

Counteraction of HMGB1 at ss-dsDNA junctions maintains liquidity of protamine-DNA co-condensates

Corresponding Author: Professor Huan-Xiang Zhou

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Ahlawat et al. describes the in vitro DNA condensing behaviour of HMGB1, Protamine and DNA, using optical tweezers, microfluidics and confocal microscopy. The noteworthy results are that HMGB1 localizes to ssDNA/dsDNA junctions, and can promote reannealing on retraction. This is in line with the known preference of HMGB1 for deformed/deformable NA substrates. The authors build on their previous work on Protamine (which forms tangles on tethered lambda DNA and solid aggregates in bulk measurements) to show that HMGB1 can also act to convert tangles into reversible bridges (by force-distance measurements) and to liquify aggregates (by FRAP). Crucially, these properties depend on the HMGB1 acidic tail, which is to be expected given previous work in the field by other labs, over more than two decades, but the authors do a good job of citing key papers in the HMGB1 literature and placing their findings in context. The experiments are well executed, and provide sufficient evidence for their conclusions. An expert in optical tweezers may have more to say, but to my mind the results speak for themselves to a large extent and are visually compelling.

The link to biology is a little more tenuous/speculative given the system is necessarily reductionist, and mixes Protamine from salmon with HMGB1 from mouse/human, although it may be in principle OK. To what extent do the transition proteins in fish resemble HMGB1 in mouse/human? It would be good to state this up front, since HMGB proteins are diverse in the number of boxes they contain and the length of their tails.

As an aside, it is good to see a lab that actually measures the effect of the addition of a GFP tag.

Specific comments:

1) The buffer conditions should be stated up front in the main text, since the ionic strength will play a part in determining the (mainly electrostatic) phenomena being described.

2) p9: "These observations support our mechanistic interpretation of single-molecule results on tangle-to-bridge conversion. When forming liquid droplets in the ternary mixture, HMGB1 indeed appears to peel a portion of protamine molecules away from dsDNA. The need for 10% PEG in the bulk experiment could be due to an effective dilution when the dimensionality of space is increased from one (on the dsDNA in the single-molecule setting) to three (in solution)"

The bulk experiment uses short DNA, which is surely the more likely reason for the need for PEG, given HMGB1's ability to scan DNA (e.g. Iwahara, also Kaplan for other TFs).

3) p10 "In forming a layer on the surface of relaxed dsDNA, the HMG boxes of HMGB1 molecules would have to bind sites along the DNA (Figure 7, last row in the single-molecule portion). The freed acidic tails would then bind HMG boxes of additional HMGB1 molecules, and this process may propagate multiple rounds."

This seems too speculative, also the graphical abstract. The freed tails could also make cis interactions with basic residues on the back face of their own HMG boxes, or simply act to neutralise positive charge to allow closer approach of other HMGBs on the DNA.

Reviewer #2

(Remarks to the Author)

