

Balancing Activation and Repression in Stress Responses: A Bivalent Switch in Transcription Factor DREB2A

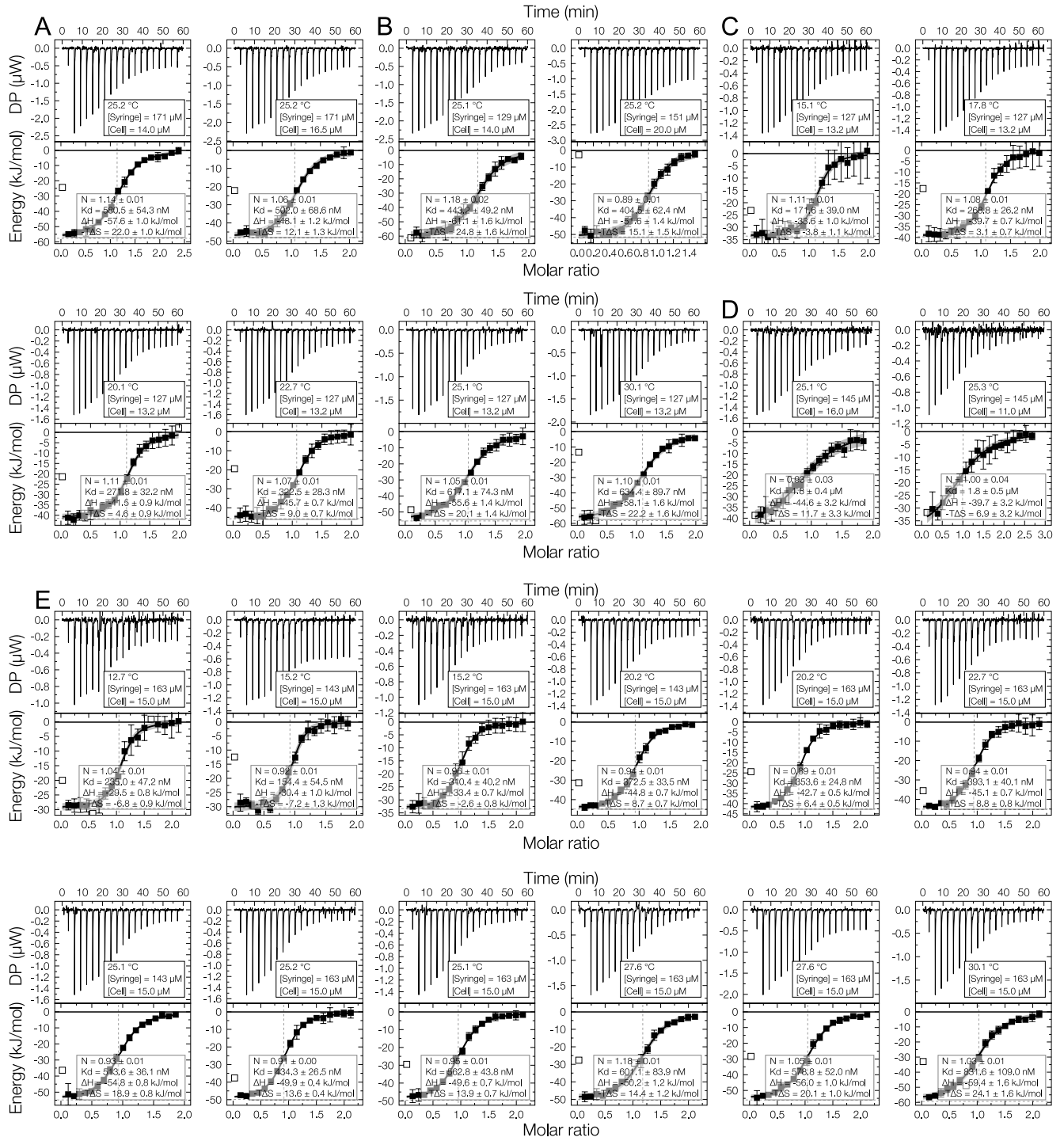
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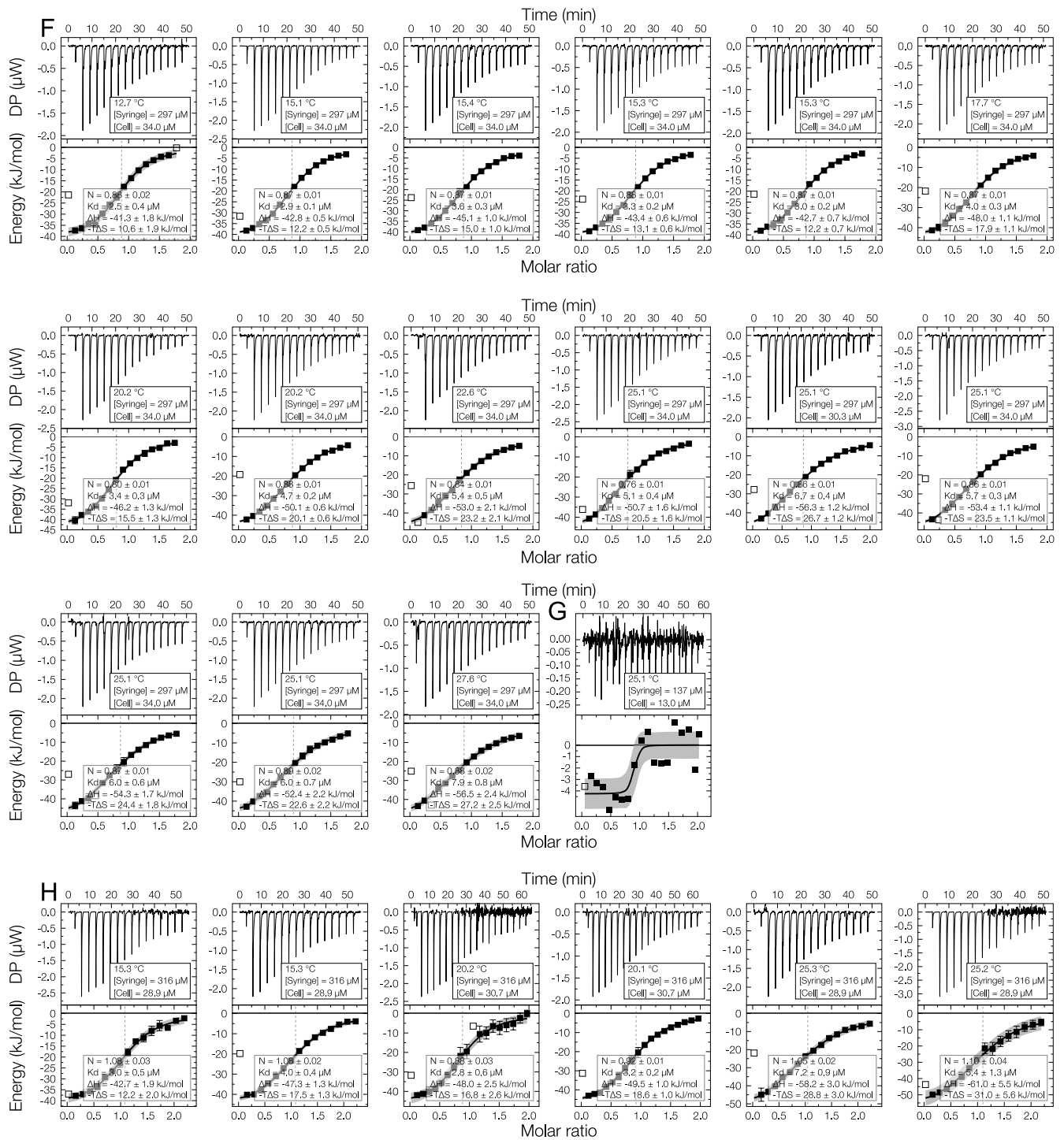
*Corresponding author

