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Supporting Information

Dissecting FOXP2 oligomerization and DNA binding

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Abstract: Protein–protein and protein–substrate interactions are critical to function and often depend on factors that are difficult to disentangle. Herein, a combined biochemical and biophysical approach, based on electrically switchable DNA biochips and single-molecule mass analysis, was used to characterize the DNA binding and protein oligomerization of the transcription factor, forkhead box protein P2 (FOXP2). FOXP2 contains domains commonly involved in nucleic-acid binding and protein oligomerization, such as a C_2H_2-zinc finger (ZF), and a leucine zipper (LZ), whose roles in FOXP2 remain largely unknown. We found that the LZ mediates FOXP2 dimerization via coiled-coil formation but also contributes to DNA binding. The ZF contributes to protein dimerization when the LZ coiled-coil is intact, but it is not involved in DNA binding. The forkhead domain (FHD) is the key driver of DNA binding. Our data contributes to understanding the mechanisms behind the transcriptional activity of FOXP2.

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Experimental Procedures

Cloning

Short constructs of human FOXP2 have been cloned into a pET28b vector (Tab. S1-S4) using the CPEC cloning technique [1]. Site-specific mutations have been introduced using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). Primers (Tab. S5) were designed using the QuikChange Primer Design Program (www.agilent.com/store/primerDesignProgram.jsp). Successful cloning of constructs was verified by sequencing (Eurofins Genomics, Ebersberg, Germany). Expression plasmids and selected templates will be made available at ADDGENE.

Expression and purification

Expression was performed in Rosetta 2(DE3) cells (Novagen, Merck) for 16 hrs overnight at 16°C after induction with 1 mM IPTG. Supplementing the LB medium with final of 4% EtOH helped to increase the protein expression levels [2]. Medium for expression of constructs containing a ZF-motif additionally was supplemented with 100 µM ZnSO4. Expression of human FOXP2 constructs was verified by western blot analysis with a-human FOXP2 antibody (AHP2020 from AbD Serotec) and on campus MALDI analysis. Expressed proteins contained a N-terminal His6-tag and a C-terminal StrepII-tag. Cells were harvested by centrifugation for 30 minutes at 4000 rcf at 4 °C and snap frozen in liquid nitrogen. Cell pellets were resuspended in lysis buffer and treated with 1 mM lysozyme followed by mechanical lysis (sonication). Benzonase (Merck) has been added to the cell suspension to remove genomic DNA. After removing the cell debris by centrifugation NaCl was added to a final of 500 mM to the supernatant to deactivate the Benzonase. Supernatant was incubated with pre-washed Ni-NTA beads (Qiagen). After washing with buffers W1, W2 and W3 proteins were eluted with buffer EB (buffers see Tab. S6). For the second affinity purification Ni-NTA elution fractions were pooled and applied to Strep-Tactin gravity flow columns (IBA GmbH, Göttingen). Strep-tag affinity purification was performed according to manual with customized buffers (Tab. S7). Strep-Tactin purification was used to concentrate the protein and exchange buffers to reaction condition. Proteins were frozen in liquid nitrogen and stored at -20°C. Protein concentration was determined from absorbance spectra recorded at a pathlength of 10 mm using the theoretical extinction coefficient calculated with the amino acid sequences of each construct with the ProtParam web tool.