- In this manuscript, the authors perform single-molecule and condensation experiments regarding the DNA interactions of protamine and HMGB1. Previously, the same research team published two papers detailing similar studies of protamine—first in JACS (2024) on condensation, and then in PNAS (2026) on single-molecule studies combined with condensation experiments. The current work is an extension of their prior studies, with the primary difference being the addition of HMGB1. Although I appreciate the attempt to build complexity by studying the interplay between two proteins rather than just one, the present manuscript is not suitable for publication. It is too preliminary, lacks mechanistic insights, and does not provide data testing the physiological relevance of the observations.
- The manuscript is difficult to read and appears to have been put together hastily. The text contains several instances of tentative statements (see examples listed below), and the authors do not attempt to support these statements through additional experimentation or quantification.
- "a thin coating covered the presumed dsDNA section"
- "adjacent (presumed) ssDNA section"
- "It looked as if the focus was formed with peeled-off ssDNA"
- "It was as if the focus moved together"
- One of the major claims of the paper is that HMGB1 can convert protamine-DNA tangles into bridges. There are two major issues with this claim. First, the authors do not fully characterize these tangles and bridges in terms of various parameters; they mainly show a few example single-molecule trajectories. I strongly recommend that they quantify their findings in a statistically meaningful way instead of asking readers to rely on vague statements. More importantly, the single-molecule data shown in Figure 3 were obtained by co-incubation with both proteins. If the authors want to claim a "conversion," they must start with a tangle formed by protamine alone and then demonstrate that the addition of HMGB1 dissolves this tangle. Furthermore, this must be demonstrated across dozens of DNA tethers to be convincing.
- The authors do not adequately justify the physiological motivation for studying HMGB1 and protamines together. They mention that in *Drosophila*, protamine-like proteins contain HMG boxes, but they cite no evidence that this is also true for mammalian systems.
- They also claim in the abstract that a liquid state enables the recruitment of repair machinery to restore the duplex structure, but they do not provide any supporting data.
- In the main text, they state that Figure 3d shows the "conversion rate." However, rates are measured in units of inverse time (time^{-1}), whereas the panel only shows the absolute number of conversions, not a true rate.
- In the introduction, they write: "It can be argued that, early in spermiogenesis when protamines are first expressed, chromatin needs to be maintained in a liquid state in order for the recruitment of repair factors." They do not provide any supporting reference for this argument. In fact, the sentence that follows ("Indeed, premature synthesis of protamine 1 in mouse spermatids resulted in precocious condensation of nuclear DNA.") does not provide any support, as it has nothing to do with DNA repair.
- In the introduction, they write: "Finally protamine condenses double-stranded DNA (dsDNA) into hyperstable 'tangles',¹⁶ leading to global transcription silencing." This sentence implies that their observation of tangles, reported in their JACS paper (Reference 16), has a causal link to global transcription silencing, but I see no actual support for this. When do the tangles appear during spermiogenesis, and when is transcription globally silenced relative to that event?
- In the introduction, they write: "These observations led us to suggest that the presence of ssDNA tracks during histone-to-protamine transition might contribute to maintaining the liquidity of DNA condensates." How are these single-stranded tracks generated, and how are they relevant to HMG proteins?
- In the introduction, they write: "...while protamine produces a large condensing force (~ 10 pN) in l-DNA held at low stretch". It is not clear what is meant here. Furthermore, is 10 pN considered "large" within the context of this study?
- In the Results section, they write: "To mimic strand breaks during the histone-to-protamine transition in spermiogenesis, we overstretched l-DNA by dual-trap OT to unpeel ssDNA tracks from nicks." While a single-strand break is equivalent to a nick—meaning preexisting nicks in the DNA can certainly mimic a single-strand break—a nick does not inherently result in a single-stranded region. Therefore, their justification for this experimental scheme is questionable.
- In the Results section, they write: "Moreover, HMGB1 foci promoted DNA strand separation but protamine foci hindered it." I could not find where this is demonstrated in the data.
- In the Results section, they write: "...such that pretransition condensates were dominated by ssDNA but post-transition spread solely involved dsDNA." It is unclear what specific transition they are referring to here.

Reviewer #3

(Remarks to the Author)

This manuscript titled "Counteraction of HMGB1 at ss-dsDNA junctions maintains liquidity of protamine-DNA co-condensates" investigates ssDNA and dsDNA interactions with protamine and HMGB1 using primarily single-molecule force-fluorescence assays. The authors show that protamine and HMGB1 exhibit different behaviors on force-stretched DNA with regions of ds and ssDNA. Overall, the experiments in the paper seem to be of high quality and the scope of work is substantial. We have some experimental and analysis concerns that need to be addressed but otherwise it seems like a solid paper.

Major concerns:

- Many terms are used throughout the paper without clear definition. While we *think* we know what you mean for most, the following terms must be clearly defined: "bridges", "tangles", "bends", "liquid-like", "solid-like", "wetting transition", "solid-like tangles to liquid-like bridges"

- Relatedly...Bends, tangles and bridges: These should be clearly identified on every relevant graph, and explained in the methods how they are identified and quantified. In many places the text described these features and we had trouble interpreting which regions of the force-extension were which.
- An experimental reference WLC curve for “naked” DNA should be included in figure S1. It is presumed throughout the text that the dashed line is basically your control condition but you haven’t experimentally shown this yet. Furthermore, the statement about S1 changes in flexibility and/or contour length need to be quantified with statistics. We doubt you’re drawing these conclusions from single curves, so please provide the mean and SD for contour and persistence length for the 3 cases (naked, +HMGB, +HMGB deltaC). Would be great to have +SYTOX here as well.
- Condensate data in Figure 5 – what is the rod like structure? The sizes here don’t seem to make sense. It also seems quite rigid given the length. Even something like DNA origami tubes don’t typically stay this straight over these length scales without curves or bends. Have you eliminated the possibility that this is some experimental anomaly (e.g. microscopic dirt, bacteria, other impurity). Visually the amount of aggregates in a and c are quite different. If the concentrations are the same then even if aggregates take different forms we should be observing the same amount of material visually, right? There is also a claim in the text that “aggregates converted into droplets” – I assume that this is a misstatement or have you actually observed a single aggregate converting into a droplet? If so please include a supplemental video of that.
- Intercalators can also change force extension behavior of DNA, but I didn’t find this covered in a meaningful way (either textually or in control experiments). Our understanding here is SYTOX was used with protamine but not with HMGB1, and yet we didn’t see any controls with just SYTOX. We’re concerned that there could be misinterpretation of an effect of SYTOX with protamine – how can you be sure this is not the case? It wasn’t helped that it was very difficult to figure out which force extension curves had SYTOX and which didn’t.