Residue/Peak	V _{trans}	V _{cis}	V _{total}	% _{trans}	% _{cis}
25 °C					
Asp241 ¹⁵ N,H ^N	7552	3042	10594	71.3%	28.7%
Gly243 ¹⁵ N,H ^N	6628	2748	9375	70.7%	29.3%
Trp244 ¹⁵ N,H ^N	7591	2991	10582	71.7%	28.3%
Gln250 ¹⁵ N,H ^N	7201	3077	10278	70.1%	29.9%
Trp244 ¹⁵ N ^ε ,H ^ε	6051	2796	8846	68.4%	31.6%
Mean				70.4%	29.6%
SD				1.3%	1.3%
20 °C					
Asp241 ¹⁵ N,H ^N	8092	3300	11391	71.0%	29.0%
Gly243 ¹⁵ N,H ^N	7019	2912	9931	70.7%	29.3%
Trp244 ¹⁵ N,H ^N	8071	3226	11297	71.4%	28.6%
Gln250 ¹⁵ N,H ^N	7774	3496	11270	69.0%	31.0%
Trp244 ¹⁵ N ^ε ,H ^N	7448	3062	10510	70.9%	29.1%
Mean				70.6%	29.4%
SD				0.9%	0.9%
15 °C					
Asp241 ¹⁵ N,H ^N	8141	3296	11437	71.2%	28.8%
Gly243 ¹⁵ N,H ^N	7031	2912	9942	70.7%	29.3%
Trp244 ¹⁵ N,H ^N	8041	3217	11257	71.4%	28.6%
Gln250 ¹⁵ N,H ^N	7462	3338	10800	69.1%	30.9%
Trp244 ¹⁵ N ^ε ,H ^N	7538	3126	10664	70.7%	29.3%
Mean				70.6%	29.4%
SD				0.9%	0.9%
10 °C					
Asp241 ¹⁵ N,H ^N	8222	3351	11573	71.0%	29.0%
Gly243 ¹⁵ N,H ^N	7081	2947	10028	70.6%	29.4%
Trp244 ¹⁵ N,H ^N	8059	3327	11386	70.8%	29.2%
Gln250 ¹⁵ N,H ^N	7554	3432	10986	68.8%	31.2%
Trp244 ¹⁵ N ^ε ,H ^N	7583	3154	10737	70.6%	29.4%
Mean				70.4%	29.6%
SD				0.9%	0.9%

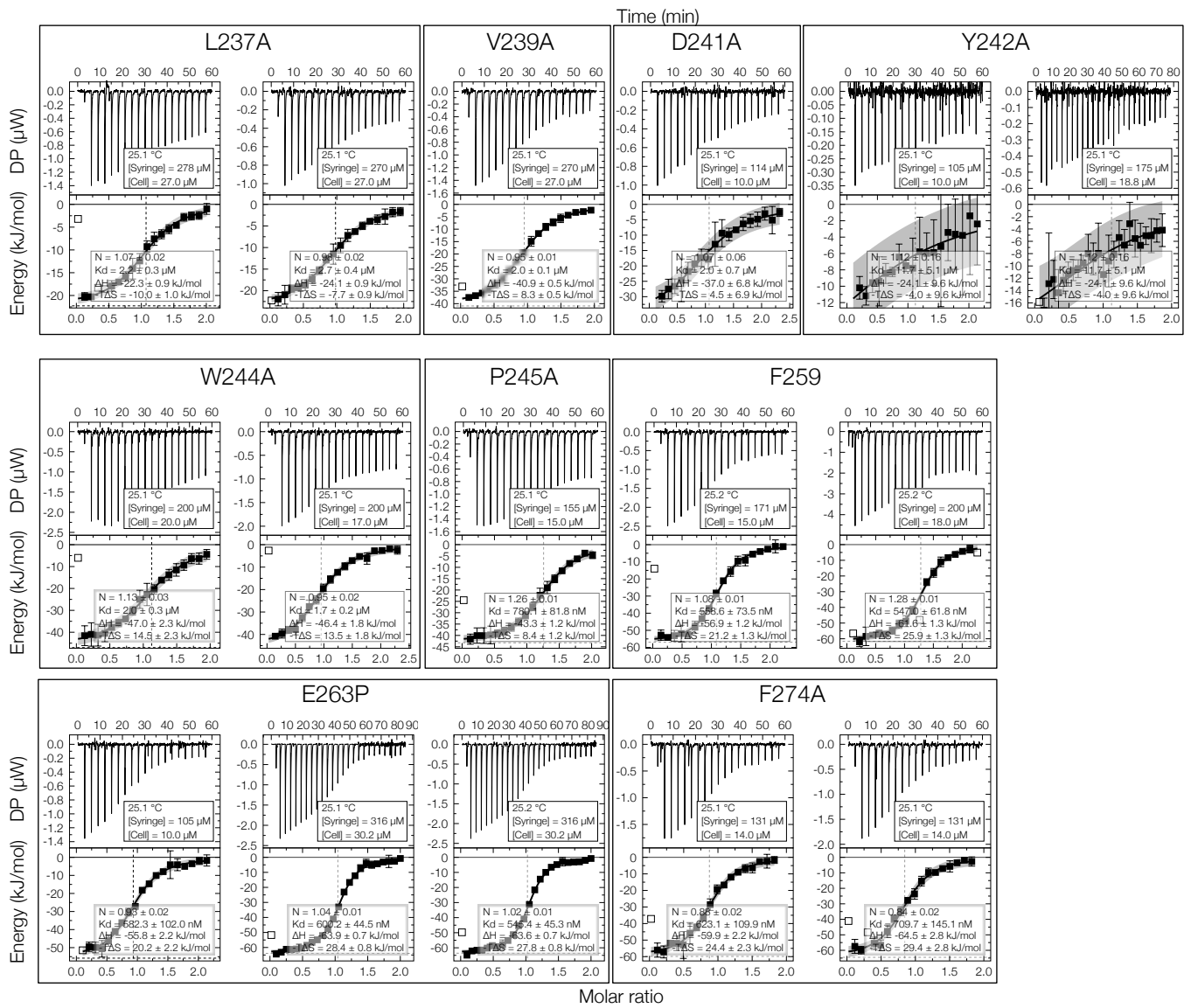
Supplementary Table S1: Unbound DREB2A *cis* and *trans* population HSQC peak volumes at different temperatures. ¹⁵N,H^N-HSQC peak volumes (V) for five peaks which could be assigned in both *cis* and *trans* states. Volumes were determined by fitting lineshapes using PINT. HSQCs (Figure S10) were recorded using a recycle delay of 6 seconds to reduce effects of potential relaxation differences between populations.



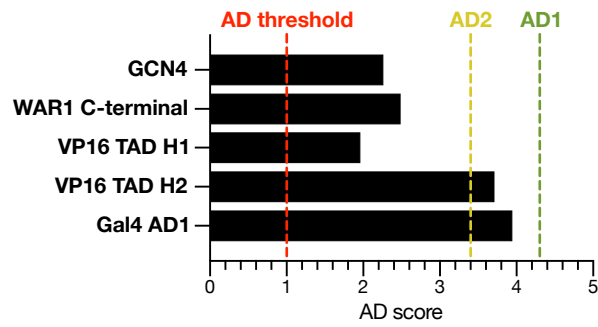
Supplementary Figure S1: Isothermal titration calorimetry data analysis. Analysis of Med25-ACID₅₃₂₋₆₈₀ ITC data used in truncation study. Top graphs show the baseline corrected data and bottom shows the one-set-of-sites model fit of integrated peaks. Experimental temperature and concentrations are given in each data plot and resulting fitting parameters are shown in the fitting plot. Error bars indicate differential power noise relative to peak area. Hollow symbols were not used in fitting. Vertical and horizontal dashed lines represent the N-value and the fitted enthalpy, respectively. From left to right: (A) DREB2A₁₅₁₋₃₃₅, (B) DREB2A₁₉₅₋₂₇₆, (C) DREB2A₂₃₄₋₃₃₅, (D) DREB2A₂₃₄₋₂₇₂, (E) DREB2A₂₃₄₋₂₇₆, (F) (next page) DREB2A₂₃₄₋₂₅₆, (G) DREB2A₂₅₅₋₂₇₆, and (H) DREB2A_{195-335,Δ244-276}.



