Supplementary Information

Structure and VP16 binding of the Mediator Med25 activator interaction domain

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Supplementary Information comprises:

Supplementary Figures 1-6

Supplementary Methods

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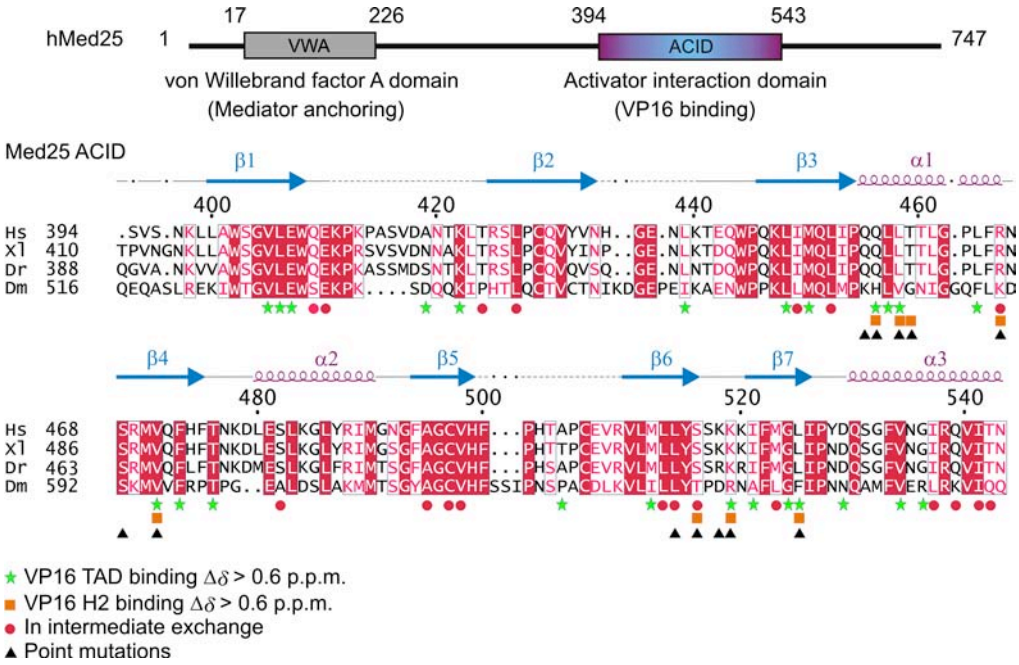
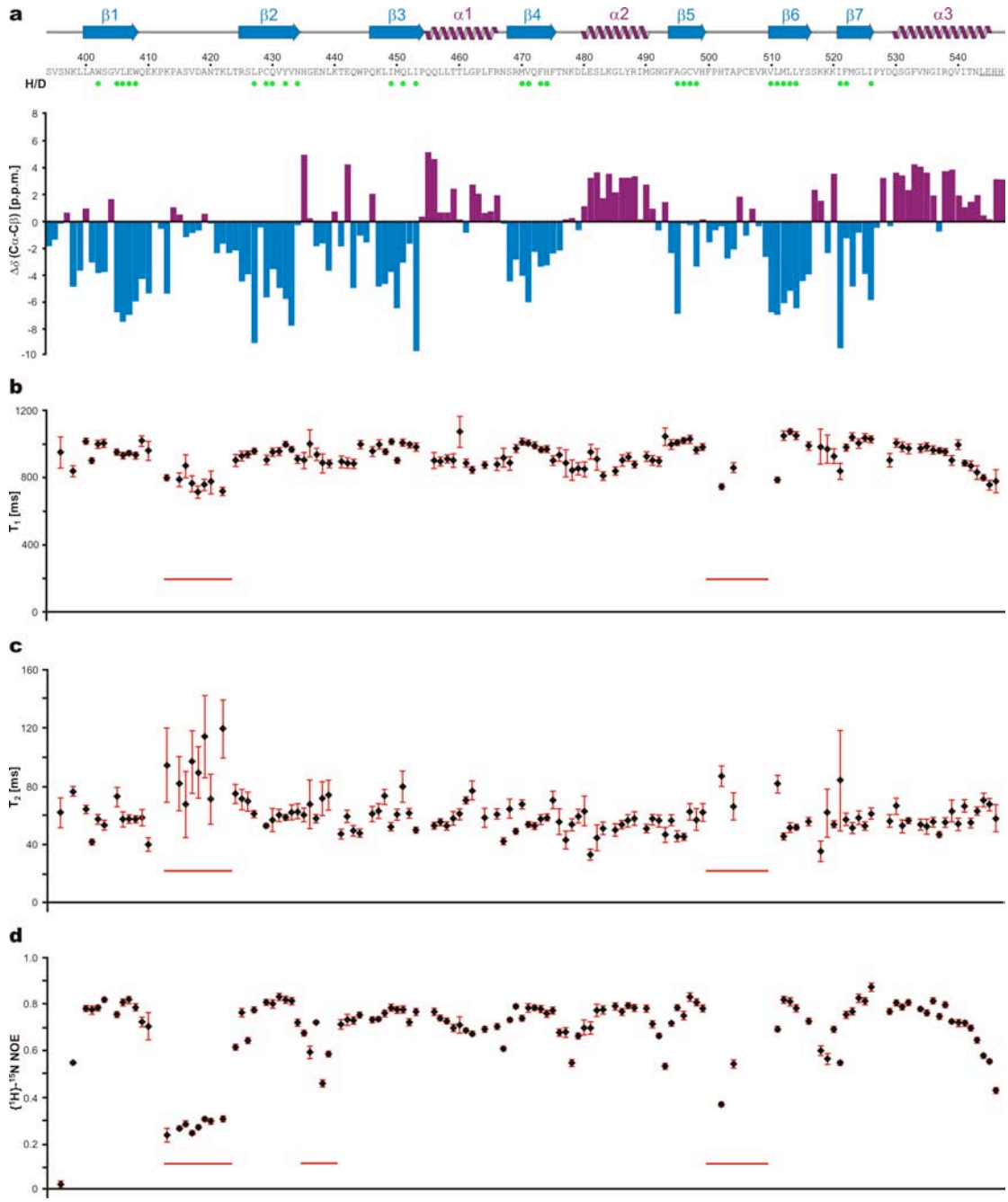


