Supporting Information

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SI Text

Materials and Data Acquisition. Because only relative movements of proteins on the contour of DNA of trajectories were recorded in our earlier studies (1), we collected new data for this study that allowed us to obtain information on the position of the protein with respect to the underlying DNA sequence.

Before labeled p53 was introduced to the flow cell, a 0.016% suspension of biotinylated beads were flowed in with a concentration and incubation time such that 2–5 beads appeared in each field of view, following Elenko (2). When the flow cell had been studded with beads, movies were taken of p53 sliding on flow-stretched λ-phage DNA, flowing 100 μL/min through a flow cell 2 mm wide, 100 μm tall, and 36 mm long p53 sliding buffer consisted of 20 mM HEPES (equilibrated to pH 7.9 with NaOH), 150 mM KCl, 0.5 mM EDTA, 2 mM MgCl2, 0.25 mg/mL BSA, and 2.5 mM DTT, p53 concentrations were between 50 and 150 pM. Fig. 2B shows a kymogram of a single p53 molecule sliding on DNA. At the end of the experiment, DNA was visualized with Sytox Orange (Invitrogen) and movies of it taken.

The beads were present at fixed locations for both the protein movies and the DNA movies, which allowed p53 trajectories to be aligned to positions on DNA (SI Text, Data Analysis) despite stage drift. This alignment additionally required lower concentrations of DNA than in earlier work, because the DNA could not be so dense as to prevent us from assigning protein particles to a distinct DNA molecule. In the previous work, stained DNA illuminated nearly the entire field of view; in the current work, DNA concentration was lowered to approximately 40 DNA molecules per field of view.

A further difference from our earlier work is that, in this study we did not impose artificial minimum trajectory lengths or durations. All trajectories that our tracking scripts identified are included in the present work’s analysis, excepting those of particles that we identified as being on the flow cell surface rather than bound to DNA. Our criterion for being stuck to the surface was having an end-to-end displacement less than half a pixel (85 nm). This distance is nearly the standard deviation in the frame-to-frame displacement of the quantum dot (QD) nearest the tether (368 bp, or 100 nm assuming the DNA is 80% stretched), and so a particle bound to DNA but not sliding on the DNA contour would be expected to move this distance within a single frame. The particles eliminated by this cutoff were clearly distinguishable from particles bound to DNA, as can be seen in Fig. 3B. The cutoff of 85 nm corresponds to an end-to-end squared displacement of 10^5 bp^2, which is where the first gray circle representing the QD nearest the tether lies. The share of particles with end-to-end squared displacements vanishes as the cutoff is approached from above, making us confident that we did not discard particles bound to DNA but trapped at a near-cognate site or otherwise immobile.

Because Brownian dynamics simulations (SI Text, Data Analysis) show no part of the DNA molecule stretched beyond 90% of its contour length, we conclude that the stretching is largely entropic rather than enthalpic—that is, bond lengths and angles and local conformations are negligibly affected by the buffer flow—we assume that the dependence of the stretching in the DNA as a function of position does not appreciably affect the sliding kinetics or binding thermodynamics of p53 to the DNA.

Data Analysis. To map the spatial positions in our movies of protein particles to positions on the contour of DNA, Brownian dynamics simulations of DNA as a tethered polymer in shear flow were performed to determine the degree of compression in the DNA as a function of the distance along the contour from the tether (3). Integrating and inverting this function yields a function that transforms positions in recorded images to positions along the sequence of λ-phage DNA.

For each p53 trajectory mapped to the DNA contour, a drift rate, v, and diffusion coefficient, D, were determined using maximum likelihood estimation (MLE). Assuming that a particle’s displacement due to drift is independent of its displacement due to diffusion, and that the particle’s displacements are all independent, the MLEs for a particle’s v and D in the absence of DNA fluctuations are derived as follows:

\[ p(\Delta x; v, D) = \exp\left(-\frac{(\Delta x - v\Delta t)^2}{4D\Delta t}\right)(4\pi D\Delta t)^{-1/2} \]

\[ L(\Delta x_1, \ldots, \Delta x_n|v, D) = \exp\left(\sum_i^{n-1} \frac{-(\Delta x_i,v - v\Delta t_i)^2}{4D\Delta t_i}\right) \prod_i^{n-1} \left(4\pi D\Delta t_i\right)^{-1/2} \]

\[ \log L = -\sum_i^{n-1} \frac{(\Delta x_i,v - v\Delta t_i)^2}{4D\Delta t_i} - \frac{1}{2} \sum_i^{n-1} \log(4\pi D\Delta t_i). \]  

where \( \Delta x_i,v \) is displacement i of the protein on DNA, which takes place over the duration \( \Delta t_i \). Taking the partial derivative of \( \log L \) with respect to the drift rate, \( v \), and setting the result equal to zero,

\[ 0 = \frac{\partial \log L}{\partial v} = \sum_i^n \frac{2(\Delta x_i,v - v\Delta t_i)}{4D\Delta t_i} - 2v = \sum_i^n \frac{\Delta x_i,v - v\Delta t_i}{\Delta t_i}. \]  

