Interaction Studies of the Human and Arabidopsis thaliana Med25-ACID Proteins with the Herpes Simplex Virus VP16- and Plant-Specific Dreb2a Transcription Factors

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Abstract

Mediator is an evolutionary conserved multi-protein complex present in all eukaryotes. It functions as a transcriptional co-regulator by conveying signals from activators and repressors to the RNA polymerase II transcription machinery. The Arabidopsis thaliana Med25 (aMed25) ACTivation Interaction Domain (ACID) interacts with the Dreb2a activator which is involved in plant stress response pathways, while Human Med25-ACID (hMed25) interacts with the herpes simplex virus VP16 activator. Despite low sequence similarity, hMed25-ACID also interacts with the plant-specific Dreb2a transcriptional activator protein. We have used GST pull-down-, surface plasmon resonance-, isothermal titration calorimetry and NMR chemical shift experiments to characterize interactions between Dreb2a and VP16, with the hMed25 and aMed25-ACIDs. We found that VP16 interacts with aMed25-ACID with similar affinity as with hMed25-ACID and that the binding surface on aMed25-ACID overlaps with the binding site for Dreb2a. We also show that the Dreb2a interaction region in hMed25-ACID overlaps with the earlier reported VP16 binding site. In addition, we show that hMed25-ACID/Dreb2a and aMed25-ACID/ Dreb2a display similar binding affinities but different binding energetics. Our results therefore indicate that interaction between transcriptional regulators and their target proteins in Mediator are less dependent on the primary sequences in the interaction domains but that these domains fold into similar structures upon interaction.

Introduction

Mediator is a multi-protein transcriptional co-regulator complex which is conserved in eukaryotes [1–3]. It acts as a bridge by transmitting signals from promoter-bound transcription regulators (activators and repressors) to the general RNA polymerase II (Pol II) machinery to regulate transcription of protein encoding genes [4,5], but its function at the molecular level is still elusive. Mediator is structurally dynamic and has a high conformational flexibility which depends on intrinsically disordered regions within some of its protein subunits [6,7]. These regions are prone to fold upon interaction with transcriptional regulators which induces structural changes in Mediator required for propagation of regulatory signals to the Pol II transcription machinery [8–10]. Mediator is evolutionary conserved from yeast to humans but individual Mediator subunits have diverged and some of them share only modest sequence homologies between different organisms [1,2,11]. In addition, the number of Mediator subunits differs between organisms, from 25 in yeast to 29 and 34 in humans and plants, respectively. The higher number of subunits in higher eukaryotes is likely related to the increased complexity of transcription regulation in multicellular organisms [2,12–15].

Med25 is one of the few Mediator subunits that are specific to higher eukaryotes and human Med25 (hMed25) has been shown to interact with several transcription factors involved in different cellular processes, including retinoid signaling by RARα, chondrogenesis by SOX9, insulin secretion in pancreatic cells by HNF4α, cellular growth and differentiation by PEA3 subfamily members and endoplasmic reticulum stress response by ATF6α [16–19]. A specific ACTivator Interaction Domain (ACID) in hMed25 has been shown to interact with the Herpes simplex virus type 1 VP16 protein and the Varicella-zoster virus protein IE62, which activate transcription of viral genes [20–23]. The structure of the hMed25-ACID (residues 394–543) has been solved by NMR and it comprises seven β-strands flanked by three helices [24,25]. The interaction between hMed25-ACID and VP16 has been studied in detail and the VP16 binding site in the ACID has been defined [20,22]. In addition to binding to the hMed25-ACID, the VP16 transcription activation domain (TAD) has been shown to interact with several general transcription factors, including TFIIA, TFIIB, TFIIF, TFIH, TBP, hTAF153/hTAF132 (Taf9), Med15 and Med17 to activate immediate early genes during lytic infection [20,26–34]. The VP16-TAD is composed of two subdomains, one N-terminal...
including residues 412–452 and one C-terminal which includes residues 452–490, which function independently and complementarily [22,31,33,36]. Similar to several other TADs (e.g. p53), the VP16-TAD is unstructured in its free, unbound state, but adopts an α-helical conformation upon binding to its target proteins [30,40]. Within the two subdomains, the formation of α-helical segments has been located to residues 429–450 and 465–480 [37].