Minor concerns:

- Figure S2 is by far the clearest evidence of the ssDNA vs dsDNA argument – I was shocked to read this so late in the paper (after I was coming up with all my doubts), and shocked it was only in SI. If this were my paper I would consider highlighting that more substantially.
- There is a super high density of information in text, figures, and legends that impedes easy comprehension. Better annotating/labeling/titling graphs and images in the figures themselves would help a lot. Also it may be possible to move some of the high-information density into discussion. These are not major but both of us commented to each other on how difficult it was to get through the paper with all of the cross referencing between text, figure, and caption.
- Scale bars are missing on some images. Every image should have a scale bar in main figures and SI.
- Places listed as “frames skipped” or “cycles not shown” should at least be explained why, and ideally be included in the whole in SI for completeness
- A figure similar to your final figure in the recent PNAS paper would be very helpful to guide readers to your interpretation of what’s happening.
- Figure 1 – all x-axes need to face the same direction, a few are inverted which makes it very confusing.
- Page 6 last paragraph “wetting” discussion does not seem like the right language, or at least needs to be clear what you mean.
- Page 5 – paragraph 1 - “characteristic dip” needs clarification and notation on figure.
- N values should be reported on relevant graphs. Distinguishing number of tethers and number of pulls is also important.
- In the introduction and throughout the text, we found it sometimes difficult to discern established fact from speculation. Particularly the last paragraph of the introduction, and some other phrases scattered throughout such as “marked a transition from ssDNA-dominated to dsDNA-dominated condensates”, “signifying the formation of tangles (hyperstable nested loops crosslinked by protamine)”, “Moreover, HMGB1 foci promoted DNA strand separation but protamine foci hindered it.”

Reviewer #4

(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.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

My original concerns have been addressed in the revision.

Reviewer #3

(Remarks to the Author)

The authors did a good job addressing our concerns, and have resolved our technical uncertainties. The supplemental video was eye opening and very nice to include. A few figures are still a bit confusing to follow the panels (e.g. Figure S7, and other figures where each individual panel is not labeled), but this is a minor concern. The updated definitions are better but there is still some room for improvement in clarity. Overall nice paper that is mostly ready for publication.

Reviewer #4

(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.

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We thank the reviewers for their constructive comments. Our point-by-point response is given below in blue.

Reviewer #1 (Remarks to the Author):

The manuscript by Ahlawat et al. describes the in vitro DNA condensing behaviour of HMGB1, Protamine and DNA, using optical tweezers, microfluidics and confocal microscopy. The noteworthy results are that HMGB1 localizes to ssDNA/dsDNA junctions, and can promote reannealing on retraction. This is in line with the known preference of HMGB1 for deformed/deformable NA substrates. The authors build on their previous work on Protamine (which forms tangles on tethered lambda DNA and solid aggregates in bulk measurements) to show that HMGB1 can also act to convert tangles into reversible bridges (by force-distance measurements) and to liquify aggregates (by FRAP). Crucially, these properties depend on the HMGB1 acidic tail, which is to be expected given previous work in the field by other labs, over more than two decades, but the authors do as good job of citing key papers in the HMGB1 literature and placing their findings in context. The experiments are well executed, and provide sufficient evidence for their conclusions. An expert in optical tweezers may have more to say, but to my mind the results speak for themselves to a large extent and are visually compelling.

We thank the reviewer for the strong endorsement of our study.

The link to biology is a little more tenuous/speculative given the system is necessarily reductionist, and mixes Protamine from salmon with HMGB1 from mouse/human, although it may be in principle OK. To what extent do the transition proteins in fish resemble HMGB1 in mouse/human? It would be good to state this up front, since HMGB proteins are diverse in the number of boxes they contain and the length of their tails.

