A sequence-specific transcription activator motif and powerful synthetic variants that bind Mediator using a fuzzy protein interface

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Although many transcription activators contact the same set of coactivator complexes, the mechanism and specificity of these interactions have been unclear. For example, do intrinsically disordered transcription activation domains (ADs) use sequence-specific motifs, or do ADs of seemingly different sequence have common properties that encode activation function? We find that the central activation domain (cAD) of the yeast activator Gcn4 functions through a short, conserved sequence-specific motif. Optimizing the residues surrounding this short motif by inserting additional hydrophobic residues creates very powerful ADs that bind the Mediator subunit Gal11/Med15 with high affinity via a “fuzzy” protein interface. In contrast to Gcn4, the activity of these synthetic ADs is not strongly dependent on any one residue of the AD, and this redundancy is similar to that of some natural ADs in which few if any sequence-specific residues have been identified. The additional hydrophobic residues in the synthetic ADs likely allow multiple faces of the AD helix to interact with the Gal11 activator-binding domain, effectively forming a fuzzier interface than that of the wild-type cAD.

Significance

How transcription activators recognize their coactivator targets is a longstanding question and is important for understanding activator specificity and synergy. Most activators are not obviously related in sequence, but they recognize a common set of coactivators, raising the question of whether these interactions are sequence-specific. We show that the yeast transcription factor Gcn4 central activation domain works via a short sequence-specific motif that can be optimized to generate powerful synthetic activators. Like many natural activators, the synthetic derivatives have redundant sequence and bind the Mediator subunit Gal11 with high affinity using a “fuzzy” protein interface. Our results suggest a mechanism to explain how a subset of natural activators use redundant sequence motifs and great flexibility in the binding interface to target unrelated coactivators.

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properties are seemingly consistent with those of SLMs, a major difference is that ADs within different transcription factors are not obviously related in sequence, whereas SLMs are readily recognized in otherwise unrelated proteins.

Yeast Gcn4, an acidic activator with tandem ADs, regulates at least 70 genes in response to metabolic stress. Gcn4 is broadly acting, regulating both TATA-containing and TATA-less genes dependent on the coactivators Mediator, SAGA, and/or TFIID (7, 43, 44). The best-characterized Gcn4 coactivator target is the Mediator subunit Gal11/Med15 that contains at least three Gcn4 activator-binding domains (ABDs), ABD1, 2, and 3 (7, 10). NMR analysis showed that the Gcn4 central AD (cAD)–ABD1 complex is dynamic, with a short half-life, mediated entirely by hydrophobic interactions between three Gcn4 residues and a shallow hydrophobic groove in Gal11 ABD1 (19). The Gcn4 cAD is intrinsically disordered but binds Gal11 ABD1 as a helix in a “fuzzy” complex in which the purely hydrophobic protein–protein interface allows the Gcn4 helix to bind Gal11 in multiple orientations. Although this fuzzy interface provides an explanation for how some ADs bind unrelated coactivators, it raises the question of whether this type of AD ever could have a specific sequence motif.

Here we show that the Gcn4 cAD does work through a short, conserved, sequence-specific motif. However, the function of this natural motif is not optimized, and its activity can be enhanced by insertion of hydrophobic residues surrounding the motif to create very powerful synthetic activators. These synthetic ADs bind Gal11 with high affinity while still using a fuzzy interface, and, as in many natural ADs, their activation function is not strongly dependent on any one residue. We present a model to explain the action of these synthetic ADs, the nature of their redundant sequence motifs, and the mechanism of fuzzy coactivator binding.

Results

Hydrophobic Residues Cannot Substitute for the Specific Gcn4 cAD Motif. NMR analysis of the Gcn4 cAD–Gal11 ABD1 complex showed that Gcn4 residues W120, L123, and F124 directly contact multiple residues within a shallow hydrophobic groove on ABD1 (19). Consistent with the structural analysis, alanine substitution showed that these three residues are essential for AD function whereas none of the 10 acidic residues within the cAD are required. Given the simple and fuzzy nature of the protein–protein interface, it was not obvious that there is a specific sequence requirement for AD function other than the spacing pattern of three hydrophobic residues, which allows alignment of these side chains on one face of a helix.

