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Ultra-large chemical libraries for the discovery of high-affinity peptide binders

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High-diversity genetically-encoded combinatorial libraries (10^8–10^13 members) are a rich source of peptide-based binding molecules, identified by affinity selection. Synthetic libraries can access broader chemical space, but typically examine only ~10^6 compounds by screening. Here we show that in-solution affinity selection can be interfaced with nano-liquid chromatography-tandem mass spectrometry peptide sequencing to identify binders from fully randomized synthetic libraries of 10^8 members—a 100-fold gain in diversity over standard practice. To validate this approach, we show that binders to a monoclonal antibody are identified in proportion to library diversity, as diversity is increased from 10^6–10^8. These results are then applied to the discovery of p53-like binders to MDM2, and to a family of 3–19 nM-affinity, α/β-peptide-based binders to 14-3-3. An X-ray structure of one of these binders in complex with 14-3-3σ is determined, illustrating the role of β-amino acids in facilitating a key binding contact.

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Drug discovery benefits from the ability to assay a large number of compounds for activity against a biomolecular target of interest. This target-based modality remains prominent in the pharmaceutical industry, and it is estimated that of all 113 first-in-class drugs approved by the FDA between 1999 and 2013, 71% were discovered using a target-based approach. While a variety of techniques are used to this end, some of the most common strategies include high-throughput screening (HTS) and fragment-based drug discovery (FBDD) for identification of small molecule binders, and protein engineering strategies for discovery of novel biologics.

In the past decade, much research attention has been devoted to discovering and engineering peptide-based binders as an emerging therapeutic modality. Given the unique niche of chemical space they occupy, possessing molecular weights in between those of small molecules (<500 Da) and biologics (up to ~150,000 Da), peptides offer a distinct profile of chemically attractive features. Peptides are synthetically accessible, amenable to chemical tailoring, and have the potential to bind the typically shallow surfaces seen in therapeutically relevant—and historically intractable—protein–protein interactions (PPIs). Importantly, chemical modifications, such as non-canonical amino acid incorporation, head-to-tail macrocyclization, and chemical stapling, can render peptides more proteolytically stable, more cell-penetrant, and even increase binding affinity relative to their natural, undervariated counterparts, which on their own tend to exhibit poor pharmacological properties.

Molecular biology-based selection techniques, such as phage display and mRNA display, are powerful tools for target-based discovery of novel peptide binders, thanks in part to their ability to examine enormous libraries (10^8–10^13 members). However, incorporation of a large number and variety of non-canonical amino acids by these methods remains challenging. Chemical combinatorial methods, such as DNA-encoded libraries (DELs) and one-bead-one-compound (OBOC) libraries, can easily overcome this hurdle; however, each of these techniques faces its own limitations. DELs are typically limited to three or four varied positions, due to inefficiencies in the chemistry used for their assembly, and as such are more often categorized as libraries of small molecules. OBOC libraries can easily incorporate more varied positions, but OBOC screening is technically challenging and typically examines only ~10^5 compounds.

Affinity selection-mass spectrometry (AS-MS) represents an alternative strategy for target-based discovery of chemically accessed peptide binders. We recently leveraged LC-MS/MS for sequencing individual synthetic peptides present in complex mixtures, and to increase the diversity of synthetic peptide libraries amenable to AS-MS, from ~10 to ~10^6. This advance was used to select improved variants of known binders from small focused libraries (10^3 members). Discovery of binders from fully randomized libraries is a much greater challenge, which may require library diversities considerably higher than are typically examined by chemical screening. In principle, commercial mass spectrometers are sufficiently sensitive to detect and sequence peptides from mixtures as complex as ~10^9 (Table 1). However, it is not obvious whether single-pass affinity selections from such libraries could provide sufficient enrichment (i.e., reduce the number of peptides sufficiently) to identify binders by LC-MS/MS-based sequencing, which is applicable to mixtures of up to several thousand peptides.

Here, we show that magnetic bead capture-based AS-MS is capable of identifying binders from libraries of up to ~10^8 random synthetic peptides. Starting with an anti-hemagglutinin monoclonal antibody (anti-HA mAb) selection target, we demonstrate that high-affinity binders can be captured with near-quantitative recovery from relevant ligand concentrations. These results translate into a selection context, where high-affinity selections-mass spectrometry (AS-MS; this work) is sufficiently sensitive to detect and sequence individual synthetic peptides present in complex mixtures as complex as ~10^9.

**Fig. 1** AS-MS enables discovery from randomized, high-diversity chemical libraries. **a** Among existing techniques for peptide binder discovery, increased chemical control over library synthesis and screening typically comes at the cost of limited library diversity. Affinity selection-mass spectrometry (AS-MS; this work), which relies on chemically accessed libraries and direct identification of active members, can investigate library diversities on the order of 10^8–10^9 while maintaining a high degree of chemical control over the synthesis and selection process. **b** A typical AS-MS workflow, which uses magnetic beads as a partitioning reagent to discriminate bound from unbound library members, and nano-liquid chromatography-tandem mass spectrometry to identify sequences of active peptides.
affinity binders containing the HA epitope are identified in proportion to library diversity over the range of $10^6$–$10^8$. Similarly conducted selections for MDM2 binding identify p53-like peptides for $10^8$-member libraries only, illustrating the utility of high-diversity synthetic libraries for identifying binders previously accessible only to molecular biology-based approaches. The power of the synthetic library approach for identifying binders not otherwise accessible is illustrated by the discovery of mixed α/β, phosphoserine-containing binders to the hub protein 14-3-3.