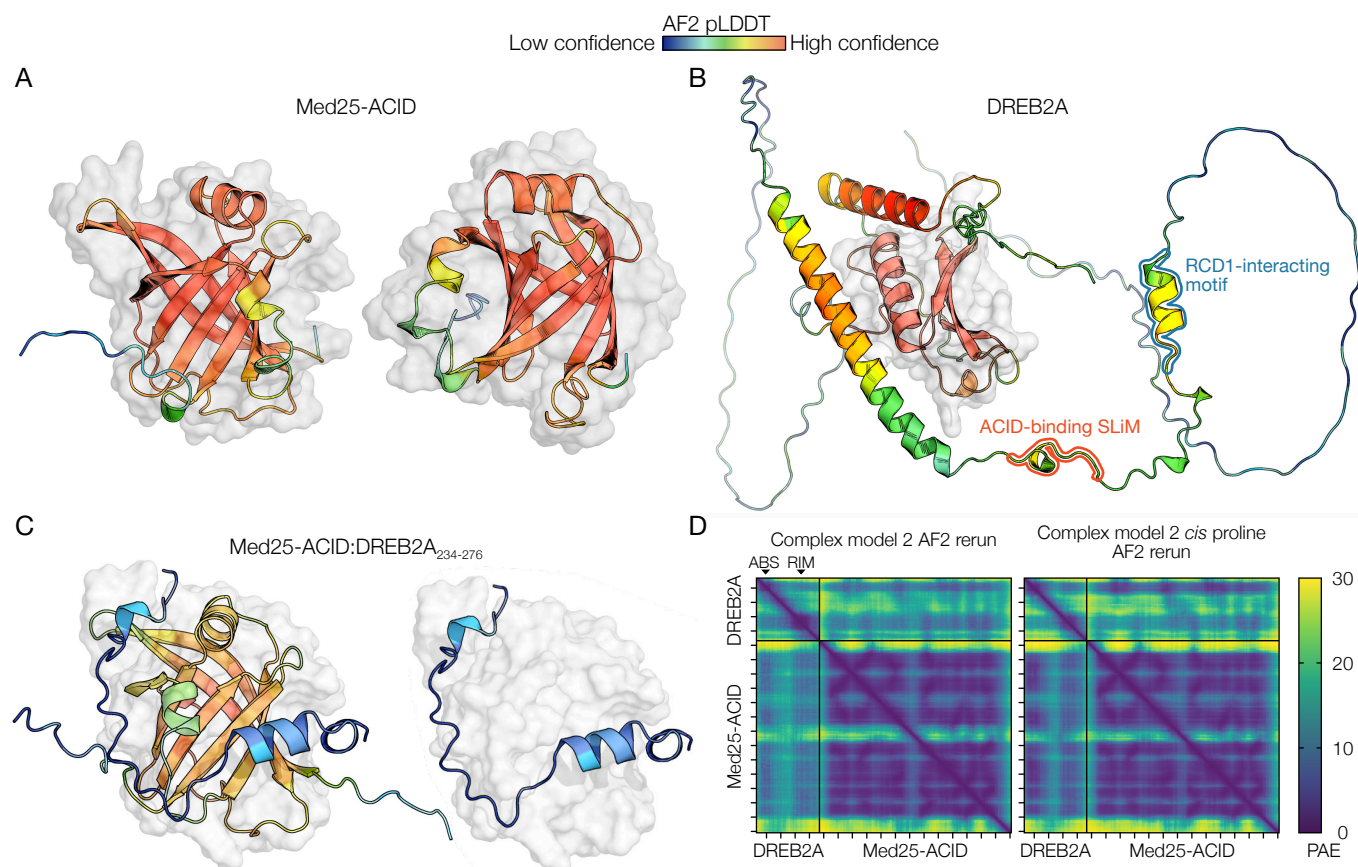
Supplementary Figure S1: Continued. Description on previous page.



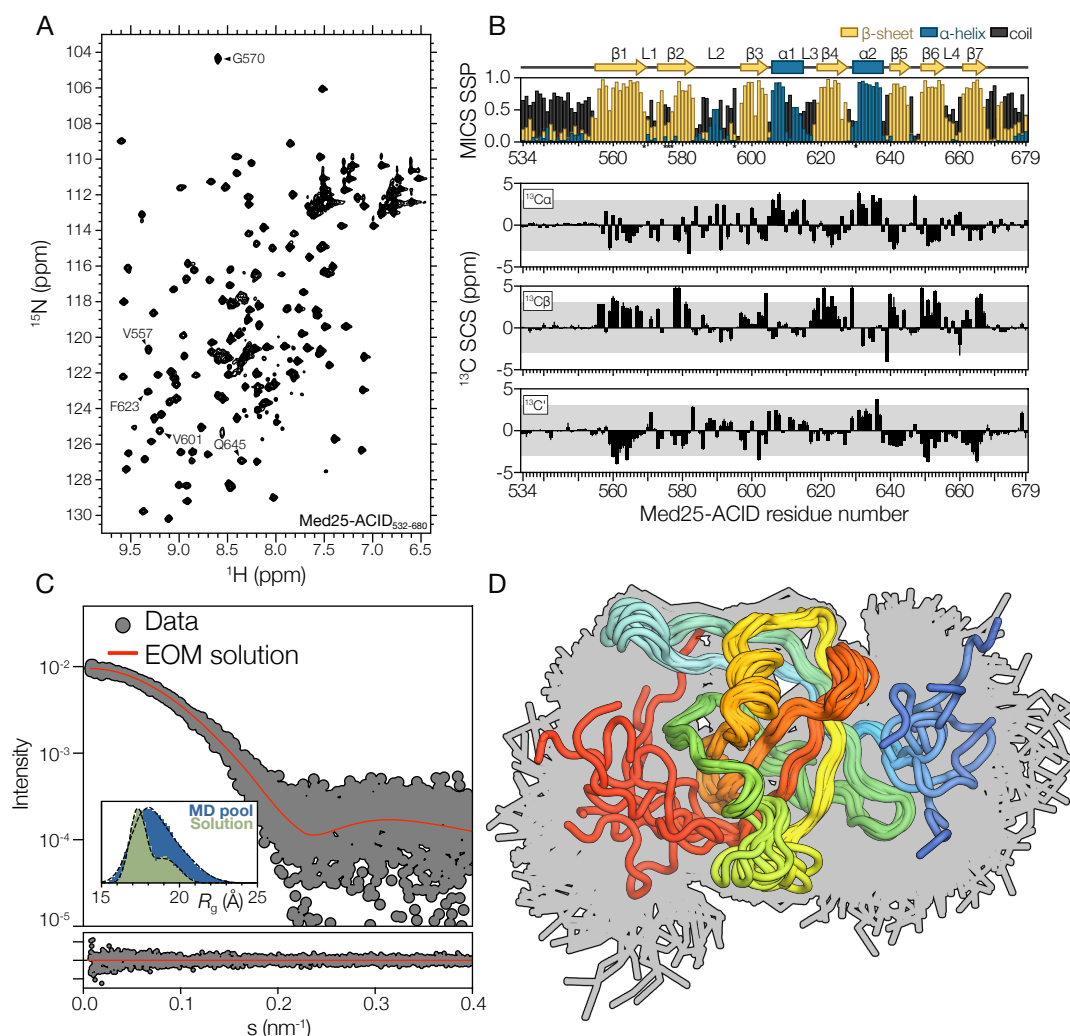
Supplementary Figure S2: Isothermal titration calorimetry of DREB2A variants. All experiments were done multiple times, but only those used for analysis are shown. Mutations were done in DREB2A₁₉₅₋₂₇₆ except for P245A which was done in DREB2A₂₃₄₋₂₇₆. The two Y242A experiments were fitted together using a global model, however fitted parameters are ambiguous. The specific variant is given above the isotherms. Error bars indicate differential power noise relative to peak area. Hollow symbols were not used in fitting.



