nature portfolio

Peer Review File

Electrostatic properties of disordered regions control transcription factor search and pioneer activity

Corresponding Author: Professor David Suter

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Sakong et al. investigate how charged regions, adjacent to canonical DNA binding domains in SOX2, affect target search in the context of chromatin. To this end, they combine single-molecule imaging in vitro with imaging and genomics analyses in stem cells. They find that the ability of pioneer factors to invade chromatin is affected by their charged regions, adjacent to the canonical DNA binding domains (referred to here as DFRs, for DBD flanking regions).

Overall, the manuscript is well written, interesting, and the science is rigorous, with only a few exceptions that are specified below. The topic is very important and timely, given strong interest in how pioneer factors access their target sites within chromatin, and how unstructured/charged regions participate in this process. The only serious issue is with the quantification of equilibrium dissociation constants (point 1). There is also a lack of evidence for the expression level and stability of transcription factors in cells (point 2). Some of the statements made based on the in vitro assays are not supported by the data, which can be addressed mainly textually by statement moderation (points 3-4). I believe that all these issues would be reasonably easy to correct, after which the manuscript should be appropriate for publication in Nature Communications.

Major points

1. Figure 3b: The quantification of the affinity of transcription factors for DNA using EMSA is invalid, and the resulting equilibrium dissociation constants (Kd) are likely wrong. Specifically, based on the EMSA in Fig. 3b, the authors concluded that the EMSA “allowed us to estimate dissociation constants of 1.6 nM for Sox2 and 2.3 nM for Sox2a.” Yet, in this EMSA, the authors used a DNA probe concentration of 2 nM, meaning that they could not physically detect Kd values under 2 nM. Practically, under these conditions, the authors are unlikely to reliably detect Kd values below 20 nM (i.e., <10-fold the DNA probe concentration). This is because under these conditions, the protein is at the “titration regime”, where Kd concentration is too close to the probe concentration. Hence, what the authors quantified was likely the concentration of the probe in each of these experiments, not Kd. If the authors wish to quantify dissociation constants using this experimental system, they could attempt to use conditions that reduce the affinity of the transcription factors for DNA (i.e., increase Kd to >10-fold above the probe concentration). This can usually be done by adding monovalent salt to the binding buffer and/or adding competitor DNA. A recommended reading on how to measure and evaluate equilibrium dissociation constants can be found in [PMID: 32758356].

2. Figure 6: Based on the ChIP-seq and ATAC-seq data in Fig. 6, the authors conclude that the DFR allows Sox2 to bind to poorly accessible chromatin and open it more effectively. Could the authors exclude the possibility that Sox2 is superior to Sox2a in recruiting cofactors that might facilitate chromatin opening? Given that most of the binding sites of Sox2 and Sox2a are non-overlapping, isn’t it fair to say that the DFR has a large effect on binding specificity, beyond what could be explained by the in vitro experiments? These aspects could be discussed, even if they cannot be reasonably addressed experimentally. More importantly, the authors should carry out immunoblotting on both lines to demonstrate that the expression level and stability of the ectopically expressed transcription factors are the same in both cell lines. This is necessary for showing that the results in Figure 6 cannot be explained by a higher expression or stability of Sox2 compared to Sox2a.

3. Fig 5. Nucleosome arrays are an inadequate model for inaccessible chromatin when assayed under the experimental conditions used here. Specifically, the authors set up some of the rationale for experiments in Fig 5 by saying that "...a target site within chromatin is expected to be less accessible than on a nucleosome because of nucleosomes stacking in a tetranucleosome conformation [REF 67-69]" (L337). Yet, this statement does not seem to be relevant for the experiments that were carried out here: nucleosome stacking is often observed in vitro in the presence of H1 histones [REF 67] or magnesium [REF 68], which were not used here. In the absence of magnesium or H1 histones, 601 arrays are largely open (e.g. see Fig 1A in Li et al 2016 [REF 67]). For the same reason, the chromatin cartoons in Fig 5 are misleading, as the 601 array is unlikely to be folded into a compact state under the conditions used. Based on data in Fig 5, the authors concluded that DFR "...enabling Sox2 to invade chromatin more effectively" (L349). But is this the result of chromatin “invasion”, rather than more binding sites that enhance scanning? If the authors wish to avoid H1 or magnesium cations in their experimental system, they should minimally avoid statements regarding chromatin folding, such as interactions between nucleosomes, "compact chromatin" (from the abstract), "invasion" into chromatin in vitro, or even a chromatin “fiber”. Such characteristics are unlikely to have a substantial relevance under the experimental conditions used.

4. Fig 4-5. All the experiments with nucleosomes (Fig 4) and chromatin (Fig 5) were done using 601 sequences that strongly position nucleosomes. This is in contrast to native sequences, where nucleosomes can slide more readily. As a result, could it be possible that some of the derived constants were overestimated or underestimated? If the authors can carry out some of the experiments without strong positioning sequences, that would be ideal; otherwise, they should at a minimum discuss this as a potential limitation.

Minor points

5. Figure 1c: Since ChIP-seq was done here, it would be appropriate to present a ChIP-seq heatmap or minimally show some sample genome tracks, as was done in Fig 6, so the reader can appreciate the quality of the data.

6. Extended Data Fig. 3: Since Sox2 and Sox2a are compared in vitro, it would be appropriate to present also the size-exclusion chromatography profile and SDS-PAGE analysis for Sox2a, as was done for Sox2. This would help exclude the possibility that variations in the apparent DNA-binding activity are attributed to differences in protein purity and solubility. Moreover, it is recommended to check the ratio between absorbance at 260 and 280 for both proteins to exclude the possibility that variations in nucleic acid contamination contributed to the differences in DNA-binding activities in vitro.

7. Line 215: “This indicates that DFRs do not impact the overall probability of interactions between freely diffusing TFs and naked DNA.” This is a rather strong and broad statement, given that only two DFRs were tested on one TF within the context of Extended Data Fig 3h. Unless the authors wish to test additional TFs and DFRs, it would seem appropriate to minimally moderate this statement to avoid the impression that they are referring to a general principle that would apply to every TF and every DFR.

Presentation

8. Extended Data Fig. 3d-e: A colour key is needed to explicitly indicate DNA length.

9. Extended Data Fig. 3g: Check if the x-axis unit should be in nm rather than bp.

10. Extended Data Fig. 3h: When referring to this figure, the text in Line 214 quotes different units than those indicated on the y-axis.

11. Figure 3i: Consider specifying in the legend what each of the constants in the figure refers to, so readers do not have to refer back to the main text every time they look at this model figure

Reviewer #2

(Remarks to the Author)
Pls see attached PDF file

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The present paper from Saking, Fierz, and Suter focuses on understanding the target search of the SOX transcription factor

family.

This is a key and long-standing question that dates back to the landmark 1970 paper by Riggs (author Ref13), who observed that the kON of LacI was about two orders of magnitude higher than the Smoluchowski limit. Since then, the TF field has been trying to understand how the ON-rates of TFs can be so high. 2 major models have been proposed. First, electrostatics. This is the model originally favored by Riggs and also others like Halfon (see https://portlandpress.com/biochemsoctrans/article/37/2/343/64235/An-end-to-40-years-of-mistakes-in-DNA-protein). Second is the model of facilitated diffusion, where a mix of 1D DNA sliding and 3D diffusion can accelerate the search. These two are not mutually exclusive. The second model forms the framework for this paper.

The problem of TF search is therefore a foundational textbook-level question, but it remains unsolved. In my opinion the present paper takes several important steps in the direction towards solving it and very much expands our understanding of TF search. There are lots of interesting observations in this paper that I won't attempt to summarize all of them, but among the things I found impressive were: 1) the nice focus on a family of SOX and the role of DFR regions, showing clearly how DFRs can affect the ON-rate; 2) the comprehensiveness of the investigation. A major strength of this paper is that it is very focused on the SOX factors and then comprehensively investigates these using a combination of genomics, in-cell single-molecule tracking, in vitro-single-molecule tracking, looking at both naked DNA and chromatinized templates, and careful modeling; 3) the findings are quite interesting and illuminating.

Overall, this is in my view an excellent paper. The comprehensiveness of the studies and the range of approaches is truly impressive, yet they are well-integrated, resulting in comprehensive insights from the orthogonal approaches. In my view, this paper is likely to be of quite general interest to a wide readership. Nevertheless, there are some issues that need to be addressed and I think there are some technical errors and problems in the present paper. However, these are all highly addressable as far as I can tell and will not overturn the major conclusions, they will just be required for correctness. I list my comments below.

COMMENTS:

1. DATA AND CODE IS MISSING: the authors provide Zenodo links to the data which is great but the links do not work. The whole point is to make the data available to the reviewer. Please correct this. Also it says the code and analysis scripts are available upon request. These need to be available as well, can you put on GitHub or Zenodo?
2. I too am convinced by facilitated diffusion, but I think the authors overstate it. They say it increases search efficiency by orders of magnitude, which sounds like >100x-1000x. This is probably not true. Theory says that if search is 50:50 3D:1D, then acceleration is half the sliding length and Hammar estimated sliding length to be 45 bp, resulting in a max acceleration of 20x. This is still significant and important, but it is not "orders of magnitude". Please correct. I would also suggest having a sentence on the slight controversy about facilitated diffusion https://portlandpress.com/biochemsoctrans/article/37/2/343/64235/An-end-to-40-years-of-mistakes-in-DNA-protein
3. Along these lines, where they estimate kON rates, can they compare directly to the Smoluchowski limit?
4. Line numbers would have helped
5. Page 2, 2nd paragraph, a "DFR-like" region also accelerates the CTCF target search by 2.5-fold in cells and is relevant for the discussion here https://www.nature.com/articles/s41589-019-0422-3
6. REF2 is an important early paper, but the key conclusions from this paper are now well-known to be wrong and has been disproven in several other papers, so probably makes sense to remove this from the reference section, since there are plenty of other papers on Sox2 that could also be cited like https://elifesciences.org/articles/22280
7. Small thing, but in Fig 2d, it is a little unclear if the proteins include the N- and C-terminal segments. It looks like Sox2 is only DBD and DFR, but there are other regions. Can you clarify if those domains are included.
8. I understand the math behind GRID, but I am skeptical of the 6-well-resolved residence times. Fitting 6 exponentials with 6 fractions involves 11 free parameters (N-1 fractions, 6 rates) from limited and noisy data does not strike me as statistically robust. Moreover, the in-cell rates are completely consistent with the in vitro rates. Lots of things can go wrong inside cells, like a Halodye getting stuck somewhere. I am not saying they should remove the GRID analysis, but they should at least include a few sentences about the uncertainty and difficulty, the limited statistical power, and the disagreement with the in vitro measurements.
a. Along these lines, a res-time of 1000s correspond a kOFF of 0.001 sec^-1. But their photobleaching rate is stated to be 0.028 s^-1. I.e. the photobleaching rate is 28x faster than the slow residence times. Which means that the error on the photobleaching rate estimation must be <2-3%. It seems completely unrealistic to get such a low error on the photobleaching estimate and therefore, it does not seem statistically robust to me to try to estimate residence times that are much much lower than the photobleaching rate.
b. It would be essential to acknowledge this and tone this down. Ideally the very slow residence times should be cross-validated with orthogonal assays.
c. The in vitro residence times are ~1 sec and ~14 sec and this is reported on page 10. The authors should compare and contrast this with the in-cell residence times.
9. DIFFUSION analysis: they fit 3 Brownian subpopulations to 1-CDFs and then they make an arbitrary threshold of 0.1 um2/s in Fig 2c and call everything below for "bound". But we know 0.1 um2/s is too high because people have done histone tracking and found the diffusion coefficient of bound chromatin to be 10x-100x slower that this. My question is why do the analysis like this, because while this is the right direction, but the analysis – unless I misunderstood in which case I do apologize – ignores several known and solved problems. Most notably defocalization and localization error. As best I can tell, the analysis ignores localization error. While eq2 is theoretically correct, it is practically incorrect. It ignores localization

error and motion blur and such. Motion blur may not be huge, but localization error must be accounted for. Defocalization is a serious issue as well, which if not taken into account will give you the wrong numbers. But this is now a solved problem. Mazza-2012 and Hansen-2018 showed how to account for both defocalization and localization error. So in short, the authors should adopt a rigorous analysis that takes into account both localization error and defocalization.
10. In eq2, the authors show an alpha on time, but this is lot in eq 3. Do the authors try to fit alpha? If so, how subdiffusive are things?
11. When the authors do the pseudo-ON-rate analysis, they do not clearly describe where the equations come from. Notably, REF2 did such an analysis but the equations in that paper are wrong. The incorrect math of REF 2 from fixed in Loffreda et al. https://www.nature.com/articles/s41467-017-00398-7 Can you specify where the equations come from?
12. The in vitro experiments are extremely comprehensive and well-done. I think this is such a major strength of this paper. All their analysis and modeling assumes that they see sliding, but if I am not mistaken, they never directly observe sliding? Sliding has been directly observed in prior work. Maybe the DNA is too short here to observe it. If they cannot directly observe sliding, they should comment on it in the main text and make clear that prior work has observed it but here it is just assumed to be true.
13. The presentation is in general well-done and comprehensive. The SI tables are great and the methods well-described for the most part. That said, the kinetic parameters for the GRID analysis appear to be missing and seem like a full SI table for the in-cell SMT parameters would be required.
14. Top of page 11, where they discuss non-specific binding times, here they may want to compare to https://www.nature.com/articles/ncomms8357 which nicely studied this.
15. Page 11, can they directly compare to the Smoluchowski limit for kON rates here? The whole reason to suggest facilitated diffusion is the higher than Smoluchowski, so this seems like a great place to calculate what they expect and compare it here.
16. I love Fig 3i. Strongly consistent with 1D sliding and facilitated diffusion.
17. Fig 4e, but more generally, when you do residence time fitting of multiple populations, you will undercount slow ones and overcount fast ones. Suppose you have 2 TFs in a nucleus. One TF binds for 1000 sec. The other TF binds for 0.5 sec ON and then diffuses and then back ON for 0.5, and does this 500 times over the 1000 sec. Then in the 1000sec movie, one TF gave 1 binding event and the other TF gave 500 binding event trajectories, giving apparent fractions of 1/501 vs. 500/501, but in reality it was 50:50. Do you correct for this? If not, the equation to do so can be found in https://www.sciencedirect.com/science/article/pii/S2589004222020521#sec4 under section “Analysis of trajectories from slow 2 Hz SPT movies”
18. A bit more discussion of how 1D sliding works on nucleosomal DNA could be nice.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have sufficiently addressed the points I raised and provided reasonable explanations where they chose not to make changes. In my view, the paper is ready for publication.

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed all of my concerns. I recommend publication of this work at Nature Commun.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
As I wrote in my first review, this is an excellent paper that addresses a long-standing and deeply interesting question using new approaches and it very effectively applies a live-cell SMT, in vitro SMT, and genomics integrated approach. The results are compelling and make sense. As I wrote in my first review, Fig 3i is especially compelling. I have reviewed many papers for Nat Comm over the last 5 years and among the ones that were eventually published, I think the present paper is near the top. I really think it is an excellent and compelling study that is likely to be of wide interest.

But it does have a significant weakness, which is the rigor of the live-cell SMT analysis. I pointed these out in my first review, but the authors have chosen to largely ignore them. This is disappointing and the issues will be obvious to experts in the field and turn them off what is otherwise an outstanding paper.
As far as I can tell from the authors response, they agree with my concerns (e.g. diffusion analysis is not correct), but rather than correcting it, they chose to keep the problematic analysis in the main figures and then more correct analyses to the SI. Likewise, we know from dozens of “chromatin dynamics studies” (e.g. using H2B tracking) that the diffusion coefficient of

chromatin is not 0.1 um^2/s/.

So I would like to ask the authors to do one of two things: 1) Maybe I am wrong, in which case, please run reaction-diffusion simulations, add defocalization and localization noise, and demonstrate that the diffusion analysis works well. Or 2) replace it with more correct analyses.

Regarding my comments in the last round.
Comments 1-7 have been addressed.

Comment 8 has not really been addressed. I remain skeptical of the 6 states and my request was to discuss the uncertainty in one sentence in the main text, which the authors have not done. They have added some discussion to the discussion section but they have not really addressed my concern.
In 8a, the authors misunderstand my concern about relative error. I do not think you can accurately fit a residence time with a photobleaching rate being similar. I remain extremely skeptical about the GRID results and the authors have chosen not to explicitly discuss the uncertainty in the main text which is disappointing.

The authors have also chosen not to address Comment 9.
In comment 9 I pointed out that eqn 2 (now eqn 3) is incorrect for data. MSD should be 4st^alpha + loc error + motion blur. This is a solved issue. Why do it incorrectly?
The authors report an alpha of 0.8 for the DNA bound fraction. Again, we know this is incorrect. Is there a realistic polymer model with an exponent of 0.8? Rouse says 0.5. Fractal globule says 0.4. Recent work, spanning several orders of magnitude suggests the exponent is more like 0.3-0.4 https://www.biorxiv.org/content/10.1101/2025.05.10.653248v1.full and another paper says something similar https://www.biorxiv.org/content/10.1101/2025.09.24.678119v1
Again, why do this incorrectly in the main text and correctly in the SI (Fig S3d).
It is true that there are several papers that use 0.1 um^2/s as the cut-off for the chromatin diffusion, but we know this is a short-hand and we know this is incorrect. If you do not account for localization error, you will dramatically overestimate the bound Diff Coef.
It does the field a disservice to propagate incorrect approaches and it also makes the paper look bad. This is an extremely impressive paper, but since you open with the in vivo SMT and do these analyses incorrectly, it will turn off expert readers from what is otherwise a fantastic paper.
The “fit diffusion coefficients from each trajectory, then fit the distribution of coefficients” was benchmarked in Figure 3 of https://elifesciences.org/articles/33125 and the performance is terrible For example, it can easily get the Bound fraction wrong by 30-55 percentage points.
I would like to insist that the authors either remove the currently incorrect diffusion analysis, or validate that their approach is indeed correct and accurate by performing reaction-diffusion simulations, adding localization noise to their simulated data, applying their analysis methodology to it, and demonstrating what they are doing is indeed correct. It is possible that I am missing something and I would be happy to be persuaded that I am, but in its current form, the analysis appears to be the “distribution of diffusion coefficient analysis” that was shown in 2018 to not work at all. Given that alternative more accurate approaches are in Fig S3d, it is really difficult to understand why they want to keep doing it incorrectly?

Comment 10: Again, I have big concerns about the statistical robustness. If you fit the alpha of each trajectory, you have a power law. To robustly fit a power law you need at least 2 log in both dimensions https://www.science.org/doi/10.1126/science.1216142 For an alpha of 0.8, this would be about 3 log in time. So trajectories should contain ca. 1000 frames, which I would imagine is not the case.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors have fully addressed my comments and concern and I strongly recommend the publication of this excellent paper without delay and without going back to reviewers.
I appreciate the changes the authors have made, and I really think this is an unusually excellent and interesting paper, that I am sure will be well received by the field.

SMALL THINGS
Fig. S3c is a bit unconventional. To my knowledge, you should use all the displacements/jumps unless you think you are missing fast moving ones due to motion blur. I might suggest to remove it but it is not a big deal.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Sakong et al. investigate how charged regions, adjacent to canonical DNA binding domains in SOX2, affect target search in the context of chromatin. To this end, they combine single-molecule imaging in vitro with imaging and genomics analyses in stem cells. They find that the ability of pioneer factors to invade chromatin is affected by their charged regions, adjacent to the canonical DNA binding domains (referred to here as DFRs, for DBD flanking regions).

