Design of Protein–Peptide Interaction Modules for Assembling Supramolecular Structures in Vivo and in Vitro

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ABSTRACT: Synthetic biology and protein origami both require protein building blocks that behave in a reliable, predictable fashion. In particular, we require protein interaction modules with known specificity and affinity. Here, we describe three designed TRAP (Tetratricopeptide Repeat Affinity Protein)–peptide interaction pairs that are functional in vivo. We show that each TRAP binds to its cognate peptide and exhibits low cross-reactivity with the peptides bound by the other TRAPs. In addition, we demonstrate that the TRAP–peptide interactions are functional in many cellular contexts. In extensions of these designs, we show that the binding affinity of a TRAP–peptide pair can be systematically varied. The TRAP–peptide pairs we present thus represent a powerful set of new building blocks that are suitable for a variety of applications.

Many cellular processes are mediated by protein–protein interactions. Creating new protein interactions or modifying existing protein interactions is a particularly appealing approach with which to control signal transduction, protein degradation, and metabolic flux.1−5 For these applications, we require protein–protein interaction modules that are modular and functional in vivo. Moreover, one would ideally have sets of protein–protein interaction pairs that are mutually compatible so that multiple cellular processes can be controlled simultaneously. A number of groups have focused on designing heterodimeric coiled coils as interaction modules.6−10 However, there are a limited number of robust and well-defined building blocks that can be used to create artificial protein interactions in vivo. Such protein–protein interaction modules can also be used for many in vitro nanotechnology applications such as protein origami and stimuli-responsive nanomaterials.11,12 To be generally useful for in vivo assembly purposes, it is essential that a designed interaction module exhibit minimal cross-reactivity with itself, with a partner from a different interaction pair, or with any cellular component.

Our goal is to create a series of robust well-defined protein interaction modules that can be used to assemble protein interactions in vivo and in vitro. We base our designs on tetratricopeptide repeat (TPR) affinity proteins, or TRAPs, which function naturally as protein interaction modules and recognize short extended peptides. Repeat proteins are an appealing scaffold for protein design because they are small, modular, and stable, and they have previously been engineered to recognize numerous cellular proteins.13−16 Unlike previous designs, however, our aim is to recognize a protein or peptide sequence that does not exist in nature. Thus, our goal requires that we alter both the protein and the peptide sequence.

We present the successful design and characterization of three TRAP–peptide interactions. We show that the TRAPs display minimal cross-reactivity with each other or with cellular proteins. Therefore, they are fully functional in vitro and in vivo. We also demonstrate that the TRAP–peptide interactions can be used to assemble protein interactions in E. coli. An advantage of our designs is that neither the TRAPs nor the peptides display any self-homodimerization. Our designs have a clear advantage over DNA or peptide-based assemblies, which can form elaborate user-defined structures in vitro but are typically not functional within cells.17

RESULTS AND DISCUSSION

The TPR–Peptide Interaction. The TPR is a 34-amino-acid helix–turn–helix motif.18 Most TPR modules contain three TPR repeats, which is the minimal number of repeats required to mediate protein–protein interactions.19,20 Figure 1 shows a crystal structure and cartoon of a natural three TPR module bound to its cognate peptide, MEEVD. We chose to use this natural TPR–peptide module as our design template because it is extremely well characterized.21,22 In particular, we highlight three features of our design template that we leverage in our approach. First, the TPR requires a free C-terminus on the peptide for binding.21 We maintain this feature in all of our designs because it limits the potential of the TPRs to cross-react with cellular proteins. Second, an important feature of TPR modules and all repeat proteins in general is that the residues that specify the fold are separate from the residues that
specify the function. This enables us to alter the TPR’s binding affinity and specificity without significantly changing its structure or stability. Third, the TPR binding interface is modular in that it consists of five pockets, one for each of the amino acids in the peptide (schematized in Figure 1B). For clarity, we label each peptide position and their corresponding binding pockets 1−5 in Figure 1B. Henceforth, we will refer to either the peptide position or the binding pocket by these numbers.

