The 9aaTAD Is Exclusive Activation Domain in Gal4

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Abstract

The Gal4 protein is a well-known prototypic acidic activator that has multiple activation domains. We have previously identified a new activation domain called the nine amino acid transactivation domain (9aaTAD) in Gal4 protein. The family of the 9aaTAD activators currently comprises over 40 members including p53, MLL, E2A and other members of the Gal4 family; Oaf1, Pip2, Pdr1 and Pdr3. In this study, we revised function of all reported Gal4 activation domains. Surprisingly, we found that beside of the activation domain 9aaTAD none of the previously reported activation domains had considerable transactivation potential and were not involved in the activation of transcription. Our results demonstrated that the 9aaTAD domain is the only decisive activation domain in the Gal4 protein. We found that the artificial peptides included in the original Gal4 constructs were results of an unintended consequence of cloning that were responsible for the artificial transcriptional activity. Importantly, the activation domain 9aaTAD, which is the exclusive activation domain in Gal4, is also the central part of a conserved sequence recognized by the inhibitory protein Gal80. We propose a revision of the Gal4 regulation, in which the activation domain 9aaTAD is directly linked to both activation function and Gal80 mediated inhibition.

Introduction

The prototypical activators of transcription, such as Gcn4 and Gal4 transcription factors, have been used in molecular biology to study gene regulation in eukaryotes [1–6], following the discovery of the first activator of transcription in prokaryotes, arac [7]. Earlier, the Gcn4, Gal4 and p53 activation domains have been designated as acidic activators [8–11]. Nevertheless, the next studies have demonstrated also the importance of the hydrophobic amino acids for Gcn4 [12–15] and p53 activators [16–23].

There are four activation domains reported for Gal4 protein. Early Gal4 studies reported one weak N-terminal activation domain AD-I (148–196 aa; 13-fold less efficient than the intact Gal4) and one strong C-terminal activation domain AD-II (763–823 aa; as efficient as the intact Gal4) [24]. Similarly to the Gcn4 acidic activator, authors correlated the presence of
multiple acidic amino acids with activation function. Initially, a deletion of the 33 amino acid long C-terminus of the Gal4 protein had only a minor effect on the Gal4 ability to activate transcription (as 85% activity was detected in the construct pMA230, Gal4 region 1–848 aa) [24]. In the next study, the same authors showed that their mutant was fully independent on galactose stimulation [25].

However, the results from Melcher and Johnston studies [26,27] clearly showed that the Gal4 C-terminal 34 aa long end represented another activation domain AD-III, which provided the interaction with coactivators of transcription and also with inhibitory protein Gal80. The Gal4 loss-of-function mutant (1–852 aa, deletion of the Gal4 C-terminal 29 aa long end) called gal4-64 supported these findings [28–30].

In the following Gal4 study from Ptashne, a shorter activation domain AD-III (840–857 aa) was declared as the major activation domain in Gal4 protein [31]. However, the shorter activation domain AD-III did not include the region recognized by inhibitory protein Gal80. Furthermore, the authors reported previously that the Gal4 constructs without the activation domain AD-III were strong activators of transcription (AD-II construct CD15XX, 1–823 aa; AD-II construct CDCD13, 1–844 aa) [24].

We have reported a new activation domain for the Gal4 transcription factor, the nine amino acids transactivation domain, 9aaTAD [32]. The activation domains 9aaTAD are created by a tandem of two hydrophobic clusters that are interspersed by a hydrophilic region. As highlighted previously [32], the hydrophobic clusters of the activation domain 9aaTAD are consistent with a broad evidence of hydrophobic patterns reported for Gcn4, p53 and other transcription factors [12–17,22]. The 9aaTAD pattern was reported and the online prediction could be found on www.piskacek.org ([MDENQSTYG] {KRHCGP} {ILVFWM} {KRHCGP} {CGP} {CGP} [ILVFWM] {CGP} {CGP}, Swissprot syntax, and the twelve specific refinement criteria RC1-12) [32,33]. Occasionally, the 9aaTAD function could be significantly enhanced by the adjacent amino acids (one aa at the C-terminus and up to 4 aa at the N-terminus, which included usually one or more hydrophobic amino acids e.g. as shown for the Msn2 orthologs [32,34]). Therefore the activation domains 9aaTAD could be up to 14 amino acid long, when the contribution of the adjacent amino acids is discernible.

The position of the Gal4 9aa TAD activation domain did not correspond to any of the previously reported activation domains AD-I, AD-II or AD-III from Ptashne lab. Rather, the position of the activation domain 9aaTAD correlated with the Gal80 binding region and with the region missing in the Gal4 loss-of-function mutant gal4-64 (1–852 aa) [26,27].

In this study, we revised function of all reported Gal4 activation domains. Surprisingly, we found that beside of the activation domain 9aaTAD none of the previously reported activation domains was functional. Our results showed that the 9aaTAD domain is the only decisive activation domain in the Gal4 protein.

Materials and Methods

Constructs

The construct pBTM116-HA was generated by an insertion of the HA cassette into the EcoRI site of the vector pBTM116 (HA cassette nucleotide sequence: TGG CTG—GAATTA—GCC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT GTC GAG ATA—GAATTC, which render in amino acids sequence: W L—E L—A T M A Y P Y D V P D Y A V E I—E F). The constructs G1-G45 and H1-H45 were generated by PCR and subcloned into pBTM116 EcoRI and BamHI sites. All construct have a spacer of three amino acids inserted into the EcoRI site; peptide -NNN- (NNN cassette: GAATTC—AATAATAAT, which render in peptide: EF—NNN). All
constructs were sequenced by Eurofins Genomics. Further detailed information about constructs, primer sequences are available on the request.