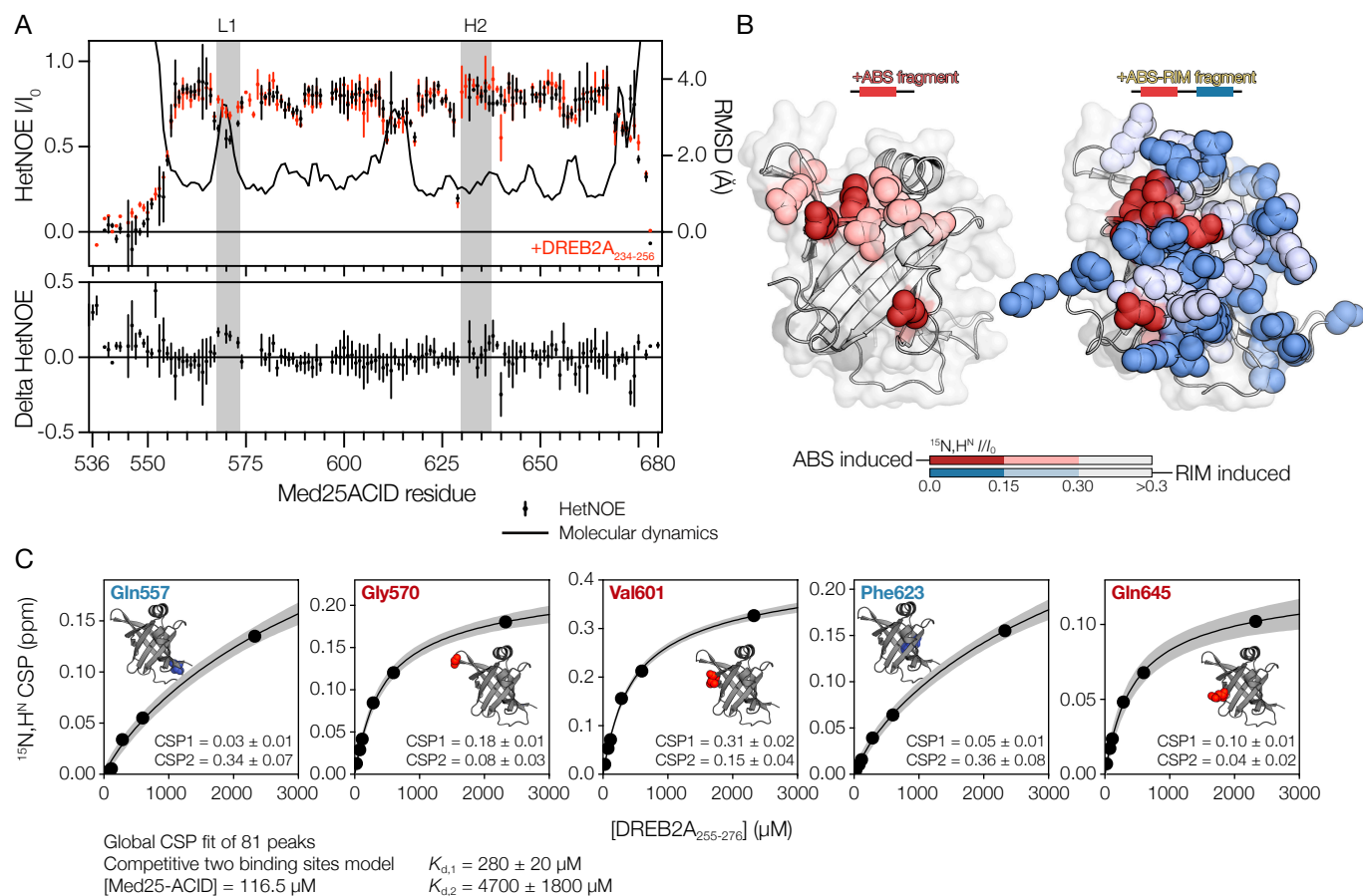
Supplementary Figure S3: Activity scores of control activation domains. Activation domain assay scores for synthetic TFs containing known ADs. The maximum tile scores of the regions comprising AD1 and 2 in DREB2A are shown.



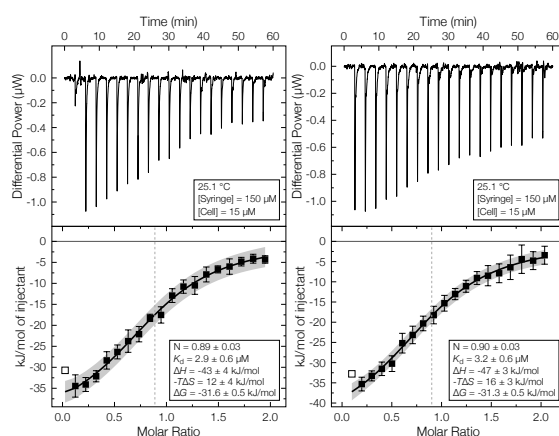
Supplementary Figure S4: AlphaFold2 predictions and confidence mapping. AlphaFold2 predictions of (A) *At*Med25-ACID (Uniprot: Q7XYY2) (B) *At*DREB2A (Uniprot: O82132) colored according to the pLDDT confidence score from the EMBL AF2 database. The ABS and RIM motifs are shown in red and blue, respectively. (C) ColabFold prediction of the Med25-ACID:DREB2A₂₃₄₋₂₇₆ complex structure. Shown with (left) and without (right) Med25-ACID cartoon representation. (D) AF2 predicted aligned errors (PAEs) of the ABS-RIM DREB2A fragment in complex with Med25-ACID obtained using the previously predicted model 2 as template. PAE matrices are shown for both *trans* and *cis* proline structures. The *cis* proline structure was generated using Gromacs by performing a 1ns MD simulation using a backbone angle restraint to obtain the *cis* conformation. Yellow color indicates low confidence in the relative position of two residues. Values in the off-diagonal blocks provide an indication of complex structure confidence at specific positions.



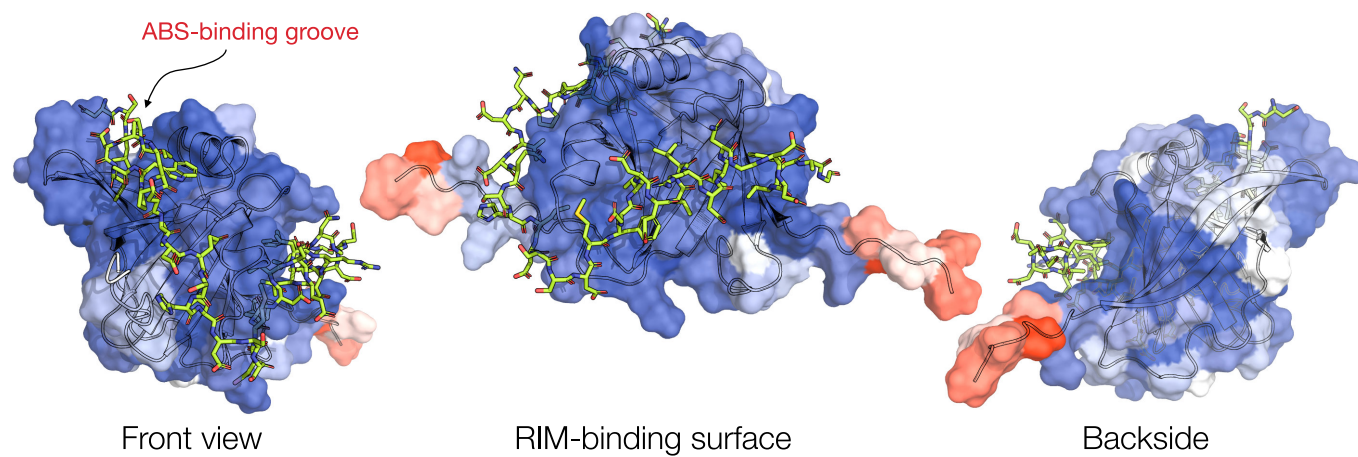
Supplementary Figure S5: Characterization of the *At*Med25-ACID domain structure using NMR and SAXS. (A) 15N-HSQC spectrum of free Med25-ACID₅₃₂₋₆₈₀. (B) Med25-ACID ¹³C^α, ¹³C^β, and ¹³C' secondary chemical shifts calculated using the SBiNLab and POTENCI web tools. The grey bars represent ±3 ppm. The MICS analysis from Figure 2A is shown above. (C) SAXS scattering data analyzed with EOM using a structure pool generated from a 1.7 μs MD simulation. The EOM solution ensemble scattering curve is shown in red and the residuals are shown below. (D) Structures from the EOM solution ensemble superimposed on the structure pool. The shown structures are truncated by 17 residues in the N-terminus (blue).