To create three TRAP−peptide pairs, we redesigned three of the binding pockets on the TRAP and the corresponding three positions on the peptide. The residues in each binding pocket are shown in Figure 2A and Supporting Information Figure 1. To achieve the goal of creating TRAP−peptide pairs with the highest specificity and least cross-reactivity, we used both positive and negative design strategies.

We previously created a TRAP pocket 5, which preferentially binds bulky nonpolar residues (such as Trp) over smaller nonpolar residues (such as Leu, Ile, and Val) at peptide position 5. We sought to create a pocket with the inverse binding specificity. In other words, we sought to identify a pocket 5 that preferentially binds Leu, Ile, or Val and discriminates against Trp. To accomplish this goal, we created a library in which we varied the three residues in pocket 5 to be any combination of the hydrophobic amino acids Leu, Ile, Val, and sulfur atoms colored red and yellow, respectively. Each amino acid in the peptide is labeled with its one-letter code. The N and C termini of the TRAP are indicated. (B) Schematic representation of the TPR−peptide interaction. Each residue in the peptide is depicted as a blue sphere; its corresponding binding pocket in the TRAP is depicted as a gray crescent. The peptide residues are numbered 1 to 5 (N to C terminus). Each pocket is also numbered to correspond with the peptide position it recognizes.

Figure 1. The TRAP−peptide design template. (A) Crystal structure of a natural TPR module bound to its cognate peptide (TPR2A from the protein heat shock organizing protein bound to the peptide sequence MEEVD, PDB ID: 1ELR). The TPR module is shown as a gray ribbon. The peptide is shown as blue sticks with oxygen and sulfur atoms colored red and yellow, respectively. Each amino acid in the peptide is labeled with its one-letter code. The N and C termini of the TRAP are indicated. (B) Schematic representation of the TPR−peptide interaction. Each residue in the peptide is depicted as a blue sphere; its corresponding binding pocket in the TRAP is depicted as a gray crescent. The peptide residues are numbered 1 to 5 (N to C terminus). Each pocket is also numbered to correspond with the peptide position it recognizes.

Figure 2. Three distinct TRAP−peptide interaction pairs. (A) We alter three pockets and their corresponding peptide positions in our design strategy. Schematic representation of the design template’s secondary structure and sequence. The design template contains three repeats (numbered 1−3 from the N to the C terminus). The first helix in each repeat is labeled “A” and colored gray. The second helix in each repeat is labeled “B” and colored white. The solvating helix, colored black and represented by “S” is also labeled. The residues in the three TRAP pockets are presented by three different colors. Please note that pocket 3 in both TRAP 2 and TRAP 3 did not require any compensatory mutations to accommodate the modified peptide (refer to Supporting Information Figure 2 for more details). (C) Pulldown assay demonstrating that each TRAP specifically recognizes its cognate peptide. GST-tagged peptides pull down TRAP proteins and are analyzed on a gel. In each lane, the position of the GST-tagged peptide is indicated by the open arrow, and the pulled down TRAP is indicated by the closed arrow. The identity of the peptide in each experiment is indicated above the lane with a cross; the identity of the TRAP is indicated below the schematic. We also indicate the identity of the residues in the designed TRAPs that differ from the design template.

We previously created a TRAP pocket 5, which preferentially binds bulky nonpolar residues (such as Trp) over smaller nonpolar residues (such as Leu, Ile, and Val) at peptide position 5. We sought to create a pocket with the inverse binding specificity. In other words, we sought to identify a pocket 5 that preferentially binds Leu, Ile, or Val and discriminates against Trp. To accomplish this goal, we created a library in which we varied the three residues in pocket 5 to be any combination of the hydrophobic amino acids Leu, Ile, Val,
Phe, and Met (TRAP pocket 5 is colored magenta in Figure 2A and Supporting Information Figure 1). We also created a peptide library containing small hydrophobic residues (Leu, Ile, and Val) at peptide position 5 (Figure 2A, Supporting Information Figure 1A). We screened the TRAP library against the peptide library using the in vivo split GFP assay described previously. Briefly, GFP is split into two halves, which have no binding affinity for each other. Fluorescence is reconstituted only if the two halves are fused to two proteins or peptides that interact. We fused the TRAP library to the C-terminal half of GFP (CGFP) and the peptide library to the N-terminal half of GFP (NGFP) (Supporting Information Figure 2A). We coexpressed the libraries in E. coli, and we identified the TRAPs that gave rise to the highest fluorescence.