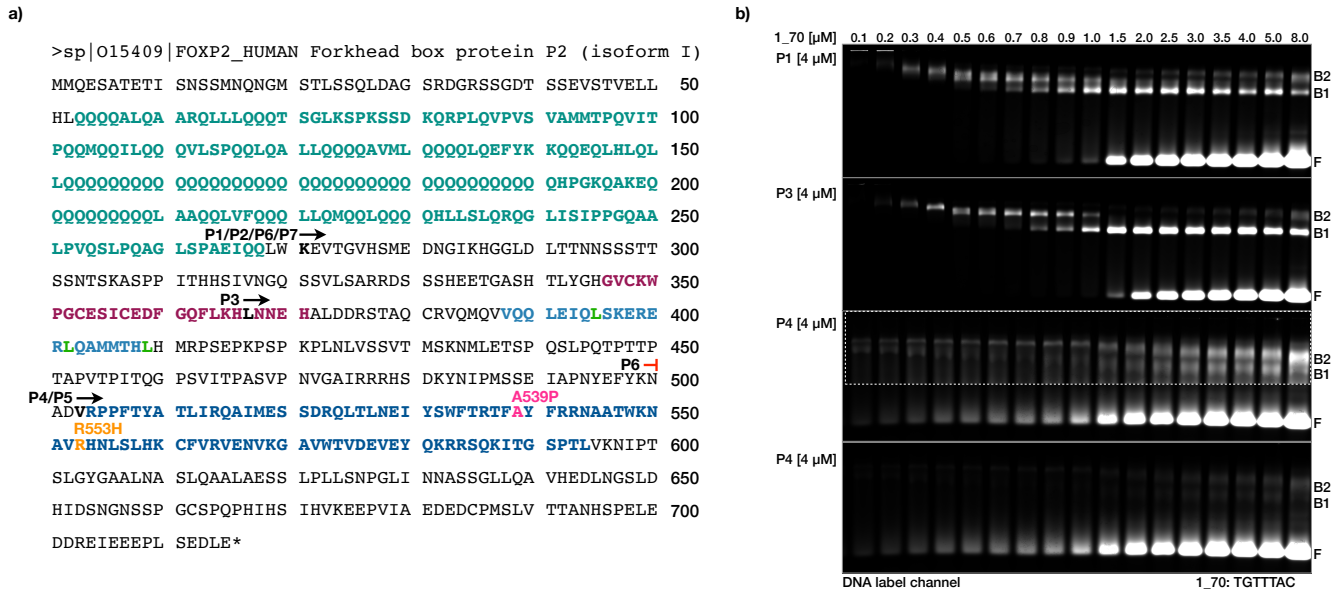

Figure S1. Truncated human FOXP2 protein constructs interacting with DNA. (a) Amino acid sequence of full-lengths human FOXP2 isoform 1 [uniprot entry O15409-1; HUMAN_FOXP2]. Sequence showing polyglutamine tract (petrol) C2H2 zinc-finger motif (purple), leucine zipper domain (light blue) and the DNA-binding forkhead domain (navy blue). Black arrows above the respective sequence mark the site of the start of truncated FOXP2 constructs P1-P7. End of C-terminally truncated construct P6 is indicated by a red sign. Amino acid substitution A539P (pink), mutation R553H (orange) and the three leucine-to-alanine substitutions in the zipper domain of P2 leucine zipper (green) are depicted. **(b)** Laser-scanned images of 5% agarose gels on which FOXP2 constructs P1, P3 and P4 incubated with increasing concentrations of Cy5-labeled DNA (1_70) containing the consensus binding site TGTTTAC were electrophoresed. Area within dashed line was contrast enhanced. F = free DNA, B1, B2 = bands attributed to protein-DNA complexes.

DNA protein interaction analysis using EMSA

For the EMSA analysis a 5% agarose gel (UltraPure agarose, Invitrogen) was prepared using 1x TA (40 mM Tris pH 8.0, 20 mM acetic acid) buffer. Before the actual electrophoresis experiment gel was pre-equilibrated (30min at 100 V) with 1x TA running buffer. Samples of protein-DNA mixtures have been incubated for 30 min, prior to loading onto the gel. Electrophoresis was performed for 45 min at 150 V, constantly cooled by an ice bath. Gels were scanned with a Typhoon laser scanner (Typhoon FLA 9500, GE Healthcare) in the Cy5 or Cy3 channel at a resolution of 25 µm. Gel images scanned in different channels were superimposed using ImageJ [3].