**Assessment of enzyme activities**

The β-galactosidase activity was determined in the yeast strain L40 [35,36]. The strain L40 has integrated the lacZ reporter driven by the lexA operator. In all hybrid assays, we used 2μ vector pBTM116 for generation of the LexA hybrids. The yeast strain L40, the Saccharomyces cerevisiae Genotype: MATa ade2 his3 leu2 trp1 LYS::lexA-HIS3 URA3::lexA-lacZ, is deposited at ATCC (#MYA-3332). For β-galactosidase assays, overnight cultures propagated in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) were diluted to an A600 of 0.3 and further cultivated for two hours and collected by centrifugation. The crude extracts were prepared by vortexing with glass beads for 3 minutes. The assay was done with 10 ul crude extract in 1ml of 100 mM phosphate buffer pH7 with 10 mM KCl, 1 mM MgSO4 and 0.2% 2-Mercaptoethanol; reaction was started by 200 ul 0.4% ONPG and stopped by 500 ul 1 M CaCO3. The average value of the β-galactosidase activities from three independent experiments is presented as a percentage of the reference with the standard deviation (means and plusmn; SD; n = 3). We standardized all results to previously reported Gal4 construct HaY including merely the activation domain 9aaTAD with the activity set to 100% [34].

**Western blot analysis**

The crude cell extracts were prepared in a buffer containing 200 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (v/v), separated by SDS-PAGE, and blotted to nitrocellulose. The immuno-detection of proteins was carried out using mouse anti-HA antibody (#26183, ThermoFisher Sci) or mouse anti-LexA (#306–719, EMD Millipore Corp). The secondary antibodies used were anti-mouse IgG antibodies conjugated with horseradish peroxidase (#A9044, Sigma Aldrich). The proteins were visualized using Pierce ECL (#32106, ThermoFisher Sci) according to the manufacturer’s instructions.

**Results**

**Functional and non-functional activation domains in Gal4 activator**

To we revised function of all reported Gal4 activation domains, we had generated a set of the Gal4 constructs (Figs 1, 2 and 3). The activities of all Gal4 constructs in this study were tested in LexA hybrid assay (the LexA is E.coli DNA binding domain and the Gal4 DBD is S.cerevisiae Gal4 DNA binding domain, both generally used for the generation of fusion hybrids; 2μ vector pBTM116 was used for all constructs; strain L40 with integrated lacZ reporter driven by lexA operator was used to assay the activation of transcription). The expression of the Lex-A-Gal4 protein hybrids were proved by western blotting (S1 Fig).

We generated LexA hybrids with all four activation domains of the Gal4 protein, which were reported previously [24,31,32]; the construct G10 (1–238 aa) with the activation domain AD-I (148–238 aa), the construct G42 (766–848 aa) with the activation domains AD-II (766–848 aa), the construct G405 with the activation domain AD-III (840–857 aa), and the construct G577 with the activation domain 9aaTAD (857–871 aa). For the most active C-terminal Gal4 region 766–881 (including the activation domain AD-II, the acid activation domain AD-III and the activation domain 9aaTAD) we generated LexA hybrid series of deletions (766–881, 766–871 and 766–863 aa; constructs G81, G49 and G50).
The constructs G577, G81, and G49, which all included the activation domain 9aaTAD, have the capacity to activate transcription (Fig 1). Surprisingly, the constructs G10, G42, G405 and G50, with activation domains AD-I, AD-II or AD-III, had no substantial activity.

Artificial peptides in the original Gal4 constructs activated transcription

From the data above, it was not clear why the Gal4 activation domains AD-I, AD-II and AD-III were inactive in our constructs. By the inspection of the previously reported data, we identified artificial peptides in the original constructs as a result of an unintended consequence of cloning [24]. Therefore we have generated a set of the Gal4 constructs with and without the artificial peptides (H41, H43, H45, H47 versus H42, H44, H46, and H48). In addition, we generated G42, G44, G46, and G48 constructs without the HA tags (the standard version of LexA hybrid without any modification of the original BTM116 vector). To avoid any cloning artefacts, we have inserted a peptide spacer of three Asparagines (NNN) between the LexA DNA binding domain and the Gal4 fragments.

We have observed that the three constructs with the artificial peptides including the H41 (Gal4 sequence 766–848 aa with RVWN HYRDV peptide), H43 (Gal4 sequence 766–844 aa...
with GIPD HYRDV peptide) and H47 (Gal4 sequence 766–823 with PEFR RVWN HYRDV peptide) strongly activated transcription (Fig 2). The activities of the corresponding constructs without the artificial peptides (constructs H42, H44 and H48) were dramatically reduced or completely diminished. This observation was even more pronounced in the constructs without the HA-tags (constructs G42, G44, G46, and G48). The weak activities were observed in two out of four constructs with the HA-tags (for the constructs H42, H48 but not for H44 and H46 constructs). All constructs without the HA-tags had none substantial activity, therefore the weak activity of the construct H42 (and even less in H48 construct) was considered as an elevated basal level, which extended to 15% of the Gal4 C-terminal construct G49 and 10% of the