The best TRAP–peptide pair that we identified bound peptides with Val at peptide position 5 about 2-fold better than peptides with Trp at peptide position 5 (Supporting Information Figure 2A). We will refer to this interaction as TRAP 1 and peptide 1 (or MEEVV). Although TRAP 1 binds small nonpolar residues better than large nonpolar residues, we wanted to achieve greater specificity between the different TRAP–peptide pairs.

Therefore, we sought to modify additional pockets and their corresponding peptide positions. Our strategy here exploits charge complementarity at TRAP pockets 2 and 3 (TRAP pockets 2 and 3 are colored orange and green, respectively, in Figure 2A and Supporting Information Figure 1). Because the TRAP and the peptide libraries were small, we screened different TRAP–peptide combinations in vitro using pull-down assays. For more details about these libraries, refer to Supporting Information Figure 2. Through this screen, we identified two TRAP–peptide pairs: TRAP 2, which binds to peptide 2 (or MERVW), and TRAP 3, which binds to peptide 3 (or MRRVW). These additional modifications greatly improved the discrimination of TRAP 1 for peptide 2 and peptide 3 (to ~7 fold and 200 fold, respectively).

High Specificity TRAP–Peptide Interactions. Figure 2B shows a summary of the TRAP–peptide interactions and indicates changes to either the TRAP or the peptide relative to the design template. For molecular engineering applications, it is critical that we know the affinity and specificity of the TRAP–peptide pairs. Therefore, we sought to characterize the affinity of each TRAP for each peptide using two complementary approaches—pull-down assays, which allow a side-by-side functional comparison, and isothermal titration calorimetry (ITC), which provides quantitative thermodynamic data. Representative pull-down assays and ITC titrations are shown in Figure 2C and D, respectively. The ITC data is summarized in Table 1.

Both assays clearly demonstrate that each TRAP recognizes its cognate peptide with affinities in the low micromolar range (Table 1, Figure 2C). These affinities are weaker than many designed protein interaction modules, such as DARPinPs, in which nanomolar or stronger affinities are frequently achieved. However, the affinity of our designs is characteristic of many natural peptide-binding protein modules such as SH3, PDZ, and SH2 domains, which function in signal transduction and other cellular processes. Protein interaction modules with similar affinities have also been used for many in vitro biotechnology applications. Therefore, we believe that the affinity of our TRAP–peptide pairs will be sufficient for numerous applications.

Moreover, each TRAP displays a clear preference for its cognate peptide. For clarity, we will discuss the specificity of the TRAP–peptide interactions in terms of pairs. Two of the three TRAP–peptide pairs display very little cross-reactivity. The TRAP 1–TRAP 3 pair is extremely specific. TRAP 1 discriminates against peptide 3 200 fold and TRAP 3 discriminates against peptide 1 about 100 fold (Table 1). The TRAP 2–TRAP 3 pair is also quite specific. TRAP 2 discriminates against peptide 3 about 50 fold, and TRAP 3 discriminates against peptide 2 about 100 fold. The TRAP 1–TRAP 2 pair is the least specific. TRAP 1 discriminates against peptide 2 about 7 fold, and TRAP 2 discriminates against peptide 1 about 25 fold.

To better convey the functional specificity of our interaction modules, we calculate the amount of each TRAP bound to each peptide at a concentration of 10 μM (Figure 3). We chose this concentration because it is reasonably obtainable in vitro and in vivo. It also results in high occupancy for each cognate interaction. At lower concentrations, the TRAPs will display even greater discrimination against noncognate peptides. The TRAP 1–TRAP 3 pair displays minimal cross-reactivity at this concentration, as does the TRAP 2–TRAP 3 pair. TRAP 1 and TRAP 2 display some cross-reactivity; nevertheless, this interaction pair may still be suitable for applications that require a low concentration of the peptide. In summary, we believe that the specificity of the TRAP 1–TRAP 3 pair and the TRAP 2–TRAP 3 pair will be sufficient for most in vivo and in vitro applications.

