VIP Activator Mapping

DOI: 10.1002/anie.200902669

A High-Resolution Interaction Map of Three Transcriptional Activation Domains with a Key Coactivator from Photo-Cross-Linking and Multiplexed Mass Spectrometry**

Chinmay Y. Majmudar, Bo Wang, Jenifer K. Lum, Kristina Håkansson, and Anna K. Mapp**

Transcriptional activators play a central role in gene regulation by stimulating the assembly of the transcriptional machinery at a promoter in a signal-responsive manner.[1] Because of the significant role that misregulated or malfunctioning transcriptional activators play in human disease, there has been a strong effort towards the identification of molecules that inhibit the ability of an activator to function through binding interactions with the transcriptional machinery.[2–4] One difficulty associated with this strategy is that distinct transcriptional activators often share binding partners within the transcriptional machinery; thus, the design or discovery of molecules specific for a particular transcription factor is quite challenging.[5] For example, the activators p53, c-Myc, and the viral oncoprotein E1A all interact with the coactivator and chromatin modifier TRRAP, yet it is not known if these activators utilize a shared binding surface for activation or if there are several distinct binding sites within the coactivator.[6–8] Covalent cross-linking has been used successfully to identify coactivator binding partners of activators, but high-resolution analysis for binding-site identification has not been reported.[9–15] A real challenge to high-resolution analysis is the acid-rich composition of many eukaryotic activators, a characteristic that renders them difficult to analyze by standard cross-linking and mass spectrometric strategies.[16–20]

Herein we describe a multiplexed mass spectrometric strategy to produce a high-resolution map of the binding sites of transcriptional activators for the first time (Figure 1). We focused on three prototypical activators, Gal4, Gcn4, and VP16, in complex with the key coactivator Med15(Gal11). The results of these experiments in combination with genetic experiments demonstrated that Gal4 and Gcn4 target the same binding surfaces within Med15, whereas the VP16-derived activator utilizes a distinct binding surface. This finding suggests that some degree of inhibitor specificity for a given activator or activator class may indeed be achievable. Further, this general approach can be applied to a broader range of activator–coactivator binding partners to expand the high-resolution interaction map of transcriptional activators.

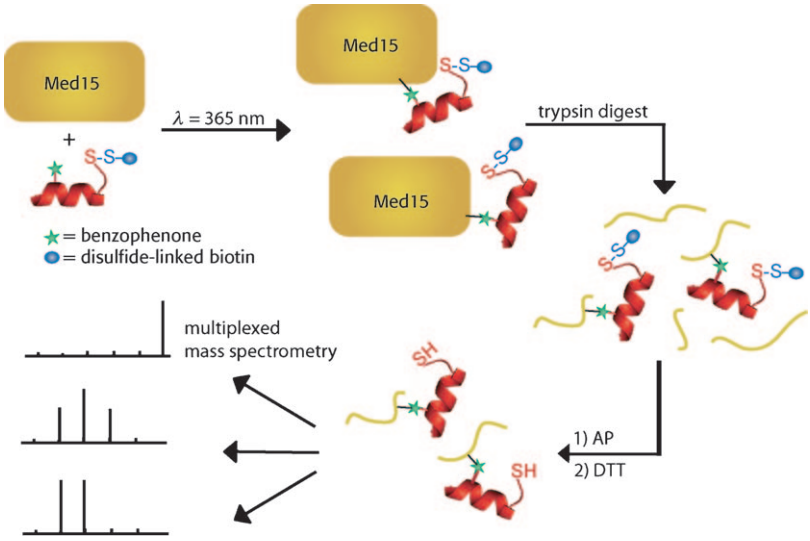

Figure 1. Schematic representation of the mapping of binding sites for transcriptional activation domains (TADs) within a key coactivator. Each TAD (red helix) contains a photoactivatable amino acid (green star) as well as biotin (blue sphere) linked by a disulfide bond. Following incubation with the coactivator Med15 and irradiation to initiate cross-linking, the mixture is trypsinized, and the cross-linked species are subjected to affinity purification (AP) with a neutravidin column. The cross-linked fragments are eluted under reducing conditions and then analyzed by high-resolution multiplexed mass spectrometry. DTT = dithiothreitol.

