

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

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Supplementary Information

Supplementary Table 1. Sequences and domain features of sperm nuclear basic proteins in flies.

Protein ^a	Sequence and annotation ^b	#R/K #D/E ^c
Transition protein-like		
Tpl94D (Q8IMZ6)	MGSVLRSRDQPRDSVAYRNF ^{FL} TVYRNQHKQMSSNDALLSASRE ^W GTLSQAQRNR ^Y ANMKD SPKRGTKNITENAAARRK ^I TKKNSRSKR ^I SQRR ^I QDKGSAYKPLTLNRSYVIRKSLVPSR G ^{FL} NLVQFFQEIHSEMPRSLAVKEAVSS ^W CAMNADQRG ^I FISDL	10
tHMG1 (Q9VCZ5)	MSDCGARPKKPM ^{SA} F ^{FL} MLWMNSTGRKHIKAEHPDFSVQEVSVKGGEM ^W RAMADEDKIV ^W QE SATTAMA ^{EY} KEK ^L KQWNFPKEH ^R FSDTQCICSSNTNQCPTLFVYDTMDDSMTPIC ^W RKCLS KTRCLH	4
tHMG2 (Q9VCZ4)	MSDYGARPKKPM ^{SA} F ^{FL} MLWMNSTGRKNIRAEHPDFSVQEVSVKGGEM ^W RAMADEHKIV ^W QE SASKAMA ^{EY} KEK ^L EKWN ^{AF} KEH ^Q TESFPHIYEAPLSSRF ^S SKTNQRPTLFVYDSKDEAMAP IC ^W RTCFSKAKCFH	3
CG30356 (A1Z7I6)	MGRAVKNNRVRSAHCNSAAKATSISGHSMLKQNNDCNGRNPFFQ ^{FL} AYFRKCSNNCLGHL PSDRVTLIAGKV ^W NYMSLSEKEP ^F IAAARRFN ^Y TYRS ^W RSRKVNWVLAQLR KSAA ^E EECRP QAQWMLMNLKSWQESVVRNLLDL ^W DHNQN	5
Protamine-like		
ProtA (Q9V3S3)	MSSNNVNECKSLWNGIISISAKDESPKGLTEMCNHP ^I RRAPQKCKPMKSCAKPRRKAACA KATRPVKCAPRQKCSK QGPVTNNAYLN ^{FV} RSFRKKHCNLKPRELIAAKA ^W ARLSEN ^R KDR ^Y RRMACKVTTSERHKRRRICQ ^Y	16
ProtB (Q9VJR1)	MSSNNVNECKSLWNGIISISAKDESPKGLTEMCNHP ^K RRAPPKCKPMKSCAKPRRKAACA KATRPVKCAPSQKCSK QGPVTNNAYLN ^{FV} RFFRKKHCDLKPQELIAEA ^W AKA ^W AELPEHR KDR ^Y RRMACKVTTSERHKRRRICK	16
Mst77F (P16909)	MSNLKQKDSKPEVAVTKSVKTYKKSIEYVNSDASDIEEDINRAEDEYASSSG ^{EV} NFLRDF KKRYGEYYSNNEIRRAETR ^W NEMSFRHRCQ ^Y SAEPLDTFHVEPNVSSSLQ ^R SSGEHRM HSEISGCADTFFGAGGSNSCTPRKEN KCSKPRVRKSCPKPRAKTSKQRRSCGKPKPKGAR PRKACPRPRKKMECGKAKAKPRCLKPKSSKPKCSM	21
Prt99C (Q9VAF7)	MGRKRGRKEYCPPIYKRQKVARVTNNGYLN ^{FEM} TEYKKRFYGLSPQDMVHYAAQ ^W TQLSM AEKEA ^F KS ^K PKSTITLKSPAQYVACEMKSDVAGGQQSSCQRQSPSARLRESE RRSSRSKT LCSAKNRQ^RGKPKPQ^SSKRRL SHMGS ^{AV} AYIHFLRK ^F Q ^R KNTELRTIDLLKTATRLWCR LPERHRHA ^F ERPLWIVTIGKS	13
CG14835 (Q9VS70)	MDYSYSIGSKSPVFISKAPYN ^{FL} REYRLGCDCKNIPAVTLVKEARNA ^W HALSDQEQSLE ^F EEVPYLMAQ ^F GPSIESAMRLTVNVLS ^P QQVTVQRTSDVQSS KRRICRGNAKTHRKKRQ^Q KMGATS^RKRSPGIGSTRCTRYTSQFHWENPPL	13
ddbt (A2VEN4)	MARSQSKMIRAAMVDDPNMVAWL ^P YLN ^{FL} RFLKRN ^F YPR ^T DLRRL ^L QVGLIR ^W IALSDA QKRL ^F EPERILAR ^V ARRQ ^R NKRRRL ^L RRARHGQKGRGAVRRPIYDSRPKPRRRKPK	23 1