In the N-terminal TAD, a nine-amino acid sequence (DFDLDMLGD) has been identified as playing a key role for VP16 transcription activity [35,41]. In addition, this nine-amino acid motif has also been identified in several other transcription factors and is proposed to bind to a common co-factor. This is the case for VP16, p53, HSF1, NF-KB and NFAT1, which all contain this motif and have been shown to interact with the TAFy31 (TAF9) protein [42].

The A. thaliana Med25 (aMed25) was originally identified as PHOTOCYTHEM AND FLOWERING TIME 1 (PTF1), which function in a phyB pathway to regulate the expression of the FLOWERING LOCUS T (FT) in response to suboptimal light conditions [43–45]. More recently, PTF1 was identified as Med25 and has been shown to be involved in regulation of jasmonate (JA) signaling and different stress response pathways [12,46,47]. Med25 interaction with specific transcription factors results in both positive and negative regulatory effects. MYC2 interaction with Med25 is involved in activation of JA-responsive gene expression; while Med25 interaction with ABI5 had a negative regulatory effect on regulation of Abscisic acid mediated gene expression, [48]. We have previously shown that the aMed25-ACID interacts with three transcription factors; the dehydration responsive element binding protein 2A (Dreb2a), the Myb-like transcription factor and the zinc finger homeodomain 1 protein [46]. Dreb2a interacts with cis-acting dehydration-responsive promoter elements and activates genes involved in drought- and salt stress responses [49,50]. The transcription activation domain of Dreb2a has been localized to residues 254–335 [51] but the minimal domain required for interaction with aMed25-ACID in yeast 2-hybrid assays was localized to residues 168–254 [46] and is thus distinct from the Dreb2a TAD. The target for the Dreb2a activation domain is therefore likely to be another, yet not identified Mediator subunit. In a previous study we found that a fragment of Dreb2a (residues 168–335; Dreb2a168–335) which includes both the TAD and the Med25 interaction domain is unstructured in the free state and was required to bind aMed25-ACID with a high affinity in vitro [52]. In the same study we also showed that hMed25-ACID was able to interact with Dreb2a, which is remarkable in two aspects. First, the ACID from A. thaliana and human share low sequence similarity (16%) and secondly, Dreb2a is a plant-specific transcription factor belonging to the large AP2/ERBP (ethylene responsive element binding protein) transcription factor family [53–55].

In the present study we use GST pull downs- and surface plasmon resonance ( SPR) experiments to show that the VP16-TAD interacts with the aMed25-ACID. Furthermore, using NMR chemical shift perturbation (CSP) and isothermal titration calorimetry (ITC) combined with available information about the well-studied interaction between hMed25-ACID and VP16-TAD, we show that the two unrelated transcription factors Dreb2a and VP16 interact with overlapping regions in the ACIDs of both human and plant Med25. Our results suggest that the Med25-ACIDs from human and A. thaliana retain a conserved structure and function regarding activator binding, despite a low level of homology in primary sequence. This highlights the importance of studying cross-species interactions between transcriptional regulators and their target proteins.

**Results**

**aMed25-ACID interacts with the Herpes Simplex virus VP16 transcription factor**

Previous studies have shown that hMed25-ACID interacts with the VP16-TAD [24,25,36,37] and we have previously reported that hMed25-ACID unexpectedly interacts with the plant-specific Dreb2a transcription factor, even though hMed25-ACID and aMed25 show very low sequence homology. In order to study if also aMED25-ACID and VP16 can interact, we carried out binding experiments between the aMed25-ACID and the VP16-TAD using pull-downs and SPR. In our experiments we used two constructs of the GST-VP16-TAD protein to test the binding properties of the different VP16 subdomains. One construct contained the N-terminal functional subdomain (TADn) comprising residues 412–452. The second construct (VP16-TAD) contained both subdomains (residues 427–485; Figure 1A). GST pull-down experiments were carried out with the aMed25-ACID, but also with the hMed25-ACID as a positive control (Figures 1B and 1C). Western blotting using antibodies against GST and aMed25-ACID revealed binding of aMed25-ACID with both VP16-TAD-constructs (Figure 1B). Next, we tested if the interaction between aMed25-ACID and the VP16 TADs could be inhibited by the A. thaliana transcription factor Dreb2a, which we have previously shown to interact with the aMed25-ACID [46,52]. For these pull-down experiments we used a 6 x his-tagged, C-terminally extended domain of Dreb2a, which comprised both the aMed25-binding domain (BD; 168–253) and the activation domain (AD; 254–335) (Figure 1A). We carried out the binding reactions as described above, but this time aMed25-ACID and Dreb2a168–335 were pre-incubated together before they were added to GST-VP16-TADs pre-bound to glutathione beads. Proteins bound to the beads were identified by Western blotting using anti-GST and anti-Med25 antibodies. As shown in Figure 1D, aMed25-ACID did not bind to the VP16-TADn when it had been pre-incubated with Dreb2a168–335. Similar results were obtained when using VP16-TAD (data not shown). The finding that Dreb2a168–335 interferes with the binding of aMed25-ACID to the VP16-TAD indicates that both Dreb2a and VP16 interact with overlapping regions on the aMed25-ACID.