The largest class of eukaryotic transcriptional activators is the acid-rich or amphipathic class, the most extensively studied members of which are Gal4, Gcn4, and VP16.[1] The descriptor "amphipathic" refers to the primary amino acid sequence of their transcriptional activation domain or TAD,

[*] Dr. C. Y. Majmudar,[+] B. Wang,[+] Dr. J. K. Lum, Prof. Dr. K. Håkansson, Prof. Dr. A. K. Mapp
Department of Chemistry, University of Michigan
930 N University Avenue, Ann Arbor, MI (USA)
Fax: (+1) 734-615-8553
E-mail: amapp@umich.edu

[+] These authors contributed equally.

[**] A.K.M. thanks the NIH (GM65330) and Novartis (Novartis Young Investigator Award) for support of this research. K.H. is the recipient of an NSF Career Award (CHE-05-47699). We also thank the Michigan Proteome Consortium for mass spectrometry analysis and the University of Michigan High Throughput Protein Lab for the development of protein expression constructs.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200902669.

the domain responsible for contacting coactivators within the transcriptional machinery and thereby initiating transcription (Figure 2a). The coactivator Med15, a component of the

a) Gcn4/105–134) TPMFEYENLEDNSKEWTSL*FDNDIPVTTDDC

Gal4(854–874) CGMFNTTTMDDVYNYL*FDDEDT

VP2 DFDLDMLGD*FDLDMLGC

b)

TAD	Med15-cross-linked peptides	Detected by	Confirmed by
Gal4	V^{74}AVMR^{78}	ESI, MALDI	ESI MS/MS, MALDI MS/MS
	R^{160}QLTPQQQQLVNQMK^{174}	ESI	ESI MS/MS
	L^{207}LTPQDMEAAK^{217}	ESI, MALDI	ESI MS/MS
Gcn4	V^{74}AVMR^{78}	MALDI	MALDI MS/MS
	L^{207}LTPQDMEAAK^{217}	ESI	ESI MS/MS
VP2	V^{74}AVMR^{78}	MALDI	MALDI-MS/MS
	N^{79}TYNTRK^{85}		
	R^{160}QLTPQQQQLVNQMK^{174}		
	L^{207}LTPQDMEAAK^{217}		
	I^{222}HQQLLFKAR^{231}		
	A^{345}LRK^{348}		

c) GACKIX domain

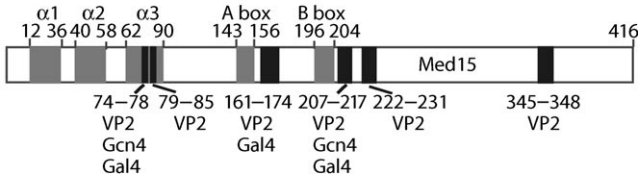

Figure 2. Cross-linking of Med15 with the TADs of Gal4 and Gcn4 and a sequence, VP2, from VP16. a) TAD sequences. Stars indicate the Phe residues that were replaced with *p*-benzoyl-L-phenylalanine. The underlined Cys residues are the positions of biotin attachment. b) Results of multiplexed mass spectrometric analysis of the cross-linked products. See the Supporting Information for the relevant MS/MS spectra. c) Schematic representation of the amino terminus of Med15, with predicted TAD-binding motifs indicated in gray and cross-linked sites identified in vitro indicated in black.

Mediator complex, has been implicated by a variety of techniques to be a target of amphipathic TADs.[13,15,21–24] The amino-terminal half of the protein appears particularly important for the function of Gal4, Gcn4, and VP16.[22–24] What is not known, however, is if these TADs utilize a shared binding surface or if each has a distinct binding site within the coactivator. Owing to the similarity of Med15 to several metazoan coactivators and the ability of Gal4, Gcn4, and VP16 to activate transcription across species,[25,26] TAD–Med15 interactions should serve as an outstanding model for the elucidation of the mechanism of coactivator recruitment and the development of a versatile approach for the determination of TAD–coactivator binding sites.