### Results

**AS-MS recovers high-affinity ligands from high dilution.** Identification of binders from high-diversity libraries would necessitate their efficient recovery at high dilution. To assess the utility of magnetic bead-based affinity capture for this purpose, we investigated the recovery of a number of peptide binders with varying affinity for anti-HA mAb clone 12ca5 (12ca5), which recognizes the linear motif DXDDYAA (Supplementary Figs. 1–6; Supplementary Table 1). In these experiments, streptavidin-coated magnetic beads (1 mg; 0.13 nmol IgG binding capacity) functionalized with biotinylated 12ca5 were incubated with mixtures of 12ca5-binding peptides (either 1 nM/peptide, 100 pM/peptide, or 10 pM/peptide; 1 mL scale). The magnetic beads were isolated, washed with buffer, and treated with chemical denaturant to elute bound peptides. A portion of this eluate (~2%) was subjected to nLC-MS analysis, and recovery was calculated using normalized MS response. To calculate recovery, a separate dose-response curve was generated for each peptide, since their MS responses varied by ~5-fold (Supplementary Fig. 7).

Under the conditions examined, only the two highest affinity binders were significantly retained, with recoveries of 75% and 25% obtained for ~4 and ~25 nM-affinity binders, respectively (Fig. 2a; Supplementary Figs. 8 and 9). This result is consistent with the work of Sannino and coworkers, who observed low recoveries for micromolar affinity ligands from high dilution experiments (Fig. 2b). Selections against 12ca5 (0.13 nmol) were then performed with the 2 × $10^6$-member library, at 10 pM/member concentration (1 mL scale; 10 fmol/peptide, 20 nmol total library), with the goal of identifying sequences similar to the HA epitope (selections were performed in triplicate; Supplementary Note 1). Selection eluates were concentrated by solid phase extraction, and analyzed by nLC-MS/MS (theoretical loading of a retained peptide: 8 fmol). From these selections, just one peptide that matched the library design was identified with a sequencing score (average local confidence (ALC) score) ≥ 80 (MDLVDFYADK; Supplementary Table 2). This sequence had five residues in common with the HA epitope, DXDDYAA, including the hot spot residues Asp4, Asp7, Tyr8, and Ala9 required for high-affinity 12ca5-binding (Supplementary Figs. 13–21).  

We endeavored to understand the enrichment achieved by the selection, where enrichment is defined as:

$$\text{(sequenced binders/total sequenced peptides)}_{\text{isolated}} / \text{(binders/total peptides)}_{\text{assayed}}$$

or equivalently

$$\text{(sequenced binders)}_{\text{isolated}} / \text{(binders)}_{\text{assayed}} * \text{(total peptides)}_{\text{assayed}} / \text{(total sequenced peptides)}_{\text{isolated}}$$

Achieving high enrichment in a single selection step would be essential for discovering rare binding molecules by AS-MS, since synthetic libraries cannot be propagated to facilitate sequential rounds of selection. Since only a single peptide was sequenced from the selection eluate, a maximum enrichment of 2 × $10^6$ was achieved. The actual enrichment may be significantly lower, if additional binders were present in the library, but not recovered and sequenced. For example, the expected number of DXDDYAA (A/S)-containing sequences in the 2 × $10^6$-member library is 152 (although not all of these are necessarily high-affinity binders), suggesting the actual enrichment could be as low as ~1.3 × $10^4$ (Supplementary Note 2).

### Table 1 Mixtures of up to $10^9$ peptides contain sufficient material for MS-based sequencing.

| Library size | Member concentration (pM) | Moles per member (fmol) |
|--------------|---------------------------|-------------------------|
| $10^6$       | 1000                      | 1000                    |
| $10^7$       | 100                       | 100                     |
| $10^8$       | 10                        | 10                      |
| $10^9$       | 1                         | 1                       |

At a given scale (1 mL), maximum diversity is a tradeoff between the amount of individual peptide required for nLC-MS/MS sequencing, and the solubility limit of the total peptide mixture (1 mM). Even more peptides might be examined concurrently at a fixed total concentration by increasing the selection volume.
To check whether additional peptides were recovered by the selection at lower abundance, we performed a selection at 200 pM/member, such that less-abundant peptides could cross the precursor selection threshold (Supplementary Fig. 22). Increasing amounts of the selection eluate were analyzed by nLC-MS/MS, with sample loadings varied from 8 fmol/member (selection threshold: ~5% recovery) to 75 fmol/member (selection threshold: ~0.5% recovery). At the lowest sample loading, just one peptide was identified (MNDLVDYADK), identical to that observed in the original selection. As sample loading was increased to 75 fmol/member, two additional epitope-containing peptides were identified: PDVDHTWQDK and ENDWQDYSHK. However, recovery of these sequences came at the cost of identifying additional non-motif-containing peptides: a total of 32 from 3 replicate selections. These results suggest that with respect to recovery of binders, little benefit is conferred by higher library concentration, and that 8 fmol/member sample loading avoids detection of peptides present in the selection eluate at lower abundance. Accounting for these additional sequences yields an enrichment of \(1.1 \times 10^3\), assuming 152 DXXDY(A/S)-containing peptides (Eq. (2)), which is on the order of enrichments reported for individual rounds of mRNA (~10^3)\(^3^9\), phage (~10^3)\(^1^4\), or cell surface display (~10^3)\(^4^0\),\(^4^1\).