Here, the index \( i \) is over the largest non-overlapping set of \( \Delta x_i,v / \Delta t_i \), which are the final and initial frames of each trajectory \( j \), so:

\[ v = \frac{\sum_j^{all traj.} x_{j,final} - x_{j,initial}}{\sum_j^{all traj.} t_{j,final} - t_{j,initial}}. \]  

We now take the partial derivative with respect to the diffusion coefficient, \( D \), and equate to zero:

\[ 0 = \frac{\partial \log L}{\partial D} = \frac{n}{2} \sum_i^n \frac{(\Delta x_i,v - v\Delta t_i)^2}{4D^2\Delta t_i} - \frac{1}{2} \sum_i^n \frac{1}{D} = \sum_i^n \frac{(\Delta x_i,v - v\Delta t_i)^2}{\Delta t_i} - 2nD \]

\[ D = \frac{1}{2n} \sum_i^n \frac{(\Delta x_i,v - v\Delta t_i)^2}{\Delta t_i} \]

\[ D = \frac{1}{2n} \sum_i^n \frac{\Delta x_i,v^2 - 2\Delta x_i,v\Delta t_i + \Delta t_i^2}{\Delta t_i}. \]
The observed displacements, $\Delta x_i$, are in fact the sum of displacement from protein diffusion, $\Delta x_{i,p}$, and displacement from DNA fluctuations, $\Delta x_{i,d}$. Substituting $\Delta x_{i,p}$ with $\Delta x_i - \Delta x_{i,d}$ in Eqs. S2 and S4, and substituting $\Delta x^2$ with $2\Delta x_{i,p} - \Delta x^2_{i,b}$ in Eq. S4, yields the following:

$$D = \frac{1}{2n} \sum_i^n (\Delta x_i + 2\Delta x_{i,p} - 2\Delta x_{i,d} - \Delta x^2_{i,b}) = 2\Delta x_{i,p} - 2\Delta x_{i,d} - \Delta x^2_{i,b}.$$ \[S5\]

Separating the terms under the sum in the expression for $v$ gives

$$v = \sum_i^n \Delta x_i + \sum_i^n \Delta x_{i,d} - \sum_i^n \Delta t_i.$$ \[S6\]

The second term in Eq. S7 vanishes because the displacements due to DNA fluctuations, $\Delta x_{i,d}$, have mean zero, and so the drift rate is simply that given in Eq. S2. In the equation for $D$ [S6], the DNA displacements are likewise independent of the protein displacements, $\Delta x_{i,p}$, and the drift, $\nu \Delta t_i$, so the sums of the cross terms $2\Delta x_{i,p} \Delta x_{i,d}$ and $2\Delta x_{i,d} \nu \Delta t$ also go to zero. Eliminating these terms and separating into four remaining sums yields

$$D = \frac{1}{2} \left( \frac{1}{n} \sum_i^n \Delta x_i^2 - \frac{1}{n} \sum_i^n \Delta x_{i,d}^2 - \frac{1}{n} \sum_i^n \Delta x^2_{i,b} \right) + \frac{1}{n} \sum_i^n \Delta t_i^2,$$ \[S8\]

This is equivalent to Eq. 2. The third and fourth terms are known from the expression of $v$ in Eq. S2 and from observed $\Delta x_i$ and $\Delta t_i$. The second term in Eq. S8 is equivalent to

$$v = \frac{1}{2} \sum_i^n \Delta x_i^2 - \frac{1}{n} \sum_i^n \Delta x_{i,b}^2,$$ \[S9\]

where $\Delta t_i$ is the number of displacements with duration $\Delta t$ in the trajectory, and $\Delta x_{i,b}$ are displacements owing to DNA fluctuation. Trajectories of DNA fluctuations were measured in previous work (4) by examining the trajectories of QDs covalently attached to λ-phage DNA at known positions. The expression in Eq. S9 is thus the expected contribution of DNA fluctuations to the apparent diffusion of the protein (SI Text, Interpolations of DNA-Fluctuation Variance and Distributions), the effect of which on our data and estimated diffusion coefficients is shown in Fig. S3.

Once a p53 particle’s diffusion coefficient had been determined, the diffusion coefficient was assigned to every midpoint of the particle’s trajectory’s displacements (Fig. 3C, dots). Data from the third of the DNA farthest from the tether was discarded owing to the large amplitude of DNA fluctuations beyond that point. The DNA was divided into segments with a width chosen equal to the mean end-to-end distance of remaining trajectories, approximately 2.9 kb (Fig. 3C, dashed lines). The mean of all the diffusion coefficients assigned to positions within each segment was calculated and then compared with the predicted diffusion coefficient based on theoretical energy landscapes. This method is equivalent to calculating the mean of $D$ over all particles contributing to a segment, weighted by the number of displacements each particle contributed.