The interactions between different Med25-ACID and VP16-TAD proteins were further analyzed using SPR. The GST-VP16-TAD and GST-VP16-TADn subdomains were immobilized on a CM5 sensor chip using amine coupling and binding was assessed for both the hMed25-ACID (as positive control) and the aMed25-ACID. The sensogram profiles were similar for all four interactions, displaying both rapid association and dissociation kinetics. The binding kinetics was obtained by monitoring the changes in absorbance at 230 nm. The dissociation constants (K_d) were calculated to 3.4 µM and 1.8 µM for the aMed25-ACID/VP16-TADn and the aMed25-ACID/VP16-TAD interactions, respectively. The K_d values obtained for the hMed25-ACID/VP16-TADn (5.4 µM) and the hMed25-ACID/VP16-TAD (2.8 µM) interactions were in the similar range. Furthermore, the K_d value for interaction between the hMed25-ACID and the VP16-TAD that we obtained in our experiments is comparable to those previously reported (1.6 µM) by Wagner et al., using ITC [56].

**NMR-studies of interactions between Dreb2a and hMed25-ACID or aMed25-ACID**

We have previously shown that both hMed25-ACID and aMed25-ACID interact with the A. thaliana Dreb2a transcription factor Dreb2a168–335. In the present study we used GM12244 cells (a human cell line) expressing Dreb2a168–335 to carry out in vivo NMR-studies of interactions between Dreb2a and aMed25-ACID. As shown in Figure 2A, the dissociation constants (K_d) were calculated to 2.4 µM and 1.8 µM for the aMed25-ACID/VP16-TADn and the aMed25-ACID/VP16-TAD interactions, respectively. The K_d values obtained for the aMed25-ACID/VP16-TADn (5.4 µM) and the hMed25-ACID/VP16-TAD (2.8 µM) interactions were in the similar range. Furthermore, the K_d value for interaction between the hMed25-ACID and the VP16-TAD that we obtained in our experiments is comparable to those previously reported (1.6 µM) by Wagner et al., using ITC [56].
A. *A. thaliana* and human Med25-ACIDs interacts with the Herpes Simplex virus VP16 transcription factor. A) Illustration of the protein domains used in this study. ACID, activator interaction domain. TAD and AD, transcription activation domains. BD, *A. thaliana* Med25-ACID-binding domain. B) GST pull-down assay using purified recombinant proteins. aMed25-ACID was incubated with glutathione beads pre-bound to GST-VP16-TADs and the proteins were visualized by immunoblotting with anti-GST and anti-Med25 antibodies. Lane 1, aMed25-ACID (input); Lane 2, GST-VP16-TADn (input); Lane 3, GST-VP16-TAD (input); Lane 4, aMed25-ACID (bound); Lane 5, aMed25-ACID + GST-VP16-TADn; Lane 6, aMed25-ACID + GST-VP16-TAD (bound). The input lanes represent 25% of the load used for each pull-down experiment. C) GST pull-down assay using recombinant proteins. GST-VP16-TADs were pre-bound to glutathione beads and incubated with hMed25-ACID (18 kDa). Proteins were separated on a 15% SDS-PAGE and stained with Coomassie blue. Lane 1, hMed25-ACID (input); Lane 2, GST-VP16-TADn (input); Lane 3, hMed25-ACID (bound); Lane 4, hMed25-ACID + GST-VP16-TADn (bound); Lane 5, GST-VP16-TAD (input); Lane 6, GST-VP16-TAD (input); Lane 7, hMed25-ACID (bound); Lane 8, hMed25-ACID + GST-VP16-TAD (bound). D) GST pull-down assay to study competition between binding of Dreb2a168–335 and VP16-TAD to aMed25-ACID. Dreb2a168–335 and aMed25 were pre-incubated and then added to GST-VP16-TADn bound to GST-beads. Lane 1, aMed25-ACID (input); Lane 2, GST-VP16-TADn (input); Lane 3, aMed25-ACID + GST-VP16-TADn (bound); Lane 4, aMed25-ACID pre-incubated with Dreb2a168–335 and GST-VP16-TADn (bound). The input lanes represent 25% of the load used for each pull-down experiment.