Taken together, these results suggest that magnetic bead affinity capture can facilitate selections from complex mixtures of synthetic peptides at a concentration of 10 pM/member. In conjunction with nLC-MS/MS peptide sequencing, very high enrichment for sequenced peptides is enabled by the use of a precursor selection threshold, to detect only those peptides that are significantly recovered by the selection (here, a DXXDY(A/S)-containing peptide—present in highest abundance). Peptides recovered in lower abundance (here, mostly non-motif-containing peptides) are present in the selection eluate, but not sequenced (Supplementary Table 4). As described below (‘Parallel selections distinguish non-specific binders’), the majority of these peptides are non-specific binders.

**Enrichment is maintained as diversity increases from 10^6-10^8.** To investigate whether comparable selection performance could be achieved using higher-diversity libraries, we assayed \(2 \times 10^7\)- and \(2 \times 10^8\)-member libraries in selections for 12ca5 binding. In theory, these libraries should contain 10- and 100-fold more DXXDY(A/S) peptides compared to the \(2 \times 10^6\)-member library. If selections from the higher-diversity libraries performed comparably, then they should recover all of these additional DXXDY (A/S)-containing peptides, with no increase in the proportion of non-binding peptides. For each library, selections were performed in triplicate, with library concentration maintained at 10 pM/member and using 0.13 nmol of selection target, as above.

From the \(2 \times 10^7\)-member library, a representative selection identified 23 peptides that matched the library design (ALC ≥ 80), 14 of which (61%) contained DXXDYA or DXXDYS (Fig. 2b; Supplementary Table 2). An additional two sequences closely resembled 12ca5 binders—KVLDYDYAWK and YDDRHYADTFK—and may correspond to inaccurate sequence assignments. Selections from the \(2 \times 10^8\)-member library identified 156 total peptides (ALC ≥ 80), 109 of which (70%) contained either DXXDYA or DXXDYS (Fig. 2b; Supplementary Tables 2-3). These results illustrate that selections identified binders from the higher-diversity libraries without loss in recovery, since the expected ~10 and ~100 DXXDY(A/S)-containing peptides were identified, and without loss in enrichment, since DXXDY(A/S)-containing peptides comprised the majority of selected peptides in each case (100%, 61%, and 70% for \(2 \times 10^6\), \(2 \times 10^7\), and \(2 \times 10^8\)-member libraries, respectively). Therefore, we concluded that
single-pass AS-MS could be applied to libraries of at least 10^8 random peptides without loss in performance.

**Recovery of binders drops from libraries beyond 10^8 members.** We next set out to determine whether libraries of diversity beyond 10^8 would also be amenable to AS-MS. To access 10^9 random synthetic peptides on a convenient lab scale, we used 20 µm TentaGel resin beads (vs. 30 µm beads, above) to prepare a library of design (X)_{20}K (X = all 1-α amino acids except for cysteine and isoleucine; 5.4 g: 1.3 × 10^9 beads) (Supplementary Fig. 23). As above, a portion of the beads was set aside prior to cleavage from resin to give a 10^8-member library for side-by-side comparison.

The 10^8-member library performed comparably to the 10^9-member library prepared on 30 µm TentaGel beads (above), yielding 257 total peptides (ALC ≥ 80), 183 of which (71%) contained either DXXDYA or DXXDYS. In contrast, selections from the 10^9-member library identified only 34 peptides (ALC ≥ 80), 21 of which (62%) contained DXXDYA or DXXDYS (Supplementary Table 5). For the 10^9-member library, selections were performed at 2 pM/memberto maintain solubility; however, the lower sequence identification rate cannot be attributed to material losses alone, since selection from a 10^9-member performed at 2 pM/membere identified 131 DXXDY(A/S) sequences (vs. 183 from the selection at 10 pM/memb; Supplementary Table 5). Analysis of pooled eluates from replicate selections from the 10^9-member library yielded no additional sequences, providing further evidence that material losses were not responsible for the reduction in sequence identification rate (since similar populations of DXXDY(A/S)-containing sequences are recovered by replicate selections) (Supplementary Tables 3 and 6).

Since affinity selections involve a large number of potential binders competing for a limited number of binding sites, it is possible that many weaker binders present in the library reduce the individual recoveries of all ligands. Two experiments were performed to address whether competition was responsible for the lower recovery of DXXDY(A/S) peptides from the 10^9-member library. First, we performed a selection using 10-fold higher stoichiometry of 12ca5 (1.3 nmol) relative to library (2 fmol/memb). If competition were limiting recovery of binders, this experiment should have recovered additional DXXDY(A/S)-containing peptides. Instead, their recovery was abrogated entirely (Supplementary Table 7). Second, to determine the frequency of binders that would be required for competition to become significant, we studied the effect of exogenous competitors on the recovery of DXXDY(A/S) peptides from the 10^9-member library. In all, 4 nM– or 3 µM–affinity competitors required concentrations of 100 nM or 100 µM, respectively, to attain the recovery of DXXDY(A/S) peptides (Supplementary Fig. 24; Supplementary Table 8). These concentrations correspond to 5 × 10^4 or 5 × 10^5 peptides (present at 2 pM/peptide), suggesting that: (1) the expected ~10^5 DXXDY(A/S) peptides in the 10^9-member library would not compete for selection target; and (2) in order for µM–affinity binders to compete with DXXDY (A/S) peptides, they would need to comprise 5% of the 10^9-member library.