### Table 1. Dissociation Constants (in μM) for Each TRAP–Peptide Pair As Determined by ITC

<table>
<thead>
<tr>
<th>Peptide</th>
<th>TRAP 1</th>
<th>TRAP 2</th>
<th>TRAP 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEEVV</td>
<td>1</td>
<td>42.1</td>
<td>&gt;500</td>
</tr>
<tr>
<td>MERVW</td>
<td>6.8</td>
<td>1.7</td>
<td>&gt;500</td>
</tr>
<tr>
<td>MRRVW</td>
<td>250</td>
<td>83</td>
<td>7.5</td>
</tr>
</tbody>
</table>

*For interactions in which we were unable to detect binding, we estimate the Kᵦ by assuming the c value is equal to 1 (C = M·N·c/Kᵦ, where M is the total macromolecule concentration in the ITC cell and N is the stoichiometry). The diagonal of the table, indicated by bold font, represents the Kᵦ for each cognate TRAP–peptide pair. Each value represents the average of two or three measurements. Standard deviations for biological replicates are typically ±20–30%.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Binding specificity of each TRAP for its cognate and noncognate peptides. Plots show the fraction of peptide bound to each TRAP. We calculate the fraction bound peptide based on the dissociation constants in Table 1 at a peptide concentration of 10 μM. The identity of each TRAP is indicated below the bar graph. Pink bars represent peptide 1 (MEEVV). Blue bars represent peptide 2 (MERVV). Green bars represent peptide 3 (MRRVW).
Affinity of the TRAP–Peptide Interaction Can Be Tuned Rationally. It may also be desirable to have a series of protein–protein interactions with varying affinities. They can be used to perturb the strength of a particular protein–protein interaction in a natural or engineered network.

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\text{Figure 4. Rational modulation of TRAP 2’s binding affinity. Left: Schematic representation of each TRAP, its cognate peptide, and peptide variants that differ only in the identity of the C-terminal amino acid (shades of red). Right: Comparison of the TRAP–peptide pairs in pull-down assays. In each lane, the position of the GST-tagged peptide is indicated by the open arrow, and that of the pulled down TRAP is indicated by the closed arrow. Panel A shows data for TRAP*; Panel B shows data for TRAP 2; Panel C shows data for TRAP 3. The sequence of the peptide is indicated above each lane. GST containing no peptide is used as a control and is indicated in each panel.}
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We previously described a TRAP with a modified pocket 5 that has a preference for large nonpolar residues at peptide position 5 (represented as TRAP* in Figure 4A).24 We incorporate this pocket into TRAP 2. We can use the specificity of this pocket to modulate the affinity of TRAP 2 rationally. Specifically, we created TRAP–peptide pairs with decreased binding affinity by varying the size of the amino acid at peptide position 5 (Figure 4B). We also attempted to modulate the binding affinity of TRAP 3 because it incorporates the same binding pocket 5. However, we were unable to detect binding between TRAP 3 and the modified peptides in our assay because the affinity of TRAP 3 for peptide 3 is about 5 fold lower than the affinity of TRAP 2 for peptide 2 (Figure 4C).

We would like to emphasize that TRAP 1 contains a different binding pocket 5, so we cannot alter its binding affinity using the same approach. Nonetheless, these data indicate that we are able to systematically alter the binding affinity of TRAP 2 by changing a single amino acid in the peptide. Thus, our building blocks also include a protein interaction module whose affinity is tunable.