We now state that the sequences of protamines in fish and mammals share a common feature: they are all arginine-rich (p. 3, second paragraph). Indeed, similar effects on DNA condensation were reported using fish and mammalian protamines. We have now conducted additional experiments to show that human transition protein 1 (TNP1) has similar effects to HMGB1 in countering protamine (new Figure S14). In Discussion, we now point to additional similarities between transition proteins and HMGB proteins in mammals (p. 13, first paragraph).

As an aside, it is good to see a lab that actually measures the effect of the addition of a GFP tag.

We thank the reviewer for recognizing our efforts in quantifying the effects of the GFP tag.

Specific comments:

1) The buffer conditions should be stated up front in the main text, since the ionic strength will play a part in determining the (mainly electrostatic) phenomena being described.

We now state the buffer condition (10 mM pH 7 imidazole with 150 mM KCl) in the opening paragraph of Results (p. 4).

2) p9: “These observations support our mechanistic interpretation of single-molecule results on tangle-to-bridge conversion. When forming liquid droplets in the ternary mixture, HMGB1 indeed appears to peel a portion of protamine molecules away from dsDNA. The need for 10% PEG in the bulk experiment could be due to an effective dilution when the dimensionality of space is increased from one (on the dsDNA in the single-molecule setting) to three (in solution)”

The bulk experiment uses short DNA, which is surely the more likely reason for the need for PEG, given HMGB1’s ability to scan DNA (e.g. Iwahara, also Kaplan for other TFs).

We thank the reviewer for raising this valid point and now cite these papers (new refs 66 and 67).

3) p10 “In forming a layer on the surface of relaxed dsDNA, the HMG boxes of HMGB1 molecules would have to bind sites along the DNA (Figure 7, last row in the single-molecule portion). The freed acidic tails would then bind HMG boxes of additional HMGB1 molecules, and this process may propagate multiple rounds.”

This seems too speculative, also the graphical abstract. The freed tails could also make cis interactions with basic residues on the back face of their own HMG boxes, or simply act to neutralise positive charge to allow closer approach of other HMGs on the DNA.

Another good point! We now add these additional scenarios in our narrative (p. 11, second last paragraph).

Reviewer #2 (Remarks to the Author):

- In this manuscript, the authors perform single-molecule and condensation experiments regarding the DNA interactions of protamine and HMGB1. Previously, the same research team published two papers detailing similar studies of protamine—first in JACS (2024) on condensation, and then in PNAS (2026) on single-molecule studies combined with condensation experiments. The current work is an extension of their prior studies, with the primary difference being the addition of HMGB1. Although I appreciate the attempt to build complexity by studying the interplay between two proteins rather than just one, the present manuscript is not suitable for publication. It is too preliminary, lacks mechanistic insights, and does not provide data testing the physiological relevance of the observations.

We hope the substantial amount of new data and the significant revisions in response to the reviewers will convince this reviewer that the body of results in the present study provides unique mechanistic insights and has important physiological relevance. In brief, (1) we have added new experiments to confirm all preliminary observations; (2) we have added new experiments on a transition protein, TNP1, to show that TNP1 and HMGB1 exert similar effects in countering protamine, thereby yielding deep mechanistic insight into how transition proteins and other transiently expressed proteins may work with protamines during spermiogenesis. (3) We further strengthen the physiological relevance of our study by adding experiments on p53, thereby linking the effects of TNP1 and HMGB1 to the repair of double-strand breaks in spermiogenesis.

- The manuscript is difficult to read and appears to have been put together hastily. The text contains several instances of tentative statements (see examples listed below), and the authors do not attempt to support these statements through additional experimentation or quantification.
- "a thin coating covered the presumed dsDNA section"
- "adjacent (presumed) ssDNA section"
- "It looked as if the focus was formed with peeled-off ssDNA"
- "It was as if the focus moved together"

We apologize for the style of writing, and now support these statements through new experiments and additional analysis and better explanation of all results (p. 5, last paragraph to p. 6, second paragraph; new Figure 2). In brief, (1) that HMGB1 coating covered dsDNA sections is confirmed by adding an intercalating dye for imaging (new Figure 2a, b). (2) Likewise, spreading to ssDNA sections is directly shown by adding the intercalating dye during retraction (new Figure 2b). (3) The containment of peeled-off ssDNA tracts in HMGB1 foci is now supported by a correlation analysis between focus size and ssDNA section length (new Figure 2d). (4) The co-migration of foci and ss-dsDNA junctions is directly visualized in new Figure 2b, and supported by the multiple lines of evidence showing that foci are always located at ss-dsDNA junctions.