The combined results are consistent with the interpretation that selections from the 10^9-member library yield more peptides than are compatible with nLC-MS/MS sequencing. As the number of peptides in the selection eluate (the sample complexity) increases, the proportion of sequenced peptides decreases. This drop in sequencing coverage was observed previously to be particularly significant beyond 10^3 peptides. This drop in sequencing efficiency can be attributed to the sample complexity increases. 

**Parallel selections distinguish non-specific binders.** Selections from identical portions of synthetic peptide mixtures obtained by split-and-pool synthesis are readily conducted in parallel. For example, a 2 × 10^8-memberlibrary synthesized on 30 µm TentaGel (~3.7 pmol/bead) provides sufficient material for ~370 selections performed at 10 fmol/membere scale. We leveraged this capability to understand what proportion of non-HA epitope-containing peptides were common to an unrelated selection target, and therefore attributable to non-specific binding. Side-by-side selections from a 2 × 10^8-member library were conducted in triplicate against both 12ca5 (mouse IgG2b κ) and a polyclonal human IgG1 with the goal of quantifying the degree of overlap among selected peptides (Fig. 3a).

Three replicate 12ca5 selections yielded a total of 133 DXXDY (A/S)-containing peptides, all of which were specific for 12ca5, along with 65 non-motif-containing peptides (Fig. 3b; Supplementary Table 9). Of the non-motif-containing peptides, 18 sequences were performed against 12ca5 and a polyclonal human IgG1 in parallel, followed by sequential nLC-MS/MS analysis.
contained motifs that differed from the HA epitope by one position (for example, EXXDYA). Of the remaining 47 peptides, closer inspection revealed that 13 may contain mis-sequenced HA epitopes. For example, VFDQWEDFSK and YMDFVDFSEK contain the FS dipeptide fragment, which is isobaric to YA (Supplementary Fig. 25). A total of 34 peptides had no obvious sequence similarity to the DXXDY/(A/S) motif. 17 of these were also sequenced from the IgG1 selections. Examination of the LC-MS data revealed that many or all of the remaining 17 peptides were also present in the IgG1 selection eluates, but not sequenced (9 of 9 selected peptides examined; Supplementary Fig. 26). Therefore, essentially all of the non-motif-containing peptides sequenced from 12ca5 selection can be attributed to non-specific binding. Presumably, many additional non-motif-containing peptides were recovered at lower abundance and not sequenced, as for the 10⁸-member library.

**High diversity enables discovery of MDM2-binding peptides.** Having established magnetic bead-based AS-MS as a selection protocol applicable to high-diversity libraries of random synthetic peptides, we sought to benchmark its performance relative to affinity selection from genetically-encoded libraries. As a model target for this purpose, we selected MDM2, an oncogenic ubiquitin ligase that binds its substrate p53 through a FXXXWXX(L/V) motif. Phage display has identified a number of well-characterized, high-affinity MDM2 binders based on this motif\(^{15,43-45}\), and we sought to determine whether AS-MS could recapitulate these results by identifying similar sequences from synthetic libraries of comparable size and design.

To mimic previous phage display libraries, a library of design (X)\(^{12}K\), where X = all L-amino acids except cysteine and isoleucine (theoretical diversity = \(1.2 \times 10^{15}\)), was synthesized on 20 µm TentaGel resin (4.2 g; 1.3 \times 10^9 beads). Prior to cleavage from resin, this library was split to yield five distinct 2 \times 10^7-member libraries, as well as a 2 \times 10^8-member library, to investigate the importance of library size in the context of selections for MDM2 binding (Fig. 4a; Supplementary Fig. 27). The N-terminal domain of MDM2 (residues 25–109) was accessed synthetically in biotinylated form, to enable its use as a selection target in conjunction with streptavidin-coated magnetic beads (Supplementary Fig. 28).

Selections from three of the five 2 \times 10^8-member libraries against (25–109)MDM2 (0.13 nmol) yielded sequences containing the FXXXWXX(L/V) motif characteristic of MDM2-binding (Fig. 4b). In total, 16 sequences from these selections were identified (ALC ≥ 80), five of which (31%) contained FXXXWXX (L/V) (Supplementary Table 10). An additional two sequences appeared to be MDM2-binding peptides, containing the minimal FXXW motif, but were potentially mis-sequenced due to poor-quality fragmentation spectra (Supplementary Fig. 29). Selections from the 2 \times 10^7-member library did not yield motif-containing sequences, consistent with the frequency of binders identified from 2 \times 10^8-member libraries. Selections were also performed from a 10^9-member library, obtained by pooling the individual 2 \times 10^8-member libraries. These selections failed to identify

![Fig. 4 High-diversity libraries facilitate discovery of p53-like peptides.](image-url)
motif-containing sequences, consistent with the poor recovery of motif-containing sequences from 10^9-member libraries in selections against 12ca5. In summary, library diversity correlates with the number of binders identified, provided the diversity is within the technical limits of AS-MS for decoding single-pass selections.