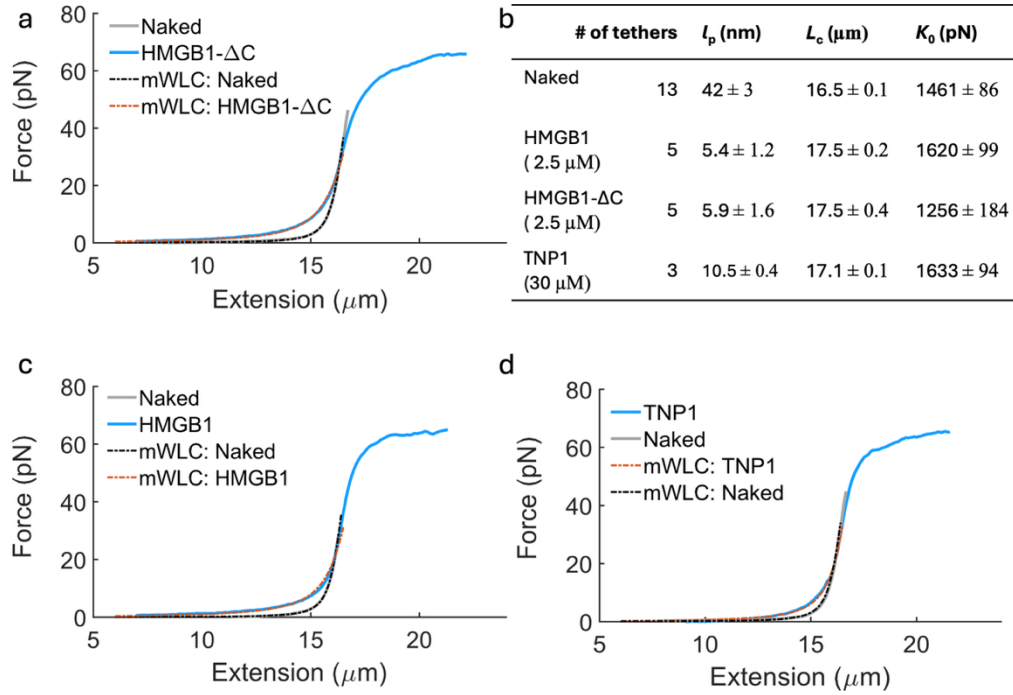
^aUniprot ID is given in parentheses after the protein name.

^bHMG boxes are underlined while Arg/Lys-rich motifs are in bold. HMG boxes were determined based on InterPro (<https://www.ebi.ac.uk/interpro/search/sequence/>)¹, Chang et al.², and the consensus F(L/M/V)...Wxxxxxxxxxx(F/Y/W). Consensus residues are shaded in green.