To localize the fragment of Med15 that interacts with activators, we designed a series of fragments that span its entire sequence and performed cross-linking reactions with isolated TADs from Gal4, Gcn4, and VP16 (VP2) containing the photoactive cross-linker *p*-benzoyl-L-phenylalanine (pBpa).[27–29] In each TAD, the position chosen for pBpa incorporation was originally a phenylalanine residue. In each case, this residue had been shown to be substitutable with other hydrophobic amino acids without detriment to function or to affinity for coactivators.[30–32] For example, Phe869 in the Gal4 transcriptional activation domain can be replaced with a cysteine residue without attenuation of transcription function.[32] The Med15 expression constructs for the cross-linking experiments were designed by using a combination of two solubility tags to maximize yield and stability.[33] For each cross-linking reaction, a solution containing the activator (10 μM) and a Med15 fragment (1 μM) was irradiated with light of wavelength 365 nm for 5 min, and the mixture was analyzed subsequently in a western blot. Consistent with previous studies that highlighted the importance of the N terminus and the middle of the protein for activator contacts, all of the TADs interacted with Med15(1-416) (see Figure 1 in the Supporting Information).[23,24] These findings suggested that at least one binding site was contained within this region. Additionally, fluorescence polarization binding experiments with this region revealed that the Gal4, Gcn4, and VP2 TADs interact with low-micromolar apparent dissociation constants (see Figure 2 in the Supporting Information). Thus, Med15(1-416) was used for all subsequent mass spectrometry experiments.

The general strategy for analysis of the cross-linked complexes to identify the TAD binding sites is illustrated in Figure 1. Particularly important was the use of a redox-sensitive biotin tag incorporated into each TAD to facilitate enrichment under conditions compatible with mass spectrometry following tryptic digest of the mixture. Second, both MALDI and ESI (the latter in both positive- and negative-ion modes), which have orthogonal mechanisms for ionization,[34–38] were employed. The complementary use of two ionization methods proved crucial for identification of the full suite of TAD binding sites. For MS/MS confirmation, high-energy collision-induced dissociation (CID) in a tandem time-of-flight (TOF/TOF) instrument was used for MALDI-generated ions, and low-energy CID in a quadrupole/Fourier transform ion cyclotron resonance (FTICR) mass spectrometer was used for ESI-generated ions. These MS/MS approaches also yielded complementary information.

Multiplexed mass spectrometric analysis revealed binding sites of Gal4, Gcn4, and VP2 within a few amino acid residues in Med15 (Figure 2b). In some cases, cross-linking could be localized to a single residue (e.g., Gal4-Met^{213}; see Figure 8 in the Supporting Information).[39] Med15(74–78) and Med15-(207–217) were detected for all three activators, which suggests that these binding sites are permissive; that is, they enable contacts with activators of different sequence compositions. In the case of the VP16-derived TAD VP2, however, we detected three additional unique cross-linked fragments, Med15(79–84), Med15(222–231), and Med15(345–348).

In the MS/MS analysis, low-energy CID of ESI-generated cationic cross-linked peptides (when detected) generally yielded more structural information than MALDI-TOF/TOF high-energy CID. For example, ESI MS/MS of triply protonated Gal4–Med15(74–78) yielded 12 backbone fragment ions, whereas MALDI-TOF/TOF analysis of the corresponding singly protonated species yielded only five (see Figure 7 in the Supporting Information). However, the use of MALDI was essential for detection of VP2–Med15 cross-linked species. Notably, in MALDI-TOF/TOF MS/MS spectra, abundant fragment peaks resulting from gas-phase

cleavage of the cross-linker to yield the free tryptic peptide from Med15 were observed. Such symmetrical cross-linker cleavage, which is highly useful for verification of the two cross-linked species, has not been reported for previous sector high-energy CID of pBpa-cross-linked peptides[40] and was also absent in our low-energy CID experiments (see, for example, Figure 11 in the Supporting Information). To our knowledge, these experiments represent the first application of combined ESI low-energy CID/MALDI high-energy CID for photo-cross-linked peptides. Alternatives to standard low-energy CID become more pressing at higher molecular weight (ca. 5 kDa) owing to the increased number of vibrational degrees of freedom into which the activation energy is dissipated.[41]

The binding sites identified by mass spectrometry were within or in close proximity to several activator-binding motifs predicted to reside within Med15 (Figure 2c). Residues 62–90 comprise helix 3 of the Med15 GACKIX module, an evolutionarily conserved activator-binding motif,[25,26] and one of the identified binding sites is within this region. Residues 143–156 and 196–204 are homologous to A and B boxes found in mammalian coactivators: sequences that have been shown to contain activator-binding sites;[42] two binding sites were identified proximal to these motifs in the MS/MS analysis.