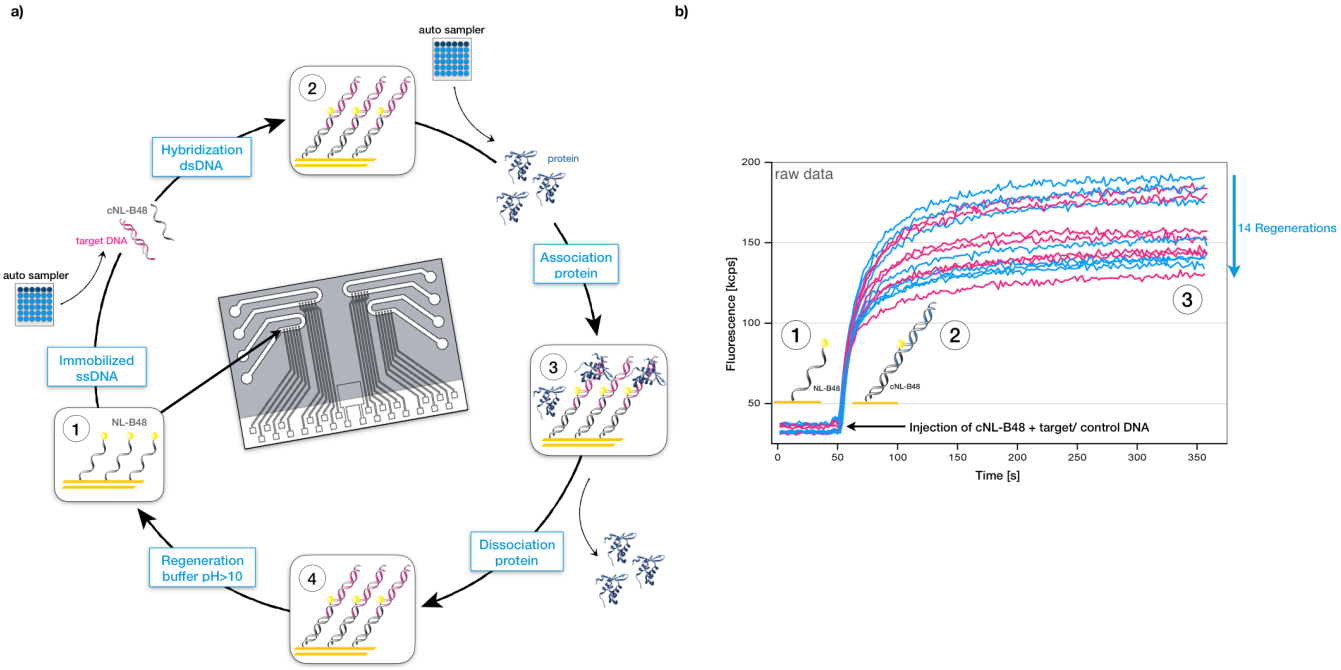

Figure S2. Experimental measurement cycle on a switchSENSE biochip. (a) Schematic of the experimental procedure: **(1)** Single-stranded anchor sequence (NL-B48) modified with fluorescent dye is immobilized on the gold surface of the biochip. Single-stranded DNA, complementary to the anchor DNA (cNL-B48), and double-stranded DNA extensions (target DNA) are hybridized for functionalizing the biochip surface. **(2)** After hybridization is completed, free excess DNA is washed off with buffer and the association of the protein (FOXP2, 2a07.pdb) can be performed **(3)**. Measuring dissociation is initiated by rinsing running buffer into the chip chamber **(4)**. When the association and dissociation measurements are finished, the chip is regenerated with a buffer at pH>10. Regenerated biochips are still functionalized with the single-stranded anchor sequence (NL-B48). Hence, the same electrode can be used again for measurements. **(b)** Fluorescence signal of hybridization and regeneration cycles. Diagram shows 14 hybridization curves (pink: target DNA, blue: control DNA) following regenerations on the chip surface (raw data). Fluorescence increase during hybridization is given in kilo counts per second (kcps).

switchSENSE interaction – experimental procedure

DNA biochip-based interaction analysis was performed at Dynamic Biosensors GmbH (Martinsried) with a DRX analyzer using a standard multi-purpose-biochip (MPC-48-1-Y1). Single-stranded anchor DNA (NL-B48) carrying a fluorescent dye (Y1) was optimized by DBS for thermal stability, absence of secondary structures, hybridization efficiency and minimal interference with FOXP2 binding experiments. Anchor DNA was hybridized with complementary DNA (cNL-B48) and an additional DNA extension with FOXP2 target or control sequence (Tab. S9). During measurements the auto sampler containing the protein samples was set to 20°C. The experiment temperature on the biochip was set to 25°C. Protein samples were diluted and measured in FOXP2 reaction buffer (10 mM HEPES pH7.4, 140 mM NaCl, 1 mM MgCl2, 0.5 µM ZnSO4, 10% Glycerol, 0.5 mM TCEP). Flow rates for association and dissociation reactions were at 50 µl/min and 200 µl/min, respectively. The biochip was regenerated after each completed association/dissociation run. Washing with regeneration solution containing 100 mM NaOH (pH13) removed dsDNA complementary DNA (with extension) and residual proteins, leaving the anchor DNA intact for a new hybridization and measurement (Figure S2). Data analysis was performed with the switchANALYSIS software from Dynamic Biosensors. The association and dissociation rate constants (kon and koff) and the respective error values were derived from a global single exponential fit model. The shown error reflects the overall error of the global fit for three concentration measurements.