**Significance and Consistency of Experimental Results.** Because the observed variation in $D$ among segments is not dramatic, we thought it especially important to see whether this variation was significant. If some segments truly have more rugged energy landscapes than others, then we expect lower-$D$ particles to be especially likely to be found in certain segments and higher-$D$ particles especially likely to be found in others. Randomizing the assignment of $D$ to particles would desegregate particles with different diffusivities, and so the difference between segments should decrease. We performed 1,000 such randomizations, and for each pair of segments $i$ and $j$, we calculated the absolute log-ratio of the two segments’ diffusion coefficients, $|\log(D_j/D_i)|$, as determined in Methods, Data analysis in the main text. Pairs of segments with significantly different $D_{\text{expt}}$ should only rarely have randomized absolute log-ratios greater than the absolute log-ratio for data where particles were assigned their observed $D$ rather than the $D$ from another, randomly selected particle. We found that 11 in 36 pairs had unrandomized absolute log-ratios greater than all but 5% of the absolute log-ratios from the shuffled data ($p < 0.05$), and that 6 of these pairs had unrandomized absolute log-ratios greater than all but 1% of the absolute log-ratios from the shuffled data ($p < 0.01$). The $p$-values of the pairs’ absolute log-ratios in $D$ are shown in Fig. S1.

Having a $D_{\text{expt}}$ that differed significantly from other segments’ $D_{\!\text{expt}}$ was imperfectly correlated with having an extreme $D_{\!\text{expt}}$. Segments 8 and 11, centered 20.4 kb and 29.3 kb from the tether, had nearly identical $D_{\!\text{expt}}$ deriving from 54, and so segment 8 differed significantly from 3 out of 8 other segments at $\alpha = 0.01$ and from another segment at $\alpha = 0.05$, while segment 11 differed significantly from no other segment. We are unsure of why this is the case; we conjecture that it might owe to segment 8’s $D_{\!\text{expt}}$ deriving from 68 particles while segment 11’s $D_{\!\text{expt}}$ derives from 54, and so segment 8’s $D_{\!\text{expt}}$ when computed with randomly reassigned $Ds$ from other segments will average over more particles’ $Ds$ and thus be less likely to take on a value far enough from the mean $D$ of all particles to produce absolute log-ratios with other segments that exceed the absolute log-ratios between segment 8 and other segments without random reassignment. If this is correct, then observing more particles would help us resolve differences between more segment pairs than we currently can.

Although 11 in 36 segment pairs differed significantly in their $D_{\!\text{expt}}$, most did not. This owes in part to many pairs having predicted landscapes of similar ruggedness and thus similar expected $D_{\!\text{expt}}$. That these pairs of segments have $D_{\!\text{expt}}$ values that cannot be solidly differentiated accords with our main result: Segments with similar theoretical $D/D_0$ are expected to have similar $D_{\!\text{expt}}$. Additionally, there is substantial individuality among the particles, as can be seen in Fig. 3C. To assess the effect on the range of $Ds$ among the particles in a segment on the segment’s $D_{\!\text{expt}}$, we employed a bootstrapping procedure. For each segment, we discarded from each particle’s set of displacements those displacements that fell outside the segment. If $N$ particles contributed to the segment, we sampled with replacement $N$ times, with sampling probability for a particle proportional to the number
of displacements contributed by the particle. We performed 10,000 such resamples for each segment. The distributions of the resulting $D_{\text{expt}}$s are shown in Fig. S4A.

To get a sense of the sufficiency of the statistics we collected, we performed an identical bootstrapping procedure, but sampling from the $N$ particles in a segment only $N/2$ times. This simulates having collected only half the data we did. The greater range in resampled $D_{\text{expt}}$ appears as cyan error bars in Fig. 3. That halving the data noticeably widens the bootstrap uncertainty estimate suggests, similarly to what was mentioned above, that more particles would allow us to sharpen our estimates of $D_{\text{expt}}$.

In addition to examining heterogeneity among particles’ observed $D$, we grouped our data according to data-collection session (a morning or an afternoon) in order to assess consistency across time and p53 aliquots. We used the same bootstrapping method described earlier in this section, but with sampling on the level of batches rather than particles. Fig. S4B shows the distributions of the bootstrapped $D_{\text{expt}}$ estimates. For some segments, the distribution is wider than is the corresponding distribution for bootstrapped $D_{\text{expt}}$ based on particle rather than batch resampling, which may mean that the differences between a few batches and average behavior owe to inherent variation in the batch. On the other hand, some segments are underrepresented in some batches, and on the level of a batch may have insufficient averaging, giving an aberrant $D$ for that particular batch-segment pair. Additionally, the smaller number of batches (10) than particles per segment ($60 \sim 70$) causes some of the distributions in Fig. S4B to not be bell-shaped.

An additional combinatorial test we performed was to randomize the location of particle trajectories on the DNA. If all the particles were undergoing uniform, position-independent Brownian motion, scrambling positional information would be expected to have little effect on the variation in $D_{\text{expt}}$ among segments. Results from five such randomizations are shown in Fig. S5. As can be seen, randomization reduces the variation in $D_{\text{expt}}$.

For every segment, the number of particles, the number of displacements, the estimates of $D_{\text{expt}}$ as determined in Methods, Data analysis in the main text, and the weighted standard deviation of particles’ $D$ are shown in Fig. 5E. As can be seen, $D_{\text{expt}}$ for a notional DNA segment is obtained from averaging over 60 \sim 70 actual p53-DNA complexes. Many particles contribute to multiple segments on the same DNA molecule (for instance, in Fig. 3A). Most DNA molecules contribute only a single trajectory or two distant trajectories, and therefore we cannot assess the extent to which different p53 particles in the same actual segment of a single DNA molecule behave uniformly relative to each other. Despite this, the large number of molecules contributing to each approximately 2.9-kb division of $\lambda$-phage DNA allows us to determine the diffusive properties of p53 particles in the aggregate within those divisions. This segment width is much larger than the error in uncertainty in particle position assignment ($\sim 500$ bp, from measurements of DNA fluctuations alone; see SI Text, Interpolations of DNA-Fluctuation Variance and Distributions), and we take this error into account when predicting segments’ aggregate $D_{\text{expt}}$.