As an initial test of whether the cAD has a specific sequence motif, we looked for another naturally occurring disordered polypeptide with the same pattern of hydrophobic residues but with a different sequence. The Gcn4 N-terminal AD (nAD; residues 1–100) is intrinsically disordered (19) and contains four clusters of hydrophobic residues important for function (19, 45). One of these clusters, found within residues 81–100 [sequence 93-VVESFF-98 (key hydrophobic residues underlined)], contains hydrophobic residues with the cAD spacing of i, i + 3, i + 4 embedded within an acidic disordered polypeptide (Fig. 14). To test whether this motif has AD function, we fused Gcn4 residues 81–100 to the Gcn4-DNA-binding domain (DBD) via a flexible Gly–Ser linker and assayed AD function in vivo at the Gcn4- and Gal11-dependent genes ARG3 and HIS4, respectively (Fig. 1B). Gcn4 mRNA was expressed under control of its natural promoter, and induction of Gcn4 translation was induced by the addition of sulfometuron methyl (SM), an inhibitor of amino acid synthesis, for 90 min before mRNA quantitation. In contrast to the WT Gcn4 cAD and full-length nAD (Table S1), Gcn4 residues 81–100 have no in vivo AD function, showing that AD activity involves more than just a simple pattern of bulky hydrophobic residues. However, changing the sequence of this hydrophobic pattern to that of the cAD [VVESFF→VWESLF (key hydrophobic residues underlined)] created a functional cAD-like polypeptide as the template for extensive mutagenesis. As a first step, all side chains within a nine-residue segment centered on the three key hydrophobic residues were individually changed to all other side chains. The resulting derivatives were assayed in vivo for AD function at ARG3. Fig. 2 graphically illustrates the effects of mutations on AD function, and quantitative results are shown in Table S1. Surprisingly, the W15, L18, and F19 residues in the starting sequence were the optimal residues for function of the motif. Replacement of W15 with nearly any other residue completely abolished AD function. Similar results for the specificity of residues L18 and F19 were observed, except that substitution of tryptophan at these positions gave ∼70–90% of the starting AD function. In sum, the three sequence-specific natural residues in the cAD motif are optimal, but the aromatic residue tryptophan...
Sequence specificity of the Gcn4 cAD motif. Residues within a nine-residue segment surrounding the cAD-like motif shown in the bracket were individually changed to all other residues, and these derivatives were tested for in vivo function at ARG3. In the chart, the starting cAD-like motif is in bold, and the key hydrophobic residues are colored red. The effects of single amino acid changes are displayed as the ability of the Gcn4 motif containing the mutation to activate transcription at ARG3, expressed as fold induction over the uninduced cAD-like AD.

In contrast to this sequence specificity, the four residues surrounding W15 are much more permissive in tolerated substitutions. About half of the substitutions at these positions produced AD function that was at least as good or was better than that of the starting cAD-like sequence, with less-optimal substitutions reducing, but not eliminating, AD function. Inspection of the tolerated residues shows that in nearly all cases substitutions of hydrophobic side chains lead to greater activity than the starting cAD-like motif. For residues Ser-20 and Ser-21, at the junction between the AD and the flexible linker, nearly all substitutions worked at least as well as serine, with hydrophobic residues generally being the most active for AD function. Taken together, our results show that the optimal Gcn4 cAD-like motif is ΦΦWΦΦΦLF, embedded within a disordered polypeptide, where Φ represents any hydrophobic residue.