To assess the functional relevance of the binding sites, we carried out reporter-gene assays of the TADs fused to a DNA-binding domain in yeast strains bearing various Med15 mutants (Figure 3). Deletion of a larger portion of Med15 (residues 1–345) attenuated the activity of all three activators, which suggests that functionally important binding sites are present in this region. Removal of a smaller portion of Med15 (residues 1–186) resulted in the reduction of activity of only VP2 and did not impact the function of either Gal4 or Gcn4. This region contains the Med15(74–78) site contacted by all three activators in vitro and the Med15(161–174) binding site targeted by VP2 and Gal4. To distinguish interactions with the helix 3 binding site (residues 74–78) and Med15(161–174), the activity of VP2 was assessed in yeast strains in which either residues 61–91 or residues 168–172 of Med15 had been removed. As shown in Figure 3, the Med15(74–78) binding site appears to be the functionally important binding site, as its removal led to a greater than 25% decrease in VP2 activity.

In additional experiments, the removal of residues 198–211 and 224–232 in Med15 resulted in a significant decrease in activity for Gal4, Gcn4, and VP2. This result indicates the importance of this region for the function of all three activators. Consistent with the cross-linking results, the deletion of residues 340–348 in Med15 only resulted in a decrease in the activity of VP2. Taken together, these results suggest a model in which VP2 can utilize at least three binding sites within Med15 for function in vivo, whereas Gal4 and Gcn4 utilize the Med15 B-box proximal binding site for function. Further, they suggest that the GACKIX domain is dispensable for Gal4 and Gcn4 function but is utilized by VP2.[43]

In conclusion, by using photo-cross-linking coupled with multiplexed mass spectrometry we have shown that amphipathic transcriptional activators of distinct sequence composition nonetheless interact in vitro with several of the same binding sites within the coactivator Med15. The complementary use of ESI and MALDI techniques was essential to maximize detection by mass spectrometry of the full suite of Med15 binding sites. Further, the combination of low- and high-energy CID provided complementary structural information for verification of assigned cross-linked species. Importantly, the high-resolution interaction map obtained from the in vitro study enabled targeted mutagenesis in vivo to discriminate functionally important binding interactions of the different activators. The in vivo experiments revealed that the VP16-derived TAD has a binding pattern that is distinct from that of Gal4 and Gcn4. In particular, only the VP16-derived TAD has a functionally important binding interaction with the GACKIX domain of the coactivator. This conclusion