Assessing Potential Cross-Reactivity with Natural Proteins in Yeast and E. coli. For the TRAPs to function properly in vivo, it is critical that they do not interact with cellular proteins, which could sequester them and preclude their interaction. To assess any potential cross-reactivity, we focused our attention on C-terminal sequences in the proteome—the “C-terminome.” We chose to limit our analysis to the C-terminomes of S. cerevisiae and E. coli because most molecular engineering applications use one of these two organisms. There are no sequences in the C-terminome of either organism that are identical to any peptide sequence known to display detectable binding to TRAP 1, TRAP 2, or TRAP 3 (Supporting Information Tables 1–3). Therefore, we reduced the stringency of our search, and identified five amino acid sequences in the C-terminome of S. cerevisiae and E. coli that have two or fewer mismatches relative to any peptide sequence known to bind to any of the TRAPs. We tested the ability of these sequences to bind to all three TRAPs using pulldown assays (see Supporting Information Figure 3, SI Note 1, Supporting Information Tables 1–3).

TRAP 2 and TRAP 3 exhibited no significant binding to any of these test sequences (Supporting Information Figure 3B and C). TRAP 1 bound some of the sequences tested, so we decided to look into them further (Supporting Information Figure 3A). We concluded that the peptides derived from yeast proteins would most likely not interfere with TRAP 1’s function because their structure and subcellular localization would preclude interaction (Supporting Information Table 4). Because some of the E. coli test sequences might be accessible to the TRAP in a cellular context (Supporting Information Table 4), we tested the ability of an E. coli extract to inhibit the TRAP 1–peptide 1 interaction in a pulldown assay. Even at high lysate concentrations, no inhibition of the TRAP–peptide interaction is observed (Figure 5A). These observations, combined with the in vivo binding data using the split GFP assay (vide infra), lead us to conclude that the TRAP–peptide pairs will not be inhibited by cellular proteins in E. coli or yeast.

Some users may wish to use the TRAP–peptide interactions in human-derived cell lines. As a first step toward determining any potential cross-reactivity with human-derived cell lines, we also attempted to compete the TRAP–peptide interactions with extracts from HEK293T and HeLa cells. We observe that the mammalian cell extracts are not able to compete any of the TRAP–peptide interactions (representative data are shown for the TRAP 2–peptide 2 interaction in Figure 5B). Therefore, we believe that our TRAP–peptide pairs will also be functional in mammalian cells.

TRAP–Peptide Interactions Can Assemble Protein Complexes in Vivo. We envision that one potential application of our TRAP–peptide interactions is to create user-specified protein interactions in living cells. Therefore, we sought to further investigate the function of the TRAP–peptide pairs in E. coli. For these experiments, we used the split GFP assay as a binding sensor.25,30,31 We fused each TRAP to CGFP and each peptide to NGFP. We coexpressed each cognate
reactions, we also sought to determine whether peptide interactions are functional in cellular contexts. In panel A, we use E. coli cell lysate to compete TRAP 1 bound to GST-tagged peptide 1 (MEEVV) using pulldown assays. Lysate concentration increases from left to right (from \( \sim 0.25 \) mg mL\(^{-1} \) to \( \sim 10 \) mg mL\(^{-1} \) total protein). In panel B, we use mammalian cell lysate from HEK293T cells (total protein concentration \( \sim 2.1 \) mg mL\(^{-1} \)) or HeLa cells (total protein concentration \( \sim 0.9 \) mg mL\(^{-1} \)) to compete TRAP 2 bound to GST-tagged peptide 2 (MERVW) using pulldown assays. As a negative control, we use GST containing no peptide as indicated in the far left lane in each gel. As a positive control, we add no cell lysate as indicated in the far left lane in each gel. All lanes contain the same amount of TRAP and GST-peptide.

TRAP–peptide pair in E. coli and measured the resulting cellular fluorescence. We chose assay conditions (i.e., inducer concentration and time point) so that protein interactions with dissociation constants in the low micromolar range will be observed.\(^{32}\) As expected, all cognate TRAP–peptide pairs function in vivo and mediate GFP assembly as indicated by their fluorescence (Figure 6).