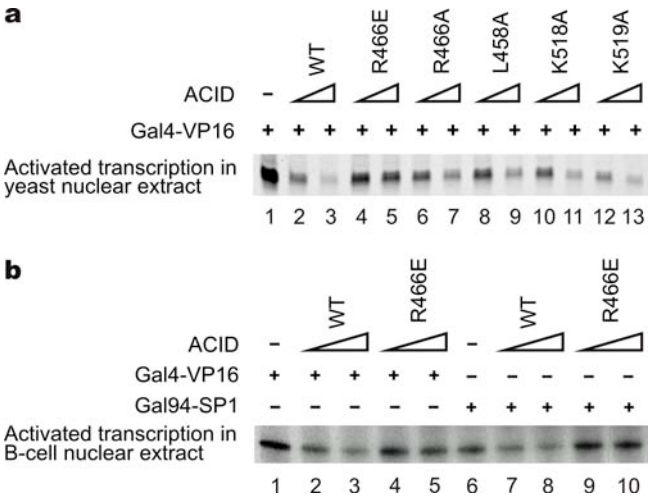
Supplementary Figure 1 Med25 domain architecture and ACID domain conservation

Sequence alignment[1] of ACID from *Homo sapiens* (*Hs*), *Xenopus laevis* (*Xl*), *Danio rerio* (*Dr*) and *Drosophila melanogaster* (*Dm*). Secondary structure elements are indicated above the sequence (spirals, α-helices; arrows, β-strands; lines, loops; dashed lines, disordered regions). Invariant and conserved residues are on red background and in red, respectively. Figure was prepared with ESPript[2]. Residues with variation in chemical shift $\Delta\delta$ (p.p.m.) > 0.6 upon VP16 TAD binding are depicted as green stars. Red spheres indicate residues with binding in an intermediate exchange regime on the NMR chemical shift time scale (broadened beyond detection). Residues affected upon addition of VP16 H2 are shown as orange squares. ACID point mutant variants are depicted as black triangles.