Creation of Short, Very Powerful ADs Based on the cAD Motif. We next asked whether the individual substitutions within the cAD-like motif that increase activity are additive for function. Based on the results of Fig. 2, synthetic ADs were created that had optimal (or nearly optimal) residues at many positions within the AD (Fig. 3 and Table S2). In most cases, multiple substitutions were additive or nearly additive for activation. For example, the starting cAD-like peptide activated ARG3 sevenfold upon SM addition, and single-residue changes showed up to threefold increased activity over the cAD-like motif. However, multiple substitutions gave a wide range of more active ADs, with several showing 30- to 90-fold ARG3 mRNA induction. None of these strong ADs bypassed the requirement for the coactivator subunit Gal11, because activated ARG3 transcription was very low in the absence of Gal11 (Table S2). Remarkably, several of these strong synthetic ADs are more active than the full-length WT Gcn4 with its tandem ADs that activate ARG3 transcription ~40-fold. The most active AD derivatives contained multiple tryptophan substitutions, with the strongest synthetic AD having the sequence motif AWVVWLFWFS. Because tryptophan is the least frequently used amino acid, such tryptophan-rich sequences are rare in nature. However, it was noted previously in a one-hybrid experiment that a short tryptophan-rich peptide derived from a tRNA synthetase has a transcription activation function when fused to a DBD (46).

To test if these powerful synthetic ADs can function within a different sequence context, we inserted selected synthetic AD motifs within the Gcn4 cAD peptide, residues 101–134, and assayed in vivo function (Table S3). The activity of the synthetic motifs was unaffected by this different context, showing that the short motif, and not the surrounding disordered sequence context, is the main determinant of function.

Highly redundant nine-residue AD signatures for yeast, mammalian, and viral activators were proposed based on sequence comparison of known activators and related factors (Fig. S1A) (47). However, we found that this motif does not correlate with AD function in our system. Using the nine-residue AD search algorithm (www.med.muni.cz/9aaTAD/index.php), we found that the Gcn4 cAD did not match the proposed motif signature, whereas Gcn4 residues 81–100, which has no AD function in our system, did match (Fig. S1B and C). Similarly, Gal4 residues 862–870 matched the proposed signature, but a segment of Gal4 containing this peptide (857–881) has little activity, demonstrating that the proposed nine-residue motif does not predict AD function in our experimental system.

The Synthetic Activators Are Unusually Stable Compared with Gcn4. It is known that the cellular levels of some transcription activators are inversely correlated with their transcription AD function; the strongest activators often are the least stable (48). Further, it has been proposed that destruction of some activators after a small number of transcription initiation events is integral to the transcription cycle, allowing expression to be shut off after the initial activation signal has ended (48-50). To test whether the activity-vs.-stability relationship holds for these Gcn4 derivatives, steady-state levels of all of the ADs were measured by Western blot, probing for SM-induced Gcn4 protein via a triple Flag tag at the
C terminus (Fig. S2). The inverse relationship between AD function and steady-state levels generally held for most single-residue substitutions shown in Fig. 2. However, most of the synthetic ADs showed clearly distinct behavior, with much higher protein levels than expected based on their strong AD function (Fig. S2B). If activator instability is an important part of biological regulation, then perhaps this unexpected stability is one reason why Gcn4 and other natural ADs are not optimized for AD function.

AD Function Correlates with Affinity for Gal11 ABD1. To investigate why the synthetic ADs strongly activate transcription, we tested whether they function by having higher coactivator-binding affinity than the natural cAD. As a representative assay for coactivator binding, we measured the affinity of the synthetic ADs for Gal11 ABD1, a well-characterized AD-binding target. Polypeptides with the synthetic motifs in the context of Gcn4 residues 101–134 were expressed as recombinant proteins and purified. We were unable to purify many of the highly tryptophan-rich peptides, because these recombinant proteins are insoluble. However, two of the synthetic ADs, M118 (AVWWSLFAS) and M120 (AFWMWLFAT), are soluble at high concentrations, allowing us to measure binding to ABD1 using isothermal titration calorimetry (ITC) (Table 1 and Fig. S3). WT Gcn4 cAD binds ABD1 with a $K_d$ of 10 μM (19). In contrast, a peptide with the Gcn4 81–100 motif (AVVESFFSS) lacking in vivo function shows no detectable ABD1 binding. As expected, the cAD-like motif used as the template for mutagenesis in Fig. 2 efficiently bound ABD1 with a $K_d$ of 5 μM. The AD derivatives M118 and M120, which respectively activate $\Delta$RG3 transcription 4.3- and 5.5-fold more strongly than the cAD motif, bind ABD1 with $K_d$s of 1.6 and 0.9 μM, respectively. Thus, the strong activator function of these two synthetic ADs correlates with a higher affinity for the Mediator subunit Gal11.

