Functional instability allows access to DNA in longer Transcription Activator-Like Effector (TALE) arrays.

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Abstract

Transcription activator-like effectors (TALEs) bind DNA through an array of tandem 34-residue repeats. Here, we examine the kinetics of DNA binding for a set of TALE arrays with varying numbers of identical repeats using single molecule microscopy. Using a new deterministic modeling approach, we find evidence for conformational heterogeneity in both the free- and DNA-bound TALE arrays. Combined with previous work demonstrating populations of partly folded TALE states, our findings reveal a functional instability in TALE-DNA binding. For TALEs forming less than one superhelical turn around DNA, partly folded open states inhibit DNA binding. In contrast, for TALEs forming more than one turn, the partly folded open states facilitate DNA binding. Overall, we find that increasing repeat number results in significantly slower interconversion between the various DNA-free and DNA-bound states. These findings highlight the role of conformational heterogeneity and dynamics in facilitating macromolecular complex assembly.

38 **Impact Statement**

39 Single molecule DNA-binding trajectories and deterministic modeling analyses
40 demonstrate a functional role for high energy partly folded states in Transcription
41 Activator-Like Effectors (TALEs) that could improve future TALEN design.

42

43 **Introduction**

44 Transcription activator-like effectors (TALEs) are bacterial proteins containing a
45 domain of tandem DNA-binding repeats as well as a eukaryotic transcriptional activation
46 domain (Kay et al., 2007; Römer et al., 2007). The repeat domain binds double
47 stranded DNA with a register of one repeat per base pair. Specificity is determined by
48 the sequence identity at positions twelve and thirteen in each TALE repeat, which are
49 referred to as repeat variable diresidues (RVDs) (Boch et al., 2009; Miller et al., 2015;
50 Moscou and Bogdanove, 2009). This specificity code has enabled design of TALE-
51 based tools for transcriptional control (Cong et al., 2012; Geissler et al., 2011; Li et al.,
52 2012; Mahfouz et al., 2012; Zhang et al., 2011), DNA modifications (Maeder et al.,
53 2013), in-cell microscopy (Ma et al., 2013; Miyanari et al., 2013), and genome editing
54 (TALENs) (Christian et al., 2010; Li et al., 2011).

55 TALE repeat domains wrap around DNA in a continuous superhelix of 11.5 TALE
56 repeats per turn (Deng et al., 2012; Mak et al., 2012). Because TALEs contain on
57 average 17.5 repeats (Boch and Bonas, 2010), most form over 1.5 full turns around
58 DNA. Many multisubunit proteins that form rings around DNA require energy in the form
59 of ATP to open or close around DNA (reviewed in O'Donnell and Kuriyan, 2006), yet

60 TALEs are capable of wrapping around DNA without energy from nucleotide
61 triphosphate hydrolysis. One possibility is that TALEs bind DNA through an
62 energetically accessible open conformation. Consistent with this possibility, we
63 previously demonstrated that TALE arrays can populate partly folded or broken states
64 (Geiger-Schuller and Barrick, 2016). While the calculated populations of partly folded
65 states in TALE repeat arrays are small, these populations are many orders of
66 magnitude larger than calculated populations of partly folded states in other previously
67 studied repeat arrays (consensus Ankyrin (Aksel et al., 2011) and DHR proteins
68 (Geiger-Schuller et al., 2018)) suggesting a potential functional role for the high
69 populations of partially folded states in TALE repeat arrays.

70 Consensus TALEs (cTALEs) are homopolymeric arrays composed of the most
71 commonly observed residue at each of the 34 positions of the repeat (Geiger-Schuller
72 and Barrick, 2016). In addition to simplifying analysis of folding and conformational
73 heterogeneity in this study, the consensus approach simplifies analysis of DNA binding,
74 eliminating contributions from sequence heterogeneity and providing an easy means of
75 site-specific labeling.

76 Here we characterize DNA binding kinetics of cTALEs using total internal
77 reflection fluorescence single-molecule microscopy. We find that consensus TALE
78 arrays bind to DNA reversibly, with high affinity. Analysis of the dwell-times of the on-
79 and off-states reveals multiphasic binding and unbinding kinetics, suggesting
80 conformational heterogeneity in both the free and DNA bound state. We develop a
81 deterministic optimization analysis that supports such a model, and provides rate

82 constants for both conformational changes and binding. Comparing the dynamics
83 observed here to previously characterized local unfolding suggests that locally unfolded
84 states inhibit binding of short cTALE arrays (less than one full superhelical turn around
85 DNA), whereas they promote binding of long arrays (more than 1 full superhelical turn).
86 Whereas local folding of transcription factors upon DNA binding is well documented
87 (Spolar and Record, 1994), local unfolding in the binding process has not. Our results
88 present a new mode of transcription factor binding where the major conformer in the
89 unbound state is fully folded, requiring partial unfolding prior to binding. The critical role
90 of such high energy partly folded states is an exciting example of functional instability.
91

92 **Results**

93 **cTALE design**

94 Consensus TALE (cTALE) repeat sequence was design previously described
95 (Geiger-Schuller and Barrick, 2016). To avoid self- association of cTALE arrays, we
96 fused arrays to a conserved N-terminal extension of the PthXo1 gene. Although the
97 sequence of this domain differs from a TALE repeat sequence, the structure of this
98 domain closely mimics four TALE repeats (Gao et al., 2012; Mak et al., 2012) and is
99 required for full transcriptional activation (Gao et al., 2012). In this study, all repeat
100 arrays contain this solubilizing N-terminal domain.
101

102 **cTALE local instability promotes population of partly folded states**

103 Figure 1A depicts types of partly folded states of a generic repeat protein. In the
104 fully folded state, all repeats are folded, and all interfaces are intact. In the end-frayed
105 states, one or more terminal repeats are unfolded and all interfaces, except the
106 interface(s) between the unfolded and adjacent folded repeat(s), are intact. In the
107 internally unfolded states, a central repeat is unfolded and all interfaces, except the
108 interfaces involving the unfolded repeats, are intact. In the interfacially ruptured state, all
109 repeats are folded but one interface is disrupted due to local structural distortion.
110 Figure 1B shows calculated free energy differences between various partly
111 folded states and the fully folded repeat array for two different RVDs (NS and HD) in an
112 otherwise identical consensus sequence background, using the intrinsic and interfacial
113 engeries we determined previously (Geiger-Schuller and Barrick, 2016). The distribution
114 of partly folded states is calculated for different 20-repeat arrays containing two types of
115 TALE arrays (with the NS RVD in red and with the HD RVD in blue) as well as
116 consensus ankyrin arrays (cAnk in black). For cTALE arrays, end frayed states are
117 within a few *RT* of the folded state, internally unfolded states are highest in energy, and
118 interfacially ruptured states fall energetically between end frayed and internally unfolded
119 states.
120 Changing the RVD sequence affects the distribution of these partly folded states:
121 HD repeat containing arrays are more likely to internally unfold or interfacially rupture
122 than NS repeat containing arrays. However, both types of cTALEs are more likely to
123 populate many of these partly folded states than cAnk is to populate even the lowest
124 energy partly folded state, the end frayed state. Thus, compared to ankyrin repeats,

125 cTALEs are locally unstable, meaning they are likely to form partly folded states. As
126 these states disrupt the superhelix, they may facilitate DNA binding.
127
128 **Single-molecule studies of cTALE binding to DNA**
129 To ask if cTALE local instability is relevant for DNA binding kinetics, DNA binding
130 trajectories were measured using single molecule total internal reflection fluorescence
131 (smTIRF). Figure 2A shows a schematic of the smTIRF experiments performed to
132 measure DNA binding. For site-specific cTALE labeling, R30 is mutated to cysteine in a
133 single repeat. Position 30 is frequently a cysteine in naturally occurring TALEs (in earlier
134 folding studies, arginine was chosen in the consensus sequence to avoid disulfide
135 formation) (Geiger-Schuller and Barrick, 2016). This cysteine was Cy3 (FRET donor)-
136 labelled using maleimide chemistry, and was attached to biotinylated slides via the C-
137 terminal His_6 tag and α-Penta•His antibodies. At salt concentrations below 300 mM
138 NaCl, cTALEs aggregate. Because DNA binding is weak at high salt concentrations,
139 measuring binding kinetics in bulk at high salt is not possible. However, tethering
140 cTALEs to the quartz slide at high salt prevents self-association, even at the low salt
141 concentrations required to study DNA binding kinetics. A histogram of $NcTALE_8$ (8 NS-
142 type repeats and the N-terminal domain) labeled via a cysteine in the first repeat shows
143 a single peak at zero FRET efficiency, as expected for donor-only constructs (Figure
144 2B).
145 To test for DNA binding to tethered cTALE constructs, we added Cy5 (FRET
146 acceptor)-labeled 15 bp-long DNA ($Cy5.A_{15}/T_{15}$) to tethered $NcTALE_8$. This results in a

147 new peak at a FRET efficiency of 0.45, indicating that DNA binds directly to cTALE
148 arrays. As DNA concentration in solution is increased, the peak at 0.45 FRET efficiency
149 increases in population (Figure 2C-D), suggesting a measurable equilibrium between
150 free and bound DNA rather than saturation or irreversible binding. In support of this,
151 single molecule time trajectories show interconversion between bound and unbound
152 states, providing access to rates of binding and dissociation.

153 As expected for reversible complex formation, the peak at 0.45 FRET efficiency
154 can be competed away by adding mixtures of labeled and unlabeled DNA to pre-formed
155 cTALE-labelled DNA complexes (schematic shown in Figure 2E; pre-formed complex
156 shown in Figure 2F, competition data shown in Figure 2G-H). Challenging pre-formed
157 complexes with a mixture of 5 nM unlabeled DNA and 15 nM labeled DNA results in a
158 slight decrease in the population of the peak at 0.45 FRET (compare Figures 2F and
159 2G). Challenging with a mixture of 50 nM unlabeled DNA and 15 nM labeled DNA
160 further decreases the peak at 0.45 FRET (Figure 2H).

161

162 **cTALE arrays display multiphasic DNA-binding kinetics.**

163 In addition to the short smTIRF movies used to generate smFRET histograms
164 from many molecules, long movies were also collected to examine the extended
165 transitions of individual molecules between the low- and high-FRET (0 and 0.45) (Figure
166 3A-B). A transition from low to high FRET (0 to 0.45) indicates that the acceptor
167 fluorophore on DNA moved close enough to the donor on the protein for FRET and is
168 likely a binding event. A transition from high to low FRET (0.45 to 0.0) indicates the

169 acceptor fluorophore on DNA moved too far away from the donor on the protein for
170 FRET and is likely an unbinding event. Low-FRET states show low colocalization with
171 signal upon direct excitation of the acceptor, confirming that high-FRET states are DNA-
172 bound states and low-FRET states are DNA-free states (Figure 3- figure supplement 1).
173 These long single molecule traces show both long- and short-lived low- and high-FRET
174 states, indicating that kinetics are multi-phasic (Figure 3A-B). Binding events (transitions
175 from low to high FRET) become more frequent as bulk DNA concentration increases
176 (compare representative traces at 1 nM dsDNA to 15 nM dsDNA; Figure 3A and Figure
177 3B). Cumulative distributions generated from dwell times in the low FRET state at a
178 given DNA concentration are best-fit by a double-exponential decay, indicating a
179 minimum of two kinetic phases associated with binding events (Figure 3C). Cumulative
180 distributions generated from dwell times in the high FRET state are best-fit by a double-
181 exponential decay, indicating that there are a minimum of two kinetic phases for
182 unbinding as well (Figure 3D).