### LexA - Gal4 C-terminal constructs with and without Artificial Peptides

| Constructs                        | ID   | Artificial Peptides          | Transactivation in % of HaY construct |
|-----------------------------------|------|-----------------------------|---------------------------------------|
| LexA-Gal4 C-termini 9aaTAD        | G49  | NEIT ASKIDGNNS KPLSPG WTDQT AYNA. TM DD VY NY FD D. | 120 ±28                               |
| LexA-HA-Gal4 + peptide (766-848 aa) | H41  | NEIT ASKIDGNNS KPLSPG WTDQT AYNA. | GIPD HYRDV. 63 ±10                   |
| LexA-HA-Gal4 (766-848 aa)         | H42  | NEIT ASKIDGNNS KPLSPG WTDQT AYNA. | 18 ± 1                                |
| LexA-Gal4 (766-848 aa)            | G42  | NEIT ASKIDGNNS KPLSPG WTDQT AYNA. | 1 ± 1                                 |
| LexA-HA-Gal4 + peptide (766-844 aa) | H43  | NEIT ASKIDGNNS KPLSPG WTDQT GIPD HYRDV. | 43 ± 6                                |
| LexA-HA-Gal4 (766-844 aa)         | H44  | NEIT ASKIDGNNS KPLSPG WTDQT. | 4 ± 1                                 |
| LexA-Gal4 (766-844 aa)            | G44  | NEIT ASKIDGNNS KPLSPG WTDQT. | 1 ± 1                                 |
| LexA-HA-Gal4 + peptide (766-833 aa) | H45  | NEIT ASKIDGNNS RN. | 2 ± 1                                 |
| LexA-HA-Gal4 (766-833 aa)         | H46  | NEIT ASKIDGNNS. | 1 ± 1                                 |
| LexA-Gal4 (766-833 aa)            | G46  | NEIT ASKIDGNNS. | 1 ± 1                                 |
| LexA-HA-Gal4 + peptide (766-823 aa) | H47  | NEIT PEFR RVWN HYRDV. | 127 ±12                               |
| LexA-HA-Gal4 (766-823 aa)         | H48  | NEIT | 14 ± 1                                |
| LexA-HA-Gal4 (766-823 aa)         | G48  | NEIT | 1 ± 1                                 |
| LexA-HA-Gal4 9aaTAD HaY (860-871 aa) | H50  | TM DD VY NY LD FD D. | 100 ±11                               |

**Fig 2. Artificial peptides in the original Gal4 constructs activated transcription.** To distinguish natural and artificial activity, a sets of LexA-Gal4 hybrid constructs were generated with and without artificial peptides presented in original constructs, an unintended consequence of cloning [24]. The LexA-Gal4 hybrid constructs assayed in L40 strain for transactivation activity are shown. The average value of the β-galactosidase activities from three independent experiments is presented as a percentage of the reference with standard deviation (means and plusmn; SD; n = 3). We standardized all results to previously reported Gal4 construct HaY including merely the activation domain 9aaTAD with the activity set to 100% [34]. The protein sequences are partially given by amino acid single letter code. Single dot means end of protein sequence, tree points mean continuing of the sequence, which is not more shown. The regions of Gal4 protein in the constructs are noted and graphically presented.

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**LexA - Gal4** constructs with and without the activation domain **AD-III** and **9aaTAD**

impact of adjacent amino acids

| Constructs | ID | LexA | Gal4 DBD (93-100 aa) | AD-III (840-857 aa) | 9aaTAD (862-871 aa) | Transactivation in % of HaY construct |
|------------|----|------|----------------------|---------------------|---------------------|-------------------------------------|
| LexA-Gal4  | G81| LexA | _NSKPLSPG WTdQ TAYNAFgitGTTGmFTTTM | DD VY N YL FD | DEDTPNPKKE | 114 ±11 |
| LexA-Gal4  | G49| LexA | _NSKPLSPG WTdQ TAYNAFgitGTTGmFTTTM | DD VY N YL FD | D. | 198 ±27 |
| LexA-Gal4  | G50| LexA | _NSKPLSPG WTdQ TAYNAFgitGTTGmFTTTM | DD. | 29 ± 2 |
| LexA-Gal4  | G42| LexA | _NSKPLSPG WTdQ TAYNAF | DD. | 1 ± 1 |
| LexA-Gal4  | G405| LexA | WTdQ TAYNAF | DD. | 1 ± 1 |
| LexA-Gal4  | pRJR200# | LexA | LLTGLVQ | WTdQ | 195 ±16 |
| LexA-Gal4  | U39| LexA | LLTGLVQ | WTdQ | 162 ±14 |
| LexA-HA-Gal4 | H577| LexA | TTTM | DD N YL FD | D. | 177 ±39 |
| LexA-HA-Gal4 | HaY | LexA | TM | DD N YL FD | D. | 100 ±11 |
| LexA-HA-Gal4 | Hdd | LexA | TM | DD | 1 ± 1 |

**Fig 3. The activation domain AD-III did not activate transcription.** The LexA-Gal4 hybrid constructs with activation domains AD-III, 9aaTAD and their artificial variants were assayed in L40 strain for activation of transcription. Gal4 DBD is Gal4 DNA binding domain. The red marks part of the artificial activation domain 9aaTAD (1/2 9aaTAD) and the blue marks part of the natural activation domain 9aaTAD (1/2 9aaTAD). Both these parts generated strong activators in the fusion with the Gal4 DNA binding domain, Gal4 DBD (83–100 aa), which served as other part of the artificial activation domain 9aaTAD (the second 1/2 9aaTAD). The artefact explained previous cloning errors of the reported construct pRJR200 with activation domain AD-III [31]. The impact of proximal amino acids on the function of the activation domain 9aaTAD was tested. The related constructs with and without activation domains AD-III and 9aaTAD are shown again to overview their activities (G81, G49, G59, G42, G405). The average value of the β-galactosidase activities from three independent experiments is presented as a percentage of the reference with standard deviation (means and plusmn; SD; n = 3). We standardized all results to previously reported Gal4 construct HaY including merely the activation domain 9aaTAD with the activity set to 100% [34]. The regions of Gal4 protein in the constructs are noted and graphically presented. The artificial activation domain AD-II is not more shown in this figure. The protein sequences are partially given by amino acid single letter code. Single dot means end of protein sequence, tree points mean continuing of the sequence, which is not more shown.