Overall, the manuscript is well written, interesting, and the science is rigorous, with only a few exceptions that are specified below. The topic is very important and timely, given strong interest in how pioneer factors access their target sites within chromatin, and how unstructured/charged regions participate in this process. The only serious issue is with the quantification of equilibrium dissociation constants (point 1). There is also a lack of evidence for the expression level and stability of transcription factors in cells (point 2). Some of the statements made based on the in vitro assays are not supported by the data, which can be addressed mainly textually by statement moderation (points 3-4). I believe that all these issues would be reasonably easy to correct, after which the manuscript should be appropriate for publication in Nature Communications.

Major points

1. Figure 3b: The quantification of the affinity of transcription factors for DNA using EMSA is invalid, and the resulting equilibrium dissociation constants (Kd) are likely wrong. Specifically, based on the EMSA in Fig. 3b, the authors concluded that the EMSA "allowed us to estimate dissociation constants of 1.6 nM for Sox2 and 2.3 nM for Sox2a." Yet, in this EMSA, the authors used a DNA probe concentration of 2 nM, meaning that they could not physically detect Kd values under 2 nM. Practically, under these conditions, the authors are unlikely to reliably detect Kd values below 20 nM (i.e., <10-fold the DNA probe concentration). This is because under these conditions, the protein is at the "titration regime", where Kd concentration is too close to the probe concentration. Hence, what the authors quantified was likely the concentration of the probe in each of these experiments, not Kd. If the authors wish to quantify dissociation constants using this experimental system, they could attempt to use conditions that reduce the affinity of the transcription factors for DNA (i.e., increase Kd to >10-fold above the probe concentration). This can usually be done by adding monovalent salt to the binding buffer and/or adding competitor DNA. A recommended reading on how to measure and evaluate equilibrium dissociation constants can be found in [PMID: 32758356].

We agree with the reviewer's concern—this is precisely why we originally described the K_d values as "estimated." However, we recognize that even with this qualification, the text may imply a level of precision that is not supported under the experimental conditions used. We have therefore revised the sentence to read:

> "… to estimate nanomolar DNA affinities for the Sox TFs (Fig. 3b, Supplementary Fig. 4d), consistent with published values [ref 41]."

We would like to emphasize that the primary purpose of this EMSA experiment was to verify the functionality of the recombinant transcription factors, not to obtain precise dissociation constants. More accurate quantification of binding affinities is provided by the single-molecule data presented later in the manuscript.

2. Figure 6: Based on the ChIP-seq and ATAC-seq data in Fig. 6, the authors conclude that the DFR allows Sox2 to bind to poorly accessible chromatin and open it more effectively. Could the authors exclude the possibility that Sox2 is superior to Sox2a in recruiting cofactors that might facilitate chromatin opening? Given that most of the binding sites of Sox2 and Sox2a are non-overlapping, isn't it fair to say that the DFR has a large effect on binding specificity, beyond what could be explained by the in vitro experiments? These aspects could be discussed, even if they cannot be reasonably addressed experimentally. More importantly, the authors should carry out immunoblotting on both lines to demonstrate that the expression level and stability of the ectopically expressed transcription factors are the same in both cell lines. This is necessary for showing that the results in Figure 6 cannot be explained by a higher expression or stability of Sox2 compared to Sox2a.

We agree with the reviewer that equal protein levels are critical to compare TF ChIP profiles. We thus FACS-sorted the cell lines according to Sox TF abundance (based on SIR fluorescence intensity after Halo-labeling) to ensure comparable expression levels of Sox2 and Sox2a across the two cell lines. This sorting strategy provides precise, quantitative control of transcription factor expression. We explain this strategy more clearly in the main text, an updated Fig. 6a, Supplementary Fig. 8a, and Table S13:

> "... and subsequently performed fluorescence-activated cell sorting (FACS) to select cells with near-identical TF expression levels (Fig. 6a, Supplementary Fig. 8a, Table S13; see Methods)."

> " Cell sorting strategy for ChIP-seq and ATAC-seq: mESCs expressing Halo-Sox2 or -Sox2a were treated with SiR647-Halo ligand and FACS-sorted using indicated gating windows (black) corresponding to near-identical TF expression levels (see also Table S13)."

We appreciate the reviewer's suggestion to consider potential differences in cofactor recruitment as the origin for differential binding dynamics. While we cannot fully exclude the possibility that the DFR contributes to differential cofactor engagement, our interpretation is that the DFR primarily enhances chromatin target search efficiency rather than altering sequence specificity. This view is supported by the motif analysis (Supplementary Fig. 1d), which shows similar motif preferences for Sox2 and Sox2a, and by the single-molecule assays in vitro, which demonstrate enhanced search dynamics DFR_{Sox2}, in the absence of any additional cofactors. Nevertheless, we acknowledge that DFR-mediated effects in vivo could extend beyond search kinetics to include cofactor interactions.

We now specifically mention this within the Discussion section:

> "Of note, our experiments focused solely on interactions of individual TFs with DNA or chromatin in the absence of other factors. Potential DFR-mediated changes in protein-protein interactions, in particular potential differences in cofactor recruitment, were not evaluated in our work, and we cannot fully exclude that such effects might contribute to the observed differences between the Sox TF variants in vivo."

3. Fig 5. Nucleosome arrays are an inadequate model for inaccessible chromatin when assayed under the experimental conditions used here. Specifically, the authors set up some of the rationale for experiments in Fig 5 by saying that "...a target site within chromatin is expected to be less accessible than on a nucleosome because of nucleosomes stacking in a tetranucleosome conformation [REF 67-69]" (L337). Yet, this statement does not seem to be relevant for the experiments that were carried out here: nucleosome stacking is often observed in vitro in the presence of H1 histones [REF 67] or magnesium [REF 68], which were not used here. In the absence of magnesium or H1 histones, 601 arrays are largely

open (e.g. see Fig 1A in Li et al 2016 [REF 67]). For the same reason, the chromatin cartoons in Fig 5 are misleading, as the 601 array is unlikely to be folded into a compact state under the conditions used. Based on data in Fig 5, the authors concluded that DFR "...enabling Sox2 to invade chromatin more effectively" (L349). But is this the result of chromatin “invasion”, rather than more binding sites that enhance scanning? If the authors wish to avoid H1 or magnesium cations in their experimental system, they should minimally avoid statements regarding chromatin folding, such as interactions between nucleosomes, "compact chromatin" (from the abstract), "invasion" into chromatin in vitro, or even a chromatin “fiber”. Such characteristics are unlikely to have a substantial relevance under the experimental conditions used.

We agree with the reviewer that full chromatin compaction is only reached with millimolar concentration of Mg^{2+} or inclusion of H1. However, in our experiments, the imaging buffer contained 100 mM KCl and 20 mM HEPES (with Na^{+} as the counterion), yielding a total monovalent ion concentration of 120 mM. Under these conditions, chromatin adopts a partially compacted state, as demonstrated by single-molecule FRET measurements that monitor nucleosome stacking (see e.g. Fig. 5C from Mivelaz et al., Mol. Cell 2020, or Fig. 1 from Korolev et al., Biophys J. 2010). Thus, nucleosome stacking is present in our conditions (albeit to a lesser degree compared to high Mg^{2+} conditions), and chromatin invasion can take place. Moreover, our statements about chromatin invasion are also supported by results obtained in living cells, where we observe higher propensity of Sox2 to bind chromatin with low accessibility, compared to Sox2a.

Together, we agree that the degree of chromatin compaction is sensitive to buffer composition, and we have clarified this point in the Results and Methods sections with the following additions:

> “We then imaged Sox2 and Sox2a binding dynamics to these chromatin fibers. Of note, under our imaging buffer conditions, corresponding to an ionic strength of 120 mM monovalent cations, chromatin is partially compacted [ref 37,79].”

> “The imaging buffer IB3 contained 100 mM KCl and 20 mM HEPES (with Na^{+} as the counterion), yielding 120 mM monovalent cations. Under these conditions, chromatin adopts a partially compacted state, as revealed by single-molecule FRET [ref 37,79].”

4. Fig 4-5. All the experiments with nucleosomes (Fig 4) and chromatin (Fig 5) were done using 601 sequences that strongly position nucleosomes. This is in contrast to native sequences, where nucleosomes can slide more readily. As a result, could it be possible that some of the derived constants were overestimated or underestimated? If the authors can carry out some of the experiments without strong positioning sequences, that would be ideal; otherwise, they should at a minimum discuss this as a potential limitation.

We agree that nucleosomes assembled on native sequences display more dynamic positioning and are generally less uniformly phased or saturated compared to those formed on 601 sequences. This could, in principle, influence the derived kinetic constants under native chromatin conditions.

However, the use of 601 sequences offers important experimental advantages, particularly the ability to precisely control nucleosome positions and densities. This level of control is essential for dissecting the mechanistic aspects of the target search process in a reproducible manner. Notably, the key findings from our in vitro assays align well with in vivo observations, suggesting that the conclusions remain robust despite the use of strongly positioning sequences.

To clarify this limitation, we have added the following statement to the Discussion section:

> "We note that the 601WS positioning sequences, while providing precise nucleosome placement, cannot recapitulate the heterogeneity of native chromatin, where nucleosome positioning, stability and occupancy varies. However, the strong concordance with in vivo data indicates that the main conclusions extend beyond the reconstituted system."

Minor points

5. Figure 1c: Since ChIP-seq was done here, it would be appropriate to present a ChIP-seq heatmap or minimally show some sample genome tracks, as was done in Fig 6, so the reader can appreciate the quality of the data.

We apologize for the lack of clarity in Fig. 1c: the data shown does not represent ChIP-seq data, but shows charge distributions across the Sox2 and Sox17 sequences. We thus improved the figure legend to clarify the methods used to generate this plot.
We do, however use ChIP to analyze TF Motif recognition (Supplementary Fig. 1). To aid interpretation and allow readers to assess data quality, we have included representative genome browser tracks in Supplementary Fig. 1c, corresponding to the data shown in Supplementary Fig. 1d.

6. Supplementary Fig. 3: Since Sox2 and Sox2a are compared in vitro, it would be appropriate to present also the size-exclusion chromatography profile and SDS-PAGE analysis for Sox2a, as was done for Sox2. This would help exclude the possibility that variations in the apparent DNA-binding activity are attributed to differences in protein purity and solubility. Moreover, it is recommended to check the ratio between absorbance at 260 and 280 for both proteins to exclude the possibility that variations in nucleic acid contamination contributed to the differences in DNA-binding activities in vitro.