- One of the major claims of the paper is that HMGB1 can convert protamine-DNA tangles into bridges. There are two major issues with this claim. First, the authors do not fully characterize these tangles and bridges in terms of various parameters; they mainly show a few example single-molecule trajectories. I strongly recommend that they quantify their findings in a statistically meaningful way instead of asking readers to rely on vague statements. More importantly, the single-molecule data shown in Figure 3 were obtained by co-incubation with both proteins. If the authors want to claim a "conversion," they must start with a tangle formed by protamine alone and then demonstrate that the addition of HMGB1 dissolves this tangle. Furthermore, this must be demonstrated across dozens of DNA tethers to be convincing.

We acknowledge that the word “convert” was an imprecise choice. What is of direct physiological relevance is that the condensed state of DNA is solid-like tangles when only protamine is present, but changes to liquid-like bridges when HMGB1 and protamine are both present. We now replace “convert” with the word “change”. That said, we now quantify differences between tangles, bridges, and bends by quantifying the rupture force and apparent unfolding length associated with each mode (new Figure S5). In addition, although we no longer describe the change as conversion, we have performed experiments suggested by the reviewers: first forming tangles in the protamine-only channel and then transferring to a channel where HMGB1 and protamine are both present, now showing bridges (new Figure S7). Lastly, in response to reviewer 3, we demonstrate the “conversion” of individual protamine-dsDNA aggregates into liquid droplets upon adding HMGB1 (new Video S1).

- The authors do not adequately justify the physiological motivation for studying HMGB1 and protamines together. They mention that in *Drosophila*, protamine-like proteins contain HMG boxes, but they cite no evidence that this is also true for mammalian systems.

We have expanded on the rationale (p. 12, last paragraph). In short, the interplay of transition proteins and other transiently expressed proteins with protamines is crucial for spermiogenesis, but the mechanism is enigmatic. Our study used HMGB1 as a model for transiently expressed proteins in mammals. Moreover, we have now conducted additional experiments to show that human transition protein 1 (TNP1) has similar effects to HMGB1 in countering protamine (new Figure S14). In Discussion, we now point to additional similarities between transition proteins and HMGB proteins in mammals (p. 13, first paragraph). Our study thus provides deep mechanistic insight into how transition proteins and other transiently expressed proteins may work with protamines during spermiogenesis.

- They also claim in the abstract that a liquid state enables the recruitment of repair machinery to restore the duplex structure, but they do not provide any supporting data.

To support this hypothesis, we have now done single-molecule experiments to show that p53, a recruiter of repair factors, colocalizes with HMGB1 and protamine at ss-dsDNA junctions (new Figure S15; p. 11, first paragraph). Our bulk experiments corroborate the colocalization of p53 and HMGB1 (new Figure S16).

- In the main text, they state that Figure 3d shows the “conversion rate.” However, rates are measured in units of inverse time (time^{-1}), whereas the panel only shows the absolute number of conversions, not a true rate

Thank you for pointing out this inappropriate choice of words. Instead of “conversion rate”, we now use the phrase “fraction of tethers exhibiting bridges instead of tangles.”

- In the introduction, they write: "It can be argued that, early in spermiogenesis when protamines are first expressed, chromatin needs to be maintained in a liquid state in order for the recruitment of repair factors." They do not provide any supporting reference for this argument. In fact, the sentence that follows ("Indeed, premature synthesis of protamine 1 in mouse spermatids resulted in precocious condensation of nuclear DNA.") does not provide any support, as it has nothing to do with DNA repair.

We have rephrased this sentence and added supporting references (#31 and 32).

- In the introduction, they write: "Finally protamine condenses double-stranded DNA (dsDNA) into hyperstable “tangles”,¹⁶ leading to global transcription silencing." This sentence implies that their observation of tangles, reported in their JACS paper (Reference 16), has a causal link to global transcription silencing, but I see no actual support for this. When do the tangles appear during spermiogenesis, and when is transcription globally silenced relative to that event?