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tional activators upon interaction with its targets [39,59–61]. For example, the VP16-TAD forms helical segments upon interaction with Tfb1, PC4, and hTAFII31 [30,37,38,60]. Such potential helical segments can also be predicted to form in Dreb2a (Figure S1). Finally, we mapped the significant chemical shifts and the residues that were undetectable already at the first titration point onto a ribbon diagram of the hMed25 NMR structure (PDB ID 2XNF) [36] (Figure 3C). From this diagram we can observe that the β-barrel that forms the hydrophobic pocket in hMED25-ACID is the most affected during ligand titration. This hydrophobic pocket has previously been described to play an important role in the hMed25-ACID/VP16-TAD interaction [56]. Interestingly, some residues that display significant chemical perturbations in our titration experiments (T424, Q451, Q456, L458, C497, H499, L525) have been described to constitute part of the interaction surface with VP16-TAD [24,36,56]. Q451 has been

Figure 2. Binding kinetics for VP16-TADn and VP16-TAD to A.thaliana and human Med25-ACID. A) SPR sensogram for association and dissociation of hMed25 to GST-VP16-TADn. B) SPR sensogram for association and dissociation of aMed25 to GST-VP16-TADn. C) Binding curves plotted from the sensograms in A and B. The dissociation constants (K_d) are indicated. D) SPR sensograms for association and dissociation of hMed25 to GST-VP16-TAD. E) SPR sensograms for association and dissociation of aMed25 to GST-VP16-TAD. F) Binding curves plotted from the sensograms in D and E. The dissociation constants (K_d) are indicated. Green curves represent aMed25 and blue curves represent hMed25.

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Figure 3. NMR studies of interactions between Dreb2a and hMed25-ACID or aMed25-ACID. A) $^1$H-$^{15}$N HSQC spectra of hMed25-ACID in the absence (blue) and in presence of 0.2 (cyan), 0.4 (green), 0.6 (yellow), 1 (orange) or 2 equivalents (red) of Dreb2a168-335. Inset shows an intermediate exchange shift of a resonance corresponding to residue Q451 with a curved-like shift. B) Chemical shift changes ($\Delta$) obtained from NMR experiments shown in (A) plotted against the residue number of hMed25-ACID. The position of the seven $\beta$-strands and three $\alpha$-helices is...
indicated under the residue numbers. Residues undergoing major chemical shift changes (Δδ > 0.10 ppm) are labeled with the respective residue number and asterisks indicate residues which have been previously reported to be part of the interaction surface with VP16-TAD [36,56]. Dotted line corresponds to the threshold value of 0.10 (two chemical shift standard deviations). G) NMR structure of hMed25-ACID (PDB ID 2MNP) [36]. Residues undergoing significant chemical shifts upon addition of Dreb2a168–335 from figure (B) are enlarged and highlighted in gold and labeled with residue name and number. Resonances which are broadened beyond detection by interaction with Dreb2a168–335 are colored in light yellow. The seven β-strands are indicated in the left view. D) Comparison of the fraction of peaks which are affected to differing degrees by interaction of Dreb2a168–335 with Med25-ACID proteins, based on spectra presented in figure 3A and 3E. Color coding indicates peaks which are unaffected (blue), broadened beyond detection (light yellow), Δδ > 0.1 (gold), Δδ < 0.1 (white). E) H\textsuperscript{1}N HSQC spectra of aMed25-ACID in the absence (blue) and in presence of 0.2 (green), 0.5 (yellow), 1 (orange) or 2 equivalents (red) of Dreb2a168–335. Inset 1 shows a cross-peak illustrating fast exchange with a curved-like shift. Inset 2 shows fast exchange shifts of two resonances (indicated by arrows). F) Similar plot as (B) but using data obtained from NMR experiments showed in (E). Δδ was plotted against the number of peaks corresponding to the number of residues of aMed25-ACID. No peak assignment is available for aMed25-ACID, therefore an identification of residues undergoing significant chemical shift changes is not possible. doi:10.1371/journal.pone.0098575.g003