To address the reviewer's suggestion, we now provide size-exclusion chromatography (SEC) profiles, SDS-PAGE analyses, and UV absorbance spectra for Sox2a alongside those for Sox2 and Sox17 in Supplementary Fig. 4a-c. These data confirm the comparable purity and solubility across all proteins. In addition, the 260/280 absorbance ratios (Table S10) are within the expected range, excluding the possibility that nucleic acid contamination contributed to the observed differences in DNA-binding activity.

7. Line 215: "This indicates that DFRs do not impact the overall probability of interactions between freely diffusing TFs and naked DNA." This is a rather strong and broad statement, given that only two DFRs were tested on one TF within the context of Supplementary Fig 3h. Unless the authors wish to test additional TFs and DFRs, it would seem appropriate to minimally moderate this statement to avoid the impression that they are referring to a general principle that would apply to every TF and every DFR.

We agree with the reviewer that the original statement was too broad, given that it was based on a limited set of constructs. We have therefore revised the sentence to refer specifically to DFR_{Sox2} and DFR_{Sox17}, which more accurately reflects the scope of our data:

> "This indicates that neither the DFR_{Sox2} nor the DFR_{Sox17} impact the overall probability of interactions between freely diffusing TFs and naked DNA."

Importantly, we have now performed additional experiments with new single-molecule data for Sox17, showing that Sox17 exhibits target search behavior on naked DNA similar to that of Sox2a (which carries DFR_{Sox17}). These results further support our conclusion and

demonstrate its consistency across two distinct DFRs. Accordingly, Fig. 3, and Supplementary Fig. 4,5 were updated.

Presentation

8. Supplementary Fig. 3d-e: A colour key is needed to explicitly indicate DNA length.

We have added a color key to explicitly indicate DNA length as requested.

Of note, Supplementary Fig. 3d-e is updated to Supplementary Fig. 5.

9. Supplementary Fig. 3g: Check if the x-axis unit should be in nm rather than bp.

We have corrected the x-axis unit to nm, as the original label was incorrect.

Of note, Supplementary Fig. 3g is updated to Supplementary Fig. 5.

10. Supplementary Fig. 3h: When referring to this figure, the text in Line 214 quotes different units than those indicated on the y-axis.

The discrepancy between the y-axis unit and the quoted text has been corrected. The y-axis now correctly includes bp^{-1}.

Of note, Supplementary Fig. 3h is updated to Supplementary Fig. 5.

11. Figure 3i: Consider specifying in the legend what each of the constants in the figure refers to, so readers do not have to refer back to the main text every time they look at this model figure.

We agree that including constant definitions in the figure legend improves clarity. We have updated the caption to read:

> "h, Scheme of the 1D target search model: initially, a TF binds nonspecifically to DNA with k_a. Once bound, the TF slides along the DNA with a speed of D_{1D} for τ_d until it dissociates from DNA. Upon reaching the target site, the TF either recognizes the target with k_r, or bypasses it. s_L: sliding length. K_R: target recognition constant."

Reviewer #2 (Remarks to the Author):

In this manuscript, Sakong et al. investigated the effect of electrostatic interactions in the DBD flanking regions (DFRs) of two IDR-containing pioneer TFs, Sox2 and Sox 17, on their DNA-/chromatin-binding dynamics both in vivo and in vitro. Importantly, they found that the differentially charged DFRs lead to distinct target search efficiency, as well as distinct search mechanisms between naked DNA and chromatinized DNA. These findings are significant not only in providing novel insights into the target search process of Sox TFs beyond the well-established facilitated diffusion model (which might very well be applicable to other TFs as well), but also sheds fundamental insights into the biophysical underpinnings of IDR-mediated TF-chromatin interactions in general.

Overall, the multi-disciplinary approaches adopted in this work are well-designed, the experimental evidence (comprising of single-molecule imaging, biochemical and genomic data) are solid and nicely complement each other, and the conclusions are convincingly supported by the data. I did not spot any major issue regarding logic, experimental design or data interpretation/argumentation. I, however, have a few issues concerning some of the experimental details, data analyses, further elaboration of the significance of certain findings, as well as several presentation-related issues, for the authors' consideration.

1. In the time-lapse imaging experiments where a 60-ms illumination is interspersed with varying dark times to obtain imaging intervals of 120, 240, 480 and 960 ms, it is unclear how the authors ensured that a certain bound molecule detected after a time-lapse is still the same molecule as before the light was switched off? Could it be that the original molecule unbinds and another molecule binds to the same binding site during the dark time, which would affect the subsequent analysis of the binding dynamics. An explanation on how this is avoided would be needed. Also, it might be useful to provide illustrative schematics for the various time-lapse illumination patterns, indicating the durations of each "on" and "off " intervals.

We thank the reviewer for this important question regarding potential ambiguity in time-lapse single-molecule imaging. We agree that, in isolation, a long imaging interval (e.g., 960 ms) could raise the concern that a molecule detected after a dark interval might not be the same one observed before the interval. However, this is precisely why we employed multiple time-lapse conditions with increasing intervals (120, 240, 480, and 960 ms), each interleaving a 60-ms illumination with a dark period. These conditions were carefully designed to yield overlapping time windows across which binding events could be sampled.

Each time-lapse condition captures a distinct kinetic regime, and by combining them, we ensure comprehensive temporal coverage. Shorter intervals (e.g., 120 ms) are sensitive to fast off-rates and minimize the chance of missed unbinding/rebinding events. Longer intervals (e.g., 960 ms) allow us to detect longer-lived events that would be underestimated in high-frame-rate acquisition due to photobleaching. Crucially, the datasets from each tl-condition overlap in their effective observational time windows, which ensures that any event falling within an intermediate lifetime range is sampled by at least one of the tl-settings.

To clarify this approach, we also updated 'In vivo smHILO microscopy setup' section in Methods:

> "Our multi-time interval approach interleaves 60 ms illumination with dark periods of 120, 240, 480, and 960 ms. This design creates overlapping observation windows that capture both fast off-rates and long-lived binding events, ensuring comprehensive sampling of all intermediate lifetimes."

Furthermore, in our experiments, spot density was sufficiently low to reliably apply the nearest-neighbour algorithm for trajectory reconstruction. The probability of a different molecule rebinding to the same location in the next frame is highly unlikely given our parameters across all the movies: the effective area of each spot is 17 px^2, while the average nucleus area is 12,100 px^2, with an average of 17 spots appearing per frame.

To further clarify our experimental design, we now include a schematic illustration of the time-lapse illumination patterns, indicating the durations of the “on” and “off” periods for each condition (new Supplementary Fig. 2a).

2. The SMT measurements, coupled with GRID analysis, identified 6 well-resolved bound states for the TFs studied, with residence times ranging from 100s of ms all the way up to 1000 s. This is substantially more than the 2-4 binding modes that are commonly observed for many other types of DNA-binding proteins, and could potentially represent something significant as a consequence of the complex chromatin-interaction landscape of Sox TFs. However, this significance is not adequately explained or elaborated on in either the Results or Discussion sections.

We thank the reviewer for raising this point. We have now elaborated on the significance of the six distinct binding states observed in our SMT-GRID analysis in the Discussion section, as follows:

> “In vivo GRID analysis revealed six Sox TF–chromatin binding states spanning from 0.18 s to 380 s in line with recent studies investigating TF binding dynamics that also revealed complex residence time distributions [ref 34,58,96-98]. As supported by our in vitro measurements, intermediate residence times (10-20 s) likely correspond to specific DNA binding, while shorter-lived states (sub-second to a few seconds) reflect transient nonspecific interactions with DNA, nucleosomes, or higher-order chromatin. The longest-lived states (>50 s) are consistent with previously reported extended TF residence on regulatory chromatin [ref 99,100] and may involve cofactor binding, transcriptional complex formation, or chromatin remodeling.”

3. The two main parameters used for quantifying DNA-binding from SMT measurements are the residence time and bound fraction. While these are certainly two commonly adopted and useful parameters, their utilities are not without limitations. For example, a molecule exhibiting many repeated transient binding events (each lasting a short duration) might end up being bound for a similar total duration as another molecule that undergoes only a few but long-lived binding events. In such situations, a useful alternative parameter would be the total fraction of time the molecule stays bound under each mode, which can simply be calculated as the product of residence time and the corresponding bound fraction, normalized against the total time the molecule spends in all binding modes (see a recent e.g. in Engl et al., Nature Commun. 2024). This parameter can hence more reliably report the partitioning of the TF molecules among the different binding modes, which might add a useful dimension to the binding landscape characterized in the work.

We thank the reviewer for this insightful comment. We have now introduced an alternative metric, ‘fractional occupancy’, to capture the total time a molecule spends in each binding mode. Fractional occupancy is defined as the product of the residence time and the GRID amplitude for each state, normalized by the sum across all states. The resulting spectra are shown in Supplementary Fig. 2e, and we quantified the occupancy of short-lived (<1 s) and long-lived (>10 s) binding events in Supplementary Fig. 2f.

Accordingly, the main text now reads:

> "We also calculated fractional occupancies [ref 55-57], i.e. the fraction of total bound time spent in each state (Supplementary Fig. 2e; see Methods). This revealed that Sox2 (0.66) and Sox17b (0.60) spend more time in long-lived states (>10 s) than Sox2a (0.47) and Sox17 (0.47) (Supplementary Fig. 2f, Table S2)."

Additionally, the 'GRID analysis' section in the Methods section has been updated as follows:

> "To generate the fractional occupancy spectrum (F_i) (Supplementary Fig. 2e), each GRID-determined state's amplitude (A_i) was weighted by its residence time (τ_i), and the resulting products were normalized by their total sum:
>
> $$F_i = A_i \cdot \tau_i / \sum_{j=1}^{100} A_j \cdot \tau_j \qquad \text{(Eq. 1)}"$$

More minor issues:

• Pg. 2 and 3: The acronym DFR was defined twice in the main text (in addition to that in the Abstract), whereas the terms DBD and mESC were not defined.