To more rigorously investigate the function of the TRAP–peptide interactions, we also sought to determine whether in vitro binding specificities are recapitulated in vivo. We coexpressed each TRAP fused to CGFP with each peptide fused to NGFP and measured the resulting cellular fluorescence. As expected, the TRAP 1–TRAP 3 and the TRAP 2–TRAP 3 pairs exhibit the least cross-reactivity in vivo (Figure 6). The TRAP 1–TRAP 2 pair exhibits some specificity, but not to the extent of the least cross-reacting pairs. Thus, the in vivo measurements mirror the in vitro binding data (compare Figures 3 and 6). These data indicate that the TRAP–peptide pairs function in E. coli as anticipated: namely, that they maintain their specificity even when presented in different contexts. If any of the TRAPs or peptides significantly bound cellular proteins, these data would not be observed.

**Conclusions.** The technology we describe here represents a significant step toward the development of reliable building blocks for synthetic biology and nanotechnology. Previous efforts have primarily focused on the development of tools to control transcription or translation.\(^{33}\) Only a few examples of post-translational building blocks have been described because the principles that govern protein–protein interactions are generally not well understood.\(^{20,33}\) Nonetheless, the engineering of more complex phenomena in living cells will require more tools to manipulate cellular processes.

A key feature of our designs is that the TRAP–peptide pairs display relatively little cross-reactivity with one another. For instance, one of our pairs has greater than 200 fold specificity. Many natural protein interaction modules are known to be promiscuous and only maintain specificity through temporal or spatial segregation.\(^{27}\) Indeed, the specificity of our best pairs is reminiscent of the specificity that has been achieved previously for SH2 domains using affinity clamps, which effectively increase the surface area of the protein interface through the use of a second protein interaction domain.\(^{34}\) Similarly, the specificity of SH3 domains has been improved through the use of N-substituted substrates.\(^{27}\) Here, we achieve similar specificities without significantly modifying the TRAP or using unnatural amino acids.

Here, we present the successful design and characterization of three TRAP–peptide pairs that can be used to create or modify cellular processes. The TRAP–peptide pairs can mediate protein assemblies in E. coli, and they display minimal cross-reactivity with each other and with cellular proteins in E. coli, S. cerevisiae, and mammalian cells. We also show that we can readily tune the affinity of one of the interactions in a defined manner. This work represents the most versatile and well-characterized set of designed building blocks to date. We believe that our protein interaction modules will be useful for engineering numerous protein assemblies in vitro and in vivo.

**METHODS**

**Cloning.** TRAP genes were cloned into the plasmid pPROEX HTa (Invitrogen) and modified to include an in frame BamHI cloning site. Peptide genes were cloned as GST fusions in a pGEX 4t3 cloning vector (GE Healthcare Life Sciences) containing a 30 amino acid

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**Figure 5.** TRAP–peptide interactions are functional in cellular contexts. In panel A, we use E. coli cell lysate to compete TRAP 1 bound to GST-tagged peptide 1 (MEEVV) using pulldown assays. Lysate concentration increases from left to right (from \( \sim 0.25 \) mg mL\(^{-1} \) to \( \sim 10 \) mg mL\(^{-1} \) total protein). In panel B, we use mammalian cell lysate from HEK293T cells (total protein concentration \( \sim 2.1 \) mg mL\(^{-1} \)) or HeLa cells (total protein concentration \( \sim 0.9 \) mg mL\(^{-1} \)) to compete TRAP 2 bound to GST-tagged peptide 2 (MERVW) using pulldown assays. As a negative control, we use GST containing no peptide as indicated in the far left lane in each gel. As a positive control, we add no cell lysate as indicated in the far left lane in each gel. All lanes contain the same amount of TRAP and GST-peptide.

**Figure 6.** TRAP–peptide pairs are functional in E. coli as detected by a split GFP assay. We fuse each TRAP to CGFP and each peptide to NGFP. We measure the resulting fluorescence of cells expressing each TRAP–peptide combination. Pink bars represent peptide 1 (MEEVV). Blue bars represent peptide 2 (MERVW). Green bars represent peptide 3 (MRRVW). The identity of the TRAP is indicated below each bar graph. The data set is normalized relative to the most fluorescent sample for each TRAP. Background fluorescence is subtracted using a control sample expressing each TRAP with NGFP containing no peptide. Bar graphs represent the average of 2–3 biological replicates, and error bars are standard deviations.
The TRAP library described in Supporting Information Figure 2A was assembled using Klenow extension of overlapping primers as described previously.38 Three positions in the TRAP (263, 298, and 301) were randomized using the degenerate codon DTS, where D represents the bases A, T, or G; S represents the bases G or C; and T represents the base T. The total theoretical library size is 216. The resulting gene library was cloned into the pMRBAD-link-CGFP vector using NcoI and AatII restriction sites as described previously.39 The peptides were cloned into pET11a-link-NFGF using XhoI and BamHI sites. The split GFP plasmids were used without modification as described previously.39