Complex formation of Med25-ACID proteins and Dreb2a studied by ITC

To obtain further insights into the binding mechanisms between hMed25-ACID and aMed25-ACID with Dreb2a, we performed ITC experiments. 20 μM Med25-ACID and 200 μM Dreb2a168–335 were used for these titrations. The calorimetry data was evaluated using the standard model of bimolecular complex formation for single-site binding. We obtained thermodynamic data from the binding curve-fittings as shown in Table 1. Based on the obtained affinity constants (K\textsubscript{D}), the K\textsubscript{P} values were calculated to 5.4 μM and 3.0 μM for the hMed25-ACID/Dreb2a168–335 and the aMed25-ACID/Dreb2a168–335 interactions, respectively. The binding free energy values obtained for both the hMed25-ACID/Dreb2a168–335 and aMed25-ACID/Dreb2a168–335 interactions, were small (~7 kcal/mol), which is characteristic for weak and transient protein-protein interactions, such as those typically observed between transcriptional regulators and their target proteins [62]. Even though the binding affinities and free energies that we observed for complex formation were similar to each other (Figure 4A, B), the binding mechanisms differed based on the large difference between the enthalpic and entropic contributions from the formation of each of the complexes (Figure 4C, Table 1). The comparison between the binding energetics from the two different interaction events showed large differences; the aMed25-ACID/Dreb2a168–335 interaction was more enthalpy-driven compared to the hMed25-ACID/Dreb2a168–335 interaction (Figure 4C). The negative enthalpy change, which originates from favorable weak interactions at the protein-protein surface (i.e. hydrogen- and van der Waals bonds) was almost 2-fold larger for the aMed25-ACID/Dreb2a168–335 interaction compared to the corresponding values for the hMed25-ACID/Dreb2a168–335 interaction. In addition, the unfavorable change in entropy, which may be caused by folding of Dreb2a168–335 into a more well-defined structure upon complex formation, was around 4-fold larger for aMed25-ACID/Dreb2a168–335 compared to the corresponding values for the hMed25-ACID/Dreb2a168–335 interaction (Figure 4C). These results suggest that the complex formation between Dreb2a168–335 and aMed25-ACID involves larger conformational rearrange-
ments compared to those that occur upon binding between hMed25-ACID and Dreb2a168–335.

From our NMR results on these interactions, we deduced that binding of Dreb2a168–335 affects large regions in both of the Med25-ACID proteins. However, we could not directly describe the different binding mechanisms that were deduced from our ITC experiments. Similar unfavorable entropy changes were obtained in a study between the transcriptional coactivator β-Catenin and the unstructured transcription factor Lef-1 [63]. The process of binding between two or more proteins—which confers functionality—, is the result of several complementary attributes at the protein’s interfaces, ranging from the amino acid composition, hydrophobicity, electrostatic interactions and hydrogen bonding. All these attributes determine the energetics of the protein-interfaces and the free energy contributions have to be kept in balance for stable complex formation. This process involves a balance between entropy and enthalpy changes where the unfavorable changes might be compensated in the regions that are not part of the binding site [64–67], which might explain the differences in binding energetics between hMed25-ACID/Dreb2a and aMed25-ACID/Dreb2a. Therefore, even though the plantspecific transcription factor Dreb2a is able to bind to the hMed25ACID, it might still give rise to a different and specific functional fold upon complex-formation with the aMed25-ACID which might be required for proper functionality during activation of transcription in Arabidopsis. Similar ITC experiments using VP16 were not carried out since GST cleavage of the fusion protein resulted in very low yields wherefore the protein concentrations required for performing ITC experiments could not be obtained.

Discussion

We have used a series of protein-protein interaction methods to show and characterize the binding between Med25-ACID from human and Arabidopsis, with the Herpes simplex virus VP16- and plant specific Dreb2a transcription factors. Several studies have already described the interaction between hMed25-ACID and VP16-TAD, and the binding site has been mapped [24,36,56]. To our knowledge, this is the first time that human herpes simplex virus VP16-TAD has been reported to interact with Arabidopsis Med25-ACID. Our earlier findings showing that hMed25-ACID interacts with the Arabidopsis transcription factor Dreb2a [52], were here studied in more detail using NMR and ITC. Our NMR experiments showed that the hMed25-ACID interaction surface for Dreb2a168–335 overlaps partially with the previously reported VP16-TAD binding site [24,36,56]. VP16 and Dreb2a are unrelated transcription factors that display an amino acid sequence identity of only 11% [Figure S1]. Notably, we found
that six amino acids in the Dreb2a sequence are identical and aligns to a region in VP16-TAD containing nine amino-acids (DFDLDMLGD), which has previously been reported as important for interaction with several regulatory proteins [41,68]. This nine amino-acid sequence has also been identified in a range of transcription factors and might represent a novel motif which can be recognized by a common co-factor. Such is the case for VP16, p53, HSF1, NF-kB and NFAT1 which both contain the nine amino-acid sequence and interact with the general co-activator TAF9 (TAFII31) [30,42,69]. However, this motif is absent in the two other transcription factors, Myb-like and ZFHD1, which we identified as interacting with Med25-ACID in the same two-hybrid screen were we found Dreb2a [46]. This is not surprising, since we found that ZFHD1 and Myb-like are involved in stress-response pathways, while Dreb2a in addition is involved in regulation of light-quality pathways downstream of the PhyB receptor.