To improve clarity and consistency, we now define DFR only once in the Abstract and once in the main text, and we have removed the redundant definition. Similarly, DBD is now defined upon first use in the main text. As noted by the reviewer, mESC was already defined in the main text as "mouse embryonic stem cells (mESCs)."

• Pg. 2: "Among Sox TFs, Sox2 exhibited the most positively charged DFR and the highest mitotic chromosome binding." Please specify what does "chromosome binding" refers to here, *e.g.* binding affinity, residence time, fraction of molecules bound, *etc*.

We thank the reviewer for this comment. The term *"mitotic chromosome binding"* is used here in accordance with prior work, specifically Raccaud et al., 2019, where the Mitotic Bound Fraction was introduced as a quantitative measure of this phenomenon. What mitotic chromosome binding refers to is the fraction of TF molecules associated with mitotic chromosomes. In Raccaud et al., this was calculated by dividing the fluorescence intensity of the TF molecules on mitotic chromosomes by the total fluorescence intensity in the cell. To remain consistent with the established terminology in the field, we have retained the original wording. To ensure clarity for readers, we now explicitly cite Raccaud et al., 2019 at the first mention of "mitotic chromosome binding" in the main text. We have now included the following in the text:

> "Among the Sox TFs, Sox2 exhibited the most positively-charged DFR, which correlated with the highest fraction of TF molecules associated with mitotic chromosomes [ref 40]."

• Pg. 11: "…the nanoscopic association rate (*k*a), previously determined as about 2×105 M-1s-1bp-1…" A reference is needed here.

To clarify the source of the nanoscopic association rate, we have now explicitly referred to the corresponding data in the manuscript:

> "…the nanoscopic association rate (k_a), previously determined as about 2×10^5 $M^{-1}s^{-1}bp^{-1}$ for the three Sox TFs (Supplementary Fig. 5e);"

This reference now clearly indicates where the value was derived from in our in vitro single-molecule tracking measurements.

• Pg. 14: "…both Sox TFs showed three different residence times on CT+, while they displayed only two nonspecific binding populations on CT- (Supplementary Fig. 5d-h)." However, Supplementary Fig. 5f and h seem to show the opposite of what is stated in the main text. Please check and reconcile.

We thank the reviewer for catching this error. Upon review, we found that the labeling in the text was inadvertently swapped. We have corrected this in the revised manuscript.

• Fig. 1: The vertical axis labels for both Fig. 1a and 1c are "charge", but they clearly denote different things; consider changing 1a to "Net charge" to distinguish it from 1c? Moreover, in Fig. 1c which shows the charge distribution across the DFR sequence, the charge at each position takes on multiple fractional values between -1 and 1. However, according to the designation in Supplementary Fig. 1a, the charges assigned to individual residues can only take on values of +1 z (K & R), +0.5 z (H), or -1 z (E & D). In that case, how could the charges shown in Fig. 1c have values other than these values (especially various fractional values other than +/- 0.5 z)? Lastly, in Fig. 1h, log (res. time) is plotted on the horizontal axis, therefore its unit should not be "s", but rather "log s". A better way to label the axis would be "log10 (res. time / s)".

As suggested, we have updated the y-axis label in Fig. 1a to read "Net charge" to distinguish it from the vertical axis in Fig. 1c. In Fig. 1h, we also corrected the x-axis label to: log10(res. time) ($\log_{10}$ s) to more accurately reflect the logarithmic scale.

Regarding the charge distribution in Fig. 1c, we appreciate the opportunity to clarify. As described in the Methods section ("Electrical charge distribution generation"), the plotted values represent sliding-window averages of the assigned residue charges, using a window of five amino acids. Therefore, the charge at each position reflects the average of up to five neighboring residues, which results in fractional values between -1 and +1 (beyond the discrete residue values of +1, +0.5, or -1). This calculation was performed using EMBOSS Pepinfo [ref 118]. We have now specified this in the figure legend:

> "c, Charge distributions for Sox2 (top) and Sox17 (bottom), calculated as sliding-window averages of residue charges over five-amino-acid segments (see Methods)."

• Fig. 2c: Please define the vertical dotted line in the figure caption.

As suggested, we have clarified the figure caption by adding the following definition:

> "The dashed vertical line denotes the diffusion coefficient threshold at D=0.1 $\mu m^2/s$."

• Supplementary Figure 5d: The gray curves in the plots are not labeled, contrary to those in panels i-l (which indicate whether each curve denotes CT+ or CT-, *etc.*).

We thank the reviewer for pointing this out. In response, we have updated the labeling in Supplementary Fig. 7d to clearly indicate the identity of the gray curves. Additionally, we discovered that panels i–l were mislabeled: the bottom subpanels were incorrectly labeled as Sox2, when they actually correspond to Sox2a. We have corrected these labels accordingly and revised the figure legend to reflect these changes.

• Lastly, a few typos/language-related issues:

Pg. 28: Under “mESC culture” section: “…5% CO2 in GMEM…” should read “…5% CO2 in GMEM…”

Pg. 32: Under “In vivo smHILO microscopy setup” section, I believe “…pixel size of 11×11 mm2” should be 11×11 µm2?

All species names should be italicized (e.g. *E. coli*)

Thank you for pointing out these typos and formatting issues. We have corrected the CO_2 formatting on ‘mESC culture’ in Methods section, the pixel size unit on ‘In vivo smHILO microscopy setup’ in Methods section (now correctly stated as 11×11 μm^2), and ensured that “E. coli” is properly italicized throughout the manuscript.

Reviewer #4 (Remarks to the Author):

The present paper from Sakong, Fierz, and Suter focuses on understanding the target search of the SOX transcription factor family.

This is a key and long-standing question that dates back to the landmark 1970 paper by Riggs (author Ref13), who observed that the kON of LacI was about two orders of magnitude higher than the Smoluchowski limit. Since then, the TF field has been trying to understand how the ON-rates of TFs can be so high. 2 major models have been proposed. First, electrostatics. This is the model originally favored by Riggs and also others like Halfon (see https://portlandpress.com/biochemsoctrans/article/37/2/343/64235/An-end-to-40-years-of-mistakes-in-DNA-protein). Second is the model of facilitated diffusion, where a mix of 1D DNA sliding and 3D diffusion can accelerate the search. These two are not mutually exclusive. The second model forms the framework for this paper.

The problem of TF search is therefore a foundational textbook-level question, but it remains unsolved. In my opinion the present paper takes several important steps in the direction towards solving it and very much expands our understanding of TF search. There are lots of interesting observations in this paper that I won't attempt to summarize all of them, but among the things I found impressive were: 1) the nice focus on a family of SOX and the role of DFR regions, showing clearly how DFRs can affect the ON-rate; 2) the comprehensiveness of the investigation. A major strength of this paper is that it is very focused on the SOX factors and then comprehensively investigates these using a combination of genomics, in-cell single-molecule tracking, in vitro-single-molecule tracking, looking at both naked DNA and chromatinized templates, and careful modeling; 3) the findings are quite interesting and illuminating.

Overall, this is in my view an excellent paper. The comprehensiveness of the studies and the range of approaches is truly impressive, yet they are well-integrated, resulting in comprehensive insights from the orthogonal approaches. In my view, this paper is likely to be of quite general interest to a wide readership. Nevertheless, there are some issues that need to be addressed and I think there are some technical errors and problems in the present paper. However, these are all highly addressable as far as I can tell and will not overturn the major conclusions, they will just be required for correctness. I list my comments below.

COMMENTS:

1. DATA AND CODE IS MISSING: the authors provide Zenodo links to the data which is great but the links do not work. The whole point is to make the data available to the reviewer. Please correct this. Also it says the code and analysis scripts are available upon request. These need to be available as well, can you put on GitHub or Zenodo?

The Zenodo links in the manuscript have now been updated to ensure functionality.

2. I too am convinced by facilitated diffusion, but I think the authors overstate it. They say it increases search efficiency by orders of magnitude, which sounds like >100x-1000x. This is probably not true. Theory says that if search is 50:50 3D:1D, then acceleration is half the sliding length and Hammar estimated sliding length to be 45 bp, resulting in a max acceleration of 20x. This is still significant and important, but it is not "orders of magnitude". Please correct. I would also suggest having a sentence on the slight controversy about facilitated diffusion https://portlandpress.com/biochemsoctrans/article/37/2/343/64235/An-end-to-40-years-of-mistakes-in-DNA-protein

We thank the reviewer for this thoughtful point. We agree that the phrase “orders of magnitude” overstated the acceleration effect of facilitated diffusion. We have revised the text to more accurately reflect theoretical and experimental estimates and also acknowledge differing views in the field:

> “This dimensionality-reduced diffusion is believed to substantially increase search efficiency compared to free diffusion alone, although the magnitude of this effect is still debated [ref 16].”

3. Along these lines, where they estimate kON rates, can they compare directly to the Smoluchowski limit?

We thank the reviewer for this comment. We do not directly observe association rates at the Smoluchovski limit, both due to experimental constraints, and because Sox2 and related factors might encounter barriers upon DNA binding and thus might not follow purely diffusion-controlled association kinetics.

We have addressed this point in more detail also in comment 15, discussing the relationship between the Smoluchowski limit, k_{on}, and $k_{s,on}$.

4. Line numbers would have helped

We have now added line numbers to the revised manuscript for easier reference.

5. Page 2, 2nd paragraph, a “DFR-like” region also accelerates the CTCF target search by 2.5-fold in cells and is relevant for the discussion here https://www.nature.com/articles/s41589-019-0422-3

We thank the reviewer for this suggestion. We have now cited the recommended paper in the main text as ‘ref 34’, in the sentence:

> “…they can also play a key role in determining chromatin binding and diffusion behavior, even in the absence of interaction partners [ref 30-34].”

6. REF2 is an important early paper, but the key conclusions from this paper are now well-known to be wrong and has been disproven in several other papers, so probably makes sense to remove this from the reference section, since there are plenty of other papers on Sox2 that could also be cited like https://elifesciences.org/articles/22280

We have replaced ‘ref 2’ at this position with the suggested reference (https://elifesciences.org/articles/22280).