**Alternative Data Analysis.** The estimation of particle diffusivity in SI Text, Data Analysis as well as in the main text treats each p53 particle as if it were undergoing normal diffusion, with a constant diffusion coefficient $D$. This $D$ aggregates the base-pair–level nonuniformity of the energy landscape experienced by a particle. We also analyzed our data using a treatment that does not attempt to assign particles a diffusion coefficient, but rather calculates the variance in all displacements by all particles in a segment.

In this treatment, we fragmented trajectories wherever they crossed segment boundaries. Then, for each segment, every displacement within a trajectory or fragment was corrected for drift and normalized by dividing it by the square-root of its corresponding duration [S11]. A trajectory fragment for which we recorded $N$ frames would have $N-1$ displacements between adjacent frames, $N-2$ displacements between frames with one intervening frame, etc., to 1 displacement with $N-1$ intervening frames. These corrected and normalized displacements were then fit to a Gaussian distribution, and the variance of the fit distribution taken as the estimate of the segment’s diffusivity, comparable to twice a diffusion coefficient, $2D$.

$$\text{drift rate} = \frac{\sum_{\text{all traj}} x_{i,\text{final}} - x_{i,\text{initial}}}{\sum_{\text{all traj}} t_{i,\text{final}} - t_{i,\text{initial}}}$$

normalized displacements
$$\left\{ (x_{i,n} - x_{i,m}) - \text{drift rate} \cdot (t_{i,n} - t_{i,m}) \right\} / \sqrt{t_{i,n} - t_{i,m}} : n > m; i \text{ over all traj}. \right.$$}

$$\left\{ \Delta x_i / \Delta t_i \right\} \sim N(\mu, 2D), j \text{ over all normalized displacements.}$$

The index $i$ is over trajectories and fragments; indices $n$ and $m$ are over frames within a trajectory or fragment; and index $j$ is over all normalized displacements. Each normalized displacement is the sum of a Gaussian random variable owing to diffusion along the DNA with mean zero and variance $2\Delta t_i$, and another Gaussian random variable owing to fluctuations of the DNA molecule on which the proteins are diffusing. To account for the increase in apparent diffusion coefficient due to DNA fluctuations we determined the DNA’s longitudinal mean squared displacement (MSD) as a function of time window $\Delta t$, as discussed in SI Text, Interpolations of DNA-Fluctuation Variance and Distributions.

After $2D$ was determined for each segment according to Eqs. S10–S12, the average $\text{MSD}_{\text{DNA}}$ over all displacements for that segment was estimated to be the share of $2D$ owing to DNA fluctuations, and was subtracted:

$$2D_{\text{protein}} = 2D_{\text{apparent}} - \frac{1}{n} \sum_j \text{MSD}_{\text{DNA}}(\Delta t_j),$$

$j \text{ over all normalized displacements.}$

We compare the sequence-dependent diffusivity using this method to that discussed in the main text and SI Text, Data Analysis in Fig. S6. The alternative diffusivity, $D_{\text{alt}} = \frac{1}{2}D$, correlates better with theoretical $D/D_0$ ($r = 0.931$) than does the diffusion coefficient $D_{\text{expt}}$ using the MLE-based approach ($r = 0.810$). We nonetheless chose to present our results using $D_{\text{expt}}$, as $D_{\text{alt}}$ is less rigorously theorized, and does not allow us to report diffusion coefficients for individual particles.

**Prediction of Energy Landscape and Local Diffusion Coefficients.** Our work bears some similarity to a single-molecule study by Harada et al. (5) that found a dependence in the dissociation kinetics of RNA polymerase from $\lambda$-phage DNA both on GC content and on the presence or absence of known promoters or promoter-like sequences. We took the additional steps, however, of quantifying the match between every site on the $\lambda$ genome and our sequence of interest, using a position weight matrix (PWM), as well as quantifying the correlation between an energy landscape based on the scored genome and the observed kinetics of the protein.
We built an effective predicted landscape \( U(x) \) as follows. Every position on \( 
abla \) DNA was scored according to a PWM for a single dimer, based on a list of known p53 binding sites. The PWM we used closely resembled those derived from six other lists based on a variety of experimental techniques (Fig. S2B). As discussed in Methods in the main text, the differences between scores are assumed to be proportional to differences between corresponding half-site energies:

\[
E_R(x) - E_S = c(PWM(x) - PWM_S), \tag{S14}
\]

where \( PWM(x) \) is the score for position \( x \), and \( PWM_S \) is the score corresponding to binding energy in the \( S \) mode. Thus, in the event that a site scores equal to the reference score, the specific and nonspecific binding energies for p53 to that site will be equal. We chose a value for \( PWM_S \) based on studies of eukaryotic transcription factor binding energies on defective versions of their consensus sequences (6). It was observed that for all the transcription factors studied, binding weakened as the consensus sites were mutated to contain one and then two mismatches (equivalent to four bits), but then became no weaker with further mutations. We therefore chose a nonspecific reference score equal to the score of the best-scoring half-site minus four bits. Varying \( PWM_S \) by a bit in either direction had little effect on our results. The choice of a four-bit threshold receives some additional justification from fluorescence-recovery-after-photo-bleaching measurements of p53 and two other eukaryotic transcription factors that found all three transcription factors’ search dynamics to be similar (7).