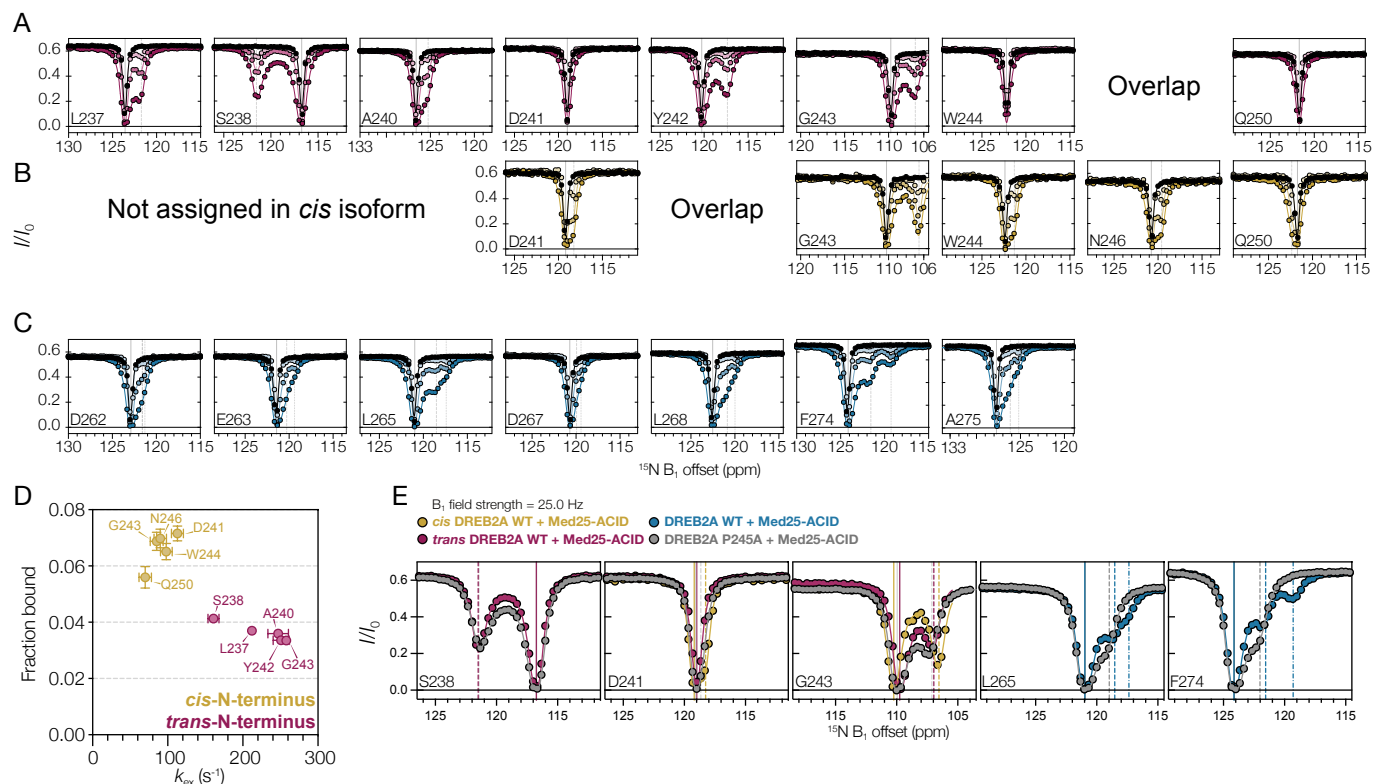
Supplementary Figure S6: Med25-ACID dynamics and peak intensity changes upon interaction with DREB2A. (A) $\{^1\text{H}\}$ - ^{15}N hetNOEs of bound Med25-ACID in complex with ABS (DREB2A₂₃₄₋₂₅₆) superimposed on the ratios obtained for unbound Med25-ACID. Differences are shown below. Grey boxes mark the positions of structures defining the ABS-binding groove. (B) Mapped relative $^{15}\text{N},\text{H}^{\text{N}}$ -HSQC peak intensities of Med25-ACID in complex with ABS (right) and ABS-RIM (left). Residues with relative intensities below 0.3 are colored and shown with spheres. CSPs induced by ABS binding are colored red, while ABS-RIM-induced additional effects are colored blue. (C) Global fit of 81 RIM (DREB2A₂₅₅₋₂₇₆) induced $^{15}\text{N},^1\text{H}^{\text{N}}$ CSPs using a competitive binding model. Example residues from each of the five regions defining the two binding sites. Structures show the location of the observed residue.



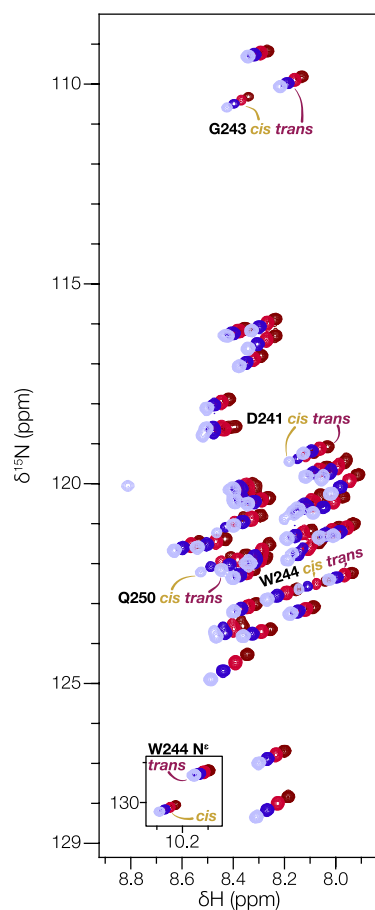
Supplementary Figure S7: Isothermal titration calorimetry results of the AtrMed25-ACID R568A variant. DREB2A₁₉₅₋₂₇₆ was used for the experiments. Error bars indicate baseline differential power noise relative to peak area. Hollow symbols were not used in fitting.



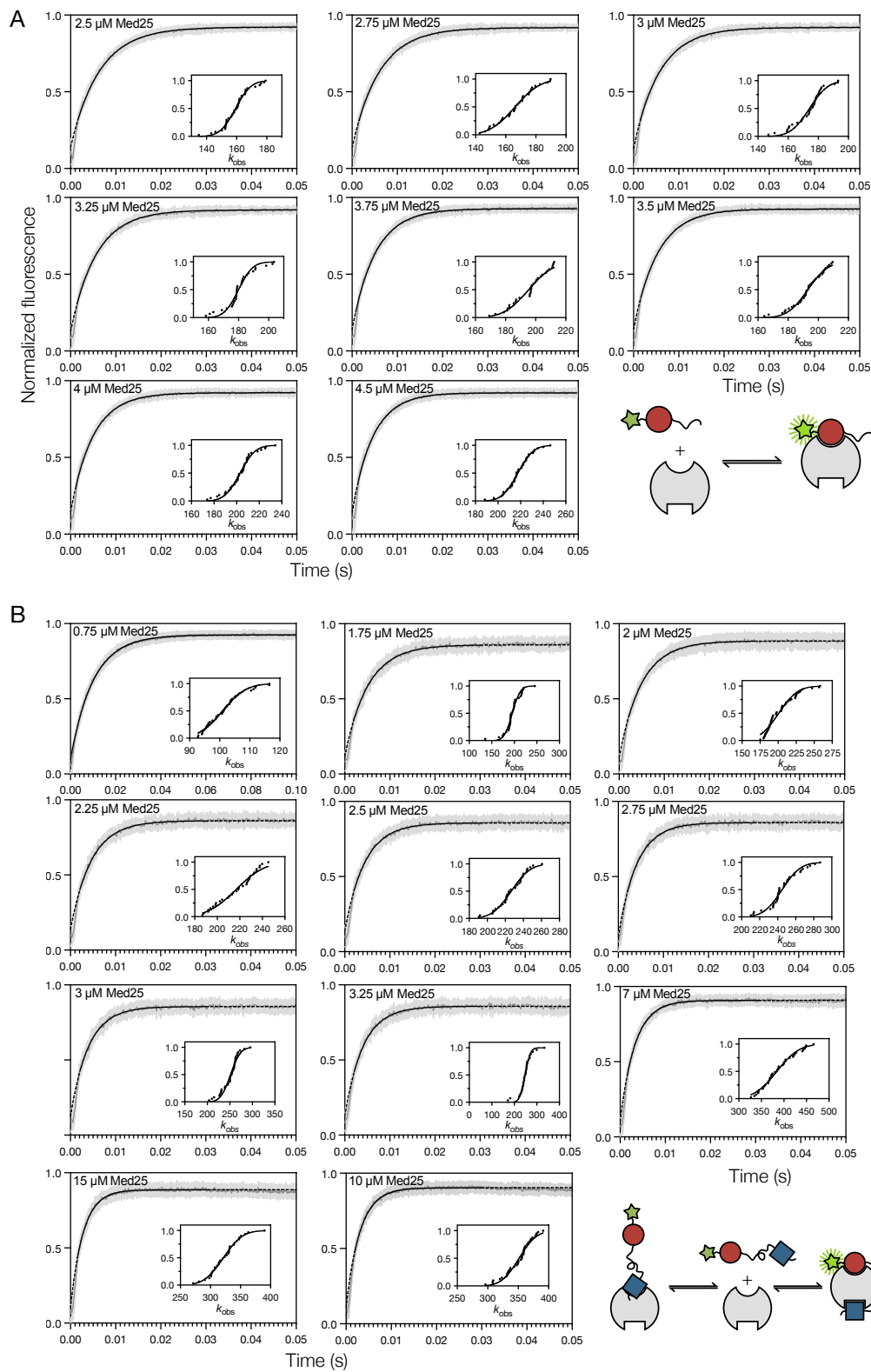
Supplementary Figure S8: *AtMed25-ACID* sequence conservation mapped on the AF2 predicted complex structure. Conservation level is colored from red (low) through white to blue (high). The shown structure corresponds to AF2 complex model 2. DREB2A is shown in green sticks.