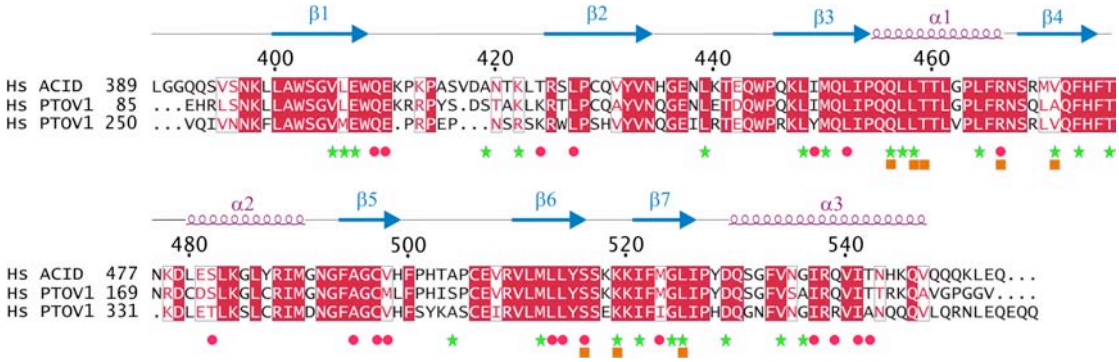
Supplementary Figure 2 NMR analysis of human Med25 ACID

(**a**) Primary and secondary structure of Med25 ACID. The last turn of the helix also contains two residues (Leu and Glu) resulting from cloning (underlined). Solvent-protected amide protons that show slow H/D exchange are indicated by green circles. Below, NMR secondary chemical shifts, $\Delta\delta$ ($^{13}C\alpha$-$^{13}C\beta$). Positive (purple) and negative (blue) values indicate α-helical and β-strand conformation, respectively.
(**b–d**) ^{15}N NMR relaxation data: (**b**) ^{15}N T_1 and (**c**) ^{15}N T_2 relaxation times, (**d**) $\{^1H\}$-^{15}N heteronuclear NOE. The error was calculated as the standard deviation of the noise in the spectrum divided by the intensity of the reference peak. Flexible regions are marked with a red line. Based on the ^{15}N T_1/T_2 ratio the ACID domain tumbles as a monomer in solution (τ_c = 12ns).



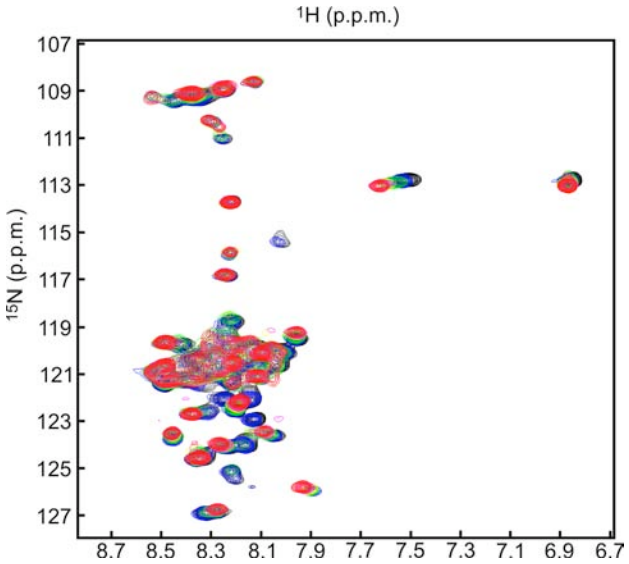
Supplementary Figure 3 *In vitro* transcription assays

(**a**) Activated transcription in yeast nuclear extract. Assays were performed as in **Figure 4b** but using 100 or 400 pmol of either wild-type ACID (lanes 2, 3) or specific ACID point mutant variants (lanes 4–13). ACID R466E variant hardly quenches transcription (lanes 4, 5) whereas ACID variants do to various extents (lanes 6–13). (**b**) The ACID surface comprising residue Arg466 interacts with activator. VP16-activated transcription (lanes 1–5) was performed as in **Figure 4d**. Comparative, human SP1-activated transcription (lane 6) was quenched by increasing amounts of wild-type ACID (530 or 850 pmol) (lanes 7, 8), whereas ACID variant R466E lost its capability to interfere with activated transcription (lanes 9, 10).