FPS mode - switchSENSE interaction analysis

Interaction analysis was performed in fluorescence proximity sensing (FPS) mode with a constant voltage of -0.1 V-0.4 V applied, which forces the surface-tethered DNA into a fixed angle. When the protein analyte binds to the DNA target, it affects the average distance of the fluorescent label from the fluorescence-quenching gold surface [4]. Besides the change in DNA orientation, a change in close proximity to the fluorescent dye or direct interaction of the protein with the fluorescent dye lead to measurable changes in the fluorescence intensity [5]. In the FPS measurements, FOXP2 protein variants were being flushed at specified concentrations over the gold electrode using microfluidic channels (experimental setup see Figure S2). In the FPS setup going from pure buffer to a specified protein concentration had a deadtime on the order of miliseconds. When the protein was flushed in, we observed fluorescence signal increases on the timescale of seconds. Hence, the concentration jump itself may be considered instantaneous, and the time dependence of the fluorescence signal directly reflects the kinetics of protein binding to the surface DNA. After the sudden increase of the protein concentration, both association and dissociation processes can take place. After flushing out the protein and replacing the bulk solution with pure buffer, only dissociation can take place. Furthermore, the microfluidic mass transport was such that the protein concentration in the bulk was not affected by the binding of protein to the surface. Hence, the bulk concentration may be considered constant and used as an input parameter to extract the rate constants for protein-DNA association and dissociation from the time dependence of the fluorescence signals by fitting a bimolecular reaction model to the data.

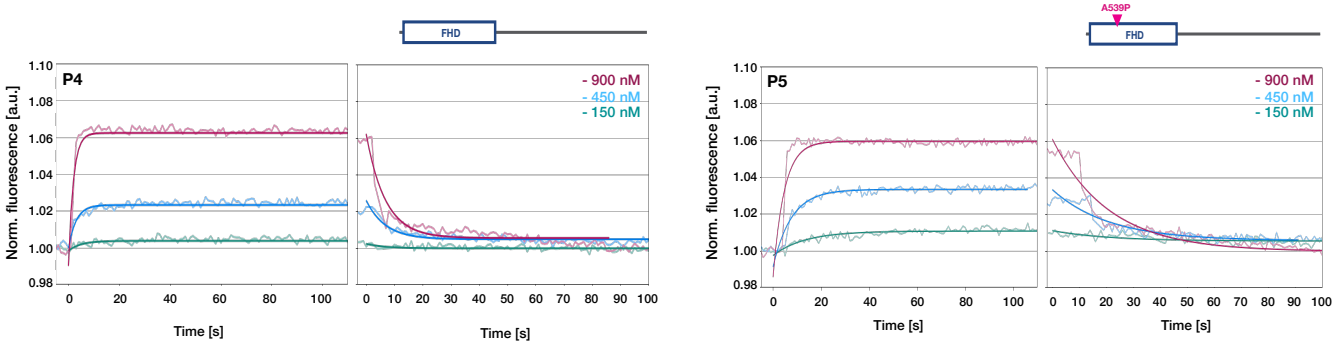

Figure S3. Concentration-dependent DNA binding kinetics of FOXP2 constructs P4 and P5. Thin solid lines: normalized fluorescence intensity over time measured during association and dissociation of protein constructs P4 (left) and P5 (right) with target DNA (see Figure 2b) attached to chip surface. Numbers give the protein concentrations. Thick solid lines: global fits to the data based on a bimolecular reaction model (see methods).