Nonetheless, we still think it is important to cite Chen et al. 2014, which remains an important contribution for establishing single-molecule imaging particularly for studies of Sox2 in mouse embryonic stem cells. However, we now cite it in the Discussion, specifically in the context of Sox2 SMT in living cells, along with other relevant papers (https://doi.org/10.1016/j.celrep.2023.112748, https://doi.org/10.7554/eLife.04236.001 and https://elifesciences.org/articles/22280).

7. Small thing, but in Fig 1d, it is a little unclear if the proteins include the N- and C-terminal segments. It looks like Sox2 is only DBD and DFR, but there are other regions. Can you clarify if those domains are included.

For Sox TFs, the DBD (HMG domain) is well characterized, and a transactivation domain has been suggested to reside in the C-terminus for most Sox TFs. However, the precise boundaries and definitions of these regions remain unclear. For this

reason, we chose not to include additional domain annotations in Fig. 1d to avoid implying a level of structural definition that is not yet established.

8. I understand the math behind GRID, but I am skeptical of the 6-well-resolved residence times. Fitting 6 exponentials with 6 fractions involves 11 free parameters (N-1 fractions, 6 rates) from limited and noisy data does not strike me as statistically robust. Moreover, the in-cell rates are completely consistent with the in vitro rates. Lots of things can go wrong inside cells, like a Halodye getting stuck somewhere. I am not saying they should remove the GRID analysis, but they should at least include a few sentences about the uncertainty and difficulty, the limited statistical power, and the disagreement with the in vitro measurements.

First of all, we would like to clarify that the GRID approach involves constraining an inverse Laplace transform to a grid of invariable decay rates with fixed spacing but variable amplitudes. This approach is thus distinct from simply fitting 6 exponential functions, as GRID makes no a priori assumptions about lifetime distributions.

Nevertheless, we initially also questioned the robustness of the approach with regards to determining residence time distributions of Sox TFs in cells. First, we thus analyzed a large number of cells, averaging 180 cells per TF and collecting ~300'000 trajectories per TF. Second, for every TF, we performed 100 independent GRID analyses, each time using data from a random subset of 80% of the cells.

For each iteration, we further filtered the resulting GRID spectra by averaging similar rates (<10% difference) into single rates ('effective GRID rates'), and discarding rates associated with low amplitude (below 0.1%). This resulted in 6 effective GRID rates consistently determined for most subsamples (see new Supplementary Fig. 2c)

Ultimately, this procedure resulted in the identification of six distinct populations of bound states (Fig. 1h). The widths of the peaks in the GRID spectra provide an estimate of the uncertainty associated with each state and the robustness of the approach.

To improve the clarity about the implemented procedure, we have updated the caption of Fig. 1h as follows:

> "h, Residence time distributions obtained from GRID analysis of 100 resampling iterations (randomly selected 80% subset of the full dataset shown in g), assembled into histograms (see Methods). The GRID amplitude indicates the bound fraction per unit of time for the corresponding residence times."

In the 'GRID analysis' section of the Methods, we have also provided a more detailed description of our data analysis:

> "For each Sox TF, 100 resampling iterations were performed. In each resampling iteration, a subset of 80% of single cells was randomly selected using a custom MATLAB code. A GRID spectrum was generated for each resampling iteration, and any GRID-determined dissociation rate with an amplitude below 0.1% was excluded. The remaining rates from all 100 resamplings were combined into a relative frequency histogram using 100 equally spaced bins (Fig. 1h)"

Finally, we wish to emphasize that longest-lived states (>50 s), which are not detected in vitro, align with previously reported extended transcription factor residence times at regulatory chromatin sites are consistent with previously reported extended TF residence on regulatory chromatin [ref 99,100] and may involve cofactor binding,

transcriptional complex formation, or chromatin remodeling. We have now discussed this further in the discussion section of the manuscript (see also below).

a. Along these lines, a res-time of 1000s corresponds to a kOFF of 0.001 sec^-1. But their photobleaching rate is stated to be 0.028 s^-1. I.e. the photobleaching rate is 28x faster than the slow residence times. Which means that the error on the photobleaching rate estimation must be <2-3%. It seems completely unrealistic to get such a low error on the photobleaching estimate and therefore, it does not seem statistically robust to me to try to estimate residence times that are much much lower than the photobleaching rate.

For the in vivo SMT experiments, we used a stroboscopic imaging sequence, i.e. a combination of 60 ms illumination followed by a defined dark time (60, 180, 420 or 900 ms), resulting in 120, 240, 480, and 960 ms imaging intervals. Our dye photobleaching rate was determined to be 0.028 s^{-1} (~36 s) under continuous illumination. Under stroboscopic imaging, the effective photobleaching rate is reduced, due to the inserted dark times. In particular, for the 960 ms interval movies, the effective photobleaching rate effectively reached 1.75 x $10^{-3}s^{-1}$ (i.e. a dye lifetime of ~570 s). This is the relevant parameter for determining the upper limit of resolvable residence times.

In the GRID spectra, the slowest off-rate resolved was 2.63 x $10^{-3}s^{-1}$ (~380 s), which lies well within the resolvable range set by this effective photobleaching rate.

Regarding the error estimation, a measured off-rate of 4.38 x $10^{-3}s^{-1}$ would be corrected to 2.63 x $10^{-3}s^{-1}$ using an effective photobleaching rate of 1.75 x $10^{-3}s^{-1}$. Assuming a 3% error in both photobleaching and measured off-rates, the resulting error in the corrected off-rate would be ~6%, below the uncertainty resulting from GRID analysis and subsampling (as described above). Together, we thus feel that photobleaching is not a major source of uncertainty in our results.

b. It would be essential to acknowledge this and tone this down. Ideally the very slow residence times should be cross-validated with orthogonal assays.

We would like to emphasize that we are not attempting to estimate residence times within a restricted range, as GRID is an unbiased approach that samples across a large space of possible rates. Importantly, in other studies where imaging was performed under similar conditions on different DNA-binding proteins, GRID did not detect such very long residence times (Engl et al., Nat. Commun., 2024; Tollenaere et al., bioRxiv, 2025). This supports that the detection of long-lived states is not an inherent artifact of the GRID method. Moreover, similarly long residence times have been reported for other TFs using alternative approaches, such as for glucocorticoid receptors using power-law fitting (Garcia et al., 2021, ref 98).

Nevertheless, we acknowledge that the low amplitude of these events limits the confidence with which they can be characterized. In particular, for Sox2a and Sox17, the GRID spectra appear more broadened and continuous. This is reflected in the manuscript where we described:

> "While the residence time distributions for Sox2 and Sox17b were narrow and well defined, Sox2a and Sox17 exhibited broader distributions (Fig. 1h), suggesting complex interactions associated with a continuum of binding energies."

To address the reviewer's concern, we have also revised the wording in the manuscript, changing "six well-resolved populations of bound states" to "six

populations of bound states" and "six distinct Sox TF–chromatin binding states" to "six Sox TF–chromatin binding states" to avoid overstating the significance of these states.

c. The in vitro residence times are ~1 sec and ~14 sec and this is reported on page 10. The authors should compare and contrast this with the in-cell residence times.

We have now elaborated on the significance of the six bound states observed in our SMT-GRID analysis in the Discussion section, as follows:

> "In vivo GRID analysis revealed six Sox TF–chromatin binding states spanning from 0.18 s to 380 s in line with recent studies investigating TF binding dynamics that also revealed complex residence time distributions [ref 34,58,96-98]. As supported by our in vitro measurements, intermediate residence times (10-20 s) likely correspond to specific DNA binding, while shorter-lived states (sub-second to a few seconds) reflect transient nonspecific interactions with DNA, nucleosomes, or higher-order chromatin. The longest-lived states (>50 s) are consistent with previously reported extended TF residence on regulatory chromatin [ref 99,100] and may involve cofactor binding, transcriptional complex formation, or chromatin remodeling."

9. DIFFUSION analysis: they fit 3 Brownian subpopulations to 1-CDFs and then they make an arbitrary threshold of 0.1 um2/s in Fig 2c and call everything below for "bound". But we know 0.1 um2/s is too high because people have done histone tracking and found the diffusion coefficient of bound chromatin to be 10x-100x slower that this. My question is why do the analysis like this, because while this is the right direction, but the analysis – unless I misunderstood in which case I do apologize – ignores several known and solved problems.

Most notably defocalization and localization error. As best I can tell, the analysis ignores localization error. While eq2 is theoretically correct, it is practically incorrect. It ignores localization error and motion blur and such. Motion blur may not be huge, but localization error must be accounted for. Defocalization is a serious issue as well, which if not taken into account will give you the wrong numbers. But this is now a solved problem. Mazza-2012 and Hansen-2018 showed how to account for both defocalization and localization error. So in short, the authors should adopt a rigorous analysis that takes into account both localization error and defocalization.

We agree that using a fixed threshold for defining "bound" versus "unbound" requires careful validation. In our initial analysis (Fig. 2c), we adopted a threshold of 0.1 μm²/s, in line with previous studies on chromatin-associated proteins, including ref 30 (https://doi.org/10.7554/eLife.75064), ref 61 (https://doi.org/10.1016/j.celrep.2023.112748), ref 62 (https://doi.org/10.1186/s13072-016-0093-1) and additional papers (https://doi.org/10.1371/journal.pcbi.1005136 added as ref 63, https://doi.org/10.7554/eLife.17667). These studies report diffusion coefficients for bound chromatin populations in the range of 0.01–0.1 μm²/s, supporting our choice as a conservative upper bound. In particular, ref 30 used the same thresholding to categorize bound fractions.

To address the reviewer's concern regarding localization error and defocalization, we performed Spot-On analysis (Hansen et al., eLife, 2018, ref 59), which accounts for axial defocalization in estimating diffusion coefficients and bound fractions. This analysis is presented in Supplementary Fig. 2, and its results are consistent with our threshold-based analysis, supporting the robustness of our findings. We initially decided to show less processed results in main figures.