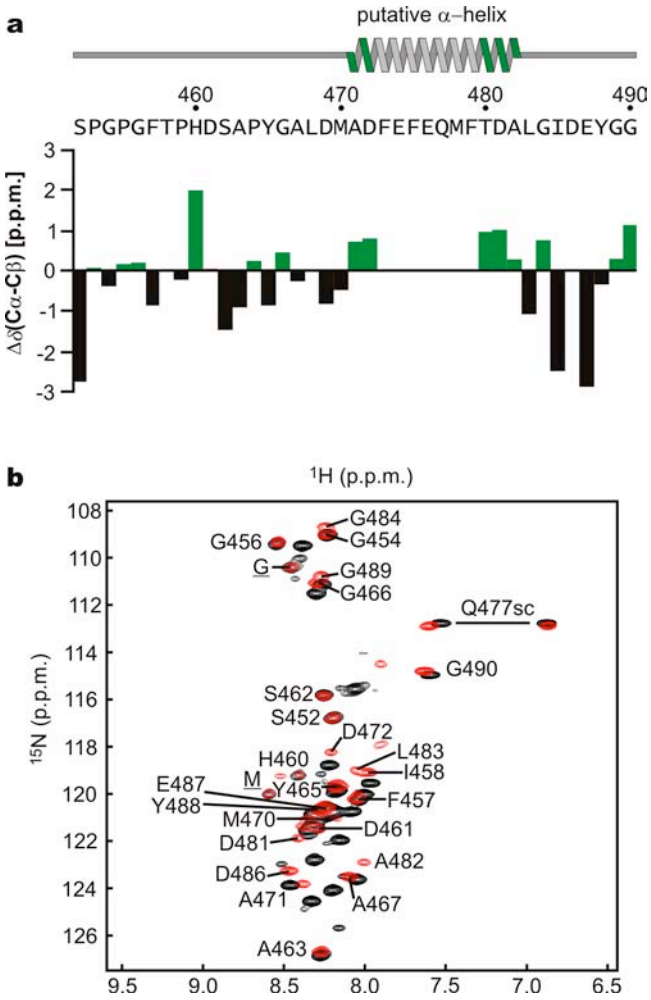
Supplementary Figure 4 Conservation of human ACID domains

Sequence alignment[1] of human ACID domains of Med25 and of both PTOV1 consecutive domains. Secondary structure elements are indicated above the sequence (spirals, α-helices; arrows, β-strands; lines, loops). Invariant and conserved residues are on red background and in red, respectively. Figure was prepared with ESPript[2]. Residues with variation in chemical shift $\Delta\delta$ (p.p.m.) > 0.6 upon VP16 TAD binding are depicted as green stars and red spheres indicate residues with binding in an intermediate exchange regime on the NMR chemical shift time scale. Residues affected upon addition of VP16 H2 are shown as orange squares (as in **Supplementary Fig. 1**).



Supplementary Figure 5 VP16 TAD–Med25 ACID interaction

Overlay of the 2 D ^{1}H,^{15}N HSQC spectra (900 MHz) of the ^{13}C,^{15}N-labeled VP16 TAD titration with Med25 ACID. Spectra are presented for the free form (black) and for molar ratios (VP16 TAD:ACID) of 1:0.2 (blue), 1:0.4 (green), 1:0.6 (yellow), 1:0.8 (magenta) and 1:1 (red). For many signals binding in an intermediate exchange regime is observed, such that signals of the bound state cannot be observed at the endpoint of the titration.



Supplementary Figure 6 Backbone assignment of VP16 H2 in complex with human Med 25 ACID suggests partial helical structure

(**a**) Primary and secondary structure of VP16 H2. NMR secondary chemical shifts $\Delta\delta$ ($^{13}C\alpha$-$^{13}C\beta$) suggest that residues Ala471–Ala482 adopt an α-helix when bound to ACID. Gaps and grey coloring of the helix indicate residues which were not detectable presumably due to substantial exchange broadening. Positive (green) and negative (black) values indicate α-helical and extended conformation, respectively.
(**b**) Overlay of the 2 D ^{1}H,^{15}N HSQC spectra for ^{13}C,^{15}N-labeled VP16 H2 free (black), and in the presence of equimolar Med25 ACID (red). Assigned VP16 H2 residues are labeled. Assigned residues from the cloning artefact GAMG are underlined.