The remaining unknown in Eq. S14 is the proportionality constant \( c \) that relates score to energy. Dissociation constants for p53 binding to the left-hand Mdm2 half-site as well as to random DNA are available from biochemical measurements (8). At our experimental conditions, p53 favors the Mdm2 half-site by a factor of 47 (8), and so for this half-site, we estimate \( E_R(x) - E_S = \log(47) \) \( k_B T = 3.9 k_B T \). Substituting this value into the left-hand side of Eq. S14, and the site’s PWM score minus \( PWM_S \) into the right-hand side gives a value for \( c \) of 0.97 \( k_B T/\text{nat} \) or 0.67 \( k_B T/\text{bit} \).

At any site \( x \), the protein may bind in four distinct modes owing to the left and right dimers being able to bind to either mode: (i) both dimers in \( S \); (ii) left dimer in \( S \), right dimer in \( R \); (iii) left dimer in \( R \), right dimer in \( S \); and (iv) both dimers in \( R \) (Fig. S2A). The statistical weight of a site \( x \) is thus the sum of the Boltzmann factors corresponding to each of the four modes:

\[
w(x) = e^{-2E_S} + e^{-(E_S + E_{X(S+x+\Delta)})} + e^{-(E_{X(S+x)})} + e^{-(E_{X(R+x)})+E_{X(R+x+\Delta)}+x)}.
\tag{S15}
\]

The constant \( c \) is a cooperativity term representing additional binding energy when both dimers are bound in specific mode. Its value was determined from Eq. S15 by substituting in energies for the left-hand and right-hand sites of the Mdm2 promoter as determined by Eq. S14 and our PWM scoring, and substituting experimental values for the \( K_d \) of the full Mdm2 site relative to the \( K_d \) for a random sequence. From this, we find \( c = -1.39 k_B T \), the negative sign indicating that the energy of a protein on a full-site that binds both component half-sites in specific mode is 1.39 \( k_B T \) lower than it would be absent any cooperativity.

A small (~10%) proportion of known p53-binding sites include a gap of 1–14 bp between half-sites. To allow gapped full-sites to be treated as such in our predicted energy landscape, \( E_R(x+\Delta) \) at each binding site was assigned as follows:

\[
E_R(x+\Delta) = \min_i (E_R(x + \Delta_0 + i) - c \log(f_i/f_0)):\n\tag{S16}
\]

where \( \Delta_0 \) is the length of a half-site, 10 bp, and thus the separation between half-site start positions in the absence of a gap. The index \( i \) is over gaps of length 0 to 14, and \( f_i \) is the frequency of gaps of length \( i \) in the dataset used to build the PWM. The second term under the minimum accounts for the suboptimal binding conformation the protein must adopt when binding to half-sites separated by a gap. As \( f_{i>0} < f_0 \), gapped full-sites suffer an energy penalty, while full-sites with zero gap suffer none.

Setting the energy scale such that \( E_S = 0 \), Eq. S15 becomes

\[
w(x) = 1 + e^{-E_R(x+\Delta)} + e^{-E_R(x)} + e^{-(E_R(x)+E_R(x+\Delta)+2x)}.
\tag{S17}
\]

A single-mode model would not include nonspecific binding and thus omit all but the final term in Eq. S17, and a model that disallowed hemi-specific binding would omit the middle two terms. From this function of the statistical weights across all positions, we may treat p53 as interacting with DNA on a “golf-course landscape,” the energy at position \( x \) of which is equal to the negative logarithm of \( w(x) \):

\[
U(x) = -\log w(x).
\tag{S18}
\]

We used the resulting effective landscape to calculate \( D_{\text{th}}, \) which is the effective energy at site \( x_i \), which is the \( i \)th site visited in trajectory \( x \). If the transition state for translocating between two sites is constant across all sites—equivalent to assuming that for any position on DNA, p53’s microscopic rates to step left and right are equal or that traps are isolated—then averaging over trajectories results in a uniform distribution of visits to all sites in a given segment, and

\[
\frac{t_s}{t_{\text{total}}} = \frac{\sum_i^n \exp(-2E_S)}{\sum_i^n \exp(-U(x_i))}.
\tag{S20}
\]

where \( U(x_i) \) is the effective energy at site \( x_i \), which is the \( i \)th site visited in trajectory \( x \). If the transition state for translocating between two sites is constant across all sites—equivalent to assuming that for any position on DNA, p53’s microscopic rates to step left and right are equal or that traps are isolated—then averaging over trajectories results in a uniform distribution of visits to all sites in a given segment, and

\[
\langle t_s/t_{\text{total}} \rangle = \frac{n \exp(-2E_S)}{\sum_i^n \exp(-U(x))}.
\tag{S21}
\]

where \( n \) is the number of sites in the segment. The right-hand side of Eq. S21 consists entirely of constants, and \( E_S \) is defined to be zero, so

\[
D = \frac{1}{n} \sum_i^n \exp(-U(x))^{-1};
\tag{S22}
\]

that is, the diffusion coefficient is diminished by a factor equal to the average of \( c \) raised to the effective energy in the segment. Because p53’s half-site-binding sequence logo is not perfectly
palindromic, \( \exp(-U(x)) \) was taken to be the mean for the forward and reverse strands.

