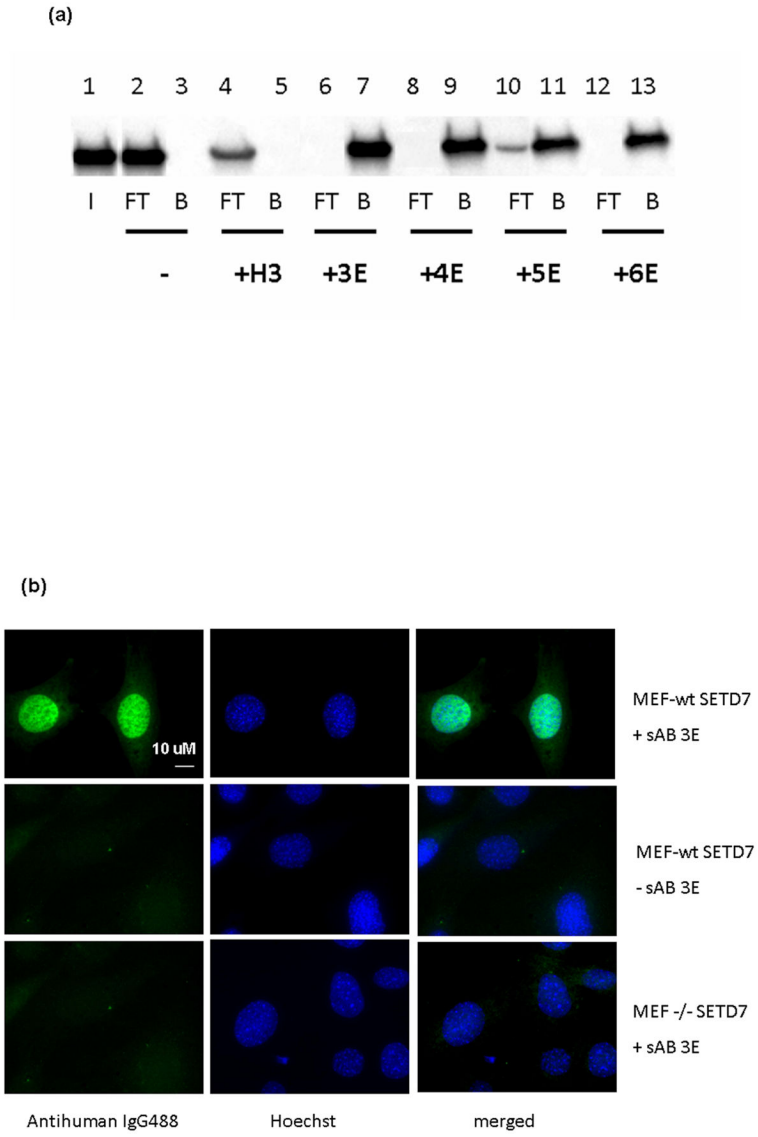


Fig. 8.

Immunoprecipitation (IP) and immunofluorescence (IF). (a) Native IP: Western Blot analysis showing antiSETD7 Fabs are able to recognize and pull down overexpressed Flag-tagged SETD7 in native form from HEK293 cells. The protein is entirely in the flowthrough (FT) (lane 2) when the cell lysate is incubated with empty beads. No band is seen in the bead fraction (B) (lane 3). Similar effect is observed with an unrelated anti-GFP Fab (H3) coated beads. The protein is observed in flowthrough (lane 4) and no band in bead fraction (lane 5). Beads coated with anti SETD7 Fabs (3E, 4E, 5E and 6E) capture Flag-tagged SETD7 in native form almost quantitatively from HEK293 lysate. (b) Immunofluorescence: Immunostainings of MEFs overexpressing wild type SETD7 (wtSETD7) with Fab3E as a primary antibody and secondary antihuman Alexa-488 conjugated IgG. Nuclear localization of wtSETD7 is observed. Hardly any signal was obtained with the negative controls −/− MEFs that proves the specificity of Fab3E.