183 The rate constant for the fast phase in DNA binding shows a linear increase with
184 DNA concentration (Figure 3E), indicating that this step involves an associative binding
185 mechanism. The slope of these rate constants as a function of DNA concentration gives
186 a bimolecular rate constant of 5.9×10^{8} $nM^{-1}s^{-1}$, close to the diffusion limit. The rate
187 constant for the slower phase (0.59 s^{-1}) is independent of DNA concentration indicating
188 a unimolecular isomerization mechanism (Figure 3E).

189 In contrast, neither of the two fitted rate constants for transitions from high to low
190 FRET (0.45 to 0.0; unbinding events) depends on DNA concentration, suggesting that

191 unbinding involves two (or more) unimolecular processes (Figure 3F). The rate
192 constants of these two phases are 1.2 s^{-1} and 0.13 s^{-1} respectively.
193 To rule out kinetic contributions of TALEs threading axially onto the ends of short
194 DNAs, binding kinetics were measured with capped double-helical DNA sites. Capped
195 DNA was generated by forming 5'digoxygenin-A_5-Cy5-A_{15} duplexed with 5'-digoxygenin-
196 T_{26} and adding a three-fold molar excess of anti-Digoxygenin. Low and high FRET dwell
197 time cumulative distributions generated from capped DNA-binding kinetics are bi-
198 phasic, similar to distributions from uncapped DNA (Figure 3- figure supplement 2). The
199 DNA concentration-independent rate constant for binding is roughly the same for
200 capped DNA as for uncapped DNA (compare $FRET_{L\rightarrow H}$ red and blue triangles in Figure
201 3- figure supplement 2), as are the dissociation rate constants (compare $FRET_{H\rightarrow L}$ red
202 and blue triangles in as well as $FRET_{H\rightarrow L}$ red and blue circles in Figure 3- figure
203 supplement 2). The rate constant for bimolecular binding of capped DNA decreases
204 compared to that for uncapped DNA (compare $FRET_{L\rightarrow H}$ red and blue circles in Figure
205 3- figure supplement 2), which is consistent with the expected decrease in the rate of
206 diffusion of the larger capped DNA. To assess the effect of molecular weight increase
207 on diffusion of capped versus uncapped DNA, Sednterp (edited by S. E. Harding, 1992),
208 a program commonly used to estimate sedimentation and diffusion properties of
209 biomolecules, was used to estimate maximum diffusion coefficients. Including the two
210 antibodies bound on the ends of capped DNA (320 kDa total) gives an estimated
211 diffusion coefficient of 4.7×10^{-7} cm^2s^{-1}, which is much lower than the estimated diffusion
212 coefficient of the uncapped DNA (1.5×10^{-6} cm^2s^{-1}). This ~3.6-fold decrease in the

213 diffusion constant for capped DNA is similar to the 6.7-fold decrease in the bimolecular
214 rate constant for binding of capped DNA (Figure 3- figure supplement 2).
215
216 **Longer cTALEs have slower DNA binding and unbinding kinetics**
217 To examine how increasing the length of the cTALE array influences DNA
218 binding, we generated Cy3-labelled constructs with 16 and 12 cTALE repeats, and
219 measured binding to a longer Cy5-labelled DNA (A_{23}/T_{23}). Because we did not observe
220 FRET for these longer constructs, a fluorescence colocalization microscopy protocol
221 was used instead (Figure 4- figure supplement 1). In this protocol, Cy3 was first imaged
222 for ten camera frames (1017.5 msec total) to identify positions of single TALE
223 molecules. Then a long time series of fluorescence images of Cy5 signal were collected
224 through directly exciting Cy5 on the DNA, and time trajectories of Cy5 signal were
225 generated from the initially identified single TALE positions.
226 Increasing the number of cTALE repeats from 8 to 12 and 16 dramatically affects
227 DNA binding kinetics. Long movies collected over a range of DNA concentrations show
228 short- and long-lived Cy5 signal on and off states, indicating a level of kinetic
229 heterogeneity similar to $NcTALE_8$ (Figure 4- figure supplement 1). Single molecule
230 traces were analyzed using a thresholding filter (see Materials and Methods and Figure
231 4- figure supplement 1) to identify states and dwell times. Cumulative distributions were
232 generated from dwell times at low Cy5 signal (unbound states, with lifetimes
233 representing binding kinetics), and at high Cy5 signal (bound states, with dwell times
234 representing unbinding kinetics). As with the eight repeat constructs, unbound

235 cumulative distributions for these longer TALE arrays are best-fit by double exponential
236 decays, particularly at high DNA concentrations (compare the cumulative distribution at
237 low DNA concentration, Figure 4- figure supplement 2A, to cumulative distribution at 5
238 nM DNA, Figure 4- figure supplement 2B). Bound cumulative distributions for longer
239 TALE arrays are best-fit by double exponential decays (Figure 4- figure supplement 2C-
240 D). All apparent rate constants are much smaller for $NcTALE_{16}$ and $NcTALE_{12}$
241 (green/black circles and triangles, Figure 4A-B) compared to $NcTALE_{8}$, indicating that
242 binding and unbinding is impeded by increasing the length of the binding surface
243 between cTALEs and their cognate DNA (Figure 4C). To address whether differences in
244 binding kinetics are related to experimental differences between colocalization and
245 FRET assays, alternating laser experiments were performed by switching between
246 FRET and colocalization detection (every 5 frames) within single molecule trajectories
247 (Figure 3- figure supplement 1). Changes in FRET and colocalization signals occurred
248 simultaneously according to single molecule time traces, showing that differences in
249 binding and unbinding kinetics of short and longer cTALEs are not due to differences in
250 colocalization and FRET assays (Figure 3 - figure supplement 1).
251
252 **A deterministic approach to modeling cTALE-DNA binding kinetics.**
253 To determine how the kinetic changes above are partitioned into underlying
254 kinetic steps in binding, we fitted various kinetic models to the cumulative distributions
255 for binding and unbinding. In addition to providing information about the mechanism of
256 binding, this approach allows us to estimate the underlying microscopic rate constants

257 and compare them for different constructs. This approach is generally applicable to
258 studies of complex single molecule kinetics. Numerical integration was used to calculate
259 the relative population of cTALE states as a function of time (Figures 5A-C and 5G-H),
260 given a binding mechanism, an associated set of rate laws, and a set of initial
261 conditions. Cumulative distributions of unbound dwell times represent the distribution of
262 times single molecules spent in the unbound state before transitioning into the bound
263 state, allowing us to split the kinetic scheme when fitting to single-molecule dwell times.
264 Among the various models tested, the model that is most consistent with the data
265 has two unbound DNA-free states and two DNA-bound states. This is consistent with
266 alternating laser experiments showing that DNA is only colocalized when cTALEs are in
267 the high FRET state (Figure 3 - figure supplement 1). This four-state model includes a
268 TALE isomerization step in the absence of DNA from a DNA-binding incompetent
269 conformation (which we refer to as TALE) to DNA-binding competent conformation
270 (which we refer to as TALE*). The DNA-binding competent TALE* conformer binds and
271 unbinds DNA (called TALE* when DNA free and TALE*~DNA when DNA-bound).
272 Before unbinding, a fraction of TALE*~DNA isomerizes to a longer-lived DNA-bound
273 state called TALE‡~DNA.
274 Based on this mechanism, the rate laws for binding are given in equations 1a -
275 1d.

276 $$\frac{d[TALE]}{dt} = -k_1[TALE] + k_{-1}[TALE^*] \quad (1a)$$

277 $$\frac{d[TALE^*]}{dt} = k_1[TALE] - k_{-1}[TALE^*] - k_2[TALE^*][DNA] \quad (1b)$$

278 $$\frac{d[TALE^* \sim DNA]}{dt} = k_2[TALE^*][DNA] \quad (1c)$$

279 $$K_{eq,DNA-free} = \frac{k_1}{k_{-1}} \quad (1d)$$

280 Since the single-molecule dwell-time histograms of the unbound states are insensitive
281 to the isomerization after DNA binding, the equation describing the time evolution of the
282 long-lived bound state ($TALE^{\ddagger}$~DNA) is not relevant to our analysis of unbound-state
283 lifetimes.

284 To determine microscopic rate constants k_1, k_{-1}, and k_2, equations 1a-1c were
285 numerically integrated in Matlab, and the fraction of TALE*~DNA as a function of time
286 was fitted to the low-FRET cumulative distributions ($NcTALE_8$; Figure 5D-E) or to the no
287 colocalization cumulative distributions ($NcTALE_{16}$; Figure 5J-K). Microscopic rate
288 constants were adjusted to reduce sum of the squared residuals between the
289 concentration of TALE*~DNA (the direct product of binding) as a function of time and
290 single-molecule cumulative distributions. In both cases, cumulative distributions at
291 different bulk DNA concentrations were fitted globally. Initial fractions of TALE and
292 TALE*~DNA were set to zero, and the initial fraction of TALE* was set to one.
293 Confidence intervals (CI) were estimated by bootstrapping (Table 1; mean and 68% CI
294 from 2000 or 8000 bootstrap iterations).

295 Rate laws for dissociation are given in equations 2a - 2d

296 $$\frac{d[TALE^* \sim DNA]}{dt} = -k_{-2}[TALE^* \sim DNA] - k_3[TALE^* \sim DNA] + k_{-3}[TALE^{\ddagger} \sim DNA] \quad (2a)$$

297 $$\frac{d[TALE^{\ddagger} \sim DNA]}{dt} = k_3[TALE^* \sim DNA] - k_{-3}[TALE^{\ddagger} \sim DNA] \quad (2b)$$

298 $$\frac{d[TALE^*]}{dt} = k_{-2}[TALE^* \sim DNA] \quad (2c)$$

299 $$K_{eq,DNA-bound} = \frac{k_3}{k_{-3}} \quad (2d)$$

300 As with the system of equations above (1a-d), the equation describing the time
301 evolution of the binding-incompetent free state (TALE) is not relevant to our analysis of
302 bound-state lifetimes.

303 To determine microscopic rate constants k_{-2}, k_{-3}, and k_3, equations 2a-2c were
304 numerically integrated in Matlab, and the fraction of TALE* as a function of time was
305 fitted to the high-FRET cumulative distributions ($NcTALE_8$; Figure 5F) or to the low
306 colocalization cumulative distributions ($NcTALE_{16}$; Figure 5L). Microscopic rate
307 constants were adjusted to reduce sum of the squared residuals between the
308 concentration of TALE* (the direct product of dissociation) as a function of time and
309 single-molecule cumulative distributions. In both cases, cumulative distributions at
310 different bulk DNA concentrations were fitted globally. The initial fraction of
311 TALE*~DNA conformer was set at one; all other initial fractions were set to zero.
312 Confidence intervals were estimated by bootstrapping (Table 1; mean and 68% CI from
313 2000 iterations).

314 Fitted curves reproduce the experimental cumulative distributions for binding and
315 unbinding (Figure 5), both for the short and long cTALE arrays, with reasonably small
316 residuals, over a range of DNA concentrations. Generally, fitted rate constants have
317 confidence intervals of 10% or smaller (Table 1).