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9aaTAD construct H577. The LexA-HA construct Hdd with the HA-tag was inactive and therefore the HA-tag could not activate transcription (Fig 3).

The artificial peptides in H41, H43 and H47 did not fit the 9aaTAD pattern and therefore are not activation domains of the 9aaTAD family.
The activation domain AD-III did not activate transcription

In order to fully justify the 9aaTAD domain function, we have generated the Gal4 constructs G49 (776–871 aa) and G50 (776–863 aa) that ended before and after the activation domain 9aaTAD. Furthermore, we have generated two different constructs including merely the activation domain 9aaTAD with two different fusion regions (boundaries) to the LexA DNA binding domain—constructs H577 (857–871 aa) and HaY (860–871 aa) (Fig 3). To avoid artefacts, we have inserted the peptide linker of three Asparagines (NNN) between LexA and Gal4 in all our constructs (LexA—HA tag—NNN—Gal4 region).

We have previously reported that fusion of the Gal4 DNA binding domain could occasionally generate strong artificial activation domains [34]. The Gal4 region (92–100 aa) might serve as a half of the artificial activation domain 9aaTAD. We generated construct U39 with a half of the artificial activation domain 9aaTAD originated from the DNA binding domain of the Gal4 (Gal4 region 92–100, peptide LVTGLFVQD) together with four amino acids of the activation domain AD-III, which represented the second half of the artificial activation domain 9aaTAD (Gal4 region 840–843, peptide WTDQ).

As shown in Fig 3, the 9aaTAD constructs H577 (857–871 aa) and HaY (860–871 aa) strongly activated transcription comparably with the most active Gal4 C-terminal segments; the constructs G49 (776–871 aa) and G81 (776–881 aa). The activity of the activation domain 9aaTAD is over hundred fold stronger than previously reported the activation domain AD-III as shown for the construct G405 (840–857 aa). The artificial activation domain (construct U39) activated transcription about two hundred fold stronger than the activation domain AD-III (construct G405) and demonstrated a cloning error in the original AD-III construct pRJR200 [31].

Discussion

Historically, the Gal4 together with Gcn4 and p53 transcription factors represented the acidic activators [1,2,24,25,31,34,37–42]. However, the broad evidence of the essential hydrophobic clusters was reported for Gcn4, p53 and other activators [12–17,22]. Recently, we have reported that both acidic activation domains of the p53 protein are in fact the activation domains 9aaTAD. Moreover, we did not find any conservation of acidic residues in the p53 activation domains 9aaTAD [34].

In this study, we found that none of the previously reported Gal4 activation domains AD-I, AD-II or AD-III was able to activate transcription. Therefore, the domain 9aaTAD is the exclusive activation domain in the Gal4 protein, which has the decisive competence to activate transcription. Thus the term acidic activation domain reported for Gal4 protein [2,25,31] and reported data disproving activation domain 9aaTAD in Gal4 protein [37,43] need urgent revision.

We demonstrated that artificial peptides included in the original Gal4 constructs [24] and fusions of the Gal4 DNA binding domain with random peptides (without proper peptide linker) [31] were responsible for the artificial transcriptional activities in numerous reported Gal4 constructs.

The original Gal4 construct pRJR200 [31] should demonstrate the presence of a strong activation domain AD-III in the Gal4 C-terminal region. In this construct, the Gal4 DNA binding domain (1–100 aa) was fused to the Gal4 region of 18 amino acids (840–857 aa) [31]. Nevertheless, we showed in this study that the same Gal4 region of 18 amino acids was unable to activate transcription (AD-III construct G405). We suspected that an artificial activation domain has been generated in the original AD-III construct pRJR200 by fusion of the Gal4 DNA binding domain with otherwise inactive the Gal4 region AD-III (840–857 aa).
We have previously reported that the fusion of the Gal4 DNA binding domain with random peptides could occasionally generate strong artificial activation domains [34]. The Gal4 region (92–100 aa) serves as a half of the activation domain 9aaTAD. The artefact could be observed in the fusion of either the Gal4 DNA binding domain (1–100 aa) or the small portion (Gal4 region 92–100, peptide LLTGLFVQD−, where hyphen marks fusion site) with the second half of the native Gal4 activation domain 9aaTAD (Gal4 region 867–871, peptide -YLFDD) or with the part of the Gal4 activation domain AD-III (Gal4 region 840–843, peptide -WTDQ).

The latter fusion (construct pRJR200#), the Gal4 DNA binding domain and four amino acids of the Gal4 activation domain AD-III were fused together without any suitable peptide spacer (Gal4 region 92–100 and Gal4 region 840–843, peptide LLTGLFVQD−WTDQ). This fusion was a powerful activator of transcription, which was two-fold stronger than the Gal4
activation domain 9aaTAD (construct HaY). The construct pRJR200# demonstrated presence of a strong artificial activation domain 9aaTAD in the original pRJR200 construct [31].

Similar artificial transactivation domain was generated previously by fusion of the Gal4 DNA binding domain (96–100 aa) and P201 activator [44]. The Gal4-P201 hybrid (peptide LFQVD—YLLPTCIP) was strong activator of transcription [44].