Table 1

Enrichment of MBP binding phage (MOS1) over control phage in a “doping” selection

Input			Output			
Titer MOS1 (cfu/ml)	**Titer SETA1 (cfu/ml)**	**Ratio of titer MOS1: SETA1**	**Rounds of sorting**	**Titer MOS1 (cfu/ml)**	**Enrichment [a]**	**Ratio[b] (MOS1:SETA1)**
2.0×10^7	2.0×10^9	$1:10^2$	2^{nd}	4.0×10^5	10	90:6
			3^{rd}	3.0×10^6	100	95:1
			4^{th}	2.0×10^7	>500	96:0
2.0×10^5	2.0×10^9	$1:10^4$	2^{nd}	3.0×10^5	5	79:17
			3^{rd}	3.0×10^6	30	92:4
			4^{th}	5.0×10^7	>100	96:0
2.0×10^2	2.0×10^9	$1:10^7$	2^{nd}	1.0×10^5	-	1:95
			3^{rd}	2.0×10^6	20	90:6
			4^{th}	6.0×10^7	>100	96:0

[a] Fold enrichment was calculated based on ratio of titer of MOS1 and SETA1 phage in output.

[b] Ratio of the positive colonies screened by colony PCR in each round. 96 colonies are screened in each round. The number of colonies harboring MOS1 phagemid increases in successive round.