318 Comparison of microscopic rate constants for 8, 12, and 16 repeats show some
319 significant differences. The bimolecular microscopic binding rate constant, k_2, is slightly
320 larger for 8 repeats than for 12 and 16 repeats (1.1, 0.31, and 0.39 $nM^{-1}s^{-1}$ for 8, 12, and
321 16 repeats respectively). However, microscopic unbinding rate constant, k_{-2}, is higher
322 for 8 repeat cTALEs (0.66 s^{-1} for $NcTALE_8$ versus 0.13 s^{-1} for $NcTALE_{12}$ and 0.299 s^{-1} for
323 $NcTALE_{16}$). Also, bound state isomerization (interconversion between TALE*~DNA and
324 $TALE^{\ddagger}$~DNA) is 5-10 times slower for 16 and 12 repeat cTALEs than 8 repeat cTALEs.
325 The value of $K_{eq,\ DNA\text{-}free}$, which is a measure of the equilibrium proportion of the
326 unbound TALE that is DNA-binding competent (TALE*) to that which is binding-
327 incompetent (TALE), is larger for cTALEs with 8 repeats ($K_{eq,\ DNA\text{-}free}$ = 1.32) than for
328 cTALEs with 12 and 16 repeats ($K_{eq,\ DNA\text{-}free}$ = 0.11 and $K_{eq,\ DNA\text{-}free}$ = 0.61, respectively).
329

330 **Discussion**

331 By measuring DNA-binding kinetics of cTALE arrays that form 0.7, 1, and 1.4
332 superhelical turns, we probe the functional relevance of locally unfolded TALE states.
333 We describe a novel method to glean mechanistic details from complex single molecule
334 kinetics. In our simplified cTALE system, we find conformational heterogeneity in both
335 DNA free and DNA-bound states. We find that association is slowed in arrays
336 containing one full turn of repeats or more. Because most natural and designed TALEs
337 contain more than a full turn of repeats, this finding has important implications for design
338 of high affinity TALE endonucleases (TALEN) molecules, suggesting that placement of
339 destabilized repeats at specific positions may increase activity.

340

341 **cTALEs containing NS RVD bind DNA with high affinity**

342 NS is an uncommon RVD in natural TALEs. Previous reports suggest that NS is

343 fairly nonspecific, but may bind with higher affinity than other common RVDs (NG, NI,

344 NN, and HD)(Miller et al., 2015). Our fitted rate constants can be used to calculate the

345 apparent K_d (K_{app}) calculated from using equation as follows:

346
$$\begin{aligned} K_{app} &= \frac{[TALE-DNA+TALE^{*}-DNA]}{[DNA][TALE+TALE^{*}]} \\ &= \frac{K_{eq,DNA-free}K_2 + K_{eq,DNA-free}K_2K_{eq,DNA-bound}}{1+K_{eq,DNA-free}} \\ &= \frac{\frac{k_1}{k_{-1}} \times \frac{k_2}{k_{-2}}(1+\frac{k_3}{k_{-3}})}{1+\frac{k_1}{k_{-1}}} \end{aligned} \quad (3)$$

347 where $K_2 = k_2/k_{-2}$.

348

349 Using fitted rate constants from Table 1 in the final equality in equation 3 gives values

350 for K_{app} of 2.5 nM for the 8 repeat cTALE array, 0.5 nM for the 12 repeat cTALE array,

351 and 1.0 nM for the 16 repeat cTALE array. Increasing the number of repeats has a

352 modest affect on the apparent K_d due to the increased population of binding

353 incompetent DNA free TALE in the 12, and 16 repeat arrays. This affinity change is

354 small compared to a previous report studying length dependence on affinity of designed

355 TALEs (dTALEs) showing the K_d of a dTALE decreased by a factor of two with the

356 addition of only 1.5 repeats (Rinaldi et al., 2017).

357 TALEs are believed to read out sequence information from one strand (Boch et
358 al., 2009). Due to the asymmetry of our DNA sequences (poly-dA base-paired with
359 poly-dT), in principle, the FRET efficiency contains information on the binding
360 orientation (and thus strand preference). However, based on the crystal structure of the
361 DNA-bound state of TAL-effector PthXo1 (Mak et al., 2012), we estimate that the
362 distance between the donor site of $NcTALE_8$ (repeat 1) to the 5' acceptor site on the
363 DNA (Cy5-A_{15}/T_{15}) should be similar for both the dA-sense or dT-sense orientations
364 (Figure 4- figure supplement 3A). Thus, the FRET data does not discriminate between
365 the two modes of binding for the eight-repeat construct. However, for the 16 repeat NS
366 RVD cTALE arrays, the PthXo1 model suggests very different distances (25 Å versus
367 73 Å for the dT-sense or dA-sense respectively, Figure 4- figure supplement 3B)
368 between the donor site (TALE repeat 14) and the acceptor site (5' Cy5-A_{23}/T_{23}). To
369 restrict the number of binding positions available to longer cTALE arrays, the 23 base
370 pair DNA used for $NcTALE_{16}$ measurements (as well as DNA depicted in Figure 4-
371 figure supplement 3B) has the same number of additional base pairs as repeats (8
372 additional repeats and 8 additional base pairs) compared to the 15 base pair DNA used
373 for $NcTALE_8$ measurements (as well as DNA depicted in Figure 4- figure supplement
374 3A). While we limited the number of available binding positions, it may be possible for
375 cTALEs to slide along DNA. However, taking into account the four repeat N-terminal
376 capping domain, there are only three available base pairs in the bound complex. Thus
377 we don't expect the distance measurements to change by more than 10Å (~3 base
378 pairs) if sliding occurs. The observation that there is colocalization but no measurable

379 FRET when $NcTALE_{16}$ is bound to DNA suggests that cTALEs containing the NS RVD
380 prefer adenine (the dA-sense mode) compared with thymine bases, consistent with
381 previous reports (Boch et al., 2009).
382
383 **Conformational heterogeneity in the unbound state may be caused by local**
384 **unfolding**
385 The cumulative distributions of dwell-times in Figure 3 provide clear evidence for
386 conformational heterogeneity in both the free and DNA-bound cTALEs. Although the
387 deterministic modeling supports such heterogeneity, puts it in the framework of a
388 molecular model, and provides a means to determine the microscopic rate and
389 equilibrium constants, such analysis provides little information about the structural
390 nature of TALE conformational heterogeneity.
391 Figure 6 shows a model of cTALE conformational change consistent with DNA
392 binding kinetics. In this model there are four TALE states. DNA-free cTALEs comprise
393 both incompetent and binding competent states. DNA-bound cTALES comprise
394 encounter and locked complexes with DNA. For 8 repeat cTALE arrays, the DNA-
395 binding competent state is more highly populated than the DNA-binding incompetent
396 state. In this reaction scheme, the DNA-binding incompetent state can be regarded as
397 an off-pathway conformation that inhibits DNA binding (Figure 6A).
398 Because the 8 repeat cTALE array does not form multiple turns of a superhelix,
399 unfolding to bind DNA is not required. In the model in Figure 6, the binding competent
400 state is the fully folded conformation, whereas the binding incompetent state includes

401 partly folded conformations. Consistent with this interpretation, increasing populations of
402 partly folded states through addition of 1M urea and through entropy enhancing
403 mutations decreases apparent binding rates of 8 repeat cTALEs (Figure 6- figure
404 supplement 1). This is also consistent with a partly folded DNA-binding incompetent
405 state in shorter cTALE arrays.

406 For 12 and 16 repeat cTALE arrays, the DNA-binding incompetent state is more
407 highly populated than the DNA-binding competent state. In the model in Figure 6, the
408 DNA-binding competent state is a high-energy conformation required for DNA binding
409 (Figure 6B-C). Because 12 and 16 repeat cTALEs are expected to form 1 and 1.4 turns
410 (excluding the N-terminal domain), we hypothesize that the binding competent state
411 includes some partly folded states that allow access to DNA. Not all partly folded states
412 open the array to access DNA; therefore, the binding incompetent state includes some
413 nonproductive partly folded states in addition to the fully folded state.

414 In arrays containing 12 or more cTALEs, the binding competent and binding
415 incompetent states likely include mixtures of many specific partly folded states. Because
416 the types of partly folded states are unknown, connecting equilibria between binding
417 competent and binding incompetent states to calculated partly folded equilibria (using
418 folding free energies similar to Figure 1) is challenging. Future work towards
419 understanding the structural characteristics of the binding competent state in TALE
420 arrays of one or more turns would inform which partly folded states to include in the
421 calculation, making this comparison meaningful. A better structural understanding of the
422 DNA binding competent state will also allow an opportunity for precise placement of

423 destabilized repeats in designed TALEN arrays which may enable more precise gene
424 editing methodologies in both clinical and basic research applications.
425
426 **TALE functional instability presents a new mode of transcription factor binding**
427 Here we demonstrate kinetic heterogeneity in DNA-bound and unbound TALE
428 arrays, and we subsequently link the observed heterogeneity to partial unfolding of
429 TALE arrays. We propose a model where binding requires partial unfolding of TALE
430 arrays longer than one superhelical turn providing a functional role for previously
431 observed moderate stability of TALE arrays. The functional instability described is
432 particularly surprising given the small population of partly folded states which we expect
433 to be DNA binding competent (partly folded states similar to internally unfolded and
434 interfacially fractured states depicted in Figure 1A). Discovery of a functional role for the
435 observed conformational heterogeneity is even more surprising, given the sequence
436 identity of each of our repeats. Sequence heterogeneity in naturally occurring TALE
437 arrays may further enable access to partly folded binding-competent states.
438 While it is well understood that many transcription factors sometimes undergo
439 local folding transition upon DNA binding (Spolar and Record, 1994; Tsafou et al.,
440 2018), the findings here indicate that for TALE arrays, the major conformer is fully
441 folded, and must undergo a local unfolding transition in order to bind DNA. Taken
442 together, these findings suggest a new mode of transcription factor binding and provide
443 compelling evidence for functional instability in TALE arrays.
444

445 **Conformational heterogeneity in the bound state**

446 Previous reports show that TALEs have multiple diffusional modes when
447 searching nonspecific DNA (Cuculis et al., 2015). Our work suggests that cTALEs have
448 multiple binding modes (encounter and locked states in Figure 6) indicating cTALEs
449 undergo a conformational change also when bound to specific DNA sequences. Table 1
450 shows that microscopic rate constants for transition into and out of longer lived locked
451 bound states become much slower in 12 and 16 repeat cTALEs compared with 8 repeat
452 cTALEs (k_{-3} and k_3). These rate constants decrease much more than the microscopic
453 unbinding rate constant (the k_{-2} values are 0.66 s^{-1}, 0.13 s^{-1}, and 0.299 s^{-1} for $NcTALE_8$,
454 $NcTALE_{12}$, and $NcTALE_{16}$ respectively) suggesting a large conformational change that
455 depends on the number of repeats. Although the model does not provide information on
456 structure of this conformational change, it is possible this conformational change
457 involves a slinky motion to decrease helical rise. Consistent with this hypothesis, crystal
458 structures of TALEs in the free and DNA-bound state show 11.5 repeats per turn in both
459 states, but the helical rise decreases upon binding (Deng et al., 2012). Another
460 possibility involves specific interaction with RVDs and bases in the major groove of
461 DNA. Crystal structures show little deformation of DNA structure, so bending of DNA
462 seems unlikely. While we can only hypothesize about the structural nature of the
463 conformational changes, deterministic simulations show that cTALEs bind DNA through
464 short encounters which occasionally become long-lived locked conformations (Figure
465 6). Taken together, these findings indicate that functional instability plays a crucial role