The position of the activation domain 9aaTAD (857–871 aa) correlates with the Gal4 region 854–875, which is necessary and sufficient for the interaction with inhibitory protein Gal80. The 21 aa long peptide derived from the Gal4 region 854–875 tightly binds to the inhibitory protein Gal80 (peptide: GMFNTTTMDDVNYLFDEDT; the Gal4 activation domain 9aaTAD was included inside of the peptide: DDVNYLFDEDT; structural data available at PDB accession code 3E1K) [45]. Similarly, related peptide derived from Gal4’s ortholog K.l.Ga9 (peptide: TQQLFNTTTMDDVNYLFDEDT; structural data available at PDB accession code 3BTS) [11] (Fig 4). Furthermore, recent report has suggested a functional link between the artificial peptides binding to the Gal80 protein and their ability to activate transcription; "Peptides selected to bind the Gal80 repressor are potent transcriptional activation domains in yeast" [46].

The Gal4 protein is known to interact with MED15 [46], TAF9 [47] and Tra1 [48,49]. Moreover, the Gal4 activator function was linked with MED15 [50–53], Tra1 [54] and SAGA/MED15 complex [55,56]. Importantly, the direct molecular and functional interaction has been reported between the Gal4 activation domain and the Sug2 regulatory protein (subunit of the 26 S proteasome) [57,58]. As a part of the Gal4 regulation, the inhibitory protein Gal80 [28,29,59–61] could be depleted by Gal3 protein in galactose dependent manner [5,62,63]. It has been also shown that the inhibitory protein Gal80 competes with the transcriptional machinery for the same Gal4 region [64,65]. Collectively, we inferred the Gal4 regulation, which involves the activation domain 9aaTAD, the Gal4 mediators of transcription and the inhibitory protein Gal80. We propose a new model of the Gal4 regulation (Fig 4) to the previously report [39].

The activation domains 9aaTAD are universally recognized by the transcriptional machinery in eukaryotes. Currently, the 9aaTAD family comprises over 40 members including Gal4, Oaf1, Pip2, Pdr1, Pdr3, Leu3, Tea1, Pho4, Gln3, Gcn4, Msn2, Msn4, Rtg3, E2A, MLL, p53-TAD-I, p53-TAD-II, HNF4 / NHR-49, FOXO3, NF-kB, NFKAT, CEBPA/E, ESX, ELF3, ETV1, KLF2/4, EBNA2, VP16, HSFI, HSF2, Hsfa, Gli3, Sox18, PIF, Dreb2a, MTF1, OREB1, WRKY45, NS1, MKL1, TRP32, VP16, EBNA2, KBP220, ECapLL, P201, AH, and B42 transcription factors. We and others have shown that the activation domains 9aaTAD have competence to activate transcription as small peptides [32,33,36,66–80]. The activation domains 9aaTAD are annotated on protein database UniProt (http://www.uniprot.org/uniprot/?query=9aatad&sort=score) and the 9aaTAD prediction service is available online (www.piskacek.org).

Supporting Information
S1 Fig. Protein expression. The protein level produced from the Gal4 constructs in L40 strain were monitored by Westernblotting. The proteins comprise LexA a HA tags with a total size of about 21 kDa. (TIIF)

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Author Contributions

Conceptualization: MP.
Investigation: MP MH MR.
Software: MP.
Supervision: MP.
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References

1. Hope IA, Struhl K. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell. 1986; 46: 885–894. PMID: 3530496
2. Ma J, Ptashne M. A new class of yeast transcriptional activators. Cell. 1987; 51: 113–119. PMID: 3115591
3. Hope IA, Mahadevan S, Struhl K. Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein. Nature. 1988; 333: 635–640. doi: 10.1038/333635a0 PMID: 3287180
4. Johnston M, Dover J. Mutational analysis of the GAL4-encoded transcriptional activator protein of Saccharomyces cerevisiae. Genetics. 1988; 120: 63–75. PMID: 3065140
5. Peng G, Hopper JE. Gene activation by interaction of an inhibitor with a cytoplasmic signaling protein. Proc Natl Acad Sci USA. 2002; 99: 8548–8553. doi: 10.1073/pnas.142100099 PMID: 12084916
6. Johnston SA, Zavortink MJ, Debouck C, Hopper JE. Functional domains of the yeast regulatory protein GAL4. Proc Natl Acad Sci USA. 1986; 83: 6553–6557. PMID: 2944111
7. Englesberg E, Iri J, Power J, Lee N. Positive control of enzyme synthesis by gene C in the L-arabinose system. J Bacteriol. 1965; 90: 946–957. PMID: 5321403
8. Stargell LA, Struhl K. The TBP-TFIIA interaction in the response to acidic activators in vivo. Science. 1995; 269: 75–8. PMID: 7604282
9. Chou S, Struhl K. Transcriptional activation by TFIIB mutants that are severely impaired in interaction with promoter DNA and acidic activation domains. Mol Cell Biol. 1997; 17: 6794–6802. PMID: 9372910
10. Dorris DR, Struhl K. Artificial Recruitment of TFIIID, but Not RNA Polymerase II Holoenzyme, Activates Transcription in Mammalian Cells. Mol Cell Biol. 2000; 20: 4350–4358. PMID: 110825199
11. Thoden JB, Ryan LA, Reece RJ, Holden HM. The interaction between an acidic transcriptional activator and its inhibitor. The molecular basis of Gal4p recognition by Gal80p. J Biol Chem. 2008; 283: 30266–30272. doi: 10.1074/jbc.M805200200 PMID: 18701455
12. Drysdale CM, Duenas E, Jackson BM, Reussser U, Braus GH, Hinnebusch AG. The transcriptional activator GCN4 contains multiple activation domains that are critically dependent on hydrophobic amino acids. Mol Cell Biol. 1995; 15: 1220–1233. PMID: 7682116
13. Jackson BM, Drysdale CM, Natarajan K, Hinnebusch AG. Identification of seven hydrophobic clusters in GCN4 making redundant contributions to transcriptional activation. Mol Cell Biol. 1996; 16: 5557–5571. PMID: 8816468
14. Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, et al. Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol. 2001; 21: 4347–4368. doi: 10.1128/MCB.21.13.4347-4368.2001 PMID: 11390663
15. Jedidi I, Zhang F, Qiu H, Stahl SJ, Palmer I, Kaufman JD, et al. Activator Gcn4 employs multiple segments of Med15/Gal11, including the KIX domain, to recruit mediator to target genes in vivo. J Biol Chem. 2010; 285: 2438–2455. doi: 10.1074/jbc.M109.071589 PMID: 19940160
16. Krois AS, Ferreon JC, Martinez-Yamout MA, Dyson HJ, Wright PE. Recognition of the disordered p53 transactivation domain by the transcriptional adapter zinc finger domains of CREB-binding protein. Proc Natl Acad Sci USA. 2016; 113: E1853–1862. doi: 10.1073/pnas.1602487113 PMID: 26976603
17. Lee CW, Aral M, Martinez-Yamout MA, Dyson HJ, Wright PE. Mapping the interactions of the p53 transactivation domain with the KIX domain of CBP. Biochemistry. 2009; 48: 2115–2124. doi: 10.1021/bi802055v PMID: 19220000
18. Denis CM, Chitayat S, Plevin F, Wang F, Thompson P, Li S, et al. Structural basis of CBP/p300 recruitment in leukemia induction by E2A-PBX1. Blood. 2012;
19. Wang F, Marshall CB, Li G-Y, Yamamoto K, Mak TW, Ikura M. Synergistic interplay between promoter recognition and CBP/p300 coactivator recruitment by FOXO3a. ACS Chem Biol. 2009; 4: 1017–1027. doi: 10.1021/cb900190u PMID: 19821614