The Strong Synthetic ADs Bind Gal11 via a Fuzzy Complex. The WT Gcn4 cAD binds Gal11 ABD1 via a fuzzy protein interface, but whether a fuzzy interface could account for the higher-affinity binding of the synthetic ADs was not known. One possible explanation for higher affinity is that the synthetic ADs “lock down” on ABD1 in a specific, higher-affinity, and less dynamic complex. An alternative possibility is that the additional hydrophobic residues in the synthetic motif allow a greater number of possible binding modes—effectively, a fuzzier, more redundant, or M120. Localized chemical-shift changes on ABD1 would be more consistent with the synthetic activators adopting a constrained conformation upon binding, rather than the fuzzy binding of the WT cAD. Instead, we see widespread chemical-shift changes spanning the same shallow groove of ABD1, similar to that observed when WT cAD binds to ABD1 (Fig. 4A). The same broad surface of ABD1 is affected by binding M118, M120, and WT cAD, with the synthetic activators inducing larger chemical-shift perturbations in ABD1, as is consistent with the increase in the number of aromatic residues in the AD and tighter ABD1 binding. The widespread chemical-shift changes on the surface of ABD1 are consistent with a fuzzy binding interface but could also be explained if the synthetic activators form longer helical regions upon binding than is the case for WT cAD.

To determine directly if the synthetic ADs bind ABD1 with a fuzzy interface, we performed NMR spin-label experiments. Analogous to the spin-label experiments previously performed with WT cAD (19), the paramagnetic spin-label TEMPO was incorporated at position 117 in the synthetic AD M120. A spin label induces loss of NMR peak intensity at sites proximal to the label. Intensity perturbations in the $^{15}$N-heteronuclear single-quantum coherence spectroscopy (HSQC) of ABD1 upon binding to spin-labeled cAD or M120 are shown in Fig. 4B plotted on the structure of ABD1. We found that the spin-labeled M120 induces perturbations over an even larger area of ABD1 than does spin-labeled cAD. The large perturbations at distal sites of ABD1 are consistent with a fuzzy binding interface for the synthetic AD M120, wherein the helical binding region adopts many orientations with respect to the binding surface of ABD1. Therefore, although the addition of large hydrophobic residues to the cAD motif generally increases both activator activity and the binding affinity to ABD1, the mutations do not result in a single, preferred bound orientation of activator and ABD1.

Unbound WT cAD was determined to be intrinsically disordered, as supported by the very narrow range of $^1$H chemical shifts in the $^{15}$N-HSQC (Fig. S4A) (19). In contrast, the more dispersed $^1$H chemical shifts of unbound M120 suggest the presence of some secondary structure even in the absence of a binding partner. Chemical-shift assignments of M120 were completed to gain structural information about the unbound synthetic AD. Comparison of measured $\alpha$ and $\beta$ chemical shifts with those predicted for a random coil protein reveals information about secondary structure (51). As expected, the $\alpha$ and $\beta$ chemical shifts of unbound WT cAD reveal no significant adoption of helical structure. However, the shifts for M120 show significant helical character for the region spanning residues 117–125 (Fig. 4C). We confirmed the increased helical propensity of M120 relative to cAD, at even lower concentrations than used for the NMR, by circular dichroism (CD) spectroscopy (Fig. S5). Far-UV CD is highly sensitive to protein secondary structure. CD scans of cAD and M120 were collected with increasing concentrations of trifluoroethanol (TFE), which stabilizes any intrinsic helical secondary structure (52). The minima at 208 and 222 nm characteristic of helical secondary structure are more prominent for M120 than for the cAD (Fig. S5C), as consistent with the NMR analysis showing the helical character of the unbound M120 peptide.