466 in cTALE DNA binding, and demonstrate the importance of conformational dynamics in
467 complex assembly.
468
469 **Materials and Methods**
470 **Cloning, expression, purification, and labeling**
471 Consensus TALE repeat constructs were cloned with C-terminal His_6 tags via an
472 in-house version of Golden Gate cloning (Cermak et al., 2011). TALE constructs were
473 grown in BL21(T1R) cells at 37°C to an OD of 0.6-0.8 and induced with 1 mM IPTG.
474 Following cell pelleting and lysis, proteins were purified by resuspending the insoluble
475 material in 6M urea, 300 mM NaCl, 0.5 mM TCEP, and 10 mM $NaPO_4$ pH 7.4.
476 Constructs were loaded onto a Ni-NTA column. Protein was eluted using 250 mM
477 imidazole and refolded during buffer exchange into 300 mM NaCl, 30% glycerol, 0.5
478 mM TCEP, and 10 mM $NaPO_4$ pH 7.4.
479 Labelling of cTALE arrays followed a previously reported protocol (Rasnik et al.,
480 2004). $NcTALE_8$ and $NcTALE_{12}$ were labeled at residue R30C in the first repeat, while
481 $NcTALE_{16}$ was labeled at residue R30C in the fourteenth repeat. 1 mg protein was
482 loaded onto 500 uL NiNTA spin column. The column as washed with 10 column
483 volumes of 300 mM NaCl, 0.5 mM TCEP, and 10 mM $NaPO_4$ pH 7.4. Tenfold molar
484 excess Cy3 maleimide dye was resuspended in 10 μL DMSO and added to column.
485 The column was rocked at room temperature for 30 minutes, then at 4°C overnight.
486 Cy3-labeled protein was eluted with 250 mM imidazole, 300 mM NaCl, 30% glycerol,

487 0.5 mM TCEP, and 10 mM $NaPO_4$ pH 7.4. Protein was stored in 300 mM NaCl, 30%
488 glycerol, 0.5 mM TCEP, and 10 mM $NaPO_4$ pH 7.4 at -80°C.

489 **Oligonucleotides**

490 Sequences used for binding studies were 5'-Cy5-A_{15}-3' and 5' T_{15}-3' duplex
491 (Cy5-A_{15}/T_{15}) for 8 repeat binding studies, and 5'-Cy5-A_{23}-3' and 5' T_{23}-3' duplex (Cy5-
492 A_{23}/T_{23}) for 12 and 16 repeat binding studies. DNA was annealed at 5 μM concentration
493 with 1.2-fold molar excess unlabeled strand in 10 mM Tris pH 7.0, 30 mM NaCl.

494 **Single-molecule detection and data analysis**

495 Biotinylated quarts slides and glass coverslips were prepared as previously
496 described (Rasnik et al., 2004). Cy3-labeled cTALEs were immobilized on biotinylated
497 slides taking advantage of neutravidin interaction with biotinylated α-penta•His antibody
498 which binds the His_6 cTALE tag. Slides were pretreated with blocking buffer (5 μL yeast
499 tRNA, 5 μL BSA, 40 μL T50) before addition of 250 pM labeled cTALE. Cy5-labeled
500 duplex DNA was mixed with imaging buffer (20 mM Tris pH 8.0, 200 mM KCl, 0.5 mg
501 mL^{-1} BSA, 1 mg mL^{-1} glucose oxidase, 0.004 mg mL^{-1} catalase, 0.8% dextrose and
502 saturated Trolox ~1mg mL^{-1}) and molecules were imagined using total internal reflection
503 fluorescence microscopy. The time resolution was 50 msec for $NcTALE_8$ and 100 msec
504 for $NcTALE_{16}$ and $NcTALE_{12}$. Collection and analysis was performed as previously
505 described (Roy et al., 2008).

506 **FRET histograms**

507 A minimum of 20 short movies were collected, and the first 5 frames (50 msec
508 exposure time) were used to generate smFRET histograms. FRET was calculated as

509 $I_A/(I_A+I_D)$ where I_A and I_D are donor-leakage and background corrected fluorescence
510 emission of acceptor (Cy5) and donor (Cy3) fluorophores. In competition experiments,
511 unlabeled DNA with the same sequence as labeled DNA was mixed at indicated
512 concentrations with labeled DNA prior to imaging.

513 **Dwell time analysis**

514 Long movies were collected with 50 msec exposure time for $NcTALE_8$ and 100
515 msec exposure time for $NcTALE_{16}$ and $NcTALE_{16}$. At least 20 representative traces at
516 each DNA concentration were selected and dwell times were determined by fitting as
517 previously described using HaMMy (McKinney et al., 2006) for FRET in $NcTALE_8$. Dwell
518 times in $NcTALE_{12}$ and $NcTALE_{16}$ colocalization experiments are determined by using a
519 thresholding procedure for Cy5 excitation (Figure 4- figure supplement 1). The algorithm
520 used to identify low and high emission states here is slightly different than previously
521 described thresholding algorithms (Blanco and Walter, 2010). To reduce the number of
522 incorrectly identified transitions arising from increased background and noise at higher
523 Cy5-labeled DNA concentrations, a thresholding algorithm with two limits was
524 implemented (see Figure 4- figure supplement 1). All FRET and colocalization data are
525 well described by models with two distinct states (0.0 FRET and ~0.45 FRET as well as
526 low colocalization and high colocalization). Dwell times of the same state (low versus
527 high FRET or low versus high colocalization) for all traces at a given DNA concentration
528 are compiled, and cumulative distribution is generated with spacing equal to imaging
529 exposure time.

530 To determine apparent rate constants using model-independent analysis,
531 cumulative distributions were fitted with single and double exponential decays (Figure 3
532 and 4). Observed rates from exponential decay fits were plotted as a function of DNA
533 concentration. Apparent rate constants were calculated as slope of DNA concentration-
534 dependent observed rates or average of DNA concentration-independent observed
535 rates.

536 **Deterministic modeling**

537 Equations 1a-1c and 2a-2c were numerically integrated using the ODE15s and ODE45
538 solver in MATLAB. Microscopic rate constants were adjusted to minimize the sum of
539 squared residuals between ODE-determined concentration of bound or free TALE and
540 single molecule cumulative distributions using lsqnonlin in MATLAB. 68% confidence
541 intervals were estimated by performing 2000 or 8000 bootstrap iterations in which
542 residuals from the best fit of the model to the data were randomly re-sampled (with
543 replacement) and re-fitted. All scripts and source data required to run this MATLAB
544 program called **De**terminstic **M**odeling for **A**nalysis of complex **S**ingle molecule **K**inetics
545 (DeMASK) are publicly available on GitHub at https://github.com/kgeigers/DeMASK.
546

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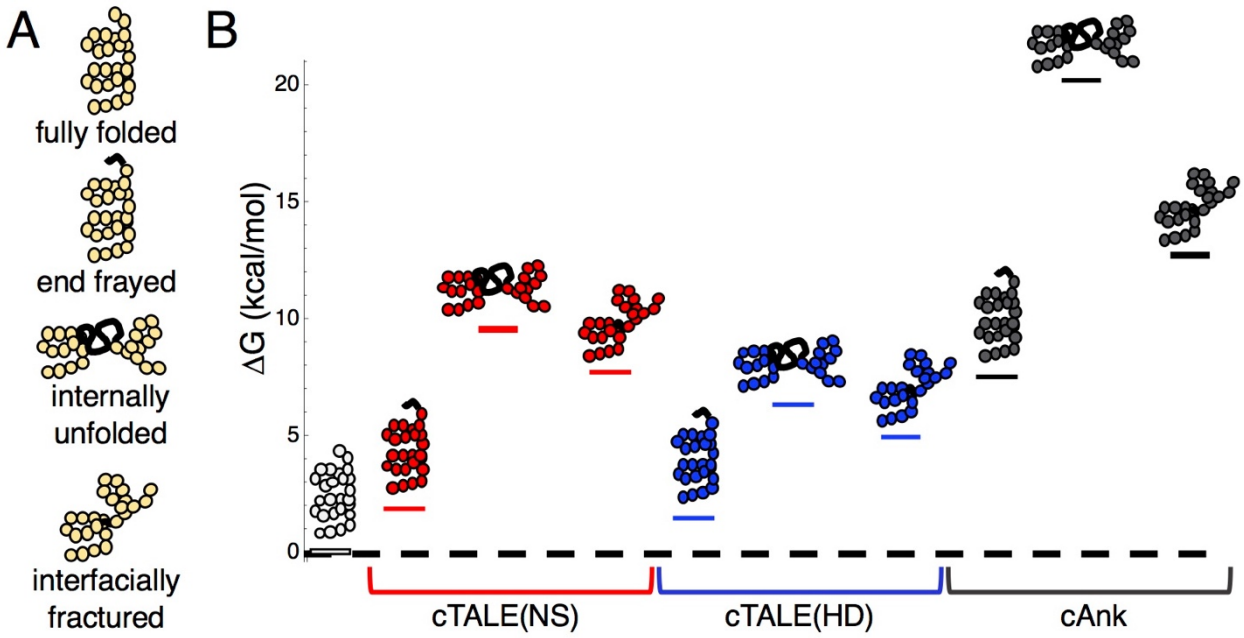


Figure 1. cTALEs populate partly folded states. (A) Cartoon of different partly folded TALE conformational states. End-frayed states have one (or more) terminal repeats unfolded. Internally unfolded states have a central repeat unfolded. Interfacially fractured states have a disrupted interface between adjacent repeats. (B) Free energies of partly folded states, calculated from previously published measurements and analysis (Geiger-Schuller and Barrick, 2016), relative to the fully folded state, for consensus TALE repeats with the NS repeat-variable diresidue sequence (cTALE(NS), red), consensus TALE repeats with the HD repeat-variable diresidue sequence (cTALE(HD), blue), and consensus ankyrin repeats (cAnk, black).