On the other hand, the human and Arabidopsis Med25-ACID homologs share only 16% identity in amino acid sequence but their secondary structure contents appear to be highly similar (Figure S3). Despite the low homology, our study shows that the ACIDs from each of the human and Arabidopsis Med25 are able to interact with both of the unrelated transcription regulators VP16 and Dreb2a in vitro. In addition, both transcription regulators seem to share interaction surfaces on the Med25-ACIDs. It is likely that both the transcriptional regulators and the Med25-ACIDs contribute to the recognition specificity for these particular protein-protein interactions. At one side, the VP16 and Dreb2a TADs both belong to the acidic activator family and share the nine amino-acid common motif found in several other TADs. On the other side, the ACIDs from human and Arabidopsis show structural similarities which might provide the interaction surfaces required for binding of the transcriptional regulators. Moreover, our study provides insight into the mechanism for interaction between Dreb2a and the Med25-ACID proteins. Our NMR experiments show that a comparable fraction of both Arabidopsis and human Med25-ACID residues are affected upon binding of Dreb2a. A detailed analysis of the interaction surfaces is hampered by strong line broadening probably due to secondary binding effects. These effects influence our NMR results, but are not detected in our ITC or SPR experiments, because of the higher protein concentrations used in the NMR experiments. Our ITC data indicate that the proteins have similar affinities but different binding energetics, which might result in distinct conformational rearrangements upon complex formation. The conformational changes appear to be larger for the interaction between the Med25-ACID and Dreb2a168–335 from Arabidopsis relative to the human Med25-ACID and Dreb2a168–335. This might be relevant for proper biological function in the normal context, where the Mediator complex in Arabidopsis needs to be triggered by interaction with Dreb2a to induce the structural rearrangements that are required to perform its function. As mentioned in the introduction, Mediator has been shown to be structurally dynamic and flexible and the binding to activators induces global structural shifts [9]. Our ITC experiments showed differences in energetics when comparing Dreb2a binding to human or Arabidopsis Med25-ACID. We therefore speculate that the plant transcription factor Dreb2a might not induce such structural shifts upon interaction with human Med25-ACID since these proteins originate from different species. It would be interesting to compare these results with the conformational changes that VP16-TAD would induce in Arabidopsis Med25-ACID. However, as already mentioned such experiments could not be performed due to difficulties to achieve sufficiently high protein concentrations. Altogether, our findings that the ACID from human and plant Med25 can interact with the unrelated VP16 and Dreb2a transcription factors, suggest that even though the Mediator complex sequences have diverged rapidly during evolution, the structure and interaction properties of its subunits remain conserved.