We now clarify these points in the revised manuscript and explicitly reference the Spot-On analysis so that readers are aware that localization and defocalization errors were taken into account:

> "In 2D HILO imaging, fast-diffusing molecules often appear motion-blurred or leave the focal plane, leading to their under-detection [ref 59,64]. To make sure that this limitation does not bias our results, we also performed Spot-On analysis of jump distance distributions, which accounts for defocalization and localization errors [ref 59]."

10. In eq2, the authors show an alpha on time, but this is lost in eq 3. Do the authors try to fit alpha? If so, how subdiffusive are things?

Indeed, when determining diffusion coefficients from MSD data, we always fit alpha.

To analyze the degree of subdiffusive behavior we have now plotted determined alpha values for all investigated TFs as the new Supplementary Fig. 3b. In particular, we sorted tracks into three bins depending on their diffusion coefficient values ($0.01 < D < 0.1$; $0.1 < D < 1$; $1 < D < 10$ $\mu m^2/s$). Each dot represents the average alpha within each bin for a given cell. The majority of molecules exhibit subdiffusive behavior with alpha ~ 0.8. Overall, Sox2 and Sox17b show somewhat stronger subdiffusive behavior than Sox17 and Sox2a, possibly resulting from stronger nonspecific DNA interactions.

We have now included this observation in the manuscript as follows:

> "Additionally, we determined the anomalous diffusion exponent α, to be ~0.8 for all the Sox TFs (Supplementary Fig. 3b; see Methods), suggesting a subdiffusive behavior. Notably, Sox2 and Sox17b exhibited slightly lower α than Sox17 and Sox2a, indicating more constrained chromatin interactions."

11. When the authors do the pseudo-ON-rate analysis, they do not clearly describe where the equations come from. Notably, REF2 did such an analysis but the equations in that paper are wrong. The incorrect math of REF 2 from fixed in Loffreda et al. https://www.nature.com/articles/s41467-017-00398-7 Can you specify where the equations come from?

In our manuscript, we define the pseudo on-rate (k_{pn}) as the time-averaged binding frequency of TFs to the whole genome, determined by the number of immobilized molecules per imaging frame (see Methods). This approach follows the method described in Raccaud et al., 2019, which is cited in the text as ref 40. To further clarify, we have updated the equation from the original version, which is described in the Methods section.

12. The in vitro experiments are extremely comprehensive and well-done. I think this is such a major strength of this paper. All their analysis and modeling assumes that they see sliding, but if I am not mistaken, they never directly observe sliding? Sliding has been directly observed in prior work. Maybe the DNA is too short here to observe it. If they cannot directly observe sliding, they should comment on it in the main text and make clear that prior work has observed it but here it is just assumed to be true.

In our work, we use the term "sliding" in the strict sense of 1D diffusion without dissociation from the DNA, but we acknowledge that this movement is not directly resolvable under our experimental conditions.

To clarify, we have added the following sentence to the text (p.12):

> "We interpret this increased search efficiency for both Sox TFs on longer DNA as evidence that 1D sliding facilitates target search. This is consistent with direct experimental evidence for TF sliding for both prokaryotic and eukaryotic TFs and DNA binding proteins [ref 19, 73-76]."

13. The presentation is in general well-done and comprehensive. The SI tables are great and the methods well-described for the most part. That said, the kinetic parameters for the GRID analysis appear to be missing and seem like a full SI table for the in-cell SMT parameters would be required.

We thank the reviewer for highlighting this point. We have now added the GRID spectra values from Fig. 1h as Table S1 in the SI to provide a complete overview of the in-cell SMT parameters. The SMT input parameters were already included in Table S14 and Table S15.

14. Top of page 11, where they discuss non-specific binding times, here they may want to compare to https://www.nature.com/articles/ncomms8357 which nicely studied this.

We have now added further information to the indicated text passage, as shown below:

> "Similarly, the nonspecific residence time of LacI in E. coli has been reported as less than 5 ms using SMT [ref 71], whereas other TFs, such as TetR or p53 exhibited longer non-specific residence times (158 ms and 900 ms, respectively) [ref 64,72]."

15. Page 11, can they directly compare to the Smoluchowski limit for kON rates here? The whole reason to suggest facilitated diffusion is higher than Smoluchowski, so this seems like a great place to calculate what they expect and compare it here.

In our *in vitro* system to directly observe TF binding via single-molecule TIRF imaging, where reactions take place on a passivated glass surface, our observed rate constants are generally below the theoretical limit for diffusion controlled association for homogeneous systems. Moreover, Sox2 variants may have to overcome energy barriers, such as adopting the correct conformation or positioning, to bind DNA upon collision. Sox2 might thus not follow solely diffusion controlled kinetics.

While we cannot directly compare our findings to the theoretical Smoluchowski limit, we can estimate how facilitated diffusion contributes to the target search process, using the DNA length-dependent increase in nonspecific (Supplementary Fig. 5c,d) and specific on-rate (Fig. 3i).

For a single 7 bp Sox2 binding site, we observe a nonspecific on-rate of $1.4 \times 10^6\ M^{-1} \cdot s^{-1}$. In the absence of sliding, this represents an upper limit for site recognition under our conditions. Going to longer DNA segments, we see a 5- (Sox2a, Sox17) to 10-fold (Sox2) increase in specific on-rate, most likely due to facilitated diffusion (see also Eq. 12).

16. I love Fig 3i. Strongly consistent with 1D sliding and facilitated diffusion.

We thank the reviewer for this encouraging comment.

17. Fig 4e, but more generally, when you do residence time fitting of multiple populations, you will undercount slow ones and overcount fast ones. Suppose you have 2 TFs in a nucleus. One TF binds for 1000 sec. The other TF binds for 0.5 sec ON and then diffuses and then back ON for 0.5, and does this 500 times over the 1000

sec. Then in the 1000sec movie, one TF gave 1 binding event and the other TF gave 500 binding event trajectories, giving apparent fractions of 1/501 vs. 500/501, but in reality it was 50:50. Do you correct for this? If not, the equation to do so can be found in https://www.sciencedirect.com/science/article/pii/S2589004222020521#sec4 under section “Analysis of trajectories from slow 2 Hz SPT movies”

We agree that from a perspective of occupancy, the long events are ‘undercounted’. In our text, we however discuss ‘fraction of binding events’ (which again are potentially fewer for long binders).

It is however interesting to see how the data looks from an occupancy point of view. We have thus determined ‘true fractions’ by scaling the exponential amplitude value by its associated time constant, as described in the mentioned paper. The new plots are shown in Supplementary Fig. 5j, Fig. 4f,h and Supplementary Fig. 7f,h.

18. A bit more discussion of how 1D sliding works on nucleosomal DNA could be nice.

We have now discussed about the 1D diffusion along nucleosomal DNA in the Discussion section:

> “We propose that Sox2 can diffuse along chromatin by exploiting nucleosome architecture. The close spatial proximity of the two DNA gyres on the nucleosome surface allows Sox2 to bypass extensive wrapped regions, while steric occlusion of the inward-facing gyre restricts sampling to exposed turns. Furthermore, spontaneous chromatin fluctuation [ref 95] transiently reveals buried DNA segments. Consequently, TFs with high nonspecific chromatin-binding ability, like Sox2, may exploit these fleeting openings to outcompete histones and invade compact chromatin more efficiently.”

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

As I wrote in my first review, this is an excellent paper that addresses a long-standing and deeply interesting question using new approaches and it very effectively applies a live-cell SMT, in vitro SMT, and genomics integrated approach. The results are compelling and make sense. As I wrote in my first review, Fig 3i is especially compelling. I have reviewed many papers for Nat Comm over the last 5 years and among the ones that were eventually published, I think the present paper is near the top. I really think it is an excellent and compelling study that is likely to be of wide interest.

But it does have a significant weakness, which is the rigor of the live-cell SMT analysis. I pointed these out in my first review, but the authors have chosen to largely ignore them. This is disappointing and the issues will be obvious to experts in the field and turn them off what is otherwise an outstanding paper.

As far as I can tell from the authors response, they agree with my concerns (e.g. diffusion analysis is not correct), but rather than correcting it, they chose to keep the problematic analysis in the main figures and then more correct analyses to the SI. Likewise, we know from dozens of "chromatin dynamics studies" (e.g. using H2B tracking) that the diffusion coefficient of chromatin is not 0.1 um^2/s/.

So I would like to ask the authors to do one of two things: 1) Maybe I am wrong, in which case, please run reaction-diffusion simulations, add defocalization and localization noise, and demonstrate that the diffusion analysis works well. Or 2) replace it with more correct analyses.

Regarding my comments in the last round.

Comments 1-7 have been addressed.

Comment 8 has not really been addressed. I remain skeptical of the 6 states and my request was to discuss the uncertainty in one sentence in the main text, which the authors have not done. They have added some discussion to the discussion section but they have not really addressed my concern.

In 8a, the authors misunderstand my concern about relative error. I do not think you can accurately fit a residence time with a photobleaching rate being similar. I remain extremely skeptical about the GRID results and the authors have chosen not to explicitly discuss the uncertainty in the main text which is disappointing.

We have now explicitly acknowledged the uncertainty of the longest-lived GRID states in the main text. The revised text is in line 126 to 130. This clarification makes clear that the slowest GRID components are interpreted qualitatively due to their proximity to the photobleaching limit.

The authors have also chosen not to address **Comment 9**.

In comment 9 I pointed out that eqn 2 (now eqn 3) is incorrect for data. MSD should be 4st^alpha + loc error + motion blur. This is a solved issue. Why do it incorrectly?

The authors report an alpha of 0.8 for the DNA bound fraction. Again, we know this is incorrect. Is there a realistic polymer model with an exponent of 0.8? Rouse says 0.5. Fractal globule says 0.4. Recent work, spanning several orders of magnitude suggests the exponent is more like 0.3-

0.4 https://www.biorxiv.org/content/10.1101/2025.05.10.653248v1.full and another paper says something similar https://www.biorxiv.org/content/10.1101/2025.09.24.678119v1

Again, why do this incorrectly in the main text and correctly in the SI (Fig S3d).