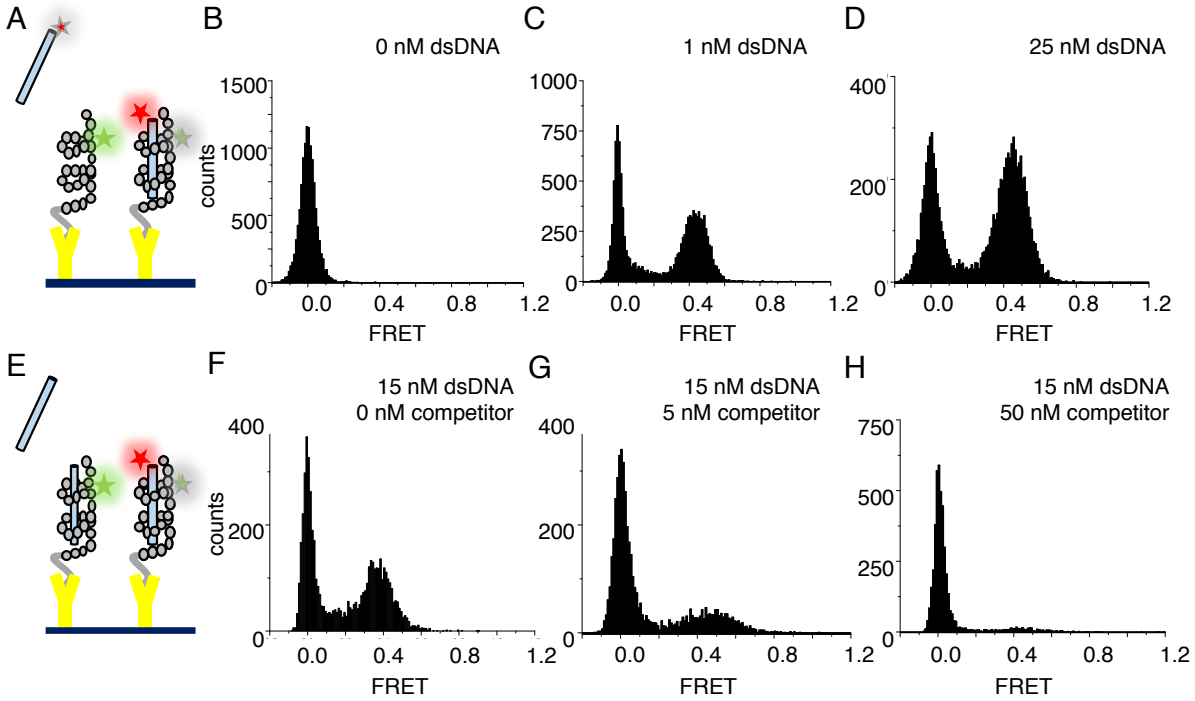
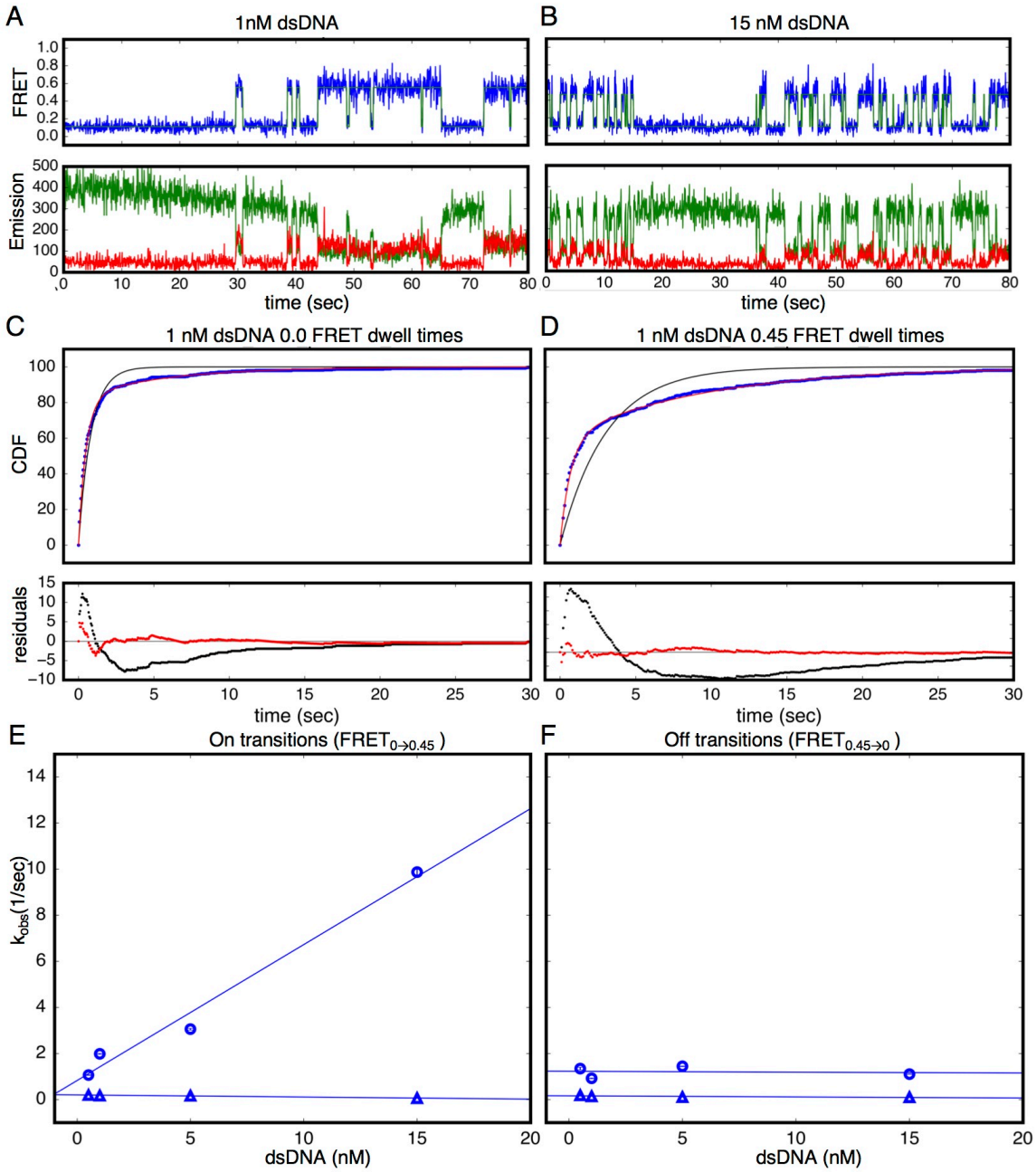


Figure 2. cTALEs bind dsDNA reversibly. (A) Schematic of single-molecule FRET assay, with donor-labelled cTALE attached to a surface, and acceptor-labelled DNA free in solution. (B-D) Single molecule FRET histograms show the appearance of a peak at a FRET efficiency of 0.45 with increasing labelled DNA, consistent with a DNA-bound cTALE. (E) Schematic of single-molecule FRET competition assay, with donor-labelled cTALE attached to a surface, and acceptor-labelled DNA as well as competitor unlabeled DNA free in solution. (F-H) Single molecule FRET histograms show the disappearance of the peak at 0.45 FRET efficiency with increasing unlabeled competitor DNA. Conditions: 20 mM Tris pH 8.0, 200 mM KCl.



688

689 **Figure 3. Single Molecule (SM) kinetics show multiple phases in binding and**

690 **unbinding kinetics.** (A-B) Long time trajectories showing transitions between low- and

691 high-FRET states (efficiencies of 0 and 0.45). The top panel shows calculated FRET

692 efficiency in blue and two-state Hidden Markov Model fit in green (McKinney et al.,

693 2006). The bottom panels show Cy3 and Cy5 fluorescence emission in green and red

694 respectively. At low DNA concentration (A), the low FRET state predominates. As DNA
695 concentration is increased (B), more time is spent in the high FRET state, because the
696 dwell times in the low FRET state are shorter. At low DNA concentrations, there
697 appears to be long- and short-lived high-FRET states. Likewise, at near-saturating DNA
698 concentrations, there appear to be long and short-lived low FRET states. (C, D)
699 Cumulative distributions of low- and high-FRET dwell times (blue circles). Fits to single-
700 exponentials (black) show large nonrandom residuals (lower panels), consistent with the
701 heterogeneity noted in (A) and (B). Double-exponentials (red) give smaller, more
702 uniform residuals. (E) Apparent association rate constants as a function of DNA
703 concentration. The apparent rate constants for the fast phase depend on DNA
704 concentration (blue circles), indicating a bimolecular step binding event. The apparent
705 rate constants for the slow phase do not depend on DNA-concentration (blue triangles),
706 suggesting an isomerization event. (F) Apparent dissociation rate constants as a
707 function of DNA concentration (phase 1 shown in blue circles, and phase 2 shown in
708 blue triangles). Neither phase shows a DNA concentration dependence, indicating a
709 dissociation and/or isomerization events. 67.4% confidence intervals are estimated
710 using the conf_interval function of lmfit by performing F-tests (Newville et al., 2014).
711 Conditions: 20 mM Tris pH 8.0, 200 mM KCl.
712 **Figure supplement 1**. Alternating laser experiments show agreement between $cTALE_8$
713 FRET and colocalization kinetics.
714 **Figure supplement 2**. cTALEs do not slide onto ends of short dsDNA.
715 **Source data 1.** List of values used to construct long time trajectories.

716 **Source data 2.** List of values used to construct CDFs.

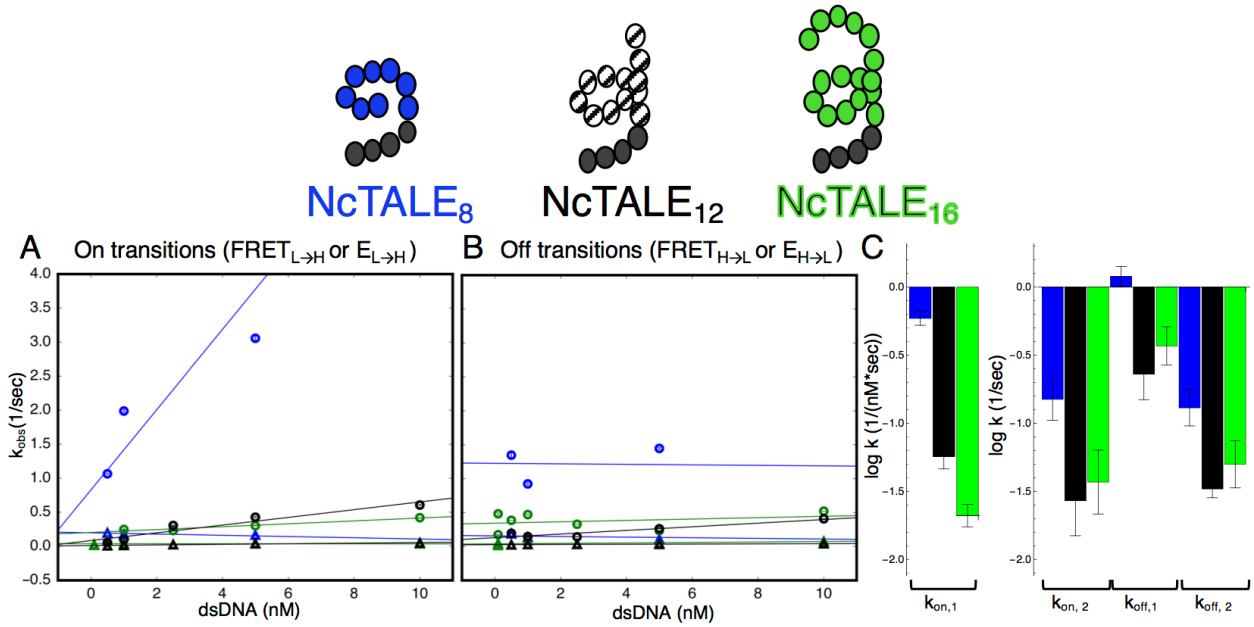
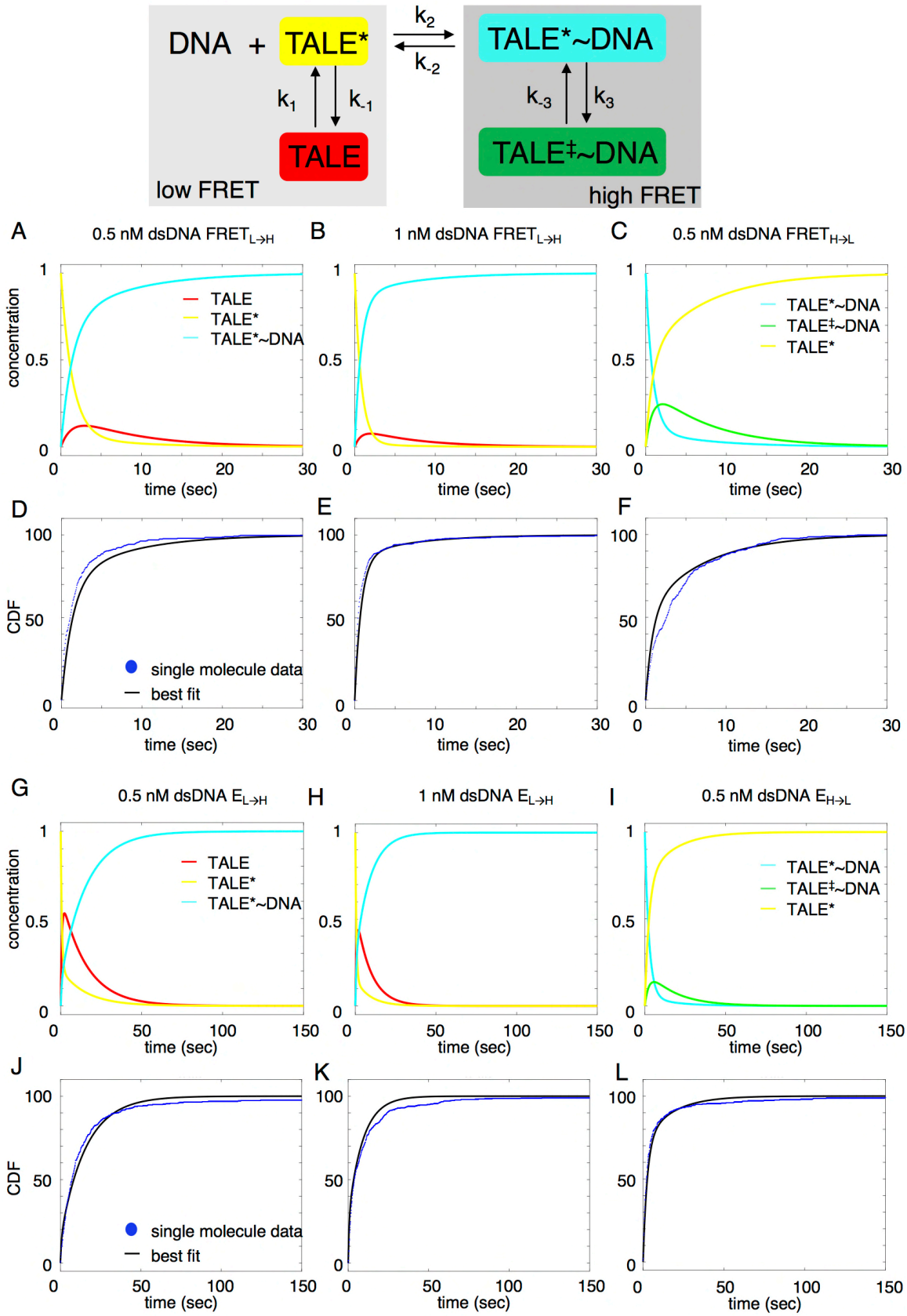