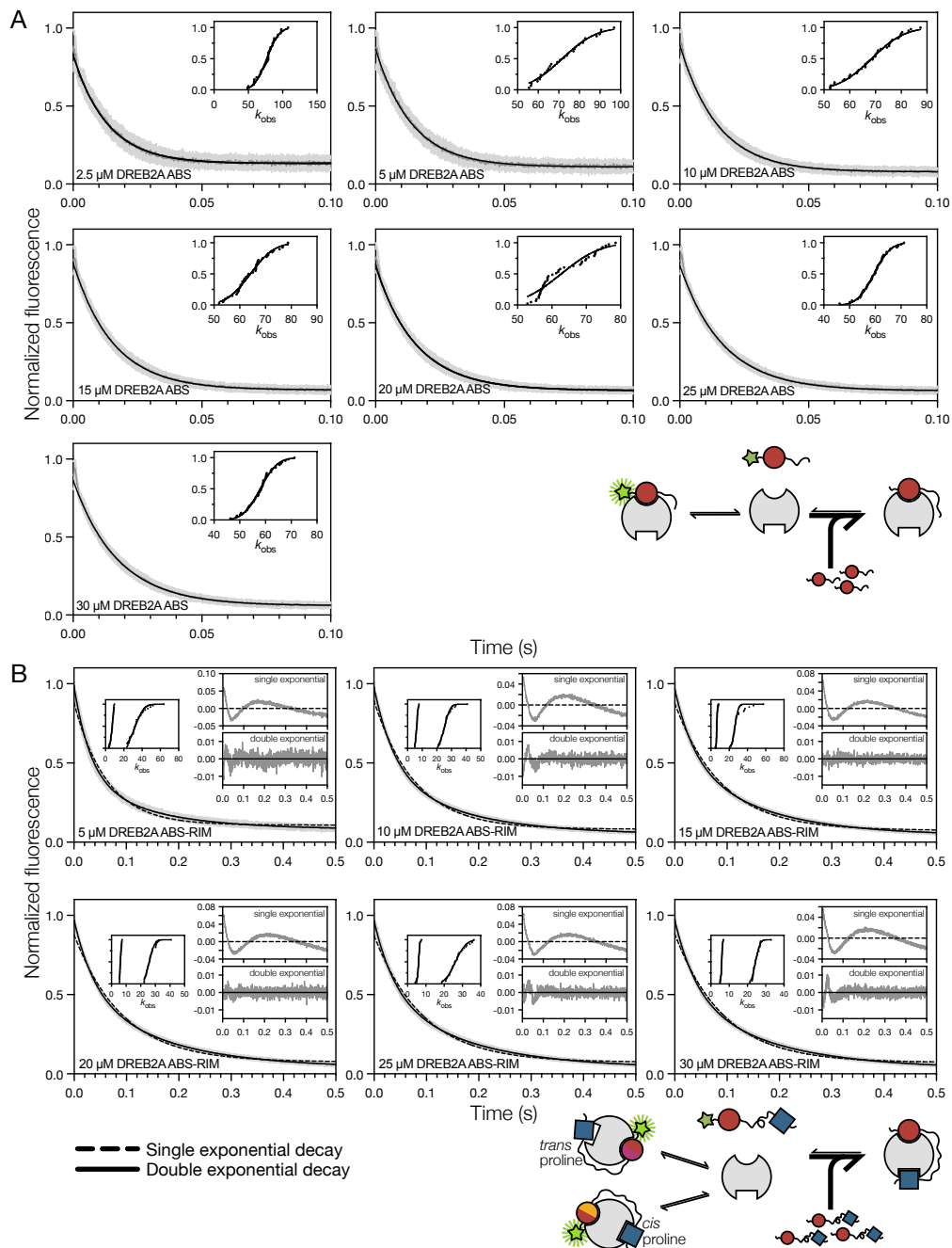
Supplementary Figure S9: ^{15}N -CEST profiles of DREB2A residues with 5% Med25-ACID used for global fitting procedures. ^{15}N -CEST profiles of ^{13}C , ^{15}N -labeled DREB2A₂₃₄₋₂₇₆ with (colored) and without (black) 5% unlabeled Med25-ACID₅₃₂₋₆₈₀ recorded at 25 °C. Data without ligand was recorded using a B_1 field strength of 12.5 Hz. For the profiles with Med25-ACID, three B_1 field strengths (6.25, 12.5 and 25.0 Hz) were used. N-terminal *trans* (A) and *cis* (B) isoform residue CEST profiles. Profiles from *trans* isoform residues Asp241, Trp244, and Gln250 are included for comparison with *cis* isoform profiles, but were not included in the global exchange model fitting. Residues N-terminal of Asp241 could not be assigned in the *cis* isoform or had too much overlap to analyze. Overlap labels indicate that the peak corresponding to the matching residue overlapped with other peaks in the HSQC, thus preventing quantitative analysis. (C) C-terminus profiles exhibiting three-state behavior. (D) Individual analysis of N-terminal peaks showing distinct behavior of *cis* and *trans* populations. (E) Selected CEST profiles of the DREB2A P245A variant (grey), with corresponding WT profiles shown. Vertical lines indicate fitted chemical shifts of the ground state (solid) and minor states (dashed and dot dashed).



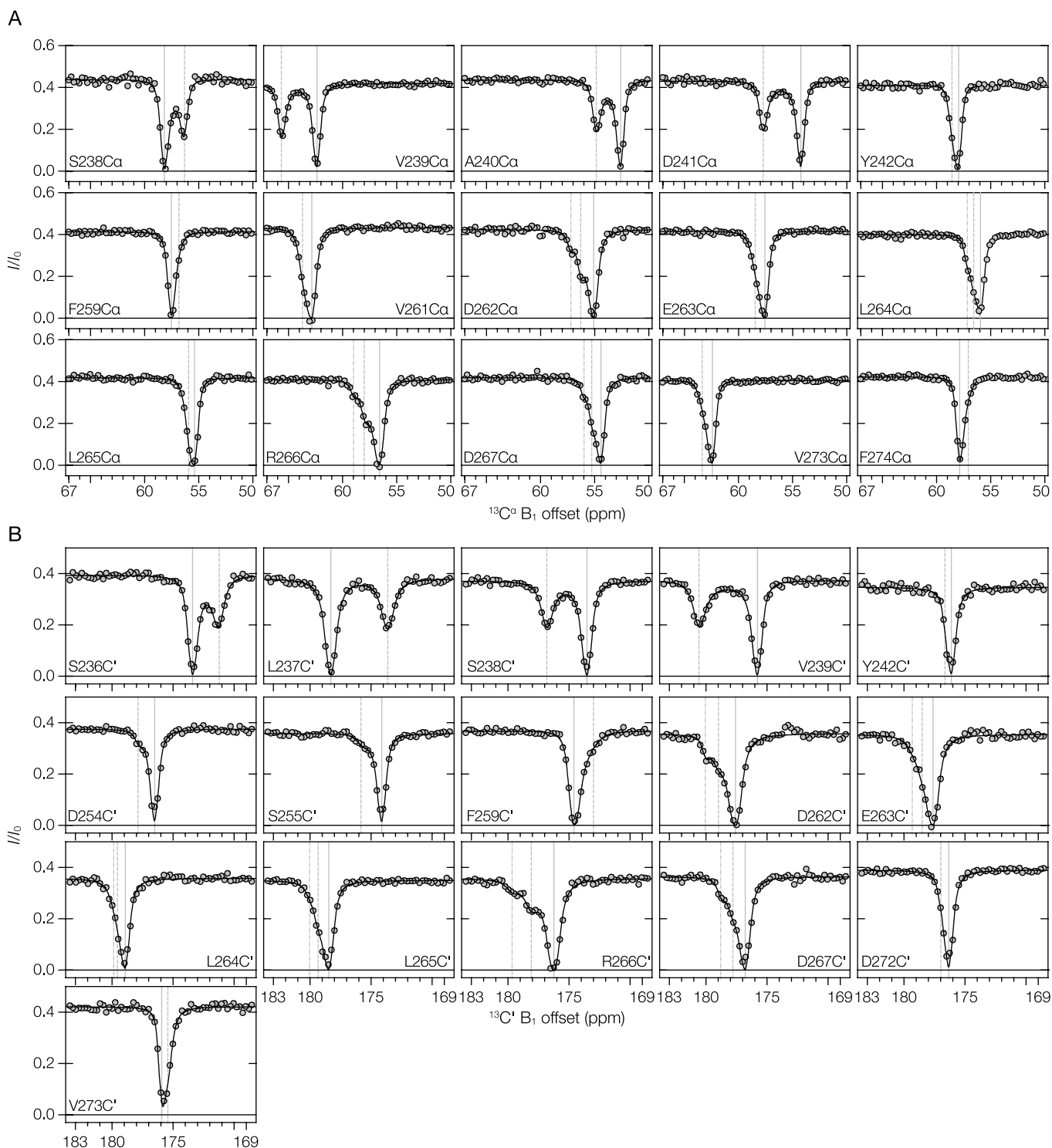
Supplementary Figure S10: Temperature titration of labeled DREB2A₂₃₄₋₂₇₆. ^{15}N , ^1H -N-HSQC spectra of DREB2A₂₃₄₋₂₇₆ in its free state, recorded in 5 °C intervals from 10 (light blue) to 25 °C (dark red). Residues used to determine *cis* and *trans* populations are highlighted and derived populations are shown in Table S1.