20. Uesugi M, Verdine GL. The alpha-helical FXXPhiPhi motif in p53: TAF interaction and discrimination by MDM2. Proc Natl Acad Sci USA. 1999; 96: 14801–14806. PMID: 10611293

21. Radhakrishnan I, Pérez-Alvarado GC, Parker D, Dyson HJ, Montminy MR, Wright PE. Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions. Cell. 1997; 91: 741–752. PMID: 9413984

22. Lee CW, Martinez-Yamout MA, Dyson HJ, Wright PE. Structure of the p53 transactivation domain in complex with the nuclear receptor coactivator binding domain of CREB binding protein. Biochemistry. 2010; 49: 9964–9971. doi: 10.1021/bi1001996 PMID: 20961098

23. Wojciak JM, Martinez-Yamout MA, Dyson HJ, Wright PE. Structural basis for recruitment of CBP/p300 coactivators by STAT1 and STAT2 transactivation domains. EMBO J. 2009; 28: 948–958. doi: 10.1038/emboj.2009.30 PMID: 19214187

24. Ma J, Ptashne M. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell. 1987; 48: 847–853. PMID: 3028647

25. Ma J, Ptashne M. The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. Cell. 1987; 50: 137–142. PMID: 3297349

26. Melcher K, Johnston SA. GAL4 interacts with TATA-binding protein and coactivators. Mol Cell Biol. 1995; 15: 2839–2848. PMID: 7739564

27. Ding WV, Johnston SA. The DNA binding and activation domains of Gal4p are sufficient for conveying its regulatory signals. Mol Cell Biol. 1997; 17: 2538–2549. PMID: 9111323

28. Douglas HC, Hawthorne DC. ENZYMATIC EXPRESSION AND GENETIC LINKAGE OF GENES CONTROLLING GALACTOSE UTILIZATION IN SACCHAROMYCES. Genetics. 1964; 49: 837–844. PMID: 14158615

29. Douglas HC, Condie F. The genetic control of galactose utilization in Saccharomyces. J Bacteriol. 1954; 68: 662–670. PMID: 13221541

30. Matsumoto K, Adachi Y, Toh-e A, Oshima Y. Function of positive regulatory gene gal4 in the synthesis of galactose pathway enzymes in Saccharomyces cerevisiae: evidence that the GAL81 region codes for part of the gal4 protein. J Bacteriol. 1990; 141: 508–527. PMID: 6989385

31. Wu Y, Reece RJ, Ptashne M. Quantitation of putative activator-target affinities predicts transcriptional activating potentials. EMBO J. 1996; 15: 3951–3963. PMID: 8670900

32. Piskacek S, Gregor M, Nemethova M, Grabner M, Kovarik P, Piskacek M. Nine-amino-acid transactivation domain: establishment and prediction utilities. Genomics. 2007; 89: 756–768. doi: 10.1016/j.ygeno.2007.02.003 PMID: 17467953

33. Piskacek M. 9aaTAD Prediction result (2006). Nature Precedings. 2009;

34. Piskacek M, Havelka M, Rezacova M, Knight A. The 9aaTAD Transactivation Domains: From Gal4 to p53. PLoS ONE. 2016; 11: e0162842. doi: 10.1371/journal.pone.0162842 PMID: 27618436

35. Miller JH. Experiments in molecular genetics. Cold Spring Harbor Laboratory; 1972.

36. Baumgartner U, Hamilton B, Piskacek M, Ruis H, Rottensteiner H. Functional analysis of the Zn(2)Cys(6) transcription factors Oaf1p and Pip2p. Different roles in fatty acid induction of beta-oxidation in Saccharomyces cerevisiae. J Biol Chem. 1999; 274: 22208–22216. PMID: 10428786