The reviewer is totally correct in noting that our JACS paper reported the observation of tangles but did not establish a causal link to global transcription silencing. We only proposed tangle formation as a potential mechanism for transcription silencing. We have now revised this sentence accordingly. Global transcription silencing occurs in the hyper-condensed sperm chromatin (p. 12, end of last paragraph, citing ref 1).

- In the introduction, they write: "These observations led us to suggest that the presence of ssDNA tracks during histone-to-protamine transition might contribute to maintaining the liquidity of DNA condensates." How are these single-stranded tracks generated, and how are they relevant to HMG proteins?

Single-stranded tracks are generated during damage repair in a step known as “resection”; we now cite refs 57 and 58 for this fact – see also our reply to a related comment below. In our previous study (ref 35), we found that the addition of ssDNA could induce a change of protamine-mediated tangles to bridges. In the present study, we observe a similar effect with HMGB1. This parallel is noted in p. 7, end of the third paragraph.

- In the introduction, they write: "...while protamine produces a large condensing force (~ 10 pN) in l-DNA held at low stretch". It is not clear what is meant here. Furthermore, is 10 pN considered "large" within the context of this study?

The paragraph in question summarizes all our main findings. We now elaborate on this specific point, in the Results section (p. 5, first paragraph) when the result is first described. In brief, as reported in several studies (new refs 59-63), a nonzero force plateau when a DNA tether is retracted to low extension is a signature of DNA condensation induced by the bound protein. As documented in our previous study (ref 35), condensation forces reported for other proteins ranged from 0.2 to 9.5 pN, so our value of 10 pN for protamine is large when compared with other proteins. Of course it is also large when compared to HMGB1, as that protein does not condense dsDNA (corresponding to a 0 condensation force). We edited the text in the Results section instead of the Introduction to avoid interrupting the flow of the text, but also in recognition of Reviewer 3's suggestion of not overloading the Introduction.

- In the Results section, they write: "To mimic strand breaks during the histone-to-protamine transition in spermiogenesis, we overstretched l-DNA by dual-trap OT to unpeel ssDNA tracks from nicks." While a single-strand break is equivalent to a nick—meaning preexisting nicks in the DNA can certainly mimic a single-strand break—a nick does not inherently result in a single-stranded region. Therefore, their justification for this experimental scheme is questionable.

Repair of double-strand breaks involves a step known as “resection”, which generates ssDNA overhangs, as documented in new refs 57 and 58. We now add this point (p. 4, last paragraph).

- In the Results section, they write: "Moreover, HMGB1 foci promoted DNA strand separation but protamine foci hindered it." I could not find where this is demonstrated in the data.

We apologize for the typo “strand separation”, when we actually meant “strand reannealing”. We have now made the correct and thank the reviewer for calling our attention. The sentence in question is a recap of the preceding paragraph, which documents the evidence on strand reannealing.

- In the Results section, they write: "...such that pretransition condensates were dominated by ssDNA but post-transition spread solely involved dsDNA." It is unclear what specific transition they are referring to here.

The transition referred to here is the wetting transition, or condensate spreading. We have now added a sentence to equate the two.

Reviewer #3 (Remarks to the Author):

This manuscript titled “Counteraction of HMGB1 at ss-dsDNA junctions maintains liquidity of protamine-DNA co-condensates” investigates ssDNA and dsDNA interactions with protamine and HMGB1 using primarily single-molecule force-fluorescence assays. The authors show that protamine and HMGB1 exhibit different behaviors on force-stretched DNA with regions of ds and ssDNA. Overall, the experiments in the paper seem to be of high quality and the scope of work is substantial. We have some experimental and analysis concerns that need to be addressed but otherwise it seems like a solid paper.

Thank you for the strong endorsement of our study.

Major concerns:

- Many terms are used throughout the paper without clear definition. While we *think* we know what you mean for most, the following terms must be clearly defined: “bridges”, “tangles”, “bends”, “liquid-like”, “solid-like”, “wetting transition”, “solid-like tangles to liquid-like bridges”

We now define these terms except for “wetting transition” in p. 5, first paragraph. For the latter, the definition is deferred to p. 6, last paragraph, where the term first appears.

- Relatedly...Bends, tangles and bridges: These should be clearly identified on every relevant graph, and explained in the methods how they are identified and quantified. In many places the text described these features and we had trouble interpreting which regions of the force-extension were which.

We now display these modes in 3 distinct colors (magenta, cyan, and blue) in stretching curves. We also describe their identification and quantification in Methods (p. 16, second last paragraph; new Figure S5).