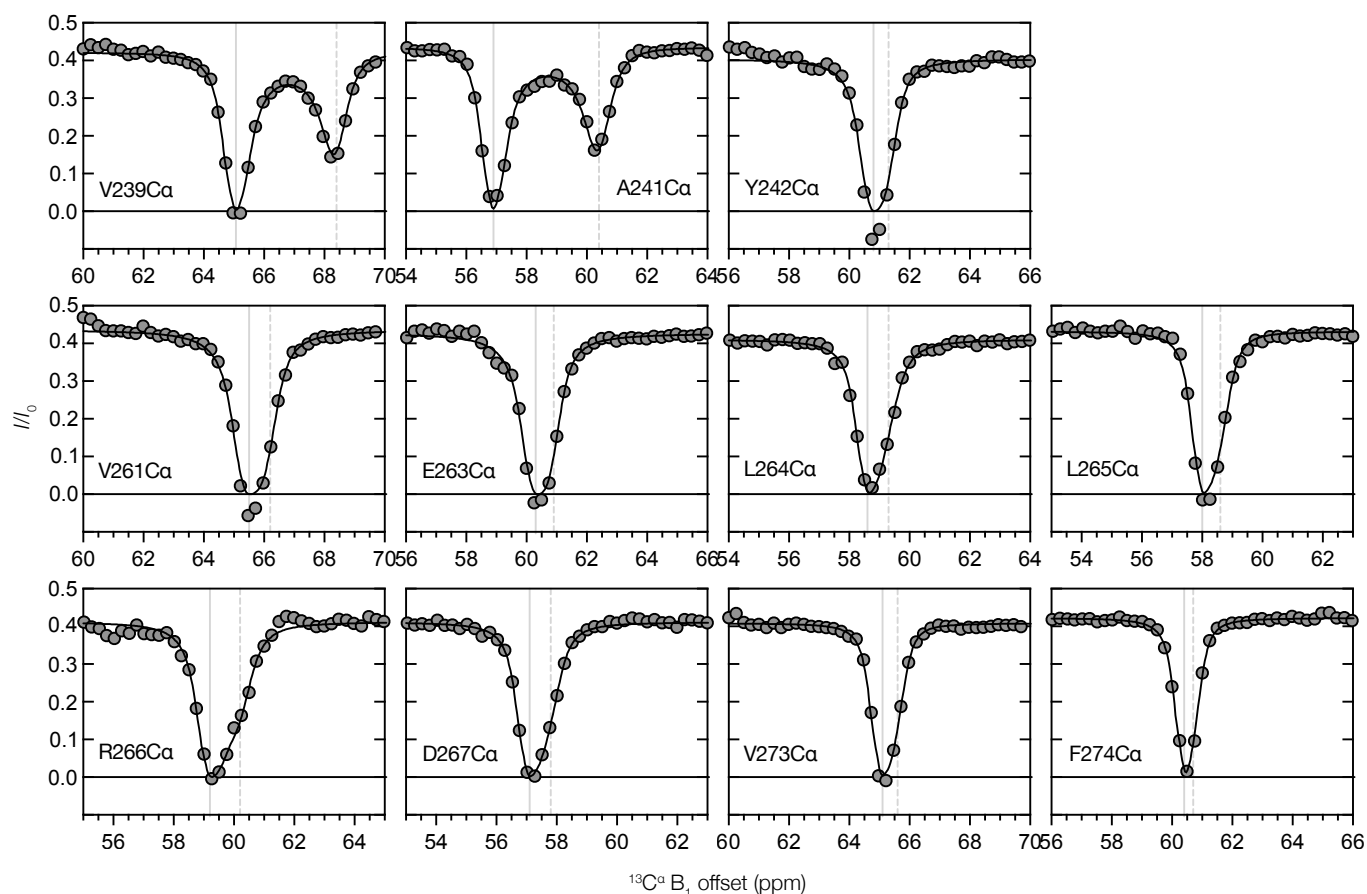
Supplementary Figure S11: Stopped-flow association kinetics of FITC-DRBE2A with Med25-ACID. FITC-DREB2A₂₃₄₋₂₅₆ (A) and FITC-DREB2A₂₃₄₋₂₇₆ (B) association data are represented as normalized and averaged traces. Insets show individual trace fitting distributions of observed rate constants. All fluorescence traces were fitted using a single exponential decay function (solid line). Dashed line indicates extrapolate fit line. For FITC-DREB2A₂₃₄₋₂₇₆, three scientific replicates of concentrations between 1.75 and 3.25 μM Med25-ACID were performed, but the differences were insignificant.



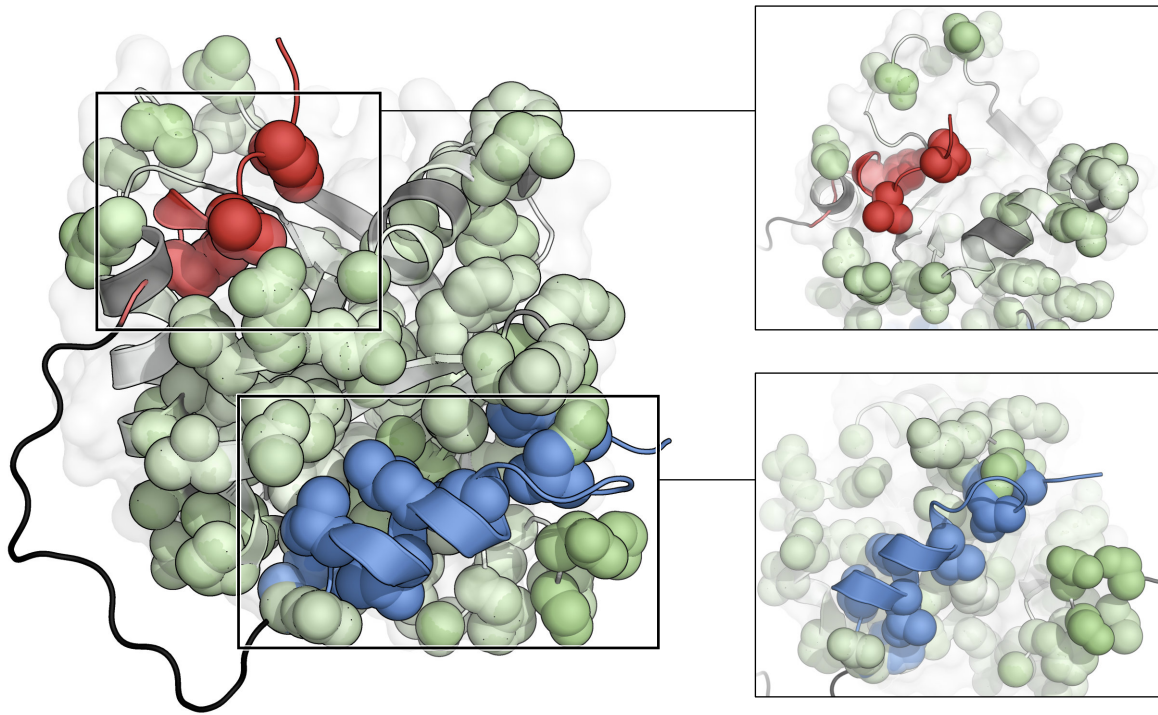
Supplementary Figure S12: Stopped-flow competitive displacement kinetics experiments of FITC-DREB2A and Med25-ACID. All fluorescence traces are represented as normalized and averaged traces. (A) FITC-DREB2A₂₃₄₋₂₅₆ dissociation data with insets showing the distribution of individual trace fits. All traces fitted using a single exponential decay function. (B) FITC-DREB2A₂₃₄₋₂₇₆ dissociation data, with insets showing double exponential decay derived observed rate constants and fit residuals of single (top, dashed line) and double (bottom, solid line) exponential decay functions.