### Materials and Methods

#### Protein expression and purification

Full-length 6 x his-GST tagged Dreb2a (Dreb2a141), 6 x his-tagged Dreb2a168–335, Arabidopsis Med25C11–280 (aMed25-ACID) and 6 x his-tagged human Med25C294–341 (hMed25-ACID) were expressed in E. coli BL21 pLysS cells and purified as described previously [46,52]. Cells for expression of isolate labeled human and Arabidopsis Med25-ACID were grown in M9 medium (22 mM Na2HPO4, 22 mM KH2PO4, 8.5 mM NaCl, 22.2 mM glucose, 1 mM MgSO4, 0.1 mM CaCl2, 29.6 mM thiamine, 40.9 μM biotin and trace elements, pH 7.4) containing 18.6 mM 15NH4Cl as the only nitrogen source. Expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM at an OD600 of 1. The cells were then grown over night at 18°C and proteins were purified as described [52], pETM30 vectors encoding VP16-TAD constructs (TADs: residues 412–452; VP16-TAD: residues 427–485) were kindly provided by Prof. Gerhard Wagner (Harvard Medical School, BCMP). The vectors were transfected into E. coli BL21 pLysS and protein expression was induced by addition of IPTG to a final concentration of 1 mM at an OD600 of 0.8 followed by incubation for four hours at 30°C. GST-fusion VP16-TAD proteins were purified by affinity chromatography on glutathione-sepharose 4B (GE Healthcare) following the manufacturer’s instructions. All purified proteins were transferred in to buffer A (20 mM Tris/HCl pH 7.5, 150 mM Na2SO4, 0.5 mM DTT) using dialysis cassettes or spin columns (Thermo Scientific). Protein samples were concentrated using ultrafiltration spin columns (Vivaspin, Sartorius) and the protein concentrations were calculated by determining their absorption at 280 nm and by using the proteins’ extinction coefficients (ε280 μM–1 cm–1: 15,470 for aMed25-ACID, 22,460 for hMed25-ACID, 66,350 for Dreb2a141, 26,930 Dreb2a168–335, 44,350 for GST-VP16-TAD and 42,860 for GST-

| Table 1. Thermodynamic data extracted from ITC measurements. |
|---------------------------------------------------------------|
| **Φ** (kcal/mol) | **ΔH** (kcal/mol) | **TAS** (kcal/mol) | **K**<sub>A</sub> (M<sup>–1</sup>) | **K**<sub>Θ</sub> (μΜ) |
|------------------|------------------|------------------|-----------------|------------------|
| hMed25-Dreb2a    | −7.18            | −10.43           | −3.24           | 1.84E5           | 5.4              |
| aMed25-Dreb2a    | −7.50            | −21.94           | −14.43          | 3.28E5           | 3.0              |

Binding parameters were determined by using a single site binding model (Origin, MicroCal). Total free binding energy change ΔG, enthalpy change ΔH, entropy factor change ΔTS, affinity constant KA and dissociation constant KD.

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VP16-TADn. The protein concentration for VP16-TADn, which lacks tyrosines and tryptophanes, was calculated based on the extinction coefficient for GST (ε280 = 42,060 M⁻¹ cm⁻¹).

**GST pull-down experiments**

For each reaction, 5 μM GST-VP16-TAD were bound to 20 μl glutathione-sepharose beads which had been pre-washed in an equilibration buffer (20 mM Tris/HCl pH 7.5, 150 mM Na₂SO₄) for one hour at 4°C. The beads were washed with equilibration buffer and pre-bound GST-VP16-TAD proteins were incubated with 5 μM of human Med25 or A. thaliana Med25 at 4°C. For the competition assays, 5 μM each of the A. thaliana Med25-ACID and 5 μM Dreb2a₁₆₈–₃₃₅ proteins were pre-incubated for 1 hour at 4°C. Unbound proteins were collected in the flow-through and proteins bound to the beads were washed three times with 1 ml of ice-cold equilibration buffer. Bound proteins were the directly eluted by boiling in sample buffer, 2.5% of the bound proteins from each reaction were separated on 15% SDS-PAGE and proteins were identified by Western blotting using anti-GST (Sigma) and anti-aMed25-ACID (Agrisera) antibodies.

**Surface Plasmon Resonance**

The SPR experiments were performed using a Biacore 3000 instrument (GE Healthcare). GST-VP16-TADs were immobilized onto a CM5 sensor chip by amine coupling according to the manufacturer’s instructions (GE Healthcare). About 500 resonance units (RU) of GST-VP16-TADs were immobilized using 10 mM sodium acetate buffer pH 4.5 and HBS-EP as running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA). Kinetics experiments were carried out by injecting decreasing concentrations of hMed25-ACID and aMed25-ACID (3 μM with 2-fold dilutions down to 0.25 μM) at a flow rate of 20 μl/min (running buffer: 20 mM Tris/HCl pH 7.5, 150 mM Na₂SO₄). Experiments were performed in duplicates and data was analyzed using the Scrubber2 (BioLogic software) and illustrated using the Graph Pad software.