It is true that there are several papers that use 0.1 um^2/s as the cut-off for the chromatin diffusion, but we know this is a short-hand and we know this is incorrect. If you do not account for localization error, you will dramatically overestimate the bound Diff Coef.

It does the field a disservice to propagate incorrect approaches and it also makes the paper look bad. This is an extremely impressive paper, but since you open with the in vivo SMT and do these analyses incorrectly, it will turn off expert readers from what is otherwise a fantastic paper.

The “fit diffusion coefficients from each trajectory, then fit the distribution of coefficients” was benchmarked in Figure 3 of https://elifesciences.org/articles/33125 and the performance is terrible For example, it can easily get the Bound fraction wrong by 30-55 percentage points.

I would like to insist that the authors either remove the currently incorrect diffusion analysis, or validate that their approach is indeed correct and accurate by performing reaction-diffusion simulations, adding localization noise to their simulated data, applying their analysis methodology to it, and demonstrating what they are doing is indeed correct. It is possible that I am missing something and I would be happy to be persuaded that I am, but in its current form, the analysis appears to be the “distribution of diffusion coefficient analysis” that was shown in 2018 to not work at all. Given that alternative more accurate approaches are in Fig S3d, it is really difficult to understand why they want to keep doing it incorrectly?

We agree that diffusion analyses should properly account for localization error, motion blur, and defocalization. We agree that the MSD-based diffusion coefficient (D) distribution analysis is suboptimal given that the trajectories are too short to resolve subdiffusing behaviors and tends to overestimate D values, although originally we believed that it would provide a minimally processed comparison of relative bound fractions across Sox TFs.

We have now removed this analysis from the Main Figure 2, and instead present a State Array (SA) analysis (Heckert et al., 2022; https://pubmed.ncbi.nlm.nih.gov/36066004/), which explicitly models localization and defocalization errors while jointly inferring trajectory-level diffusion coefficients and uncertainty estimates on a diffusion-error grid using an RBME likelihood. This approach allows us to obtain diffusion coefficient distributions for each movie, capturing the landscape of D-values that reflect DFR-dependent effects on TF diffusive behavior. Spot-On analyses are retained in the main and supplementary figures as an orthogonal validation and especially to minimally resolve overlapping diffusive states (D > 0.05) by separating them into two components. Note that we have now restricted the ‘time-point’ parameter to 8 (previously 25), as this is the conventional and recommended setting for 100 Hz SMT acquisitions and 10 frames of mean trajectory length. With this setting, we avoid over-weighting long trajectories and ensure sufficient sampling of out-of-focus or short-lived molecules in jump length histograms. Importantly, all trajectories remain included in the single-cell Spot-On analysis without restricting the jump-to-consider parameter (revised Fig. 2e-g, S3b).

The latest study on chromatin diffusion that combined multiple imaging techniques to span several orders of magnitude in time in single-particle tracking (Mazzocca et al., 2025; https://pubmed.ncbi.nlm.nih.gov/40463058/) showed that chromatin exhibits strongly subdiffusive dynamics ($\alpha \approx 0.3$), corresponding to effective diffusion coefficients in the range of $\sim 10^{-3}$–10^{-2} $\mu m^2\ s^{-1}$. It is also well aligned with previously reported values (https://pubmed.ncbi.nlm.nih.gov/28035241/; https://pubmed.ncbi.nlm.nih.gov/27764097/).

Based on this finding, we change the threshold as 10^{-2} µm² s^{-1} to define chromatin-bound Sox TFs.

Accordingly, the manuscript is updated:
from line 138 to 163 for in vivo diffusion analysis in the Results section;
from line 977 to 983 for State array analysis in the Methods section;
from line 984 to 992 for Spot-On analysis in the Methods section.

Comment 10: Again, I have big concerns about the statistical robustness. If you fit the alpha of each trajectory, you have a power law. To robustly fit a power law you need at least 2 log in both dimensions https://www.science.org/doi/10.1126/science.1216142 For an alpha of 0.8, this would be about 3 log in time. So trajectories should contain ca. 1000 frames, which I would imagine is not the case.

We agree with the reviewer and removed the anomalous diffusion exponent (α) data, as we are lacking statistical strength to determine it accurately.

Reviewer #4 (Remarks to the Author):

The authors have fully addressed my comments and concern and I strongly recommend the publication of this excellent paper without delay and without going back to reviewers.
I appreciate the changes the authors have made, and I really think this is an unusually excellent and interesting paper, that I am sure will be well received by the field.

SMALL THINGS
Fig. S3c is a bit unconventional. To my knowledge, you should use all the displacements/jumps unless you think you are missing fast moving ones due to motion blur. I might suggest to remove it but it is not a big deal.

We thank the reviewer for their appreciation of our work. For the diffusion coefficient analysis using Spot-On (data displayed in Figure 2e-g and Supplementary Figure 3b), we used all the trajectories. We decided to keep Supplementary Figure 3c in order to illustrate how state fractions shift when progressively incorporating longer trajectories.

In this manuscript, Sakong et al. investigated the effect of electrostatic interactions in the DBD flanking regions (DFRs) of two IDR-containing pioneer TFs, Sox2 and Sox 17, on their DNA-/chromatin-binding dynamics both *in vivo* and *in vitro*. Importantly, they found that the differentially charged DFRs lead to distinct target search efficiency, as well as distinct search mechanisms between naked DNA and chromatinized DNA. These findings are significant not only in providing novel insights into the target search process of Sox TFs beyond the well-established facilitated diffusion model (which might very well be applicable to other TFs as well), but also sheds fundamental insights into the biophysical underpinnings of IDR-mediated TF-chromatin interactions in general.

Overall, the multi-disciplinary approaches adopted in this work are well-designed, the experimental evidence (comprising of single-molecule imaging, biochemical and genomic data) are solid and nicely complement each other, and the conclusions are convincingly supported by the data. I did not spot any major issue regarding logic, experimental design or data interpretation/argumentation. I, however, have a few issues concerning some of the experimental details, data analyses, further elaboration of the significance of certain findings, as well as several presentation-related issues, for the authors' consideration.

1. In the time-lapse imaging experiments where a 60-ms illumination is interspersed with varying dark times to obtain imaging intervals of 120, 240, 480 and 960 ms, it is unclear how the authors ensured that a certain bound molecule detected after a time-lapse is still the same molecule as before the light was switched off? Could it be that the original molecule unbinds and another molecule binds to the same binding site during the dark time, which would affect the subsequent analysis of the binding dynamics. An explanation on how this is avoided would be needed. Also, it might be useful to provide illustrative schematics for the various time-lapse illumination patterns, indicating the durations of each "on" and "off " intervals.
2. The SMT measurements, coupled with GRID analysis, identified 6 well-resolved bound states for the TFs studied, with residence times ranging from 100s of ms all the way up to 1000 s. This is substantially more than the 2-4 binding modes that are commonly observed for many other types of DNA-binding proteins, and could potentially represent something significant as a consequence of the complex chromatin-interaction landscape of Sox TFs. However, this significance is not adequately explained or elaborated on in either the Results or Discussion sections.
3. The two main parameters used for quantifying DNA-binding from SMT measurements are the residence time and bound fraction. While these are certainly two commonly adopted and useful parameters, their utilities are not without limitations. For example, a molecule exhibiting many repeated transient binding events (each lasting a short duration) might end up being bound for a similar total duration as another molecule that undergoes only a few but long-lived binding events. In such situations, a useful alternative parameter would be the total fraction of time the molecule stays bound under each mode, which can simply be calculated as the product of residence time and the corresponding bound fraction, normalized against the total time the molecule spends in all binding modes (see a recent

e.g. in Engl et al., *Nature Commun.* 2024). This parameter can hence more reliably report the partitioning of the TF molecules among the different binding modes, which might add a useful dimension to the binding landscape characterized in the work.

More minor issues:

- Pg. 2 and 3: The acronym DFR was defined twice in the main text (in addition to that in the Abstract), whereas the terms DBD and mESC were not defined.
- Pg. 2: "Among Sox TFs, Sox2 exhibited the most positively charged DFR and the highest mitotic chromosome binding." Please specify what does "chromosome binding" refers to here, *e.g.* binding affinity, residence time, fraction of molecules bound, *etc*.
- Pg. 11: "…the nanoscopic association rate (k_a), previously determined as about 2×10^5 $M^{-1}s^{-1}bp^{-1}$…" A reference is needed here.
- Pg. 14: "…both Sox TFs showed three different residence times on CT+, while they displayed only two nonspecific binding populations on CT- (Extended Data Fig. 5d-h)." However, Extended Data Fig. 5f and h seem to show the opposite of what is stated in the main text. Please check and reconcile.
- Fig. 1: The vertical axis labels for both Fig. 1a and 1c are "charge", but they clearly denote different things; consider changing 1a to "Net charge" to distinguish it from 1c?

  Moreover, in Fig. 1c which shows the charge distribution across the DFR sequence, the charge at each position takes on multiple fractional values between -1 and 1. However, according to the designation in Extended Data Fig. 1a, the charges assigned to individual residues can only take on values of +1 z (K & R), +0.5 z (H), or -1 z (E & D). In that case, how could the charges shown in Fig. 1c have values other than these values (especially various fractional values other than +/- 0.5 z)?

  Lastly, in Fig. 1h, log (res. time) is plotted on the horizontal axis, therefore its unit should not be "s", but rather "log s". A better way to label the axis would be "$\log_{10}$ (res. time / s)".
- Fig. 2c: Please define the vertical dotted line in the figure caption.
- Extended Data Figure 5d: The gray curves in the plots are not labeled, contrary to those in panels i-l (which indicate whether each curve denotes CT+ or CT-, *etc.*).
- Lastly, a few typos/language-related issues:
  - Pg. 28: Under "mESC culture" section: "…5% CO2 in GMEM…" should read "…5% CO_2 in GMEM…"
  - Pg. 32: Under "In vivo smHILO microscopy setup" section, I believe "…pixel size of 11×11 mm^2" should be 11×11 μm^2?
  - All species names should be italicized (e.g. *E. coli*)