Supplementary Figure S13: ^{13}C -CEST profiles of ^{13}C , ^{15}N -labeled DREB2A₂₃₄₋₂₇₆ with 5% unlabeled Med25-ACID. The pulse sequences used modulate HSQC peaks as function of *i*-1 carbon saturation such that the HSQC peak corresponding to residue e.g. Trp244 would be modulated as a function of Gly243 carbon saturation. Shown profiles correspond to the atom given in labels. (A) C^α and (B) C' CEST profiles. All profiles correspond to the *trans* state of DREB2A. Due to pulse sequence details, the C^α experiment was not able to probe glycine residues.



Supplementary Figure S14: ^{13}C -CEST profiles of the DREB2A₂₃₄₋₂₇₆,P245A variant. ^{13}C -CEST profiles fitted globally using a two-state model. Residues Glu263, Leu264, Leu265, Arg266, and Asp267 displayed three-state behavior using WT DREB2A₂₃₄₋₂₇₆ but could be described using a two-state model in the P245A variant. The experiment was done using a B_1 strength of 30.0 Hz.



Supplementary Figures S15: Comparison of binding sites on human Med25-ACID with the *AtMed25:DREB2A* complex model. CSPs (green) induced by VP16, ERM and p53 binding, mapped on the human Med25-ACID domain. The CSPs of each are overlaid on the same structure. DREB2A from the predicted structure of the *AtMed25-ACID:DREB2A* complex shows the binding sites of DREB2A. The zooms show the ABS-binding groove and RIM-binding surface equivalents on the human Med25-ACID domain.

Supplementary Data 1: Estimating affinities of *cis* and *trans* isoforms based on ^{15}N -CEST determined saturation levels. Based on NMR analysis of unbound DREB2A₂₃₄₋₂₇₆ (Figure S10) we determined that $29.6 \pm 1.3\%$ of DREB2A is found in the *cis*-proline isoform, while $70.4 \pm 1.3\%$ is found in the *trans* isoform (Table S1). As these populations can be considered concentrations, we calculated the equilibrium constant ($K_{\text{eq,trans} \rightarrow \text{cis}}$) as $[\text{cisDREB2A}]/[\text{transDREB2A}] = 0.42 \pm 0.02$. To determine the binding affinity of the two isoforms, we needed the solution of the two following equations:

$$K_{\text{d,trans}} = \frac{[\text{Med25}] \cdot [\text{transDREB2A}]}{[\text{Med25:transDREB2A}]} \quad 1$$

$$K_{\text{d,cis}} = \frac{[\text{Med25}] \cdot [\text{cisDREB2A}]}{[\text{Med25:cisDREB2A}]} \quad 2$$

From the reaction scheme (Figure 5B) we know that the concentrations of DREB2A must satisfy the following equation:

$$[\text{DREB2A}]_{\text{total}} = [\text{transDREB2A}] + [\text{cisDREB2A}] + [\text{Med25:transDREB2A}] + [\text{Med25:cisDREB2A}] \quad 3$$

Since the equilibrium constant for the proline isomerization is known, we know that, at equilibrium, the concentration of free *cis*DREB2A must be:

$$[\text{cisDREB2A}] = K_{\text{eq,trans} \rightarrow \text{cis}} \cdot [\text{transDREB2A}] \quad 4$$

The CEST analysis revealed that $3.6 \pm 0.1\%$ of *trans*DREB2A and $6.8 \pm 0.1\%$ of *cis*DREB2A were in the bound state (P_{bound}) and thus the concentrations of the complexes can be expressed as a function of the total concentrations:

$$[\text{Med25:transDREB2A}] = P_{\text{bound,trans}} \cdot ([\text{transDREB2A}] + [\text{Med25:transDREB2A}]) \quad 5$$

$$[\text{Med25:cisDREB2A}] = P_{\text{bound,cis}} \cdot ([\text{cisDREB2A}] + [\text{Med25:cisDREB2A}]) \quad 6$$

These equations can be rewritten as a function of free DREB2A concentrations:

$$[\text{Med25:transDREB2A}] = 3.6\% \cdot \frac{[\text{transDREB2A}]}{1 - 3.6\%} \quad 7$$

$$[\text{Med25:cisDREB2A}] = 6.8\% \cdot \frac{[\text{cisDREB2A}]}{1 - 6.8\%} \quad 8$$

Applying equations 4, 7, and 8 in equation 3, the concentration of *trans*DREB2A can thus be expressed as a function of total DREB2A:

$$[\text{DREB2A}]_{\text{total}} = [\text{transDREB2A}] + K_{\text{eq,trans} \rightarrow \text{cis}} \cdot [\text{transDREB2A}] + 3.6\% \cdot \frac{[\text{transDREB2A}]}{1 - 3.6\%} + 6.8\% \cdot \frac{K_{\text{eq,trans} \rightarrow \text{cis}} \cdot [\text{transDREB2A}]}{1 - 6.8\%} \quad 9$$

$$\frac{[\text{DREB2A}]_{\text{total}}}{[\text{transDREB2A}]} = 1 + K_{\text{eq,trans} \rightarrow \text{cis}} + 3.6\% \cdot \frac{1}{1 - 3.6\%} + 6.8\% \cdot \frac{K_{\text{eq,trans} \rightarrow \text{cis}}}{1 - 6.8\%} \quad 10$$

$$[\text{transDREB2A}] = \frac{[\text{DREB2A}]_{\text{total}}}{1 + K_{\text{eq,trans} \rightarrow \text{cis}} + 3.6\% \cdot \frac{1}{1 - 3.6\%} + 6.8\% \cdot \frac{K_{\text{eq,trans} \rightarrow \text{cis}}}{1 - 6.8\%}} \quad 11$$

$$[transDREB2A] = \frac{500 \mu\text{M}}{1 + 0.42 + 0.038 + 0.031} = 335.9 \mu\text{M} \quad 12$$

Thus, we can now calculate the concentrations of the other DREB2A states.

$$[cisDREB2A] = K_{eq,trans \rightarrow cis} \cdot [transDREB2A] = 141.0 \mu\text{M} \quad 13$$

$$[\text{Med25}: transDREB2A] = 3.6\% \cdot \frac{[transDREB2A]}{1 - 3.6\%} = 12.7 \mu\text{M} \quad 14$$

$$[\text{Med25}: cisDREB2A] = 6.8\% \cdot \frac{[cisDREB2A]}{1 - 6.8\%} = 10.4 \mu\text{M} \quad 15$$

In order to solve the equilibrium constant equations, we need the concentration of free Med25-ACID. This could theoretically be derived from the added amount of $\sim 25 \mu\text{M}$, however the affinity is high and thus small errors in the concentration measurements, where we expect relatively large errors, would have a massive effect on the calculated affinities. Since we have determined the overall affinity of DREB2A₂₃₄₋₂₇₆ using ITC, we can apply the following equilibrium to determine the expected concentration of free Med25-ACID in our sample:

$$[\text{Med25}] + [\text{DREB2A}] \rightleftharpoons [\text{Med25}: \text{DREB2A}] \quad 16$$

$$K_d = \frac{[\text{Med25}] \cdot [\text{DREB2A}]}{[\text{Med25}: \text{DREB2A}]} \quad 17$$

$$[\text{Med25}] = \frac{K_d \cdot [\text{Med25}: \text{DREB2A}]}{[\text{DREB2A}]} \quad 18$$

where

$$[\text{DREB2A}] = [transDREB2A] + [cisDREB2A] \quad 19$$

$$[\text{Med25}: \text{DREB2A}] = [\text{Med25}: transDREB2A] + [\text{Med25}: cisDREB2A] \quad 20$$

By substituting concentrations using equation 19 and 20, we can now evaluate equation 18:

$$[\text{Med25}] = \frac{504 \text{ nM} \cdot (12.6 \mu\text{M} + 10.4 \mu\text{M})}{335.9 \mu\text{M} + 141.0 \mu\text{M}} = 0.024 \mu\text{M} \quad 21$$

Which finally enables us to solve equations 1 and 2.

$$K_{d,trans} = \frac{0.024 \mu\text{M} \cdot 335.9 \mu\text{M}}{12.7 \mu\text{M}} = 640 \text{ nM} \quad 22$$

$$K_{d,cis} = \frac{0.024 \mu\text{M} \cdot 141.0 \mu\text{M}}{10.4 \mu\text{M}} = 330 \text{ nM} \quad 23$$

Errors were obtained by evaluating the entire series of calculations 10000 times using random sampled normal distributed input variables of fraction bound percentages (P_{bound}), ITC determined affinity (K_d) and isomerization equilibrium constant ($K_{eq,trans \rightarrow cis}$). The calculation was repeated using parameters determined at 10 °C to enable better comparison with stopped-flow data recorded at 10 °C.