37. Warfield L, Tuttle LM, Pacheco D, Klevit RE, Hahn S. A sequence-specific transcription activation motif and powerful synthetic variants that bind Mediator using a fuzzy protein interface. Proc Natl Acad Sci USA. 2014; 111: E3506–3513. doi: 10.1073/pnas.1412088111 PMID: 25122681

38. Hahn S. Structure(?) and function of acidic transcription activators. Cell. 1993; 72: 481–483. PMID: 8440015

39. Himmelfarb HJ, Pearberg J, Last DH, Ptashne M. GAL11P: a yeast mutation that potentiates the effect of weak GAL4-derived activators. Cell. 1990; 63: 1299–1309. PMID: 2124519

40. Ptashne M. Two “what if” experiments. Cell. 2004; 116: S71–72, 2 p following S76. PMID: 15055587

41. Ptashne M. The chemistry of regulation of genes and other things. J Biol Chem. 2014; 289: 5417–5435. doi: 10.1074/jbc.X114.547323 PMID: 24395432

42. Strubin M, Struhl K. Yeast and human TFIIID with altered DNA-binding specificity for TATA elements. Cell. 1992; 68: 721–730. PMID: 17392977

43. Erkina TY, Erkine AM. Nucleosome distortion as a possible mechanism of transcription activation domain function. Epigenetics Chromatin. 2016; 9: 40. doi: 10.1186/s13072-016-0092-2 PMID: 27679670
44. Lu X, Ansari AZ, Ptashne M. An artificial transcriptional activating region with unusual properties. Proc Natl Acad Sci USA. 2000; 97: 1988–1992. doi: 10.1073/pnas.040573197 PMID: 10681438

45. Kumar PR, Yu Y, Stemgalnanz R, Johnston SA, Joshua-Tor L. NADP regulates the yeast GAL induction system. Science. 2008; 319: 1090–1092. doi: 10.1126/science.1151903 PMID: 18292341

46. Han Y, Kodaek T. Peptides selected to bind the Gal80 repressor are potent transcriptional activation domains in yeast. J Biol Chem. 2000; 275: 14979–14984. PMID: 10809742

47. Klein J, Nolden M, Sanders SL, Kirchner J, Weil PA, Melcher K. Use of a genetically introduced cross-linker to identify interaction sites of acidic activators within native transcription factor IID and SAGA. J Biol Chem. 2003; 278: 6779–6786. doi: 10.1074/jbc.M212514200 PMID: 12501245

48. Reeves WM, Hahn S. Targets of the Gal4 transcription activator in functional transcription complexes. Mol Cell Biol. 2005; 25: 9902–9102. doi: 10.1128/MCB.25.20.9902-9102.2005 PMID: 16199885

49. Majmudar CY, Labut AE, Mapp AK. Tra1 as a screening target for transcriptional activation domain discovery. Bioorg Med Chem Lett. 2009; 19: 3733–3735. doi: 10.1016/j.bmcl.2009.05.045 PMID: 19497740

50. Sakurai H, Hiraoka Y, Fukasawa T. Yeast GAL11 protein is a distinctive transcription factor that enhances basal transcription in vitro. Proc Natl Acad Sci USA. 1993; 90: 8382–8386. PMID: 8378310

52. Nogi Y, Fukasawa T. A novel mutation that affects utilization of galactose in Saccharomyces cerevisiae. Curr Genet. 1980; 2: 115–120. doi: 10.1007/BF00420623 PMID: 6958827

53. Hashimoto H, Kikuchi Y, Nogi Y, Fukasawa T. Regulation of expression of the galactose gene cluster in Saccharomyces cerevisiae. Isolation and characterization of the regulatory gene GAL4. Mol Gen Genet. 1983; 191: 31–38. PMID: 6958827

54. Lin L, Chamberlain L, Zhu LJ, Green MR. Analysis of Gal4-directed transcription activation using Tra1 mutants selectively defective for interaction with Gal4. Proc Natl Acad Sci USA. 2012; 109: 1997–2002. doi: 10.1073/pnas.1116340109 PMID: 22308403

55. Larsson M, Uvell H, Sandström J, Rydén P, Selth LA, Börklund S. Functional studies of the yeast med5, med15 and med16 mediator tail subunits. PLoS ONE. 2013; 8: e73137. doi: 10.1371/journal.pone.0073137

56. Larssen E, Winston F. The Saccharomyces cerevisiae Srb8-Srb11 complex functions with the SAGA complex during Gal4-activated transcription. Mol Cell Biol. 2005; 25: 114–123. doi: 10.1128/MCB.25.1.114-123.2005 PMID: 15601835

57. Chang C, Gonzalez F, Rothermel B, Sun L, Johnston SA, Kodadek T. The Gal4 activation domain binds Sug2 protein, a proteasome component, in vivo and in vitro. J Biol Chem. 2001; 276: 30956–30963. doi: 10.1074/jbc.M102254201 PMID: 11418596

58. Russell SJ, Johnston SA. Evidence that proteolysis of Gal4 cannot explain the transcriptional effects of proteasome ATPase mutations. J Biol Chem. 2001; 276: 9825–9831. doi: 10.1074/jbc.M10889200 PMID: 11152478

59. Douglas HC, Hawthorne DC. Regulation of genes controlling synthesis of the galactose pathway enzymes in yeast. Genetics. 1966; 54: 911–916. PMID: 5970626

60. Douglas H.C. GP. A gene controlling inducibility of the galactose pathway enzymes in Saccharomyces. Biochimica et Biophysica Acta (BBA)—Specialized Section on Nucleic Acids and Related Subjects. 1963; 68: 155–156.