Interferometric scattering mass photometry

Microscope coverslips (#1.5, 24x50 mm and (#1.5, 24x24 mm, Thermo Scientific Menzel) were cleaned and assembled into flow chambers as previously described [6]. Landing assays, data acquisition and image processing were performed as previously described [6]. Buffers were filtered through a 0.2 µm pore size syringe filter. For calibration standard proteins bovine serum albumin (BSA), alcohol dehydrogenase (ADH) and ß-amylase (all purchased from Sigma-Aldrich) were diluted to 10 nM in FOXP2 reaction buffer (10 mM HEPES pH7.4, 140 mM NaCl, 1 mM MgCl2, 0.5 µM ZnSO4, 10% Glycerol, 0.5 mM TCEP). For each movie of a standard protein measured, a histogram was made and fitted with Gaussians according to how many peaks are resolved (Fig. S4a). Fitted centers of these Gaussians and the corresponding masses that they are assigned to were plotted and fitted to a straight line. Resulting parameters were used as conversion between mass and measured contrast of FOXP2 constructs (Fig. S4b). FOXP2 constructs P1-P3 (green laser setup) and P4- P5 (blue laser setup) were measured at a concentration of 100 nM in FOXP2 reaction buffer.

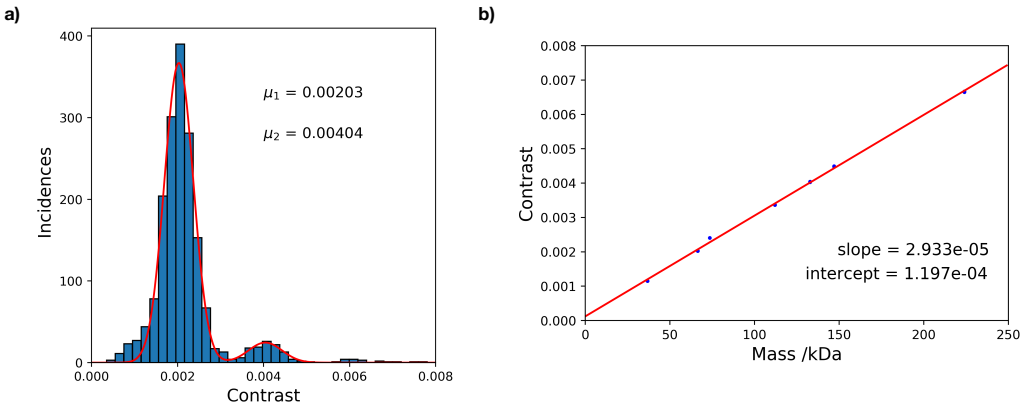

Figure S4. Mass photometry calibration measurements. Contrast of standard proteins bovine serum albumin (BSA), alcohol dehydrogenase (ADH) and ß-Amylase used for calibration. For each movie of a standard protein measured, a histogram was made and fitted with Gaussians according to how many peaks are resolved. (a) Example histogram of a BSA measurement fitted with two Gaussians according to monomer and dimer distribution. (b) Fitted centers of these Gaussians and the corresponding masses that they are assigned to were plotted and fitted to a straight line. Points used: BSA monomer and dimer (66.4 kDa and 132.8 kDa), ADH monomer, dimer and tetramer (36.8 kDA, 73.5 kDa and 147 kDa) and ß-Amylase dimer and tetramer (112 kDa and 224 kDa).