Table 2

Target (UNIPROT ID)	Pab ID	k_{on} (X 10′ M^{-1} I^{-1})	k_{off} (X 10^{-4} I^{-1})	K_D (nM)	CDR-L3 91	92	93	94	94a	94b	95	96	CDR-H1 29	30	31	32	33	34	CDR-H2 50	51	52	52a	53	54	55	56	57	58
	B01	16	0.43	2.7	Y	S	Y	G	-	-	L	I	F	S	S	Y	S	I	Y	I	S	S	S	S	G	Y	T	Y
	F02	3	0.53	17.7	Y	Y	F	S	-	-	L	I	V	Y	Y	Y	Y	I	S	I	S	P	Y	Y	G	S	T	Y
MBP (P0AEX9)	G10	9.3	0.47	5.1	S	S	S	S	-	-	L	I	V	Y	Y	Y	S	I	S	I	Y	P	Y	Y	G	S	T	S
	H12	2.6	0.71	27.3	S	Y	Y	F	-	-	P	F	F	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
	A 06	5.9	0.82	13.9	S	S	S	S	-	-	L	I	L	S	Y	S	S	I	S	I	S	S	S	S	G	S	T	S
	F04	52	0.94	1.8	S	Y	Y	Y	K	K	L	I	F	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
	B12	9.4	1.2	12.8	S	S	S	S	-	-	L	I	F	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
SETD7 (QBWTS6)	B05	51	2.4	4.7	S	L	W	G	-	-	P	V	V	S	S	S	Y	I	S	I	S	S	Y	Y	G	S	T	S
	G11	29	0.56	1.9	S	S	S	S	-	-	L	I	F	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
	H07	130	1.1	<1.0	S	F	T	S	-	-	P	I	F	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
	B09	17	2.1	12.4	S	Y	Y	Y	-	-	P	F	I	Y	Y	Y	Y	I	Y	I	S	P	Y	S	G	Y	T	S
	A07	76	0.4	<1.0	R	Y	Y	P	-	-	L	V	I	Y	Y	S	S	I	S	I	S	S	Y	Y	G	Y	T	Y
HEF1 (Q14511)	C01	4.6	0.77	16.7	S	S	S	S	-	-	L	I	I	Y	S	S	S	I	S	I	S	S	Y	S	G	Y	T	Y
	D11	2.5	0.55	22	S	Y	Y	Y	-	-	L	F	F	S	S	S	Y	I	Y	I	S	S	Y	S	G	Y	T	Y
	H01	1.8	0.3	16.7	S	S	S	S	-	-	L	I	I	Y	S	S	S	I	S	I	S	S	Y	S	G	Y	T	Y
	A04	10	0.95	9.5	G	W	S	G	-	-	L	I	V	Y	S	S	S	I	S	I	Y	S	S	S	S	Y	T	S
	B08	12	0.83	6.9	S	S	S	S	-	-	L	I	F	S	S	S	Y	I	S	I	Y	S	Y	Y	G	Y	T	S
LBD of ERR1 (P11474)	C09	13	0.34	2.6	V	S	Y	W	R	-	L	I	F	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
	F03	6.4	0.86	13.4	S	S	S	S	-	-	L	I	V	S	S	S	S	I	S	I	S	S	S	S	G	S	T	S
	G11	17	1.5	8.8	S	K	Y	Y	-	-	P	I	I	Y	S	S	S	I	S	I	Y	S	S	Y	G	Y	T	Y
	A06	32	0.34	1.1	K	P	W	G	-	-	L	V	V	Y	S	S	S	I	S	I	S	S	Y	Y	G	Y	T	S
	B09	10	0.45	4.5	G	V	D	F	-	-	P	I	F	S	Y	S	S	I	S	I	Y	S	S	S	G	S	T	S
SCAN domain (Q8IWY8)	B04	19	4.4	23.2	S	S	S	S	-	-	L	I	F	Y	S	Y	Y	I	S	I	S	S	S	S	G	S	T	S
	F08	70	3.8	5.4	K	S	M	G	-	-	L	I	F	Y	S	S	S	I	S	I	S	P	S	Y	G	S	T	Y
	H07	11	0.82	7.5	S	S	S	S	-	-	L	I	V	S	Y	S	S	I	S	I	S	S	S	S	G	S	T	S
	A01	73	0.2	<1.0	I	Y	G	S	-	-	L	I	F	S	Y	S	Y	I	Y	I	S	S	Y	S	G	Y	T	Y
GAP1 (Q6GIL8)	B04	19	0.47	2.5	S	S	P	W	G	-	P	I	I	S	S	S	Y	I	Y	I	S	S	S	Y	G	S	T	Y
	A03	35	0.28	<1.0	E	R	Y	R	W	-	L	I	F	Y	S	Y	S	I	Y	I	Y	P	Y	S	G	S	T	S
	B04	21	0.49	2.3	S	W	D	Y	G	-	L	L	F	Y	S	S	S	I	S	I	S	S	S	S	S	S	T	S
PGK (Q6GIL7)	B09	30	0.39	1.3	S	G	S	S	-	-	P	L	I	S	Y	Y	S	I	Y	I	S	P	S	S	G	Y	T	S
	B02	65	1	1.5	Y	Y	G	W	S	-	L	V	V	V	S	S	S	I	S	I	S	S	Y	S	G	S	T	Y
	A08	140	0.79	<5.6	Y	G	Q	S	-	-	L	I	F	S	Y	S	Y	I	S	I	Y	P	Y	Y	G	S	T	S
Bromo (Q7JVP4)	B08	110	0.31	<2.8	T	T	Y	N	-	-	L	I	V	S	S	Y	Y	I	S	I	Y	S	Y	Y	G	S	T	Y
	B09	0.84	0.26	31	Y	K	Y	S	-	-	L	I	V	Y	Y	S	Y	I	S	I	Y	S	Y	Y	G	Y	T	S