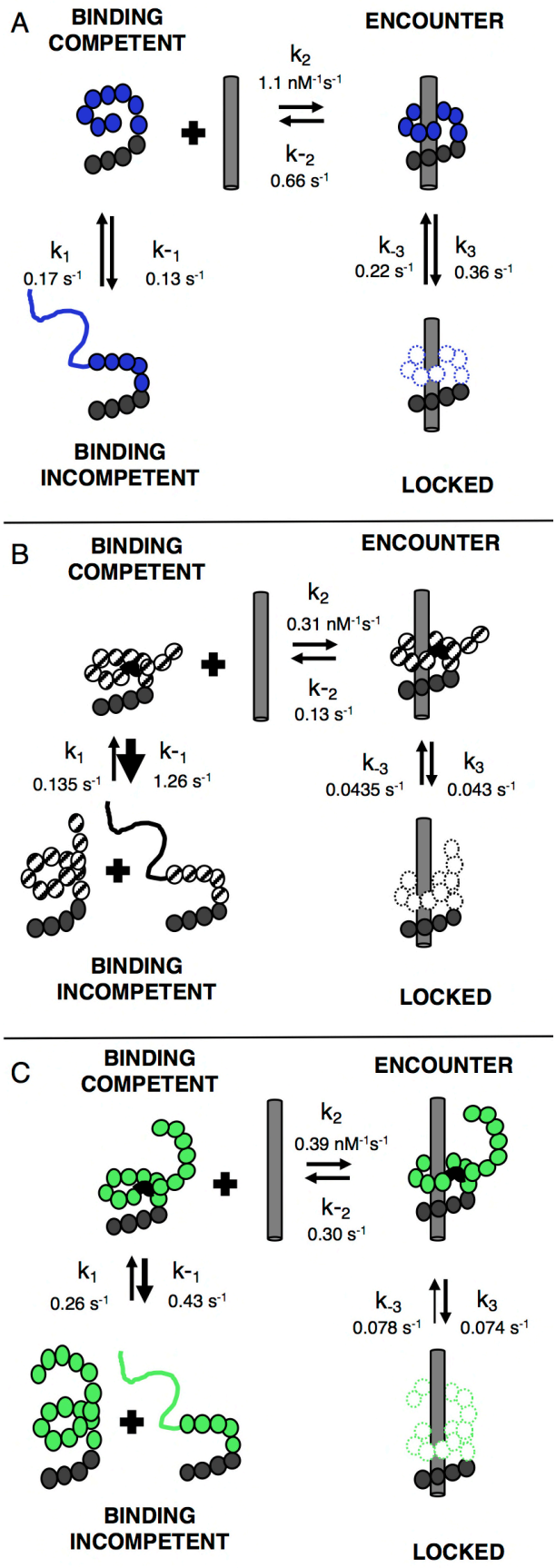
Figure 4. A 16-repeat TALE protein binds and unbinds DNA more slowly than an eight repeat protein. (A) Apparent association rate constants as a function of DNA concentration for an 8 repeat cTALE (blue), a 12 repeat cTALE (black), and a 16 repeat cTALE (green). 8 repeat TALE kinetics are measured by FRET ($FRET_{L\rightarrow H}$) while 12 and 16 repeat TALE kinetics are measured by colocalization ($E_{L\rightarrow H}$). The apparent rate constants for the fast phase of binding are DNA concentration dependent (blue, black, and green circles), indicating a bimolecular binding event. The DNA concentration-dependence is greatest (larger slope) for the 8 repeat cTALE. The apparent rate constants for the slow phase do not depend on DNA-concentration (blue, black, and green triangles), suggesting an isomerization event. (B) Apparent dissociation rate constants as a function of DNA concentration (phase 1 shown in circles, and phase 2 shown in triangles). Neither phase shows a DNA concentration dependence, indicating a dissociation and/or isomerization events. Rate constants for all phases are slower for

732 the 12-repeat construct (black) and 16-repeat construct (green) than for the 8-repeat
733 construct (blue), particularly for the bimolecular binding step. (C) Log_{10} of rate constants
734 for 8 (blue), 12 (black), and 16(green) repeat cTALEs. Units of the bimolecular binding
735 rate constant are $nM^{-1}s^{-1}$, other unimolecular rate constants have units s^{-1}. 67.4%
736 confidence intervals are estimated using the conf_interval function of lmfit by performing
737 F-tests (Newville et al., 2014). Conditions: 20 mM Tris pH 8.0, 200 mM KCl.
738 **Figure supplement 1**. Colocalization trajectories show TALE-DNA binding and
739 unbinding events.
740 **Figure supplement 2**. Bound and unbound lifetimes of 16- and 12-repeat TALE
741 proteins are consistent with multiphasic binding and unbinding.
742 **Figure supplement 3**. Distance estimates between labeling sites for $NcTALE_8$ and
743 $NcTALE_{16}$ and the 5' ends of bound DNA.
744


DNA + TALE*
k2
k-2
TALE*~DNA
k1
k-1
k-3
k3
TALE
TALE‡~DNA
low FRET
high FRET
A
0.5 nM dsDNA FRETL→H
B
1 nM dsDNA FRETL→H
C
0.5 nM dsDNA FRETH→L
concentration
TALE
TALE*
TALE*~DNA
TALE‡~DNA
time (sec)
D
E
F
CDF
single molecule data
best fit
G
0.5 nM dsDNA EL→H
H
1 nM dsDNA EL→H
I
0.5 nM dsDNA EH→L
J
K
L

745

746 **Figure 5. Deterministic simulations provide evidence for conformational**
747 **heterogeneity in the unbound state.** The model most consistent with data is shown at
748 the top. Unbound TALEs can exist in DNA-binding competent (TALE*) or DNA-binding
749 incompetent (TALE) states. DNA-bound TALEs can exist in short-lived (TALE*~DNA) or
750 long-lived ($TALE^{‡}$~DNA) DNA-bound states. Cumulative distributions of dwell-times
751 (shown as blue points) from 8 repeat single-molecule time trajectories (A-F) and 16
752 repeat single-molecule time trajectories (G-L) were analyzed with the model (best-fit
753 shown in black). (A-C and G-I) Populations of states as a function of time, generated by
754 numerical integration in Matlab. (D-F and J-L) Cumulative distributions in blue circles
755 and best fit lines are shown in black. Best-fit microscopic rate constants and 68%
756 confidence intervals are listed in Table 1.
757


A
BINDING COMPETENT
ENCOUNTER
k2
1.1 nM-1s-1
k-2
0.66 s-1
k1
0.17 s-1
k-1
0.13 s-1
k-3
0.22 s-1
k3
0.36 s-1
BINDING INCOMPETENT
LOCKED
B
BINDING COMPETENT
ENCOUNTER
k2
0.31 nM-1s-1
k-2
0.13 s-1
k1
0.135 s-1
k-1
1.26 s-1
k-3
0.0435 s-1
k3
0.043 s-1
BINDING INCOMPETENT
LOCKED
C
BINDING COMPETENT
ENCOUNTER
k2
0.39 nM-1s-1
k-2
0.30 s-1
k1
0.26 s-1
k-1
0.43 s-1
k-3
0.078 s-1
k3
0.074 s-1
BINDING INCOMPETENT
LOCKED

758

759 **Figure 6. TALEs with multiple superhelical turns must break to bind DNA.** Single-
760 molecule FRET studies and deterministic modeling support a model where TALEs exist
761 in four states: binding incompetent, binding competent, encounter complex, and locked.
762 In this model, for TALEs that form less than one full superhelical turn (8 repeats, A),
763 partly folded states are off-pathway and slow down binding. For longer TALEs that form
764 one (12, B) or more (16, C) complete superhelical turns, partial unfolding is required for
765 binding. DNA-bound TALEs form both encounter complexes and higher-affinity locked
766 conformations. Dynamics of long (12 and 16-repeat; B-C) TALEs bound to DNA are
767 significantly slower than for the shorter (8-repeat; A) TALE.
768 **Figure supplement 1**. Urea and destabilizing mutations decrease apparent binding rate
769 of $cTALE_8$.
770