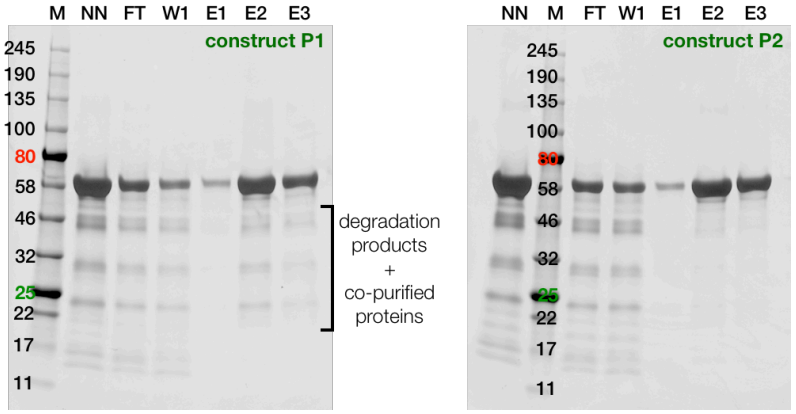

Figure S5. Strep-Tactin purification of constructs P1 and P2. Laser-scanned image (Cy5 channel) of a 4-20% gradient SDS PAGE gel stained with RotiBlue quick solution. Purifications of constructs P1 and P2, SDS PAGE preparation and digitalization in the Tyhoon scanner have been simultaneously performed. Direct comparison reveals a higher grade of low molecular impurities for construct P1 than P2 after the second affinity purification. M: Marker; NN: pooled Ni-NTA fractions loaded on Strep Tactin column; W1: wash step 1; E1-E3: elution fractions.