Like Many Natural ADs, the Synthetic AD Motifs Are Redundant. Because the synthetic AD motifs still bind Gal11 via fuzzy interfaces, one possibility is that the additional hydrophobic residues within the short motifs allow greater flexibility in binding ABD1.

Table 1. Affinity of AD derivatives for Gal11 ABD1 measured by ITC

| AD peptide | ABD1 $K_d$, μM |
|------------|----------------|
| Gcn4 cAD (101–134) | 10.1 ± 1.4 |
| Gcn4 81–100 | ND |
| Gcn4 cAD-like | 5.1 ± 0.35 |
| Gcn4 M118 | 1.6 ± 0.11 |
| Gcn4 M120 | 0.9 ± 0.09 |

Shown are the Gcn4 derivatives and their measured affinities for Gal11 ABD1. ND, not determined, too weak to measure. Data on WT cAD are from ref. 19.

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via alternative interactions. To test this possibility, we queried whether the starting tryptophan, leucine, and phenylalanine residues within the synthetic motifs are critical for function of the synthetic ADs. Fig. 5 shows that although replacing the tryptophan, leucine, and phenylalanine residues by alanine abolished the function of the WT cAD motif, these same alanine substitutions gave at most a twofold decrease in AD function in the context of the synthetic ADs. For example, in the synthetic AD M120, the tryptophan-to-alanine substitution led to a twofold decrease in function, whereas AD function was reduced by only 15–20% by alanine replacement of the leucine and phenylalanine residues. In the M118 and M121 derivatives, replacement of the tryptophan, leucine, or phenylalanine residues by alanine led to an approximately twofold decrease in activity but had little or no effect on function of the M122 motif. Such behavior is similar to that seen in some natural ADs where it has been difficult to assign important residues within the AD by mutagenesis of single residues (35, 53). Together, the results suggest that many ADs, including our synthetic ADs, contain redundant sequence motifs that allow alternative modes of binding to ABDS.

Discussion

To understand the mechanisms of transcription activation, one important goal is to determine how coactivators such as Mediator integrate signals from multiple factors to modulate transcription precisely. Although many transcription activators and coactivators are known, relatively little is understood about the mechanism and specificity of these interactions. Although a few ADs bind coactivators in complexes with relatively high affinity and specific geometry, many show dynamic binding and modest affinity in coactivator interactions. ADs often bind to several structurally unrelated coactivators, and it has been difficult to understand how ADs can adapt to different partners. Also confusing are the findings that unrelated ADs are not obviously related in sequence. What properties, if any, do ADs targeting the same coactivators have in common? All known ADs are intrinsically disordered polypeptides, and mutagenesis generally has revealed that a small number of hydrophobic residues are important for activity. However, no sequence-specific motif-encoding AD function has been identified to date.

The Gcn4 cAD binds the Mediator subunit Gal11/Med15 using a fuzzy protein–protein interface, potentially explaining how
many ADs bind to seemingly unrelated coactivators (19). If this binding mechanism is representative of a large class of ADs, then we expect that ADs will not be very specific, allowing them to bind ADs of different sequences. Similarly, ADs of this type are predicted to have fairly low information content, allowing new AD functions to develop readily during evolution. Although peptide interactions with structured proteins in other systems often are mediated through short linear motifs, no short linear motifs have yet been recognized in ADs. However, AD sequence motifs that bind via a fuzzy interface may differ from those in complexes with specific geometry.

Structural studies found that the Gcn4 cAD uses three hydrophobic residues on one face of a helix to bind Gal11 ABD1. In accordance with the conservation of these three Gcn4 residues in closely related yeasts (Fig. S6), we show here that this short motif is sufficient to activate transcription when transferred to another disordered polypeptide. Our extensive mutagenesis also showed that the sequence of the three WT cAD hydrophobic residues is optimal for AD function. Furthermore, surrounding this motif with additional hydrophobic residues generates short and very powerful synthetic ADs. These synthetic ADs bind ABD1 with up to 10-fold higher affinity, providing an explanation for their powerful AD function.