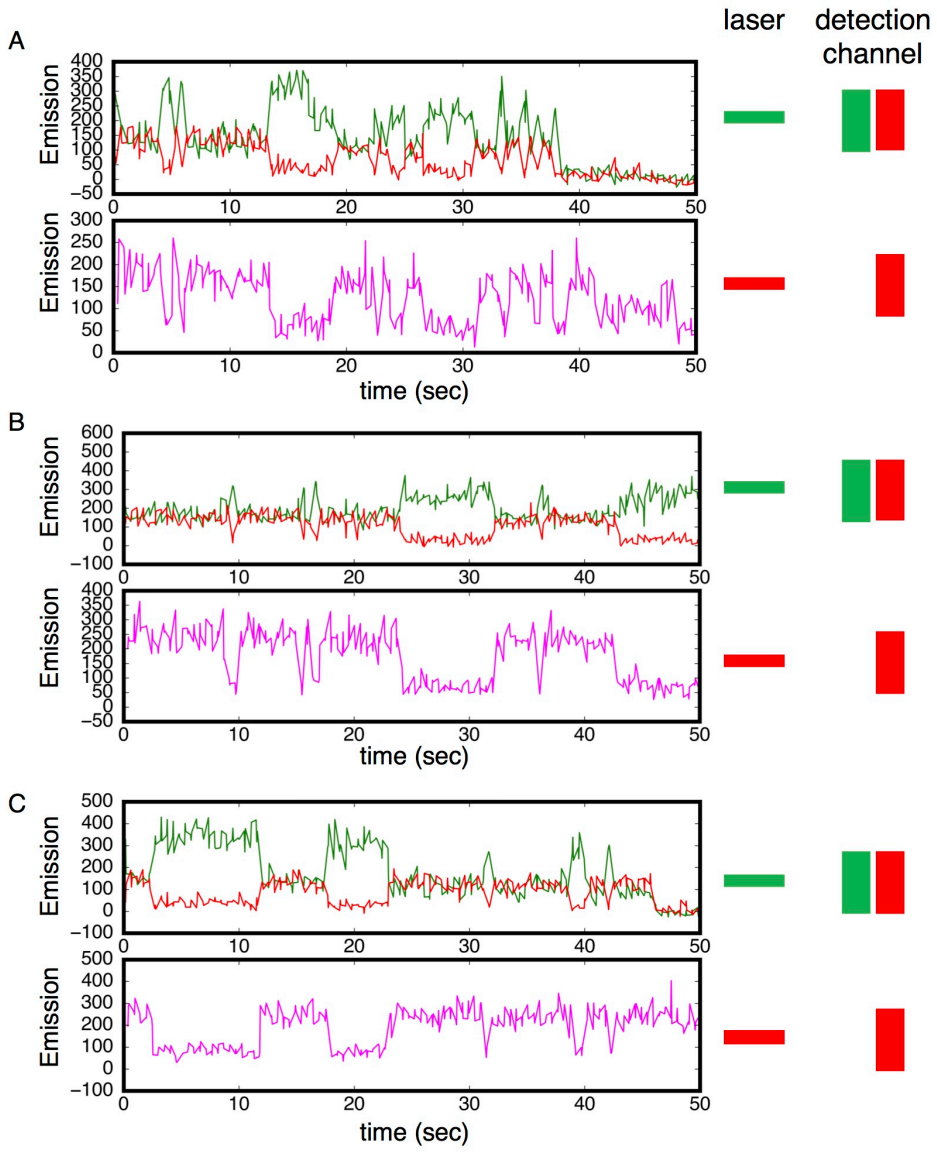
Table 1. Kinetic parameters obtained from deterministic simulation fits.

	k_1 (sec^{-1})	k_{-1} (sec^{-1})	$K_{eq,\ DNA\text{-}free}$	k_2($sec^{-1}nM^{-1}$)	k_{-2} (sec^{-1})	k_3 (sec^{-1})	k_{-3} (sec^{-1})	$K_{eq,\ DNA\text{-}bound}$ (nM^{-1})
$NcTALE_8$ [a]	0.17 [0.16, 0.18]	0.13 [0.12, 0.14]	1.32 [1.26, 1.39]	1.1 [1.08, 1.12]	0.66 [0.65, 0.67]	0.36 [0.35, 0.37]	0.222 [0.218, 0.227]	1.62 [1.58, 1.66]
$NcTALE_{12}$ [b]	0.135 [0.133, 0.137]	1.26 [1.09, 1.34]	0.11 [0.10, 0.12]	0.31 [0.28, 0.33]	0.130 [0.129, 0.131]	0.043 [0.042, 0.044]	0.0435 [0.0428, 0.0442]	0.99 [0.97, 1.00]
$NcTALE_{16}$ [a]	0.26 [0.25, 0.27]	0.43 [0.39, 0.47]	0.61 [0.57, 0.64]	0.39 [0.38, 0.41]	0.299 [0.298, 0.300]	0.074 [0.073, 0.076]	0.078 [0.076, 0.079]	0.96 [0.95, 0.97]

68% confidence intervals shown in brackets are from 2,000[a] and 8,000[b] iterations of bootstrap analysis.

771

772 **Supplemental Material**



773

774 **Figure 3- figure supplement 1. Alternating laser experiments show agreement**

775 **between cTALE$_8$ FRET and colocalization kinetics.** (A-C) Three representative time

776 trajectories. In these trajectories, excitation alternated between red and green with 250

777 msec at each color. Top panels show detection of red and green light resulting from

778 green laser excitation. High red (and low green) fluorescence result from FRET. Lower

779 panels show detection of red light resulting from red laser excitation. There is strong

780 correlation between periods of high FRET in the upper panels (high red fluorescence,

781 low green fluorescence) and periods of high red fluorescence (from DNA binding) in the

782 lower panel. Conditions: 200 mM KCl, 20 mM Tris pH 8.0.

783

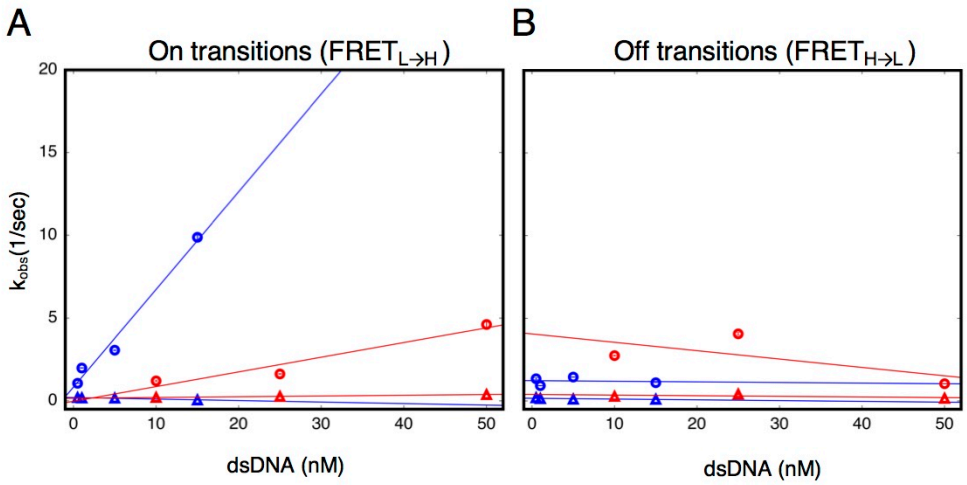


Figure 3 – figure supplement 2. cTALEs do not slide onto ends of short dsDNA. Apparent association and dissociation rate constants as a function of DNA concentration for an 8 repeat cTALE array (NcTALE$_8$) binding to uncapped (blue) and capped (red) DNA, measured by single molecule FRET dwell time analysis. (A) Rate constants for conversion from the low to the high FRET state (FRET$_{L\rightarrow H}$). Rate constants for the faster phase increase with DNA concentration (circles), indicating a bimolecular event. The DNA concentration-dependence is stronger (larger slope, k=0.59 ± 0.08 nM^{-1} sec^{-1}) with uncapped DNA than with capped DNA (k=0.09 ± 0.05 nM^{-1} sec^{-1}), which is likely a result of the faster diffusion of small, uncapped DNA (10 kDa) compared to large, capped DNA (320 kDa). Rate constants for the slower phase are not DNA-concentration dependent (triangles). (B) Rate constants for conversion from the high to the low FRET state (FRET$_{H\rightarrow L}$). Neither phase shows a DNA concentration dependent, indicating unimolecular steps. Conditions: 200 mM KCl, 20 mM Tris pH 8.0.

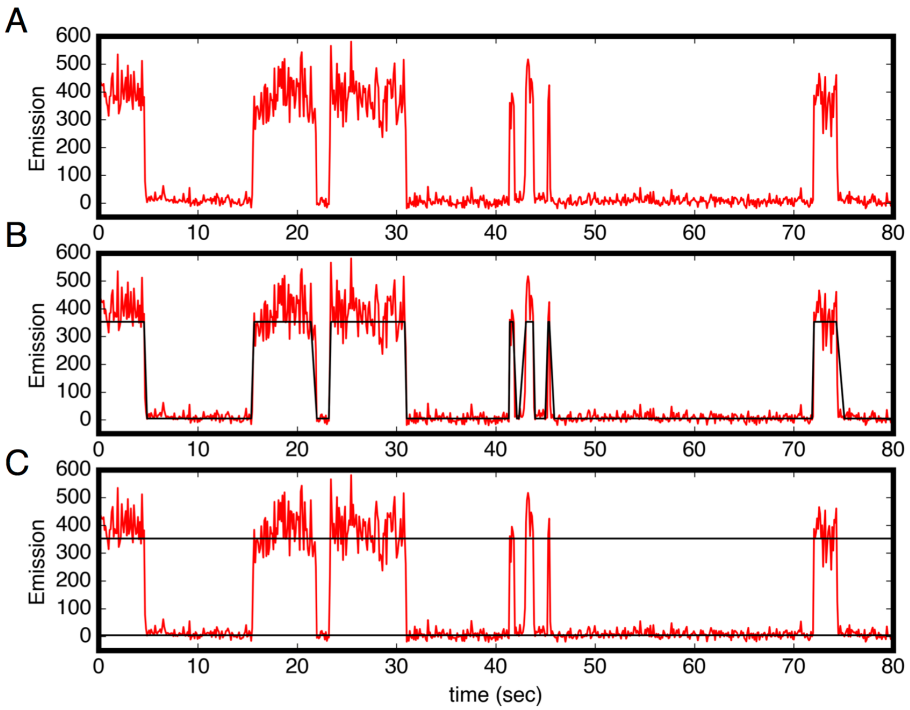


Figure 4- figure supplement 1. Colocalization trajectories show TALE-DNA binding and unbinding events. (A-C) One representative time trajectory and colocalization analysis for a sixteen repeat TALE, $NcTALE_{16}$, incubated with 1 nM Cy5-A_{15}/T_{15}. In the colocalization protocol, both the green and red lasers were used in the first ten frames (not shown) to identify single molecule locations. For all subsequent frames, Cy5-labeled DNA was continuously excited with the red laser. Red light emission images (from Cy5) were collected with time steps of 100 msec. Trajectories were generated from sites of red/green colocalization in the first ten frames, and were corrected for background Cy5 emission (red trajectories above). For each trajectory, we set two thresholds for low and high Cy5 emission (C). A molecule is designated to be in the high emission (DNA-bound) state above the high threshold, and in the low emission state below the low threshold. From this state-assignment procedure, dwell-times were

812 determined in the high (bound) and low (unbound) states (C). Conditions: 200 mM KCl,

813 20 mM Tris pH 8.0.