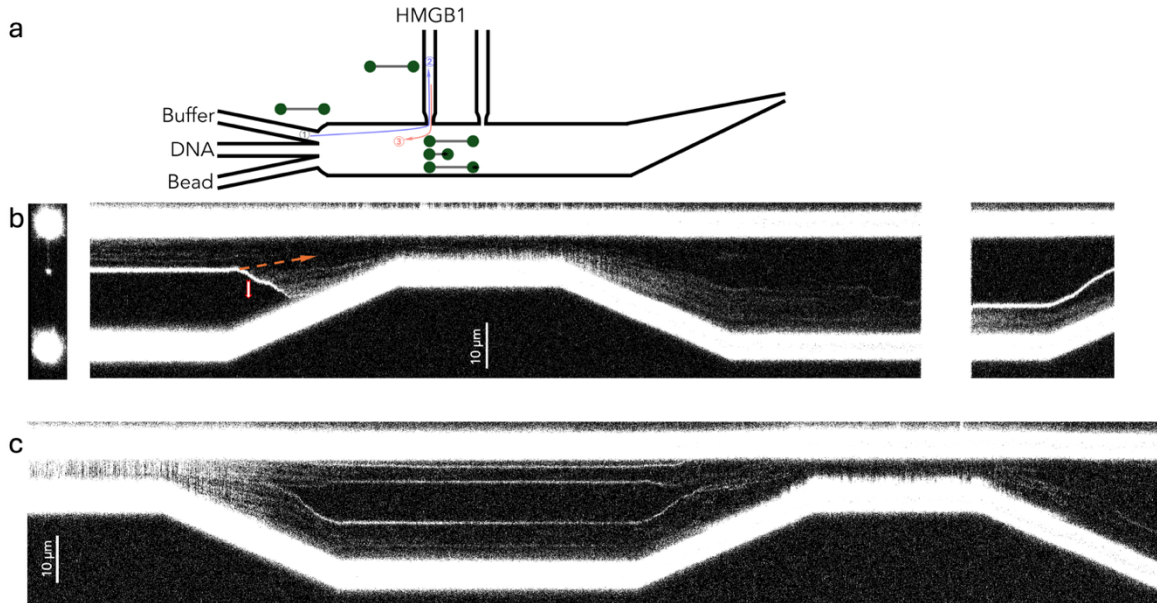
^cNumber of Arg and Lys residues (and number of Asp and Glu residues if any) in Arg/Lys-rich motifs, identified as the shortest stretch of sequence outside HMG boxes with the highest net charge and at most one D or E.

Supplementary Table 2. Statistics of force-extension curve acquisition

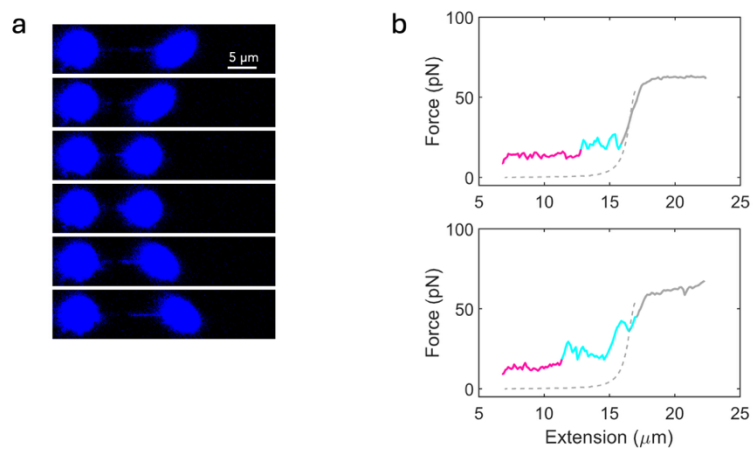
Figure #	Experimental condition	# of tethers	# of force curves	Measured properties
1d	Protamine (10 μ M)	6	6	Force-extension curves showing tangles
1f	HMGB1 (8 μ M) + GFP-HMGB1 (0.8 μ M)	9	9	Force-extension curves simultaneous with 2D scans
1h	HMGB1 (8 μ M) + GFP-HMGB1 (0.8 μ M)	5	5	Force traces simultaneous with kymographs
1k	HMGB1 (5 μ M) or buffer	20 13	52 25	Reannealing probability
3c	HMGB1 (5 μ M) HMGB1 (2.5 μ M) HMGB1 (0.25 μ M) + protamine (2.5 μ M)	29 32 28	87 96 84	# of tethers forming bridges or tangles
4d	HMGB1 (2.5 μ M) HMGB1- Δ C (2.5 μ M) + protamine (2.5 μ M)	32 23	96 69	# of tethers forming bridges or tangles
S7d	HMGB1 (2.5 μ M) + protamine (2.5 μ M)	18	54	# of tethers forming bridges or tangles
S10b	GFP-HMGB1 (2.5 μ M) + protamine (2.5 μ M)	17	51	# of tethers forming bridges or tangles