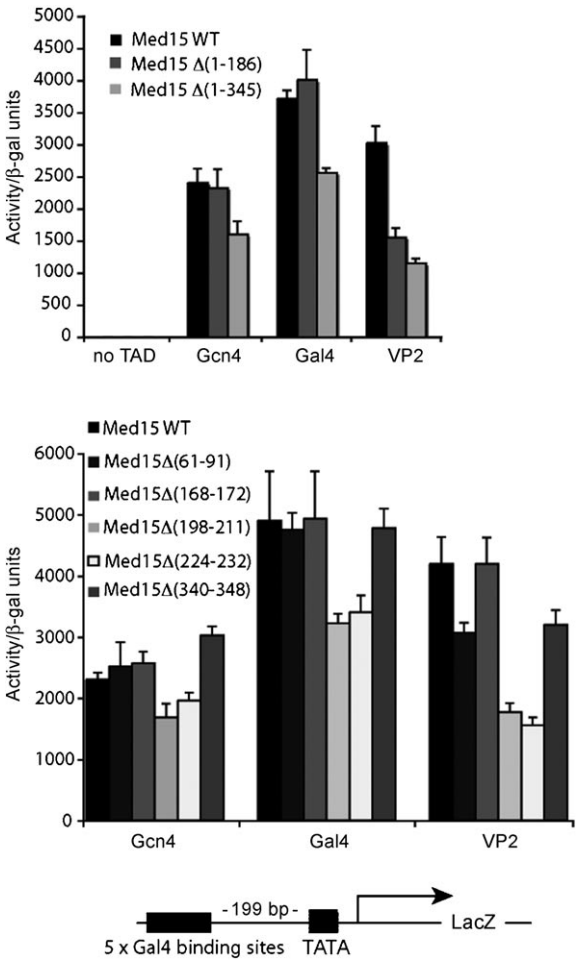

Figure 3. In vivo analysis of TAD-binding sites. Results from β-galactosidase (LacZ) assays performed in yeast strains containing plasmids expressing either wild-type Med15 (Med15 WT) or truncated forms of Med15 as well as plasmids expressing the relevant TAD fused to the minimal DNA-binding domain of Gal4. Since each TAD functions through interaction with several coactivators, transcriptional activity is attenuated rather than abolished upon removal of a key binding interaction. The reported activity is the mean value from at least three experiments (error is the standard deviation of the mean).

highlights the necessity of cellular validation of the results of in vitro binding experiments, particularly in the case of moderate-affinity interactions, such as those investigated in this study. The results of this study have important implications for future screens to identify therapeutically useful inhibitors of activator–coactivator interactions. They reveal that specificity in the attenuation of the function of a particular activator or activator class can probably be achieved by targeting an appropriate binding site.

Received: May 19, 2009
Published online: August 13, 2009

Keywords: coactivator binding sites · cross-linking · mass spectrometry · photochemistry · transcriptional activators