A closer examination of the FXXXWWX(L/V)-containing sequences identified here alongside known MDM2-binding peptides revealed sequence similarity outside of the FWL triad (Fig. 4c). Specifically, a 6-residue motif was observed among the majority of sequences: (S/T)FX(D/E)YWXXL. Each of the conserved positions corresponds to a hot spot of binding energy, as determined by mutational analysis. The ability to identify not only the FWL triad but also other significant determinants of binding affinity supports our interpretation that AS-MS is capable of matching the performance of phage display, in the context of selections against MDM2. While others have demonstrated the utility of synthetic libraries for identifying MDM2 binders, our results illustrate their utility for mapping the determinants of a protein-protein interaction.

AS-MS identifies non-canonical 14-3-3γ-binding peptides. To illustrate a key advantage of the synthetic library approach, we investigated whether AS-MS could achieve comparable selection performance from a library based on non-canonical amino acids. As a selection target, we chose the hub protein 14-3-3, which interacts with a range of disease-relevant proteins including p53, Raf kinases, and estrogen receptor α. Considerable effort has been devoted to developing modulators of these interactions, which are generally mediated by phosphorylation.

For use in selections against 14-3-3, we designed a library based on a fixed phosphoserine, flanked by eight varied positions (Fig. 5a). At each varied position, we incorporated one of 18 non-canonical amino acids—including β- and D-amino acids—encompassing a variety of polar, non-polar, charged, and aromatic side-chain functionalities (Fig. 5a) (theoretical diversity =\(1.1 \times 10^{46}\)). This library was synthesized on 30 μm Tentagel resin (2.9 g; 2 \times 10^8 beads), yielding 2 \times 10^8 members (Supplementary Fig. 30).

Side-by-side selections were performed against the 14-3-3γ isoform, and 12ca5 (negative control, to identify non-specific binders; 0.13 nmol each). These selections yielded a total of 19 sequences that matched the library design (ALC \(\geq 80\), 17 of which were unique to 14-3-3γ, and 2 of which were unique to 12ca5 (Supplementary Table 11; Supplementary Fig. 31). Extracted ion chromatograms were used to verify the absence of 14-3-3γ-uniform sequences from the 12ca5 selections (Supplementary Figs. 32 and 33). Among the 14-3-3γ-unique sequences, seven contained a C-terminal motif: (β-homoserine)-(β-alanine/β-homoserine)-(4-nitrophenylalanine)). In general, α-amino acids were not present within the sequences identified. β-amino acids were enriched at positions 3, 7, and 8, but were otherwise largely absent. A positional frequency analysis revealed additional preferences for cyclohexylalanine at the N-terminus, and a β-homoserine at position three (Supplementary Fig. 31).

Discovered peptides bind 14-3-3γ with low nanomolar affinity. To test the binding affinity of putative 14-3-3γ-binding peptides that contained the C-terminal (β-homoserine)-(β-alanine/β-homoserine)-(4-nitrophenylalanine) motif, we synthesized fluorophore-labeled forms of three selected peptides, for use in a fluorescence anisotropy binding assay (Supplementary Figs. 32–33). As a negative control, we employed a sequence identified from a 12ca5 selection. Each of the putative 14-3-3γ-binding peptides examined exhibited low nanomolar affinity for 14-3-3γ, with \(K_D\) values ranging from 3 to 19 nM (Fig. 5c). By contrast, the negative control peptide exhibited ~10,000-fold weaker binding, suggesting that the specific amino acid sequences of the 14-3-3γ-binding peptides—not the phosphoserine alone—were required for their identification.

Unlabeled forms of the non-canonical 14-3-3γ binders were assayed for their ability to compete with BiExoS, a peptide ligand derived from the Pseudomonas aeruginosa cytotoxin Exoenzyme S90, in a fluorescence anisotropy competition assay (Supplementary Figs. 38–41). This experiment would test whether the peptides bind to the amphipathic 14-3-3γ binding groove, or elsewhere on the protein. The non-canonical 14-3-3γ binders were found to compete off BiExoS with IC\(_{50}\) values ranging from 78 to 530 nM, suggesting that they indeed bind in the canonical, phosphopeptide-accepting binding channel on 14-3-3γ (Fig. 5d). By contrast, the negative control peptide showed no inhibitory activity.

β-amino acids facilitate a key binding contact with 14-3-3. As an additional means of characterizing the binding interaction of non-canonical peptides with 14-3-3γ, we crystallized 14-3-3-12 in complex with 14-3-3γ. 14-3-3γ was used in place of 14-3-3γ to facilitate crystallization, and retained most of the binding activity for 14-3-3.12 (Supplementary Fig. 42). Diffraction data were collected to a resolution of 1.8 Å, and the structure was solved by molecular replacement (Supplementary Table 12).

The 14-3-3-12 backbone adopts an extended conformation in the 14-3-3γ binding groove, flanked by two half-turns defined by thiazolylalanine4 and β-alanine8 (Fig. 5e). 4-Nitrophenylalanine6, which was selected along with thiazolylalanine and 4-fluorophenylalanine at this position, makes hydrophobic contacts with Leu218, Ile219, and Leu222 of 14-3-3γ (Supplementary Fig. 43). 4-Nitrophenylalanine9—the residue most conserved by the selection—participates in an electrostatic interaction and/or H-bond with the NH3 group of Lys122 (N–O distance=3.2 Å), and makes a hydrophobic contact with Ile168 (Supplementary Fig. 43). We speculate that the β-residues conserved at positions 7 and 8 of 14-3-3.12 provide the backbone flexibility necessary to accommodate these energetically-important interactions, which were not identified by selection from peptide libraries based on canonical amino acids.