61. Johnston SA, Salmeron JM, Dincher SS. Interaction of positive and negative regulatory proteins in the galactose regulon of yeast. Cell. 1987; 50: 143–146. PMID: 3297350

62. Jiang F, Frey BR, Evans ML, Friel JC, Hopper JE. Gene activation by dissociation of an inhibitor from a transcriptional activation domain. Mol Cell Biol. 2009; 29: 5604–5610. doi: 10.1128/MCB.00632-09 PMID: 19651897

63. Egriboz O, Jiang F, Hopper JE. Rapid GAL gene switch of Saccharomyces cerevisiae depends on nuclear Gal3, not nucleocytoplasmic trafficking of Gal3 and Gal80. Genetics. 2011; 189: 825–836. doi: 10.1534/genetics.111.131839 PMID: 21890741

64. Carrozza MJ, John S, Sil AK, Hopper JE, Workman JL. Gal80 confers specificity on HAT complex interactions with activators. J Biol Chem. 2002; 277: 24648–24652. doi: 10.1074/jbc.M201965200 PMID: 11986329

65. Weake VM, Workman JL. Inducible gene expression: diverse regulatory mechanisms. Nat Rev Genet. 2010; 11: 426–437. doi: 10.1038/nrg2781 PMID: 20421872
66. Sandholzer J, Hoeth M, Piskacek M, Mayer H, de Martin R. A novel 9-amino-acid transactivation domain in the C-terminal part of Sox18. Biochem Biophys Res Commun. 2007; 360: 370–374. doi: 10.1016/j.bbrc.2007.06.095 PMID: 17603017

67. Lindert U, Cramer M, Meuli M, Georgiev O, Schaffner W. Metal-responsive transcription factor 1 (MTF-1) activity is regulated by a nonconventional nuclear localization signal and a metal-responsive transactivation domain. Mol Cell Biol. 2009; 29: 6283–6293. doi: 10.1128/MCB.00847-09 PMID: 19797083

68. Piskacek M. Common Transactivation Motif 9aaTAD recruits multiple general co-activators TAF9, MED15, CBP and p300. Nature Precedings. 2009;

69. Piskacek M. 9aaTADs mimic DNA to interact with a pseudo-DNA Binding Domain KIX of Med15 (Molecular Chameleons). Nature Precedings. 2009;

70. Hong JY, Chae MJ, Lee IS, Lee YN, Nam MH, Kim DY, et al. Phosphorylation-mediated regulation of a rice ABA responsive element binding factor. Phytochemistry. 2011; 72: 27–36. doi: 10.1016/j.phytochem.2010.10.005 PMID: 21101110

71. Shekhawat UKS, Ganapathi TR, Srinivas L. Cloning and characterization of a novel stress-responsive WRKY transcription factor gene (MusaWRKY71) from Musa spp. cv. Karibale Monthan (ABB group) using transformed banana cells. Mol Biol Rep. 2011; 38: 4023–4035. doi: 10.1007/s11033-010-0521-4 PMID: 21110110

72. Lou S, Luo Y, Cheng F, Huang Q, Shen W, Kleiboeker S, et al. Human parvovirus B19 DNA replication induces a DNA damage response that is dispensable for cell cycle arrest at phase G2/M. J Virol. 2012; 86: 10748–10758. doi: 10.1128/JVI.01007-12 PMID: 22837195

73. Matsushita A, Inoue H, Goto S, Nakayama A, Sugano S, Hayashi N, et al. The nuclear ubiquitin proteasome degradation affects WRKY45 function in the rice defense program. Plant J. 2012;

74. Aguilar X, Blomberg J, Brännström K, Olofsson A, Schleucher J, Björklund S. Interaction studies of the human and Arabidopsis thaliana Med25-ACID proteins with the herpes simplex virus VP16- and plant-specific Dreb2a transcription factors. PLoS ONE. 2014; 9: e98575. doi: 10.1371/journal.pone.0098575 PMID: 24874105

75. Scharenberg MA, Pippenger BE, Sack R, Zingg D, Ferralli J, Schenk S, et al. TGF-β-induced differentiation into myofibroblasts involves specific regulation of two MKL1 isoforms. J Cell Sci. 2014; 127: 1079–1091. doi: 10.1242/jcs.142075 PMID: 24424023

76. Piskacek M, Vasku A, Hajek R, Knight A. Shared structural features of the 9aaTAD family in complex with CBP. Mol Biosyst. 2015; 11: 844–851. doi: 10.1039/c4mb00672k PMID: 25564305

77. Qiu Y, Li M, Pasoreck EK, Long L, Shi Y, Galvão RM, et al. HEMERA Couples the Proteolysis and Transcriptional Activity of PHYTOCHROME INTERACTING FACTORs in Arabidopsis Photomorphogenesis. Plant Cell. 2015;

78. Lee K, Goh GYS, Wong MA, Klassen TL, Taubert S. Gain-of-Function Alleles in Caenorhabditis elegans Nuclear Hormone Receptor nhr-49 Are Functionally Distinct. PLoS ONE. 2016; 11: e0162708. doi: 10.1371/journal.pone.0162708 PMID: 27618178

79. Farris TR, Dunphy PS, Zhu B, Kibler CE, McBride JW. Ehrlichia chaffeensis TRP32 is a Nucleomodulin that Directly Regulates Expression of Host Genes Governing Differentiation and Proliferation. Infect Immun. 2016;

80. Presnell JS, Schnitzler CE, Browne WE. KLF/SP Transcription Factor Family Evolution: Expansion, Diversification, and Innovation in Eukaryotes. Genome Biol Evol. 2015; 7: 2289–2309. doi: 10.1093/gbe/evv141 PMID: 26232396