Target (UNIPROT ID)	Pab ID	CDR-H3 94	95	96	97	98	99	100	100a	100b	100c	100d	100e	100f	100g	100h	100i	100j	100k	100l	100m	101
	B01	R	A	Y	E	R	H	S	Y	S	Y	Y	W	R	F	Y	G	Y	A	G	M	D
	F02	R	S	Y	G	P	Y	Q	L	Y	G	W	H	S	N	-	-	-	-	A	L	D
MBP (P0AEX9)	G10	R	F	Y	S	Y	T	Y	P	W	A	Q	W	G	Y	-	-	-	-	A	L	D
	H12	R	Y	Y	K	F	M	S	Y	Y	W	P	Y	Y	Y	Q	H	K	-	G	M	D
	A 06	R	Q	Y	W	Y	S	Y	G	Y	R	D	S	Y	Q	I	-	-	-	G	F	D
	F04	R	Y	K	P	Y	H	S	Y	N	T	G	Y	W	Y	W	Y	G	-	A	M	D
	B12	R	K	L	G	Y	K	Y	W	G	S	S	Y	F	W	Y	Y	H	-	A	L	D
SETD7 (QBWTS6)	B05	R	W	Y	Y	L	S	S	Y	Y	S	R	Y	Q	Q	T	S	Y	-	G	F	D
	G11	R	L	Q	E	G	P	R	A	W	Y	Y	W	Y	Q	R	Y	-	-	G	M	D
	H07	R	W	Y	G	Y	Y	M	R	W	S	S	Y	F	Y	Q	-	-	-	A	F	D
	B09	R	A	Y	Y	E	W	G	V	G	P	G	T	-	-	-	-	-	-	G	L	D
	A07	R	Q	S	P	D	V	F	Y	Y	Y	M	T	-	-	-	-	-	-	G	M	D
HEF1 (Q14511)	C01	R	Y	V	Y	W	G	S	Y	T	G	Y	-	-	-	-	-	-	-	G	I	D
	D11	R	S	Y	W	E	F	G	G	T	S	I	-	-	-	-	-	-	-	A	L	D
	H01	R	F	K	G	W	G	H	Y	Y	S	W	-	-	-	-	-	-	-	A	L	D
	A04	R	S	G	S	H	P	G	Y	Y	W	M	W	W	G	K	Y	T	-	G	L	D
	B08	R	G	T	Y	K	Y	S	Y	G	G	W	S	I	R	H	G	-	-	A	M	D
LBD of ERR1 (P11474)	C09	R	K	W	G	G	S	S	W	H	Y	W	Q	Y	G	S	-	-	-	A	F	D
	F03	R	Y	N	V	T	Y	W	L	Y	Y	Y	Y	E	F	P	G	Y	-	G	M	D
	G11	R	G	F	Y	V	W	F	S	S	W	S	-	-	-	-	-	-	-	A	F	D
	A06	R	P	F	K	E	V	P	G	F	Y	Y	G	G	W	Y	S	S	V	G	F	D
	B09	R	G	Y	A	W	K	Y	S	R	Y	G	Y	I	G	-	-	-	-	G	I	D
SCAN domain (Q8IWY8)	B04	R	Q	M	T	Y	M	W	G	W	P	R	-	-	-	-	-	-	-	G	F	D
	F08	R	M	Y	P	Q	G	L	W	S	Y	Y	M	W	K	G	F	W	-	A	M	D
	H07	R	N	A	Y	W	S	W	N	D	W	W	G	K	Y	G	Y	-	-	A	L	D
	A01	R	Y	H	G	S	W	W	W	K	S	K	-	-	-	-	-	-	-	A	L	D
GAP1 (Q6GIL8)	B04	R	G	G	W	V	W	Y	Y	P	Y	Y	Y	T	L	W	K	-	-	G	M	D
	A03	R	Q	I	W	G	T	G	M	Q	G	W	Y	P	Y	L	G	G	-	G	F	D
	B04	R	H	T	W	L	W	Y	Y	S	W	P	S	I	A	W	G	-	-	A	L	D
PGK (Q6GIL7)	B09	R	G	A	Y	Y	M	W	W	W	G	R	Y	-	-	-	-	-	-	G	L	D
	B02	R	T	Y	S	S	Q	V	W	P	W	G	G	Y	-	-	-	-	-	A	L	D
	A08	R	E	S	V	Y	Q	Q	Y	W	W	N	V	S	W	G	-	-	-	G	I	D
Bromo (Q7JVP4)	B08	R	Y	V	Y	S	Y	W	Y	Y	S	P	-	-	-	-	-	-	-	G	M	D
	B09	R	T	S	Y	P	F	Y	R	H	Y	S	T	-	-	-	-	-	-	A	M	D

Table 3

Affinity of sABs for the apo and bound forms of MBP

sAB ID	$K_{D\ apo}$ (nM)	$K_{D\ bound}$ (nM)
8M	144	2
13M	49	2
17M	28	0.3
2O	2.7	NB
3O	16.2	NB
4O	17.7	NB