Discussion

In this work, we demonstrate that affinity selection-mass spectrometry, using magnetic bead reagents, provides sufficient enrichment to identify high-affinity binders from randomized libraries of 10^8 synthetic peptides. With respect to accessible diversity, this advance brings synthetic libraries up to the level of molecular biology-based combinatorial libraries. Diversity is a key determinant of selection outcome, as illustrated here for the discovery of p53-like binders to MDM2, and in the field of antibody engineering. Therefore, the results described here can be expected to considerably extend the utility of synthetic libraries for discovering novel binding molecules.

The practical limit to library diversity amenable to single-pass AS-MS is 10^8, beyond which the number of binders identified decreases. Our combined results are consistent with non-specific binding as the origin of this limit, which results in the recovery of more peptides from >10^9-member libraries than can be sequenced by nLC-MS/MS with high coverage. Since diversity is limited by selection performance and nLC-MS/MS sequencing coverage, rather than peptide solubility, future work should focus on these areas. For example, multi-stage selections might be employed to improve enrichment further, and to reduce the number of peptides in a >10^6-member library sufficiently for nLC-MS/MS sequencing. Sequencing coverage might also be

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**Fig. 5 Synthetic libraries identify a 14-3-3γ-binding consensus based on β-amino acids.** a Design of a non-canonical library used in selections against 14-3-3γ. A phosphoserine was fixed in the middle, flanked by four varied positions on either side. A suite of non-canonical amino acids was incorporated at each varied position. b Sequences of putative 14-3-3γ-binding peptides (14-3-3.1, 14-3-3.6, and 14-3-3.12) identified from affinity selections, as well as a negative control peptide (non-binder or NB.1, identified as an artifact in selections against 12ca5) chosen for resynthesis and binding validation studies. Residues comprising the conserved C-term motif among 14-3-3γ binders—Nph-β-Ser-(β-Ala/β-Ser)-Nph—are indicated in blue. c Identified binders exhibited nanomolar affinities for 14-3-3γ, as determined by fluorescence anisotropy of FITC-labeled 14-3-3γ-binding peptides, NB.1, and a known 14-3-3γ-binding peptide (BiExoS; positive control). These affinities were approximately 10,000-fold higher relative to the negative control. KD values are given in b. Uncertainties correspond to 95% confidence intervals derived from nonlinear regression. d Identified 14-3-3γ-binders could compete off bound BiExoS in a fluorescence anisotropy competition assay, suggesting they bind 14-3-3γ in the canonical, amphipathic binding groove. IC50 values are given in b. e Molecular structure of 14-3-3σΔc (white surface) in complex with 14-3-3.12 (cyan sticks), based on a 1.80 Å crystal structure. The 2Fo–Fc electron density map corresponding to 14-3-3.12 is shown (blue mesh), contoured at 1σ. Abbreviations: β-Ala β-alanine; β-Thr β-homothreonine; Aad adaminobutyric acid; Aph 4-aminophenylalanine; Cha cyclohexylalanine; Cpa cyclopropylalanine; Dba diaminobutyric acid; Fph 4-fluorophenylalanine; Hyp hydroxyproline; Nph 4-nitrophenylalanine; Nva norvaline; Orn ornithine; pSer phosphoserine; Thz thiazolylalanine. Error bars correspond to standard error among three technical replicates.

Improved by the use of specialized nLC columns and extended analysis times.

The primary benefit of synthetic peptide libraries is the chemical control gained over the library design. Here, the combination of large library diversity and non-canonical amino acids led to the discovery of high-affinity 14-3-3γ-binding peptides that utilize β-amino acids to facilitate a binding contact. Given the comparatively low diversities examined by AS-MS relative to the upper bounds of genetically-encoded techniques (10⁸ vs. 10¹³), we anticipate that taking advantage of the chemical capabilities AS-MS affords—such as straight-forward non-canonical amino acid incorporation—may prove critical for more intractable targets. For example, AS-MS may be particularly suited to engineering peptide and peptoid foldamers. Interfacing non-canonical amino acid incorporation with the macrocyclic architectures that have been rendered accessible to phage and mRNA display would further expand the breadth of chemical space amenable to exploration by AS-MS. In our case, performing selections on libraries of macrocycles would require an additional, post-enrichment linearization step for reliable MS/MS-based sequencing. We envision that progress in these areas, along with improved mass spectral methods to enable investigation of libraries of even greater diversities, may ultimately facilitate discovery of fully non-natural peptide binders to historically undruggable targets.
Methods