Experimental \( D_{\text{exp}} \) was compared with predicted \( D/D_0 \) by calculating Pearson’s correlation coefficient \( r_{\text{exp}} \) for the two quantities over all the segments. Assessment of statistical significance was made using the permutation test described in Methods, Prediction of Diffusion Coefficients. Owing to the 10-bp half-site PWM having the bulk of its information content in two nucleotides three positions apart, permuting the \( PWM \) is not a viable control, as \( 10 - 3 = 7 \) out of \( 10^3 = 100 \) permuted \( PWM_S \) will closely resemble the original \( PWM \). We thus chose to permute the scores of the positions on \( \lambda \) DNA rather than permuting the \( PWM \). Each permutation of scores gives rise to a permuted \( ER(\lambda) \) and thus a control landscape \( U(\lambda) \) and corresponding control \( D/D_0 \) or \( D_{\text{exp}} \). To obtain \( p \)-values, we calculated an \( r_{\text{cal}} \) between each control \( D/D_0 \) and \( D_{\text{exp}} \). Reported \( p \) is the proportion of \( r_{\text{cal}} \) equaling or exceeding \( r_{\text{exp}} \).

**Non-specific Binding in Model Parametrization.** To parametrize our scored \( \lambda \) genome into an energy landscape, we used dissociation constants from in vitro affinity assays of p53 and 30-bp oligonucleotides bearing full-sites, half-sites, and random DNA (8). Because p53’s binding site is 20-bp long, it is possible that one or more noncognate sites are available for p53 to bind to on either side of the full- and half-sites. Indeed, oligonucleotides of only 26 bp have been used to study binding between p53 and its cognate sites (9), so it is not improbable that a 30-bp oligonucleotide can accommodate p53 binding at least four noncognate sites. If this is the case, then the apparent preference of p53 for half-site 30-mers relative to random 30-mers, of approximately a factor of 8, reflects a true preference for a single half-site over a single random site of 35:

\[
\frac{n \exp(-E_n) + \exp(-E_h)}{n \exp(-E_n)} = x_{hn} \frac{\exp(-E_h)}{\exp(-E_n)} = n(x_{hn} - 1),
\]

where \( n \) is the number of sites available on the oligonucleotide for binding, including the cognate site, \( E_h \) and \( E_n \) are half-site and noncognate binding energies, respectively, and \( x_{hn} \) is the apparent factor by which p53 prefers to bind the half-site in the specific mode relative to noncognate DNA in the non-specific mode. For values of \( n = 5 \) and \( x_h = 8 \), the true preference for half-sites is approximately four-and-a-half times greater than the apparent preference, corresponding to an energy difference of 1.5 \( k_B T \).

This energy difference is reflected in a greater value for the proportionality constant \( c \) relating the score of a site to its energy. With available binding sites flanking the cognate site, \( c = 0.97 k_B T/n \), while with three sites on either side (\( n = 5 \) in Eq. S24), it increases to 1.37 \( k_B T/n \). This has the concomitant effect of raising the energy of cooperativity between specific-mode binding in the two dimers (that is, raising the energy of the specifically-bound state) from \( \varepsilon = -1.39 k_B T \) to \(+0.19 k_B T \); that is, specific binding becomes weakly anticooperative. The increase in \( c \) amounts to a more rugged landscape, with deeper wells at half- and full-sites, while the decrease in \( \varepsilon \) causes full-site binding to become weaker. The information content of the p53 sequence logo is such that these two effects are similar in magnitude and opposite in sign, and thus largely cancel each other out. For a pair of adjacent half-sites that each score a typical 4 bits better than the score corresponding to non-specific binding, \( s_{hn} \), the energy for fully specific binding, which is the dominant effect of raising the energy of cooperativity between specifically-bound states, is \( 2 \cdot (\log(2) \text{nat}/\text{bit}) \cdot 4 \text{ bits} \cdot 0.97 k_B T/n \) for \( 1.39 k_B T = 6.8 k_B T \) in the absence of available flanking sites, and \( 2 \cdot (\log(2) \text{nat}/\text{bit}) \cdot 4 \text{ bits} \cdot 1.37 k_B T/n = -0.19 k_B T = 7.4 k_B T \). We presented results assuming no flanking sites, but the landscapes based on the availability of 4 flanking sites are very similar in the predicted local diffusion coefficients they produce: Both have a correlation coefficient of 0.81 with experimental \( D \).