- An experimental reference WLC curve for “naked” DNA should be included in figure S1. It is presumed throughout the text that the dashed line is basically your control condition but you haven’t experimentally shown this yet. Furthermore, the statement about S1 changes in flexibility and/or contour length need to be quantified with statistics. We doubt you’re drawing these conclusions from single curves, so please provide the mean and SD for contour and persistence length for the 3 cases (naked, +HMGB, +HMGB deltaC). Would be great to have +SYTOX here as well.

First off, as we have stated in the Figure 1d caption, the dashed line shown in force-extension curves is an experimental curve for naked DNA – we always acquire a stretching curve for the naked DNA tether (i.e., inside the buffer channel) before transferring it to the protein channel. We have now updated Figure S1 to also show example fits of stretching curves to the modified wormlike chain model, and include fitting statistics in a new panel (b).

Force-extension curves and fit for “+SYTOX” are presented below.

- Condensate data in Figure 5 – what is the rod like structure? The sizes here don’t seem to make sense. It also seems quite rigid given the length. Even something like DNA origami tubes don’t typically stay this straight over these length scales without curves or bends. Have you eliminated the possibility that this is some experimental anomaly (e.g. microscopic dirt, bacteria, other impurity). Visually the amount of aggregates in a and c are quite different. If the concentrations are the same then even if aggregates take different forms we should be observing the same amount of material visually, right? There is also a claim in the text that “aggregates converted into droplets” – I assume that this is a misstatement or have you actually observed a single aggregate converting into a droplet? If so please include a supplemental video of that.

These “structures” in Figure 5a (now 6a) are aggregates. They appear rod-like only because of their small size and the low resolution of the microscope. When incubated over time, aggregates grow larger, so their irregular shape becomes apparent; the same holds when we prepare samples at higher concentrations of the components. The increased amount of aggregates shown in Figure 5c (now 6c) is due to the fact that HMGB1- Δ C promotes aggregation.

We now include a video (Video S1) to show the conversion of individual aggregates into droplets.

- Intercalators can also change force extension behavior of DNA, but I didn’t find this covered in a meaningful way (either textually or in control experiments). Our understanding here is SYTOX was used with protamine but not with HMGB1, and yet we didn’t see any controls with just SYTOX. We’re concerned that there could be misinterpretation of an effect of SYTOX with protamine – how can you be sure this is not the case? It wasn’t helped that it was very difficult to figure out which force extension curves had SYTOX and which didn’t.

We did not include SYTOX when acquiring any force-extension curves to avoid any artifacts. We have noted this fact in line 8 of p. 5, and now reiterate it in Methods (p. 16, second paragraph). SYTOX and other dyes were added only for imaging purposes. That said, to satisfy these reviewers, we have now added force-extension curves in the presence of SYTOX and show them here. The results are similar to those without the dye.

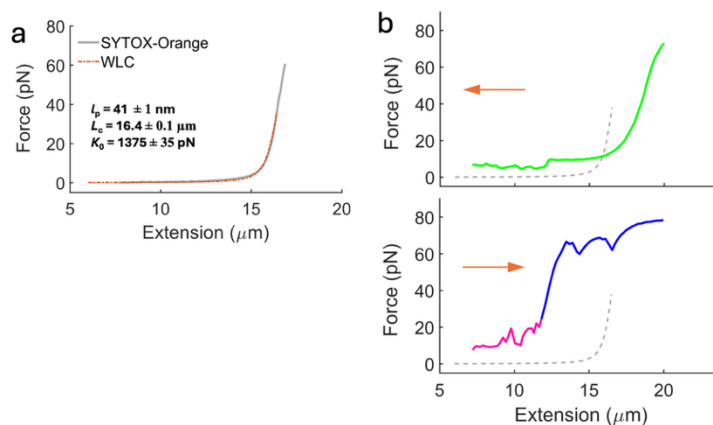


Figure R. Force-extension curves when SYTOX Orange is present. (a) Stretch curve in the presence of 0.5 μM SYTOX Orange, and fit to the WLC model. Fitting parameters from three tethers are also listed. (b) Retract and stretch curves in the presence of 10 μM protamine and 0.5 μM SYTOX Orange.

Minor concerns:

- Figure S2 is by far the clearest evidence of the ssDNA vs dsDNA argument – I was shocked to read this so late in the paper (after I was coming up with all my doubts), and shocked it was only in SI. If this were my paper I would consider highlighting that more substantially.