We found that two of the synthetic ADs function via a fuzzy protein interface. Part of the reason for the higher activity of the synthetic ADs is that the additional hydrophobic residues within the motif can provide redundant interactions with coactivator targets. This redundancy was revealed by mutations within the tryptophan, leucine, and phenylalanine residues that are essential for function of the WT cAD but are only modestly important in the context of the synthetic ADs. In the WT cAD–ABD1 complex, a single side of the helix contains the tryptophan, leucine, and phenylalanine residues that face the ABD1 hydrophobic groove in multiple orientations (Movie S1). For the synthetic ADs, the extra hydrophobic residues in the motif yield a more extensive hydrophobic surface on the helix, likely allowing the peptide to rotate along its helical axis to present multiple faces of the helix to the ABD1 hydrophobic groove (Movie S2) in addition to binding ABD1 in multiple orientations. This more extensive hydrophobic surface decreases the dependence on the original WxxLF motif. In other words, we predict that the higher affinity of the synthetic activators is generated in part by a fuzzier interface.

Our analysis also showed that the synthetic AD M120 has some helical character in the absence of a binding partner. In contrast to the disordered WT cAD, M120 AD shows a propensity for helix formation in the absence of Gal11. Thus, the strong activity of the synthetic ADs is likely a combination of the extra hydrophobic residues that make redundant contacts with ABDs and their stronger intrinsic helical character.

Our results raise the question of why the Gcn4 AD (and presumably other ADs) is not optimized for activity. The optimized synthetic motifs show that activity of the natural cAD can be increased >13-fold, with activity greater than that of full-length Gcn4. Overexpression of a strong AD is known to be detrimental and squelches transcription of other genes (54). Therefore, constitutive expression of a very strong AD may outcompete other natural ADs for their coactivator targets. In accordance with this notion, cell growth rate is ∼30% slower when Gcn4 is constitutively expressed. Also, many natural genes are synergistically activated by combinations of factors, and a very powerful activator will likely override the need for multiple inputs in gene activation. Given these considerations, there may be no biological advantage to optimizing the function of most ADs apart from viral activators.

Finally, our results suggest that multiple sequence motifs function as transcription ADs. Although the Gcn4 cAD is one specific motif with AD function, it apparently is not used in most other yeast activators. A search of the yeast proteome for the WxxLF motif finds that it occurs in 180 yeast proteins, but only four of these (Gcn4, Gal4, Sbf5, and Hr3) are predicted by sequence analysis to be sequence-specific transcription factors. Apart from Gcn4, none of the WxxLF motifs in the other transcription factors appear in known ADs. For example, the sequence WQILF is found in a helical segment of Gal4 that lies between the two known ADs and likely is involved in regulating AD function (55, 56). Therefore, we propose that multiple sequence-specific motifs embedded within disordered peptides can target the same set of ADs within coactivators. It remains to be determined how often ADs are comprised of these short motifs and whether there are other classes of ADs that function by different mechanisms.

Materials and Methods

Yeast Strains and Plasmids. Yeast strain SHY823 (Δgcn4, leu2) was transformed with the following LEU2- and Gcn4-derivative plasmids for protein expression and mRNA analysis: pRS315 (vector, no GCMN), pSH940 (WT GCMN), pSH942 (Gcn4 101–281; cAD), pSH943 (Gcn4 1–100; nAD), pSH1021 (Gcn4 81–100, or pSH1068 [Gcn4 81–100; cAD-like]). Gcn4 motif mutations were generated in pSH1068 by site-directed mutagenesis. All Gcn4 derivatives except pSH942 contained the flexible linker GSGSGS linking the AD to Gcn4 125–281 containing the DBD. All Gcn4 derivatives contained a C-terminal 3×-Flag tag. Additional Gcn4 motif mutations were made in pSH942 as noted in the text to be in the context of the cAD (Table S3). Yeast strain SHY822 (Δgcn4, Δgal11, leu2) was used to test the Gal11 dependence of the synthetic ADs (Table S2).