Software used for preparation of figures
Structural representations have been generated with PyMOL (http://www.pymol.org) and, where indicated, with MOLMOL[3]. Figures were prepared using Photoshop and Illustrator (Adobe Systems Inc.) and CorelDRAW (Corel Corporation).

Supplementary Methods

Cloning and protein expression. DNA encoding human Med25 ACID residues 394–543 was cloned into vector pET-21b (Novagen), resulting in a C-terminal His_6-tag. ACID variants were obtained by QuickChange site-directed mutagenesis (Stratagene). Preparation of pure, recombinant VP16 TAD was hampered by intrinsic disorder, which impaired soluble expression, and by the absence of tryptophan residues, which impaired detection of the protein during purification. Furthermore, conventional strategies designed to overcome these difficulties by fusing TAD to glutathione S-transferase or maltose-binding protein resulted in degraded proteins. A fusion construct of the Gal4 DNA-binding domain (residues 2–147) followed by a TEV cleavage site and VP16 TAD residues 413–490 was cloned into vector pET-21b, resulting in a C-terminal His_6-tag. DNA encoding for VP16 H2 (residues 452–490) was cloned into a modified pET9d vector, resulting in an N-terminal His_6-tagged MBP fusion construct. A TEV cleavage site was included between MBP and VP16 H2. Variants Gal4-VP16 H1 (residues 404–451), Gal4-VP16 H2 (residues 452–490), Gal4-VP16 TAD H1mt (residues 413–490, F442P), Gal4-VP16 TAD H2mt (residues 413–490, F473A F475A F479A), Gal4-VP16 H1mt (residues 404–451, F442P), and Gal4-VP16 H2mt (residues 452–490, F473A F475A F479A) were subcloned into vector pET-21b. Details of the cloning are available upon request. Proteins were produced by overexpression in *E. coli* BL21(DE3)RIL cells (Stratagene). Media were supplemented with 30 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin or 30 μg ml^{-1} kanamycin, inoculated with overnight cultures, and grown to an OD_{600} ~ 0.8 at 37°C. Expression was induced by adding IPTG to 0.5 mM and incubating at 18°C for 16 h. Cells were harvested by centrifugation and stored at –80°C. For unlabeled proteins, bacteria were grown in LB. For $^{15}N/^{13}C$- or ^{15}N-labeled proteins, bacteria were grown in M9 minimal media supplemented with ^{13}C-glucose (2 g l^{-1}) and/or ^{15}N-ammonium chloride (0.5 g l^{-1}).

Preparation of ACID variants. Cells containing ACID variants were resuspended in 20 mM sodium phosphate pH 6.5, 150 mM NaCl, 10 mM 2-mercaptoethanol, and 1:100 protease inhibitor mix containing 17 g l^{-1} phenylmethylsulfonyl fluoride, 0,0284 g l^{-1} leupeptin, 0,137 g l^{-1} pepstatin A, 33 g l^{-1} benzamidin, and lysed by sonication. After centrifugation, the protein was bound to Ni-NTA (Qiagen). After washing the column with 20 mM sodium phosphate pH 6.5, 1.15 M NaCl, and 10 mM 2-mercaptoethanol and 20 mM sodium phosphate pH 6.5, 150 mM NaCl, 10 mM imidazole, and 10 mM 2-mercaptoethanol, bound proteins were eluted with 20 mM sodium phosphate pH 6.5, 150 mM NaCl, 200 mM imidazole, and 10 mM 2-mercaptoethanol. After dilution with the same volume of 20 mM sodium phosphate pH 6.5, 50 mM NaCl, 4 mM DTT, the sample was loaded onto a HiTrap-SP cation-exchange column (GE Healthcare) equilibrated in 20 mM sodium phosphate pH 6.5, 4 mM DTT, and eluted with a linear gradient to 1 M NaCl. Protein was further purified on a Superose 6 gel filtration column (GE Healthcare) equilibrated either in 2.5 mM sodium phosphate pH 6.5, 100 mM NaCl, 4 mM DTT or 20 mM sodium phosphate pH 6.5, 100 mM NaCl, 1 mM EDTA, 4 mM DTT. NMR samples contained 0.2–0.4 mM protein and were prepared in 10% (v/v) D_2O or 100% D_2O.