In Supporting Information Figure 2B, we mutated positions 332 and 334. In Supporting Information Figure 2C, we mutated positions 305 and 308. Sequences for all the TRAPs are provided in the Supporting Information. All numbering is based on the heat shock organizing complex as described previously.36

**Protein Expression and Purification.** TRAPs were expressed as fusion proteins with an N-terminal hexahistidine tag in BL21(DE3) Gold cells. Five-milliliter starter cultures were added to 500 mL of autoinduction media and shaken at 25 °C for approximately 16 h. Cells were harvested by centrifugation, incubated in 20 mL of lysis buffer on ice (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM β-mercaptoethanol containing one EDTA free protease tablet (Roche) and 20 mg lysozyme), and lysed by sonication. Lysate was cleared by centrifugation. Nickel-NTA resin (Qiagen) was washed twice with buffer A (50 mM Tris, pH 7.4, 150 mM NaCl) containing 20 mM imidazole. Lysate was applied to the resin and allowed to bind for about 15 min at 4 °C. The resin was washed first with buffer A and then five times with buffer B (50 mM Tris, pH 7.4, 300 mM NaCl). Bound protein was eluted using buffer C containing 250 mM imidazole. The imidazole was removed by dialysis (into 50 mM Tris, pH 8.0, 300 mM NaCl, 5 mM 2-mercaptoethanol at 4 °C).

GST peptides were expressed and purified as above, except that glutathione sepharose 4B resin (GE Healthcare Life Sciences) was used and washed with phosphate buffered saline, pH 7.4 (PBS). Bound protein was eluted using PBS, pH 8.0, containing 50 mM reduced glutathione.

Where necessary, proteins were further purified by gel filtration on an S75 column in 50 mM Tris, 300 mM NaCl, and 5 mM 2-mercaptoethanol. The peak corresponding to free TRAP is symmetric and elutes at a position consistent with the TRAP being a monomer. Protein was concentrated using Amicon Ultra-15 centrifugal filters with a 10 kDa molecular weight cutoff and frozen at −80 °C until use.

**Pulldowns.** 35 Approximately 50 μg of GST peptide was incubated with glutathione sepharose 4B resin for 1 h at 4 °C. We used a volume of 200 μL for each wash and incubation step, unless otherwise noted. The resin was washed five times with PBS buffer. Then we incubated the resin with 100 μg of TRAP for 1 h at 4 °C. The resin was washed five times again with PBS buffer. Finally, we boiled the resin with 15 μL of SDS loading dye and 10 μL of water for 10 min to elute the bound TRAP and GST-peptide. We separated 15 μL of the supernatant on a 15% SDS/PAGE gel.

For cell lysate competition experiments, we substitute varying amounts of PBS buffer for BL21(DE3) Gold E. coli or mammalian cell lysate. Cell lysates from HEK293T and HeLa cells were prepared as described previously.38 E. coli cell lysate was prepared as described above except that cells expressing no protein were used and no antibiotics were added to the medium. We used Bradford assays to estimate the total protein concentration of each cell lysate. These concentrations are as follows: E. coli cell lysate is 10 mg mL⁻¹; HEK293T cell lysate is 2.1 mg mL⁻¹. HeLa cell lysate is 0.9 mg mL⁻¹.