A similar treatment for the true preference of a dimeric DNA-binding protein for binding a full-site in full-specific mode relative to a noncognate site in non-specific mode, \( \exp(-2E_h - \varepsilon)/\exp(-E_n) \), as a function of the apparent preference, denoted \( x_{hn} \), follows:

\[
\frac{n \exp(-E_n) + 2n(x_{hn} - 1)}{n \exp(-E_n)} \frac{\exp(-E_h)}{\exp(-E_n)} = x_{hn}.
\]

Rearranging and substituting in Eq. S24,

\[
\frac{n \exp(-E_n) + 2n(x_{hn} - 1)}{n \exp(-E_n)} \frac{\exp(-E_h)}{\exp(-E_n)} = n(x_{hn} - 2x_{hn} + 1).
\]

Although nonspecific binding to the oligonucleotides did not turn out to affect our results substantially, this owes to an accident of the parameters relevant to our system. Nonspecific binding of proteins to specific probes receives little attention, and yet is necessary to consider when making accurate estimates of binding preferences.

**Interpolations of DNA Fluctuation Variance and Distributions.** We used our data from earlier work (4) of QDs covalently attached to positions on \( \lambda \)-phage DNA one-third and two-thirds the distance from the tether to estimate the mean apparent diffusivity owing to DNA fluctuations, \( \langle \Delta x^2 \rangle \), in Eq. S9, \( \langle \Delta x^2 \rangle \) at position \( x \) along the contour is expected to fluctuate according to a polynomial in \( x \) with nonzero linear and quartic coefficients (10). For all time windows \( \Delta t \) up to a maximum of two seconds, we fit these coefficients to the observed variance in displacement of the QDs at \( x = 1/3L \) and \( x = 2/3L \) (\( L = \) the contour length of \( \lambda \) DNA), and an assumed zero-variance point at the tether, between frames separated by \( \Delta t \) to arrive at an expression for \( \langle \Delta x^2(\Delta t) \rangle \):

\[
\langle \Delta x^2(\Delta t) \rangle = a_1(\Delta t)^1 \cdot x + a_4(\Delta t)^4 \cdot x^4.
\]

The same QD data was used to correct estimates of \( D/D_0 \) for the uncertainty in the assignment of experimental displacements to segments owing to DNA fluctuations. We determined for each segment’s \( D/D_0 \) the proportion \( \alpha \) of the apparent population of the segment \( s \) that can be expected to originate in fact from neighboring segments \( s = 1 \) to the left and \( s + 1 \) to the right:

\[
\frac{D_{\text{corrected}}}{D_0}[s] = (1 - \alpha_{s-1} - \alpha_{s+1}) \frac{D}{D_0}[s] + \alpha_{s-1} \frac{D}{D_0}[s-1] + \alpha_{s+1} \frac{D}{D_0}[s+1]
\]

\[
\alpha_{s+1} = \int_{s+1/2}^{s+3/2} Q(x|s+\Delta s) dx + \frac{1}{w} \int_{-w/2}^{w/2} Q(x|s) dx
\]

The variable \( s \) identifies the segment whose \( D/D_0 \) is estimated; \( \alpha_{s+1} \) is the contribution to a segment’s observed population of neighboring segments \( s + 1 \). The integral is over all base pairs in the indicated segment. \( Q(x|s) \) is the distribution of longitudinal DNA displacements from equilibrium for segment \( s \), normalized such that \( \int_{-w/2}^{w/2} Q(x|s) dx = 1 \), which we obtained from the same QD measurements used to correct experimental \( D \) for DNA fluctuations. We assumed that the density of data giving rise to observed diffusion coefficients in each segment was uniform within that
segment, and so convolved the distributions of the quantum dots displacements with a uniform distribution the width of a segment, 1/w. It is worth remarking that the distribution of DNA displacements, Q, is itself a function of distance from the tether, so the convolution kernel widens as it moves farther from the tether.

To determine the distribution \( Q(x|s) \) used in Eq. S27, we constructed sample distributions of the position of the QDs at 1/3 and 2/3 the length of the DNA from the tether, about their mean positions. The variances of these distributions were used to find the coefficients of a similar polynomial as the one in Eq. S25. Interpolated distributions consisted of a linear combination of the zero-variance delta distribution assumed for the tether point, such that the variance of the interpolated distribution at a position \( s \) equaled the fitted polynomial evaluated at that position:

\[
Q(x|s) = \begin{cases} 
  b_0 Q(x|0) + (1 - b_0) Q(x|\frac{1}{3}L) & 0 < s \leq \frac{1}{3}L \\
  b_0 Q(x|\frac{1}{3}L) + (1 - b_0) Q(x|\frac{2}{3}L) & \frac{1}{3}L < s \leq \frac{2}{3}L 
\end{cases}
\]  

\[\text{Var}(Q(x|s)) = a_1 s + a_4 s^4.\]  

The QD measurements were also used to add noise to simulations, which was then subtracted out using an identical procedure as described in SI Text, Data Analysis.

Control for Specific Binding. To verify that p53 can recognize its cognate sites in our experimental conditions, we synthesized a DNA construct to which we expected p53 to bind specifically. In brief, we cloned into the pET-28b plasmid a 36-bp insert containing the p21 5′ site, p53’s strongest known functional binding element (GAACATGTCCCAACATGTTG), as well as two sites absent from pET-28b recognized by the nicking endonuclease Nt.BspQI. After extracting DNA from the transformed cells, we nicked the plasmid, treated it with an excess of a biotinylated oligonucleotide equivalent to the nicked segment, and used rolling-circle amplification with the T7 DNA replisome to produce long (>100 kb) DNA constructs with a p53 RE repeated every 5,380 nucleotides (the length of the plasmid, minus the fragment lost during the double digest, plus our insert). The resulting constructs were treated with Escherichia coli DNA polymerase I and T4 ligase to join Okazaki fragments.