Preparation of VP16 variants. Cells containing Gal4-VP16 TAD were resuspended and sonicated in buffer A (10 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, 10 μM $ZnSO_4$, 10 mM 2-mercaptoethanol, and 1:100 protease inhibitor mix). After centrifugation, the soluble fraction was purified by affinity chromatography using Ni-NTA. After washing with buffer A and 10 mM Tris, pH 8.0, 100 mM NaCl, 40 mM imidazole, 10% (v/v) glycerol, 10 μM $ZnSO_4$, 20 mM 2-mercaptoethanol, and 1:100 protease inhibitor mix, the protein was eluted with 10 mM Tris, pH 8.0, 100 mM NaCl, 200 mM imidazole, 10% (v/v) glycerol, 10 μM $ZnSO_4$, 20 mM 2-mercaptoethanol, and loaded onto a HiTrap-SP cation-exchange column (GE Healthcare) equilibrated in 20 mM Hepes, pH 7.5, 10 μM zinc acetate, 1 mM DTT. Protein was eluted with a linear gradient to 1 M NaCl. After TEV protease cleavage overnight, cleaved Gal4 protein was found in the flowthrough of a Ni-NTA column. After elution with 10 mM Tris, pH 8.0, 100 mM NaCl, 300 mM imidazole, 10 μM $ZnSO_4$, 20 mM 2-mercaptoethanol, the sample was loaded onto a HiTrap-Q anion-exchange column (GE Healthcare) equilibrated in 20 mM Tris, pH 8.0, 10 μM zinc acetate, 1 mM DTT, and eluted with a linear gradient to 1 M NaCl. His_6-tagged protease was found in the flowthrough, whereas TAD was applied onto a reversed phase chromatorgraphy column (DSC18, Sigma-Aldrich), eluted with 85% (v/v) methanol, diluted with water and lyophylized.

Cells containing VP16 H2 were resuspended and sonicated in buffer B (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1:100 protease inhibitor mix). The Ni-NTA column was washed with buffer B containing additionally either 1 M NaCl or 20 mM imidazole. Protein was eluted with 20 mM Tris, pH 8.0, 150 mM NaCl, 200 mM imidazole, 10 mM 2-mercaptoethanol, diluted with the same volume of 20 mM Tris pH 8.0, 50 mM NaCl, and 5 mM DTT, loaded on a MonoQ column (GE Healthcare) equilibrated in 20 mM Tris pH 8.0, 5 mM DTT, and eluted with a linear gradient to 1 M NaCl. After gel filtration (Superose 6, GE Healthcare) in 20 mM Tris pH 8.0, 150 mM NaCl, and 5 mM DTT, the His_6-tagged MBP was cleaved with TEV protease overnight and the tag and the His_6-tagged protease removed with a Ni-NTA column. Protein was loaded on a DSC18 column (Sigma-Aldrich), eluted with 85% (v/v) methanol, diluted with water, and lyophilized. Protein identities were confirmed by mass spectrometry.

For EMSA and transcription assays, cells were resuspended in 10 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole pH 8.0, 10% (v/v) glycerol, 10 μM $ZnSO_4$, 10 mM 2-mercaptoethanol, 1:100 protease inhibitor mix, and sonicated. After centrifugation, protein was purified on a Ni-NTA column. Gal4-VP16 TAD and its variants were eluted with 10 mM Tris pH 8.0, 100 mM NaCl, 200 mM imidazole pH 8.0, 10% (v/v) glycerol, 10 μM $ZnSO_4$, 10 mM 2-mercaptoethanol, loaded onto a HiTrap-SP cation-exchange column (GE Healthcare) equilibrated in 20 mM Hepes pH 7.5, 10% (v/v) glycerol, 10 μM zinc acetate, 1 mM DTT, and eluted with a linear gradient to 1 M NaCl. Protein was purified over a Superose 12 gel filtration column (GE Healthcare) equilibrated in 20 mM Hepes pH 7.5, 150 mM potassium acetate, 10% (v/v) glycerol, 10 μM zinc acetate, 1 mM DTT.

Supplementary References

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