mRNA Analysis. Yeast strains were grown in duplicate to an OD600 of 0.5–0.8 in 2% (wt/vol) dextrose synthetic complete Ile-Val-Leu medium at 30 °C. Cells were induced with 0.5 μg/mL 5M for 90 min to induce amino acid starvation (7), RNA was extracted and assayed in triplicate by RT-quantitative PCR, and the results were analyzed as described (7).

Quantification of in Vivo Gcn4 Levels. Cells (1.5 mL) from the cultures used for the above mRNA analysis were pelleted and washed in water. Cells were resuspended in 1x lithium dodecyl sulfate sample buffer (Life Technologies) containing 50 mM DTT and then were lysed at 95 °C for 5 min, spun for 5 min, separated on a 4–12% Bis-Tris gel with 3-(N-morpholino)propanesulfonic acid buffer (Life Technologies) and then analyzed by western blotting with antibodies against ADs (Table S1)
buffer, and then analyzed by Western blot. Gcn4-3×Flag was visualized with mouse anti-FLAG (Sigma) and Tfn2 with rabbit polyclonal anti-Tfn2 (S.H. laboratory). Western blots were quantitated with the Odyssey scanner and software (LI-COR Bioscience) using a titration of Gcn4 81–100 to standardize protein amounts. All motifs were normalized to the Tfn2 loading control.

Recombinant Protein Expression and Purification. Gal11 and Gcn4 derivatives were expressed as small ubiquitin-like modifier (SUMO) fusion proteins (Invitrogen), and the SUMO tag was removed during purification. Expression constructs and purification of Gal11 158–238 and Gcn4 derivatives were as described (17). Bacterial cells for expression of isotopically labeled proteins used in NMR were grown and peptides were purified as described (19).

CD Measurements. CD spectra were recorded using a Jasco J-810 Circular Dichroism Spectropolarimeter. Spectra were recorded at room temperature using a 1-mm quartz cell, a measurement range of 250–300 nm, a scanning speed of 10 nm/min, 1-nm bandwidth, 8-s response time, and 0.1-nm data pitch. The initial peptide concentration was 100 μM in 20 mM phosphate buffer, pH 7.0, and TFE was added in increasing volumes to final concentra-
tion of 70% (vol/vol). Peptide concentration was determined by meas-
uring absorbance at 280 nm for Gcn4 101–134 WT (ε280 = 6,990 M/cm) and M120 (ε280 = 12,490 M/cm) using a Nanodrop ND-1000 Spectrophotometer.

NMR Experiments and Resonance Assignments. NMR HSQC titration and spin-label experiments were completed on a Bruker 500 MHz AVANCE spectrometer. All experiments were collected at 25 °C in NMR buffer (20 mM sodium phosphate, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, and 5 mM DTT) with 5% (vol/vol) D2O. Chemical-shift assignments were obtained on a 1 M [15N]-M120 sample using standard triple-resonance experiments (HNCA, HNHCACB, and HNCO spectra) on a Bruker 800 MHz spec-
trometer. 15N-HSQC titration experiments were completed by adding unlabeled M118 or M120 to a 200 μM 15N-ABD1 sample to a final ratio of 5:1 synthetic ABD:ABD1, maintaining a constant concentration of 15N-ABD1. The reverse titration, adding unlabeled ABD1 to 15N-M120, was performed similarly. Paramagnetic relaxation enhancement experiments were performed as for cAD (19), with the spin-label 4-(2-iodoacetamido)-TEMPO incorporated at S117C of M120. HSQC spectra of 200 μM 15N-ABD1 and 400 μM of spin-labeled M120 were collected in the presence (reference intensity) and absence of acetic acid.

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