**NMR chemical shift perturbations experiments**

NMR titration experiments were performed on a Bruker Avance III 850 MHz spectrometer equipped with a ₁H, ₁₃C, ₁₅N TCI cryoprobe. 2D HSQC spectra were recorded for ₁₅N-labeled hMed25-ACID or ₁₅N-labeled aMed25-ACID (150 μM) in the absence and in the presence of increasing concentrations of unlabeled Dreb2a₁₆₈–₃₃₅ (molar ratio hMed25-ACID:Dreb2a₁₆₈–₃₃₅: 1:0.2; 1:0.4; 1:0.6; 1:1; 1:2) (molar ratio aMed25-ACID:Dreb2a₁₆₈–₃₃₅: 1:0.2; 1:0.5; 1:1; 1:2). For the NMR titration with Dreb2a₁₆₈, 2D HSQC spectra of 25 μM ₁₅N-labeled aMed25-ACID were recorded in its free state and in the presence of 12.5, 25 and 50 μM Dreb2a₁₆₈. The sample for each titration point was prepared independently and used at these low concentrations since Dreb2a₁₆₈ precipitates at higher concentrations. All experiments were recorded at 25°C in 20 mM Tris/HCl pH 7.5, 150 mM Na₂SO₄, 0.5 mM DTT, 10% (v/v) D₂O. Data were processed using Topspin 3.0 software (Bruker) and analyzed using the NMRview software. Assignments of backbone chemical shifts of human Med25-ACID were obtained from the Biological Magnetic Resonance bank with the accession number 17139 (www.bmrbr.wisc.edu). Chemical shift changes of ₁H and of ₁₅N were weighted using the formula Δδ = [(ΔδH)² + (0.2ΔδN)²]¹/² [57] to take the chemical shift ranges of ₁H and ₁₅N into account.

**Isothermal titration calorimetry**

Binding experiments were performed using an auto ITC₂₀₀ (MicroCal, GE Healthcare) at 25°C. Protein concentrations in the reaction chamber were 20 μM of hMed25-ACID or 20 μM of aMed25-ACID. 200 μM Dreb2a₁₆₈ was used in the syringe for the titration. For the control experiment, Dreb2a was titrated into buffer A (20 mM Tris/HCl pH 7.5, 150 mM Na₂SO₄, 0.5 mM DTT). For each experiment, we performed 19 automated injections of 2 μl each with 150 s intervals between each injection and with a stirring speed of 1000 rpm. The titrations were repeated twice. Calorimetric data were plotted and fitted using the standard single-site binding model (Origin, MicroCal).

**Supporting Information**

**Figure S1** Sequence alignment of the TADs from Dreb2a and VP16. Asterisks indicate identical amino acids. The regions in VP16-TAD which have propensity to form α-helices upon interaction with its target are indicated (435–450 and 465–485) [37]. The black box indicates the region of the nine-amino-acid sequence that has previously been reported as important for interaction of VP16-TAD with several co-factors [41,66] and which also has been identified in a range of transcription factors such as VP16, p53, HSF1, NF-κB and NFAT1 [30,42,67]. The Dreb2a sequence has six identical amino acids that align to that region in VP16-TAD (DFDLDMGLD).

(EPS)

**Figure S2** Interaction between aMed25-ACID and Dreb2a₁₆₈. 2D ₁H,₁₅N HSQC spectra of aMed25-ACID in the absence (blue) and in the presence of 0.2 (green), 0.5 (yellow), 1 (orange) and 2 equivalents (red) of Dreb2a₁₆₈. Fast transition between the free and bound state could not be analyzed. Inset 1, modest chemical shift perturbation of a resonance upon addition of 0.5 equivalents Dreb2a₁₆₈ which was undetectable at higher ligand concentrations. Inset 2, example of a cross-peak decreasing in intensity upon addition of Dreb2a₁₆₈ (bottom left) which might indicate slow exchange and an unaffected cross-peak (upper right).

(EPS)

**Figure S3** Sequence alignment of the A. thaliana and human Med25-ACID. Asterisks indicate identical amino acids.

Secondary structure content of human Med25-ACID extracted from PDB ID 2XNF and secondary structure prediction for A. thaliana Med25-ACID using Jpred3 server. Alpha helical content is highlighted in pink (H) and β-strands are highlighted in blue (B). Human Med25-ACID contains 7-strands and 3 α-helices while A. thaliana Med25-ACID is predicted to contain 7-strands and 2 α-helices.

(EPS)

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**Author Contributions**

Conceived and designed the experiments: XA JB KB AO JS SB. Performed the experiments: XA JB KB. Analyzed the data: XA KB AO JS SB. Contributed reagents/materials/analysis tools: XA JB KB AO JS SB. Wrote the paper: XA JS SB.
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