[1] M. Ptashne, A. Gann, *Genes & Signals*, Cold Spring Harbor Laboratory, New York, **2001**.
[2] A. K. Mapp, A. Z. Ansari, *ACS Chem. Biol.* **2007**, *2*, 62.
[3] C. Y. Majmudar, A. K. Mapp, *Curr. Opin. Chem. Biol.* **2005**, *9*, 467.
[4] P. P. Pandolfi, *Oncogene* **2001**, *20*, 3116.
[5] The coactivator CBP interacts with more than 300 activators: H. M. Chan, N. B. La Thangue, *J. Cell Sci.* **2001**, *114*, 2363.
[6] C. A. Kulesza, H. A. Van Buskirk, M. D. Cole, J. C. Reese, M. M. Smith, D. A. Engel, *Oncogene* **2002**, *21*, 1411.
[7] J. Park, S. Kunjibettu, S. B. McMahon, M. D. Cole, *Genes Dev.* **2001**, *15*, 1619.
[8] P. G. Ard, C. Chatterjee, S. Kunjibettu, L. R. Adside, L. E. Gralinski, S. B. McMahon, *Mol. Cell. Biol.* **2002**, *22*, 5650.
[9] H. J. Lin, T. Kodadek, *J. Pept. Res.* **2005**, *65*, 221.
[10] A. Z. Ansari, S. S. Koh, Z. Zaman, C. Bongards, N. Lehming, R. A. Young, M. Ptashne, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14706.
[11] C. T. Archer, L. Burdine, T. Kodadek, *Mol. Biosyst.* **2005**, *1*, 366.
[12] C. E. Brown, L. Howe, K. Sousa, S. C. Alley, M. J. Carrozza, S. Tan, J. L. Workman, *Science* **2001**, *292*, 2333.
[13] J. Fishburn, N. Mohibullah, S. Hahn, *Mol. Cell* **2005**, *18*, 369.
[14] J. Klein, M. Nolden, S. L. Sanders, J. Kirchner, P. A. Weil, K. Melcher, *J. Biol. Chem.* **2003**, *278*, 6779.
[15] W. M. Reeves, S. Hahn, *Mol. Cell. Biol.* **2005**, *25*, 9092.
[16] A. Sinz, *Mass Spectrom. Rev.* **2006**, *25*, 663.
[17] M. Trester-Zedlitz, K. Kamada, S. K. Burley, D. Fenyo, B. T. Chait, T. W. Muir, *J. Am. Chem. Soc.* **2003**, *125*, 2416.
[18] S. M. Lamos, C. J. Krusemark, C. J. McGee, M. Scalf, L. M. Smith, P. J. Belshaw, *Angew. Chem.* **2006**, *118*, 4435; *Angew. Chem. Int. Ed.* **2006**, *45*, 4329.
[19] V. H. Wysocki, G. Tsaprailis, L. L. Smith, L. A. Breci, *J. Mass Spectrom.* **2000**, *35*, 1399.
[20] N. B. Cech, C. G. Enke, *Anal. Chem.* **2000**, *72*, 2717.
[21] M. J. Swanson, H. Qiu, L. Sumibcay, A. Krueger, S. J. Kim, K. Natarajan, S. Yoon, A. G. Hinnebusch, *Mol. Cell. Biol.* **2003**, *23*, 2800.
[22] Y. C. Lee, J. M. Park, S. Min, S. J. Han, Y. J. Kim, *Mol. Cell. Biol.* **1999**, *19*, 2967.
[23] J. M. Park, H. S. Kim, S. J. Han, M. S. Hwang, Y. C. Lee, Y. J. Kim, *Mol. Cell. Biol.* **2000**, *20*, 8709.
[24] C. J. Jeong, S. H. Yang, Y. Xie, L. Zhang, S. A. Johnston, T. Kodadek, *Biochemistry* **2001**, *40*, 9421.
[25] M. Novatchkova, F. Eisenhaber, *Curr. Biol.* **2004**, *14*, R54.
[26] J. K. Thakur, H. Arthanari, F. Yang, S. J. Pan, X. Fan, J. Breger, D. P. Frueh, K. Gulshan, D. K. Li, E. Mylonakis, K. Struhl, W. S. Moye-Rowley, B. P. Cormack, G. Wagner, A. M. Näär, *Nature* **2008**, *452*, 604.
[27] R. Ahrends, J. Kosinski, D. Kirsch, L. Manelyte, L. Giron-Monzon, L. Hummerich, O. Schulz, B. Spengler, P. Friedhoff, *Nucleic Acids Res.* **2006**, *34*, 3169.
[28] G. Dorman, G. D. Prestwich, *Biochemistry* **1994**, *33*, 5661.
[29] A. Wittelsberger, D. F. Mierke, M. Rosenblatt, *Chem. Biol. Drug Des.* **2008**, *71*, 380.
[30] C. M. Drysdale, E. Duenas, B. M. Jackson, U. Reusser, G. H. Braus, A. G. Hinnebusch, *Mol. Cell. Biol.* **1995**, *15*, 1220.
[31] J. L. Regier, F. Shen, S. J. Triezenberg, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 883.
[32] A. Z. Ansari, R. J. Reece, M. Ptashne, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 13543.
[33] J. DelProposto, C. Y. Majmudar, J. L. Smith, W. C. Brown, *Protein Expression Purif.* **2009**, *63*, 40.
[34] W. M. Bodnar, R. K. Blackburn, J. M. Krise, M. A. Moseley, *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 971.
[35] M. D. Stapels, D. F. Barofsky, *Anal. Chem.* **2004**, *76*, 5423.
[36] M. Getie, C. E. Schmelzer, A. S. Weiss, R. H. Neubert, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2989.
[37] K. F. Medzihradszky, H. Leffler, M. A. Baldwin, A. L. Burlingame, *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 215.
[38] A. Sinz, S. Kalkhof, C. Ihling, *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1921.
[39] Within a given binding site, Met and Arg are hot spots for pBpa cross-linking owing to the presence of methyl or methylene groups adjacent to heteroatoms: a) O. Jahn, H. Tezval, J. Spiess, K. Eckart, *Int. J. Mass Spectrom.* **2003**, *228*, 527; b) A. Wittelsberger, B. E. Thomas, D. F. Mierke, M. Rosenblatt, *FEBS Lett.* **2006**, *580*, 1872.
[40] O. Jahn, K. Eckart, O. Brauns, H. Tezval, J. Spiess, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12055.
[41] E. M. Marzluff, J. L. Beauchamp in *Large Ions: Their Vaporization, Detection, and Structural Analysis*, Wiley, New York, **1996**.
[42] D. H. Kim, G. S. Kim, C. H. Yun, Y. C. Lee, *Mol. Cell. Biol.* **2008**, *28*, 913.
[43] This conclusion is consistent with the reported GACKIX dependence of the Oaf1 TAD with sequence homology to VP2: J. K. Thakur, H. Arthanari, F. Yang, K. H. Chau, G. Wagner, A. M. Näär, *J. Biol. Chem.* **2009**, *284*, 4422.