We appreciate the reviewers' recognition that Figure S2d presents the clearest evidence for the localization of condensates at ss-dsDNA junctions. Following their suggestion, we now move this panel to a new main-text figure, Figure 2. We also present additional data in this new figure.

- There is a super high density of information in text, figures, and legends that impedes easy comprehension. Better annotating/labeling/titling graphs and images in the figures themselves would help a lot. Also, it may be possible to move some of the high-information density into the discussion. These are not major but both of us commented to each other on how difficult it was to get through the paper with all of the cross referencing between text, figure, and caption.

We have now improved the annotation of most figures and have removed some text from Introduction.

- Scale bars are missing on some images. Every image should have a scale bar in main figures and SI.

We now make sure that all images have a scale bar.

- Places listed as “frames skipped” or “cycles not shown” should at least be explained why, and ideally be included in the whole in SI for completeness

We now present these skipped frames and cycles in new Figure S3.

- A figure similar to your final figure in the recent PNAS paper would be very helpful to guide readers to your interpretation of what’s happening.

The final figure, Figure 8, in this manuscript, is intended to serve a similar purpose as the final figure in our recent PNAS paper.

- Figure 1 – all x-axes need to face the same direction, a few are inverted which makes it very confusing.

We corrected Figure 1 to have all x axis values going from small to large.

- Page 6 last paragraph “wetting” discussion does not seem like the right language, or at least needs to be clear what you mean.

We now explicitly define what a wetting transition is.

- Page 5 – paragraph 1 - “characteristic dip” needs clarification and notation on figure.

We now use a block arrow to indicate this dip in the figure, cite our previous work clarifying this dip, and recap this clarification in the figure caption.

- N values should be reported on relevant graphs. Distinguishing number of tethers and number of pulls is also important.

We now collect the number of tethers and number of force curves in new Table S2.

- In the introduction and throughout the text, we found it sometimes difficult to discern established fact from speculation. Particularly the last paragraph of the introduction, and some other phrases scattered throughout such as “marked a transition from ssDNA-dominated to dsDNA-dominated condensates”, “signifying the formation of tangles (hyperstable nested loops crosslinked by protamine)”, “Moreover, HMGB1 foci promoted DNA strand separation but protamine foci hindered it.”

We have striven to distinguish fact and speculation in the revision, as illustrated by how we dealt with the 3 quoted examples. For “marked a transition from ssDNA-dominated to dsDNA-dominated condensates”, as explained in our response to the reviewers’ comment above regarding “characteristic dip”, we now clarify that this characterization was established in ref. 35. For “signifying the formation of tangles (hyperstable nested loops crosslinked by protamine)”, we have revised the sentence to make it clear that the first half of the quoted text related to the defining feature of tangles, but the second half (the portion in parentheses) is speculation. For “Moreover, HMGB1 foci promoted DNA strand separation but protamine foci hindered it,” as we explained in our response to reviewer 2, we made a typographical error: it is strand reannealing, not separation.

Reviewer #4 (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

We thank the reviewers for their endorsement of our paper for publication. Our response to the minor remaining issues is given below in blue.

Reviewer #1 (Remarks to the Author):

My original concerns have been addressed in the revision.

Reviewer #3 (Remarks to the Author):

The authors did a good job addressing our concerns, and have resolved our technical uncertainties. The supplemental video was eye opening and very nice to include. A few figures are still a bit confusing to follow the panels (e.g. Figure S7, and other figures where each individual panel is not labeled), but this is a minor concern. The updated definitions are better but there is still some room for improvement in clarity. Overall nice paper that is mostly ready for publication.

We thank the reviewers for the positive assessment. We have resolved the remaining minor points as follows:

- **Figure Panels (Supplementary Figure 7 and others):** We have updated the legend of Supplementary Figure 7a to explicitly clarify that the panels represent a time sequence. Although we do not label the individual panels, the legend explicitly differentiates the sequence (“first panel,” “middle three panels,” and “last panel”). We also audited all other figures to ensure that individual unlabeled panels are called out in the figure legends or main text.
- **Clarity of Definitions:** To remove any remaining ambiguity, we have expanded and refined the descriptions of “tangles” and “bridges” in the first paragraph of Page 5. We added contextual details to ensure that these terms are immediately clear to the reader.

Reviewer #4 (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.