**ITC.** ITC experiments were carried out in an iTC200 (MicroCal) with a 10 kDa molecular weight cutoff and frozen at −80 °C until use. Protein concentrations were determined on an HP8453 UV–vis spectrophotometer (Agilent) in triplicate. The extinction coefficients for TRAP 1 (20 860 M⁻¹ cm⁻¹), TRAP 2 (22 350 M⁻¹ cm⁻¹), and TRAP 3 (22 350 M⁻¹ cm⁻¹) were determined by the ProtParam tool of ExPASy (web.expasy.org/protparam). Immediately prior to each experiment, all proteins were centrifuged at 10 000 rpm for 10 min and then degassed for 10 min in a MicroCal Thermo Vac Degasser. Approximately 500–1200 μM TRAP was titrated into a cell containing 30–100 μM GST-tagged peptide. Control experiments in which TRAPs were titrated into either GST alone (GST subtraction) or PBS buffer (buffer subtraction) were conducted. All experiments were conducted with a 0.5 μL initial injection followed by 20 2.0 μL injections with a 250 s spacing between injections. The syringe stirred at 1000 rpm at a temperature of 25 °C for the duration of the experiment. The heats from each thermogram were integrated and fit to a 1:1 binding model in Origin 7E (OriginLab). Typically, the stoichiometry for each measurement was 1 ± 0.1. Each measurement was repeated in duplicate or triplicate.

**Circular Dichroism.** All circular dichroism measurements were conducted in PBS buffer (pH 7.4) on an Aviv circular dichroism spectrometer Model 215. The concentration of each TRAP was ~10 μM and determined as indicated above. Wavelength scans of each TRAP display characteristics of an α-helix with minima at 208 and 222 nm. We conducted thermal denaturation experiments by monitoring the absorbance at 222 nm as a function of temperature. We recorded measurements from 4 to 75 °C with 1 °C steps and a 1 min of equilibration time at each step (see Supporting Information Figure 4).

**Split GFP Assay.** TRAP libraries were cotransformed with NFGP-peptide in BL21(DE3) Gold cells and plated on LB agar containing 50 μM IPTG, 0.05% arabinose, 100 μg mL⁻¹ ampicillin, and 50 μg mL⁻¹ kanamycin. Plates were incubated overnight at 30 °C and then examined the following morning using a 1W Royal Blue LED flashlight and glasses containing a 500 nm LP filter (NIGHT-SEA). We picked the brightest colonies, extracted the pMRBAD-link-CGFP plasmid by miniprep (Qiagen), and sequenced the TRAP gene (W.M. Keck Foundation Biotechnology Resource Laboratory). Hits were confirmed by retransforming individual TRAP–CGFP members with their NFGP peptide counterpart and plated on inducing plates and rescreened for fluorescence.

For quantitative measurements of split GFP formation, we cotransformed TRAP–CGFP with NFGP–peptide in BL21(DE3) Gold. Colonies were picked and inoculated in 5 mL of LB containing 100 μg mL⁻¹ ampicillin and 50 μg mL⁻¹ kanamycin overnight at 30 °C. The cultures were diluted 1/100 in 100 mL of LB containing the same concentration of antibiotics and grown at 30 °C until they reached an OD600 of 0.5. They were induced with 50 μM IPTG and 0.05% arabinose and grown for an additional 6 h at 20 °C. Thirty-milliliter aliquots of the cells were harvested and frozen at −20 °C until future use.

To measure the fluorescence of cells expressing the split GFP components, we washed the cells twice by centrifugation and resuspension in 5 mL of PBS (pH 7.4). After the final wash, the cells were diluted to an OD600 of ~0.3–0.5 in PBS. The OD was recorded and used to normalize the fluorescence intensity. We measured the fluorescence of each sample in duplicate on a PTI fluorimeter (slit widths = 4 nm; excitation = 468 nm; emission = 505 nm). Following OD normalization, we further normalized the data set relative to the most fluorescent sample for each TRAP. We also subtracted background fluorescence using a control sample expressing each TRAP with NFGP containing no peptide.

### ASSOCIATED CONTENT

#### Supporting Information

We include experimental sections about the analysis of the C-terminome, the protein sequences, thermal denaturation data, and more detailed information about the design of the TRAP–peptide pairs. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00415.
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