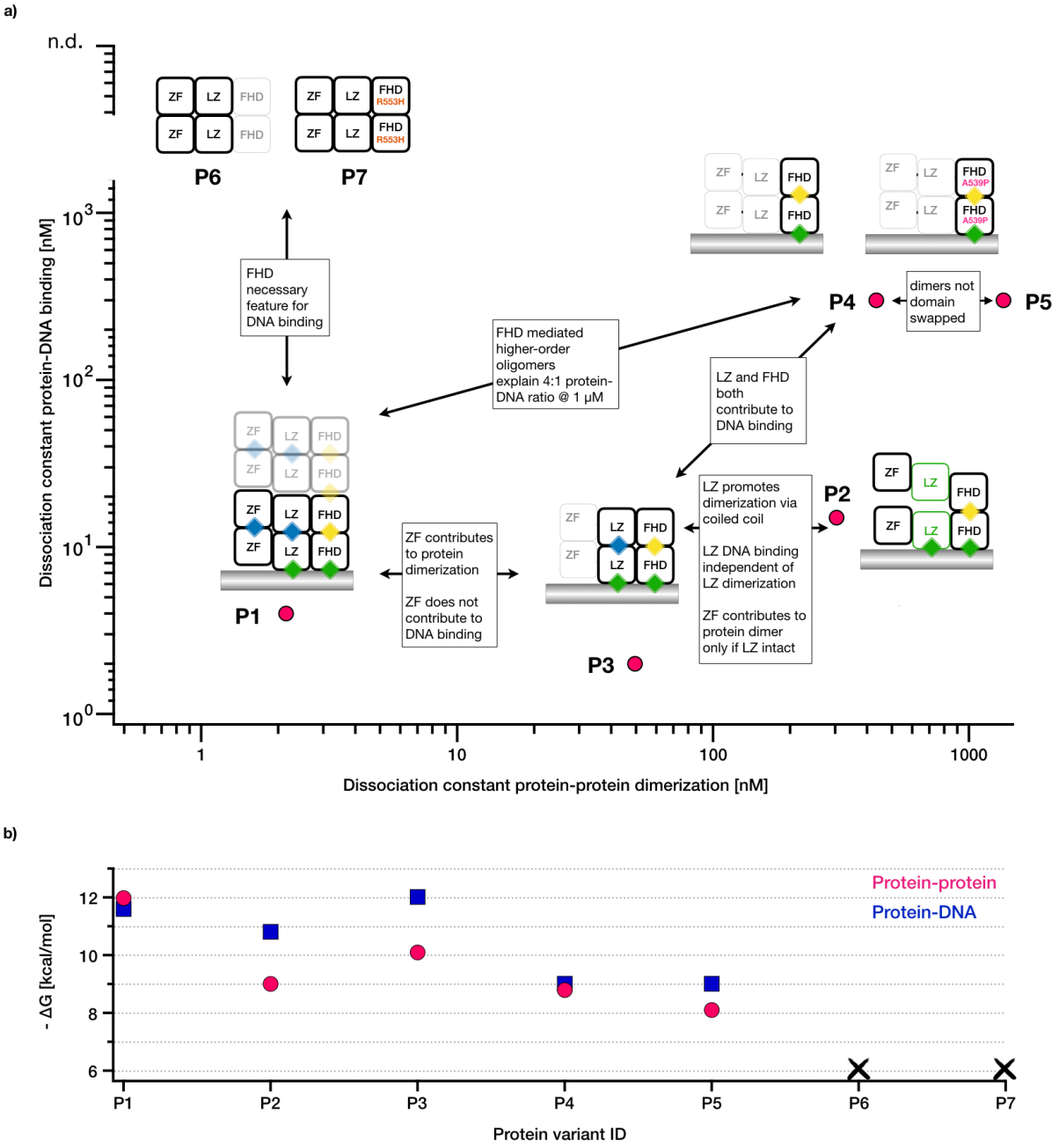

Figure S6. Summary of protein-protein and protein-DNA dissociation constants and interaction free energies. a) Red circles: dissociation constants as obtained from analyzing on- und off rates of the protein constructs via FPS and from analyzing monomer-dimer distributions as seen in mass photometry measurements. N.d. = not determined. Diamonds indicate attractive interactions. **b)** Free energies as computed from the dissociation constants.

Table S1. Sequence of the expression plasmid pET28b with protein construct P1 (ZF-LZ-FHD). Upper case indicates coding sequence with N-terminal His6-tag and C-terminal StrepII-tag.

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Table S2. Sequence of the expression plasmid pET28b with protein construct P3 (LZ-FHD). Upper case indicates coding sequence with N-terminal His6-tag and C-terminal StrepII-tag.