Materials. H-Rink Amide-ChemMatrix resin was purchased from PCAS BioMatrix Inc. (St-Jean-sur-Richelieu, Quebec, Canada). In all, 30 μm TentaGel M NH2 microspheres (M30352; 0.20–0.25 mmol/g amine loading) were purchased from Rapp Polymere (Tübingen, Germany). In all, 20 μm TentaGel S NH2 microspheres (TMN-9909-P; 0.2-0.3 mmol/g amine loading) were purchased from Peptides International (Louisville, KY). Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Bzl)-OH, Fmoc-Glu(Bzl)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trr)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Ph-OH, Fmoc-Pro-OH, Fmoc-Ser(Bu)-OH, Fmoc-Thr(Bu)-OH, Fmoc-Trp (Boc)-OH, Fmoc-Tyr(Bu)-OH, and Fmoc-Val-OH were purchased from Advanced ChemTech (Louisville, KY). Fmoc-D-Asp(Bu)-OH, Fmoc-D-Gln(Trr)-OH, Fmoc-D-Leu-OH, and Fmoc-D-Lys(Boc)-OH were also purchased from Advanced ChemTech (Louisville, KY). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxid-hexafluorophosphate (HATU) was purchased from P3 Biosystems (Louisville, KY). 4-[(R)-N-(1H-9Fluoren-9-y1)-methoxyformimidoyl]-2,4-dimethoxybenzyl-phenoxycetic acid (Fmoc-Arg amide linker), Fmoc-Lys(Ala)-OH, Fmoc-β-Ala-OH, and Fmoc-Lys(Ala)-OH, and di tert-butyl dicarbamate were purchased from Chem-Impex International (Wood Dale, IL). Biotin-(PEG)4-NHS ester and biotin-(PEG)4-propionic acid were purchased from ChemPep Inc. (Wellington, FL). HBTU (0.95 mmol), and diisopropylethyl amine (DIEA; 2.9 mmol, 500 μL) were added to individual portions of resin. Couplings were allowed to proceed for 20 min. Resin portions were recombined, and washed with DMF. Fmoc removal was carried out by treatment of the resin with 20% piperidine in DMF (1x flow wash; 2x 5 min batch treatments). Following removal of N-terminal Fmoc group, resins were washed 3 x 5 mL with DMF, then 3 x 5 mL with DCM.

Manual solid-phase synthesis of non-canonical peptides. Manual, batch synthesis of peptide-carboxamides was carried out on a 0.05 mmol scale, using H-Rink amide-ChemMatrix resin (0.45 mmol/g). Resin was weighed into disposable fritted syringes (Torvag), washed 3x with DMF, then swirled for 1 min. After each synthesis was complete, resins were washed with DCM (5x) and dried under reduced pressure.

Manual fast-flow peptide synthesis. Manual fast-flow synthesis of peptide-carboxamides was carried out using H-Rink amide-ChemMatrix resin (0.18 mmol/g, 0.03 mmol scale for N-terminal domain of MDM2; 0.45 mmol/g, 0.1 mmol scale for all other peptides). Syntheses were carried out at 90 °C; amide bond formation was effected in 8 s and Fmoc removal was carried out in 8 s with 20% (v/v) piperidine in DMF. Individual cycle times were allowed to proceed for 20 min. After each synthesis was complete, resins were washed with DCM (5x) and dried under reduced pressure.

Split-and-pool synthesis of peptide libraries. Split-and-pool synthesis was carried out on 30 μm TentaGel resin (0.26 mmol/g) for 106 member libraries, and 20 μm TentaGel resin (0.26 mmol/g) for 105 member libraries. Splits were performed by suspending the resin in DMF and dividing it evenly (via pipet) among 18 plastic fritted syringes on a manifold. Couplings were allowed to proceed as follows: solutions of Fmoc-protected amino acids, HATU (0.38M in DMF; 0.9 eq relative to amino acid), and DIEA (1.1 eq for histidine; 3 eq for all other amino acids) were each added to individual portions of resin. Couplings were allowed to proceed for 20 min. Resin portions were recombined, and washed with DMF. Fmoc removal was carried out by treatment of the resin with 20% piperidine in DMF (1x flow wash; 2x 5 min batch treatments). Resin was washed again with DMF.

Magnetic bead preparation. In all, 100 μL portions of MyOne Streptavidin T1 Dynabeads (10 mg/mL; 1 mg: 0.15 mmol IgG binding capacity) were transferred to 1.7 mL plastic centrifuge tubes, and placed in a magnetic separation rack (New England Biolabs, cat# S1506S). The beads were washed three times with blocking buffer (1 mL 0.1% BSA or 10% FBS, 0.02% Tween 20, 1x PBS), and then treated with portions of biotinylated target protein (~1.2 eq). The resulting suspensions were treated with 1 mL 0.1% dithiothreitol in rotation-venting mixers and kept for 30 min at room temperature or 1 h at 4 °C. After this time, the beads were returned to the separating rack, the supernatant was removed, and the beads were washed 3x with blocking buffer.