We repeated our experiments with the same biochemical and imaging conditions, but using this synthetic construct instead of lambda phage DNA. We found p53 to bind nonuniformly to DNA; rather, we observed a periodicity in its binding, with a period corresponding to the expected separation between instances of the binding site, 5,380 bp (Fig. S8). While the binding profile is enriched for particles spaced by integer multiples of 5,380 bp, we found there to be a distribution of distances, which can be attributed to the concentration of p53 used in the experiment being sufficiently high to have multiple particles bind within some 5,380-bp segments, and thus locally saturate the p21 5′ sites. We intend to explore additional properties of p53 binding on this and other engineered constructs in a future publication.


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**Fig. S1.** Differences in \( D_{\text{expt}} \) among the segment pairs and assessment of significance. (A) For each pair of segments \( i \) and \( j \), we show the absolute log-ratios, \(|\log(D_i/D_j)|\), between the pairs’ \( D_{\text{expt}} \). (B) \( p \)-values for absolute log-ratios between segments’ \( D_{\text{expt}} \) shown in A. All particles’ \( D \)s were randomly reassigned to another particle, and \( D_{\text{expt}} \) was calculated for each segment using these reassigned particle \( D \)s. One thousand such rounds were performed. Table entries are the proportion of reassigned rounds in which the absolute log-ratio of \( D_{\text{expt}} \) between a pair of segments was greater than or equal to the absolute log-ratio for the unassigned data. (C) Graphical depiction of information in B. \( p < 0.05 \) denoted by *; \( p < 0.01 \) denoted by **.
Fig. S2. (A) Four modes of binding: (i) fully nonspecific; (ii) first dimer nonspecific, second dimer specific; (iii) first dimer specific, second dimer nonspecific; (iv) fully specific. The energy at a position $x$ in the golf-course landscape is equal to the negative logarithm of the sum of the statistical weights of these four modes. (B) Sequence logos of the p53 half-site from a variety of position weight matrices (1–7).

**Fig. S3.** Effect of DNA fluctuations on diffusion coefficients. Each particle is represented by dots in a horizontal line, as in Fig. 3C (main text). Data in Fig. 3C is reproduced in gray. As in Fig. 3C, each dot represents the center of a displacement, positioned along the horizontal axis according to the location on DNA of the displacement, and positioned vertically according to its parent trajectory’s diffusion coefficient. Gray dots correspond to data after correction for DNA fluctuations, with segments’ average diffusivity denoted by gray crosses. Green dots correspond to data before apparent diffusivity owing DNA fluctuations subtracted out per Eqs. 2 and 59, with the average diffusivity in a segment denoted by green crosses.

**Fig. S4.** (A) Violin plot of segments’ $D_{\text{expt}}$ from 10,000 bootstrap resamplings of particles contributing to the segments’ $D_{\text{expt}}$. Means and standard deviations of resampled $D_{\text{expt}}$ shown as black error bars. (B) Violin plot of segments’ $D_{\text{expt}}$ from 10,000 bootstrap resamplings of data-collection batches. Non-resampled means shown as thick red crosses (same position as bars in Fig. 3D), with standard deviations of resampled $D_{\text{expt}}$ shown as red error bars.
Fig. S5. Comparison of calculations of experimental $D_{\text{expt}}$ (black bars) with $D_{\text{expt}}$ obtained after randomizing particle positions on DNA (cyan traces).

Fig. S6. Comparison of diffusion coefficients determined using the alternative method described in SI Text, Alternative Data Analysis. Red and black bars are identical to those in Fig. 5. Gray bars are half $2D$, called $D_{\text{alt}}$, as determined by finding the variance of the fitted Gaussian distribution of normalized displacements in a segment. The correlation coefficient, $r$, between theoretical $D/D_0$ and $D_{\text{alt}}$ is 0.931; and between the MLE-based $D_{\text{expt}}$ and $D_{\text{alt}}$ is 0.831.

Fig. S7. Distribution of displacements across all analyzed segments, as a function of time window $\Delta t$. The red bars indicate the standard deviation of the distributions, and the blue trace the mean. As can be seen, the mean displacement is nearly zero, though close inspection will reveal that it increases approximately linearly with time, as is expected from hydrodynamic drag.
Verification of p53's ability to recognize target sites. We synthesized DNA constructs consisting of several (>10) repeats of a ∼5.4-kb plasmid (SI Text, Control for Specific Binding) into which we cloned the p21 5′ binding site for p53, immobilized them in our flow cell, and treated them with fluorescently labeled p53. For 35 DNA molecules, we recorded the distances between all pairs of particles on the construct, the aggregate distribution of which is shown as blue bars (bin width = 2 pixels) in A. Green arrows indicate distances corresponding to integer multiples of the plasmid length (i.e., the spacing between known p53 Res) and are found at positions locally enriched for p53 binding. We also present distances only between adjacent particles on the same construct as blue bars in B. In the absence of preferential binding, nearest neighbors would have an exponential distribution, a fit of our data to which is shown as a red trace. The difference between the fit and the measured distribution is shown as green bars. Green arrows are identical to those in A.