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Table S3. Sequence of the expression plasmid pET28b with protein construct P4 (FHD only). Upper case indicates coding sequence with N-terminal His6-tag and C-terminal StrepII-tag.

```
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```

Table S4. Sequence of the expression plasmid pET28b with protein construct P6 (ZF-LZ). Upper case indicates coding sequence with N-terminal His6-tag and C-terminal StrepII-tag.

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GCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAAAGAAGTGACTGGAGTTCACAGTATGGAAGACAATGGCATTAAACATGGAGGGCTAGACCTCACTACTAACAATTCCTCCTCGACTACCTCCTCCAAC
ACTTCCAAAGCATCACCACCAATAACTCATCATTCCATAGTGAATGGACAGTCTTCAGTTCTAAGTGCAAGACGAGACAGCTCGTCACATGAGGAGACTGGGGCCTCTCACACTCTCTATGGCCATGGAGT
TTGCAAATGGCCAGGCTGTGAAAGCATTTGTGAAGATTTTGGACAGTTTTTAAAGCACCTTAACAATGAACACGCATTGGATGACCGAAGCACTGCTCAGTGTCGAGTGCAAATGCAGGTGGTGCAACAGT
TAGAAATACAGCTTTCTAAAGAACGCGAACGTCTTCAAGCAATGATGACCCACTTGCACATGCGACCCTCAGAGCCCAAACCATCTCCCAAACCTCTAAATCTGGTGTCTAGTGTCACCATGTCGAAGAAT
ATGTTGGAGACATCCCCACAGAGCTTACCTCAAACCCCTACCACACCAACGGCCCCAGTCACCCCGATTACCCAGGGACCCTCAGTAATCACCCCAGCCAGTGTGCCCAATGTGGGAGCCATACGAAGGCG
ACATTCAGACAAATACAACATTCCCATGTCATCAGAAATTGCCCCAAACTATGAATTTTATAAAAATAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
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Table S5. Mutagenesis primer for generation of constructs P2, P5 and P7. Fw primer with mutation site depicted in bold letters.

Name	Sequence	Construct
L395A	GTGCAACAGTTAGAAATACAG**GCT**TCTAAAGAACGCGAACGT	P2
L402A	CTTCTAAAGAACGCGAACGT**GCT**CAAGCAATGATGACCCA	P2
L409A	AAGCAATGATGACCCAC**GCG**CACATGCGACCCTCAG	P2
A539P	CAGCTGGTTTACACGGACATTT**CCT**TACTTCAGGCG	P5
R533H	ACTTGGAAGAATGCAGTA**CAT**CATAATCTTAGCCTGCAC	P7

Table S6. Buffers Ni-NTA purification. Composition of buffers used in Ni-NTA purification.

Name	Composition
Lysis buffer	25 mM HEPES pH 8.0 (adjusted with KOH) 100 mM NaCl 10% glycerol 0.1% Triton X-100 1 mM MgCl2 20 µM ZnSO4 10 mM Imidazol 5 mM TCEP (freshly added)
Binding buffer	25 mM HEPES pH 8.0 (adjusted with KOH) 500 mM NaCl 10% glycerol 1 mM MgCl2 20 µM ZnSO4 10 mM Imidazol 0.5 mM TCEP (freshly added)
W1 (High salt buffer)	25 mM HEPES pH 8.0 (adjusted with KOH) 1 M NaCl 10% glycerol 0.5 mM TCEP (freshly added)
W2 (Urea buffer)	25 mM HEPES pH 8.0 (adjusted with KOH) 100 mM NaCl 500 mM Urea 10% glycerol 20 mM Imidazol 0.5 mM TCEP (freshly added)
W3 (Low salt buffer)	25 mM HEPES pH 8.0 (adjusted with KOH) 100 mM NaCl 10% glycerol 30 mM Imidazol 0.5 mM TCEP (freshly added) 10 µM ZnSO4 (optionally)
EB (Elution buffer)	25 mM HEPES pH 8.0 (adjusted with KOH) 300 mM NaCl 1 mM MgCl2 10% glycerol 500 mM Imidazol 0.5 mM TCEP (freshly added) 10 µM ZnSO4 (optionally)

Table S7. Buffers Strep-Tactin purification. Composition of buffers used in Strep-tag purification.

Name	Composition
Washing buffer	25 mM HEPES pH 7.6 (adjusted with KOH) 150 mM NaCl 1 mM MgCl2 10% glycerol 0.5 mM TCEP (freshly added) 1 µM ZnSO4 (optionally)
ES (Elution/ storage buffer)	25 mM HEPES pH 7.6 (adjusted with KOH) 150 mM NaCl 1 mM MgCl2 10% glycerol 10 mM D-Desthiobiotin 0.5 mM TCEP (freshly added) 1 µM ZnSO4 (optionally)

Table S8. DNA sequences used as substrates in EMSA experiments depicted in Figure 1 and Figure 1S. Bold letters give consensus DNA binding motifs for the FHD.

Name	Sequence	Source
1_70	CGCCTGTTACGGCATCAGGGCTTTGGTTTGGGCGGGCTGCT**TGTTTAC**CAATTGTCTCCGGCGGTAT	Sequence from [7]
2_32	GGCGGGCTGCT**TGTTTAC**CAATTGTCTCCGGC	shortened 1_70
3_32	AGTCTCGTGAACCGACAAC**TCTTGAC**TCACTG	lab
4_29	TTGCCCCCTTAAA**TATTTGC**CTAAGCCTC	Sequence from [8]

Table S9. DNA sequences used in switchSENSE experiments. Bold letters give consensus DNA binding motif for the FOXP2-FHD. Target and control sequence are taken from [7]. There referred to as binder (seq_172) and no binder (seq_53) sequence.

Name	Sequence	Description
NL-B48	AU-TAGTCGTAAGCTGATATGGCTGATTAGTC**GGAAGCA**TCGAACGCTGAT[Y1]	Anchor DNA
cNL-B48	ATCAGCGTTCGATGCTTCCGACTAATCAGCCATATCAGCTTACGACTA	complement anchor
cN52	ACAATTGGTAAACAAGCAGCCCGCCCAAACCAAAGCCCTGATGCCGTAACAG	target sequence
cNL-B48 + N52	CTGTTACGGCATCAGGGCTTTGGTTTGGGCGGGCTGCT**TGTTTAC**CAATTGTATCAGCGTTCGATG CTTCCGACTAATCAGCCATATCAGCTTACGACTA	target extension
cNB52	TTGTTTCTCTAGTCGGATGAAGTCGCTAAGGCCTTAAGGCGGCTCCACGGAT	control sequence
cNL-B48 +NB52	ATCCGTGGAGCCGCCTTAAGGCCTTAGCGACTTCATCCGACTAGAGAAACAAATCAGCGTTCGATGC TTCCGACTAATCAGCCATATCAGCTTACGACTA	extension control

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Author Contributions

K.H. and H.D. designed research; K.H. carried out protein biochemistry; K.H. and H.D. analyzed biochip data; K.H. and G.Y. performed iSCAMS experiments and analyzed data; K.H. and H.D. wrote the manuscript and prepared the figures; P.K. commented on the manuscript. All authors discussed the results and contributed to the final manuscript.