Affinity selection from random libraries. Library (typically 10 fmol/member) was incubated with 100 μL (1 mg) portions of protein-immobilized magnetic beads in 400 μL 0.1% FBS, 1x PBS, 10% DMSO (v/v). Dynabeads (1 mg) were incubated on a rotating mixer (1 h at 4 °C). Typical final conditions: 1 mg/mL magnetic beads, 10 pm/member library. The centrifuge tubes containing the bead suspensions were transferred to the magnetic separation rack. The beads were washed 3 x 1 mL w/ 1x PBS. Bound peptides were eluted with 2 x 100 μL 6M guanidine hydrochloride, 200 mM phosphate, pH 6.8. Eluates were concentrated via C18 ZipTip® pipette tips and lyophilized. Purified peptides were typically resuspended in 6 μL water (0.1% formic acid), and 5 μL were submitted for nLC-MS/MS analysis.
nLC-MS/MS analysis. **Analysis** was performed on an EASY-nLC 1200 (Thermo Fisher Scientific) nano-liquid chromatography handling system connected to an Orbitrap Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific). Samples were run on a PepMap RSLC C18 column (2 μm particle size, 15 cm × 50 μm ID; Thermo Fisher Scientific, P/N E8801). A nanoViper Trap Column (C18, 3 μm particle size, 100 A pore size, 20 mm × 75 μm ID; Thermo Fisher Scientific, P/N N1494E) was used for desalting. The standard nano-LC method was run at 40 °C and a flow rate of 300 nL/min with the following gradient: 1% solvent B in solvent A ramping linearly to 61% B in A over 40 or 60 min, where solvent A = water (0.1% FA), and solvent B = 80% acetonitrile, 20% water (0.1% FA). Positive ion spray voltage was set to 2200 V. Orbitrap detection was used for primary MS, with the following parameters: resolution = 120,000; quadrupole-pole-pole scan range = 200–1400 m/z; RF lens = 30%; AGC target = 1 × 10^5; maximum injection time = 100 ms; 1 microscan. Acquisition of secondary MS spectra was done in a data-dependent manner: dynamic exclusion was employed such that a precursor was excluded for 30 s if it was detected four or more times within 30 s (mass tolerance: 10.00 ppm); monoisotopic precursor selection used to select for peptides; intensity threshold was set to 5 × 10^6; charge states 2–10 were selected; and precursor selection range was set to 200–1400 m/z. The top 15 most intense precursors that met the preceding criteria were subjected to subsequent fragmentation. Three fragmentation modes—collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), and electron-transfer/higher-energy collisional dissociation (ETDc)—were used for acquisition of secondary MS spectra. Only precursors with charge states 3 and above were subjected to all three fragmentation modes; precursors with charge states 2 of were subjected to CID and HCD only. For all three modes, detection was performed in the Orbitrap (resolution = 30,000; quadrupole isolation; isolation window = 1.3 m/z; AGC target = 2 × 10^6; maximum injection time = 100 ms; 1 microscan). For CID, a collision energy of 30% was used. For HCD, a collision energy of 25% was used. For ETDc, a supplemental activation collision energy of 25% was used.

**Direct fluorescence anisotropy**. All direct fluorescence anisotropy affinity measurements were conducted in FA buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20, 0.1% (w/v) BSA). A 1:1 dilution series of 14-3-3y (starting at 100 μM) was performed in polystyrene non-binding low-volume Corning Black Round Bottom 384-well plates (Corning 4514) containing a fixed concentration FITC-labeled peptide (10 nM or 50 nM). Measurements were performed at ambient temperature using a Tecan Infinite F500 plate reader with the following parameters: λex = 485 (20 nm); λem = 535 (25 nm); mirror: Dichroc 510; flashes: 20; integration time: 50 μs; settle time: 0 μs; gain: manual 90; Z-position: calculated from well. The G-factor was set at 35 mP based on wells containing only the FITC-labeled peptide.

**Fluorescence anisotropy competition**. All fluorescence anisotropy competition experiments were conducted in FA buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20, 0.1% (w/v) BSA). A 1:1 dilution series of unlabeled peptides (starting at 10 μM) were performed in wells containing a fixed concentration FITC-labeled biE605 (10 nM) and 14-3-3y (20 nM). Measurements were performed at ambient temperature using a Tecan Infinite F500 plate reader with the following parameters: λem = 535 (25 nm); mirror: Dichroc 510; flashes: 20; integration time: 50 μs; settle time: 0 μs; gain: manual 90; and Z-position: calculated from well. The G-factor was set at 35 mP based on wells containing only the FITC-labeled peptide.

**X-ray structure determination**. Unlabeled 14-3-3-12 was soaked into preformed crystals of 14-3-3 SoCa, which grew in 25% PEG400, 5% Glycerol, 0.2 M CaCl2, 0.1 M HEPES pH 7.5 plus 2 mM BME within two weeks. The soaked crystal was fished after 15 days of incubation and flash-frozen in liquid nitrogen. Diffraction data was collected at 100K on an in-house Rigaku Micromax-003 (Rigaku Europe, Kemsing Sevenoaks, UK) sealed tube x-ray source and Dectris Pilatus 200K detector (DECTRIS Ltd Baden-Daettwil, Switzerland). Integration, scaling and merging were done using DIALS (CCP4i2) after which molecular replacement is done with MOLREP (CCP4i2) using DPC 4.43 as search model. A threedimensional structure of 14-3-3-12 was generated using eLBOW (Phenix) after which it was built within this structure based on visual inspection of Fo-Fc and 2Fo-2Fc electron density maps in Coot. Several rounds of model building and refinement (based on isotropic b-factors and standard set of stereo-chemical restraints: covalent bonds, angles, dihedrals, planarities, chiralities, non-bonded) were performed using Coot and Phenix.refine. See Supplementary Table 12 for data collection and refinement statistics. Structural coordinates have been deposited in the PDB (6TCH).

**Data availability**. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The source data underlying Figs. 2b, 3b, and 4b, and Supplementary Figures 12, 22–24, 27, and 30 are provided as Source data file. Source data are provided with this paper.
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