814

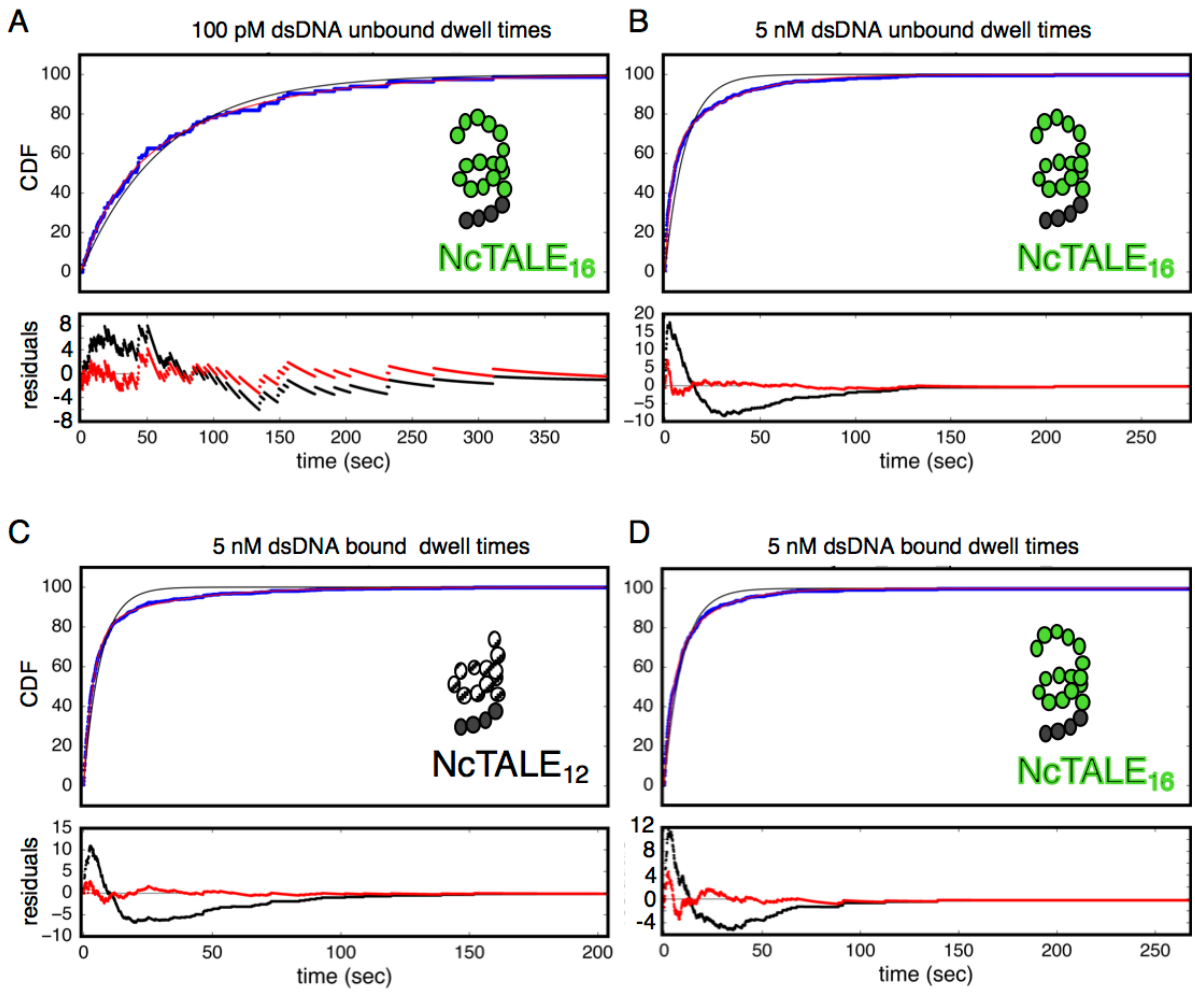


Figure 4- figure supplement 2. Bound and unbound lifetimes of 16- and 12-repeat TALE proteins are consistent with multiphasic binding and unbinding. (A, B) Cumulative distributions of low-FRET dwell times (blue circles) for a 16-repeat construct at low and high DNA concentration. Fits to single-exponentials (black) show large nonrandom residuals (lower panels) which are most pronounced at high DNA concentrations, consistent with the binding heterogeneity observed in eight repeat cTALEs (Figure 3). Double-exponentials (red) give smaller, more uniform residuals. (C, D) Cumulative distributions of high-FRET dwell times (blue circles) for 16- and 12-

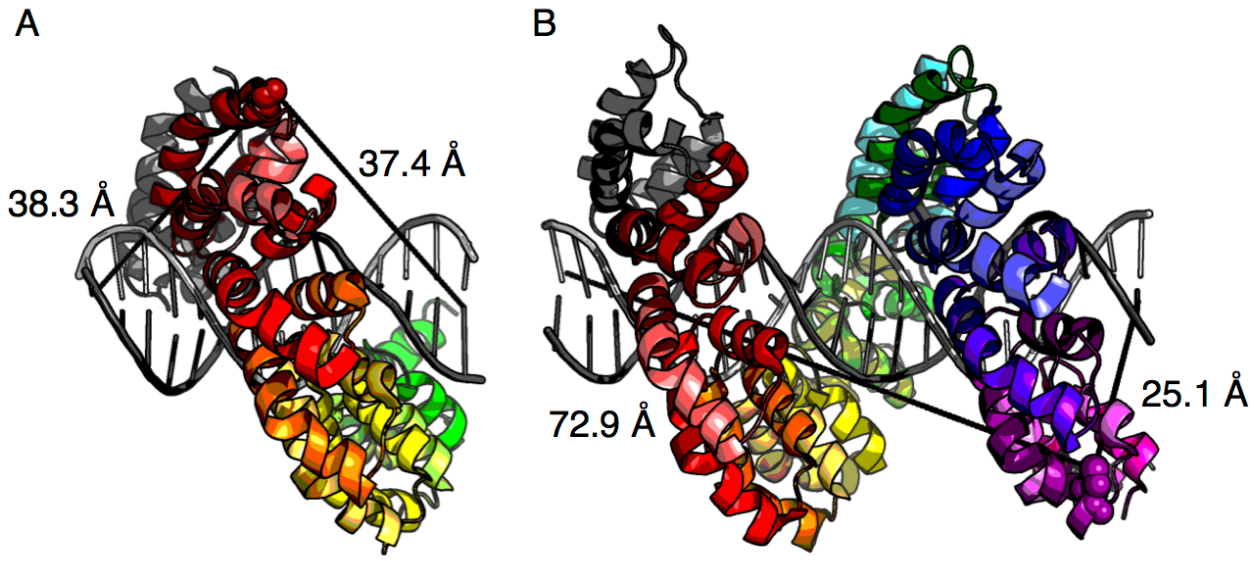
824 repeat constructs at high DNA concentration. Fits to single-exponentials (black) show

825 large nonrandom residuals (lower panels), consistent with the dissociation

826 heterogeneity observed in eight repeat cTALEs. Double-exponentials (red) give smaller,

827 more uniform residuals.

828



829

830 **Figure 4- figure supplement 3. Distance estimates between labeling sites for**

831 **$NcTALE_8$ and $NcTALE_{16}$ and the 5' ends of bound DNA.** The crystal structure of

832 PthXo1 (PDB: 3UGM) (Mak et al., 2012) in complex with DNA (colored by repeat) as a

833 model of donor-acceptor fluorophore distances . (A) The distance from C30 in the first

834 repeat (the site of labelling in $NcTALE_8$) to the 5' base of the sense strand (light grey) is

835 38.3Å; the distance to the 5' base of the antisense strand (dark grey) is 37.4Å. (B) The

836 distance from C30 in the fourteenth repeat (the site of labelling in $NcTALE_{16}$) to the 5'

837 base of the sense strand (light grey) is 72.9Å; the distance to the 5' base of the

838 antisense strand (dark grey) is 25.1Å. Note that although there is likely to be some

839 variation these distances since the DNAs used here are longer (15 and 23 bases) than

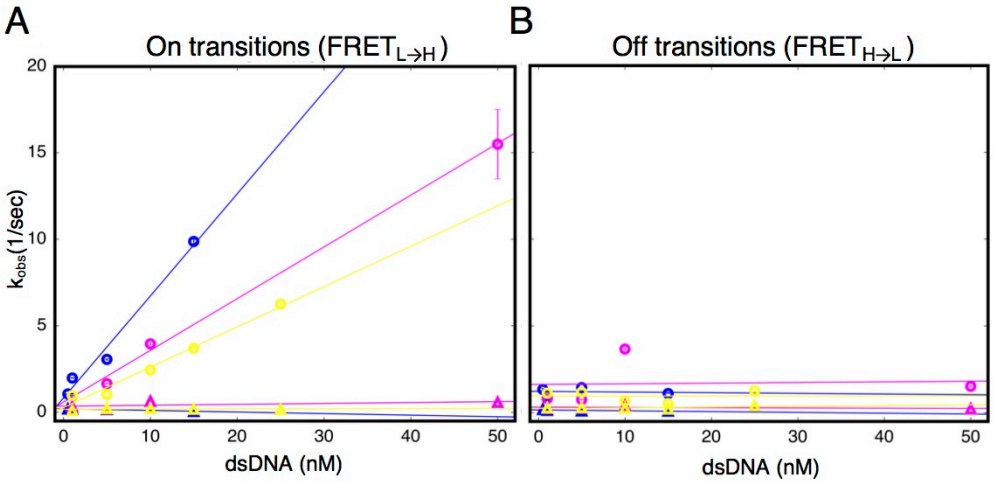
840 the TALE arrays (8 and 16 repeats, each with a four-repeat N-capping domain), the

841 differences in donor-acceptor distances for the 16 repeat construct (B) are likely to be

842 robust to registry shifts of a few bases.

843

844



845

846 **Figure 6- figure supplement 3 1. Urea and destabilizing mutations decrease**

847 **apparent binding rate of cTALE$_8$.** (A) Apparent association rate constants as a

848 function of DNA concentration for an 8 repeat cTALE in 0 M urea (blue), 1 M urea

849 (pink), and with destabilizing point mutations (yellow; mutations substitute leucine at

850 position 1 in the fourth repeat with a glycine). The DNA concentration dependence is

851 strongest (steepest slope) in the absence of urea and destabilizing point substitutions

852 (circles). Rates in the slower phase (triangles) appear unaffected by urea or mutational

853 destabilization. (B) Apparent dissociation rate constants as a function of DNA

854 concentration (phase 1 shown in circles, and phase 2 shown in triangles). Rates in the

855 both phases appear unaffected by urea and mutational destabilization. Conditions: 200

856 mM KCl, 20 mM Tris pH 8.0.

857

858 **Source Data**

859 **Figure 3- source data 1. List of values used to construct long time trajectories.**

860 Long time trajectories showing transitions between low- and high-FRET states

861 (efficiencies of 0 and 0.45) for 1 nM dsDNA (source data 1A) and 15 nM dsDNA (source

862 data 1B) depicted in Figure 3A-B.

863 **Figure 3- source data 2. List of values used to construct CDFs.** Cumulative

864 distributions of low-FRET (source data 2A) and high-FRET (source data 2B) dwell times

865 depicted in Figure 3C-D.