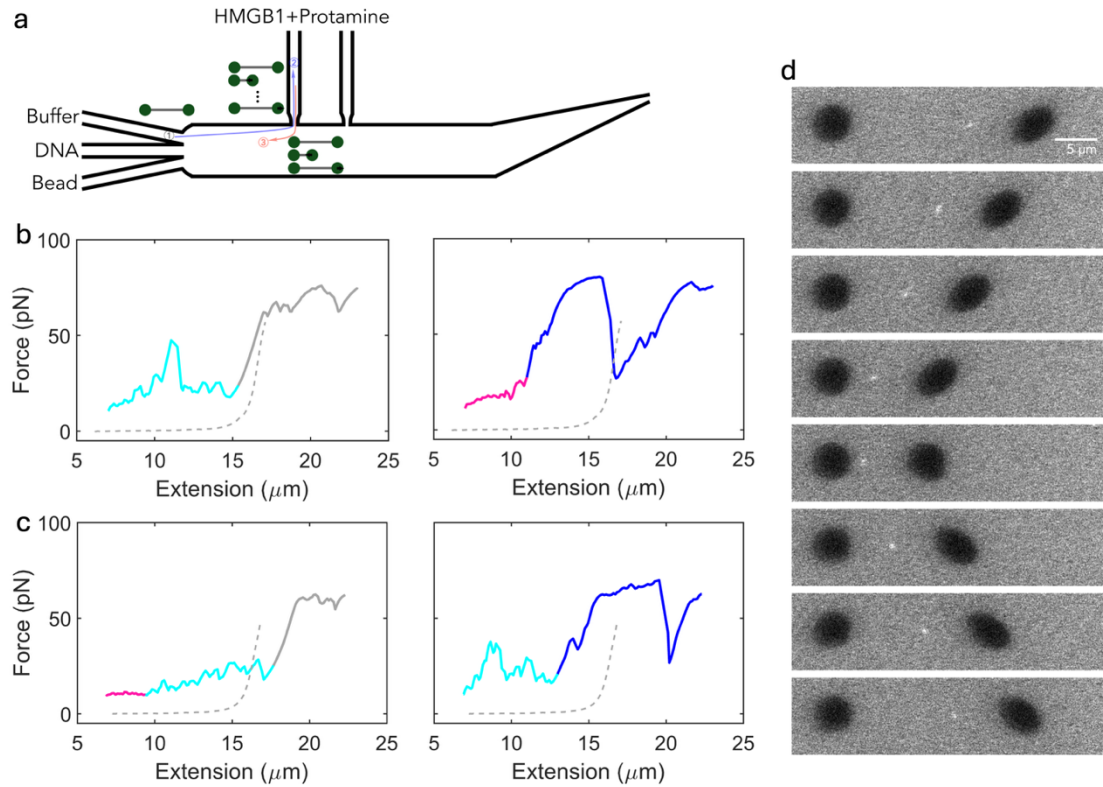
Supplementary Figure 1. Stretching curves of λ -DNA in the presence of HMGB1, HMGB1- Δ C, or TNP1 and fitting parameters. Fits to the mWLC model (equation [1]) are displayed as dashed curves. **(a)** Stretching curves in the buffer channel and in the HMGB1 channel (2.5 μ M). **(b)** Same as **(a)** but HMGB1 was replaced with HMGB1- Δ C. **(c)** Fitting parameters. **(d)** Same as **(a)** but HMGB1 was replaced with 30 μ M TNP1.



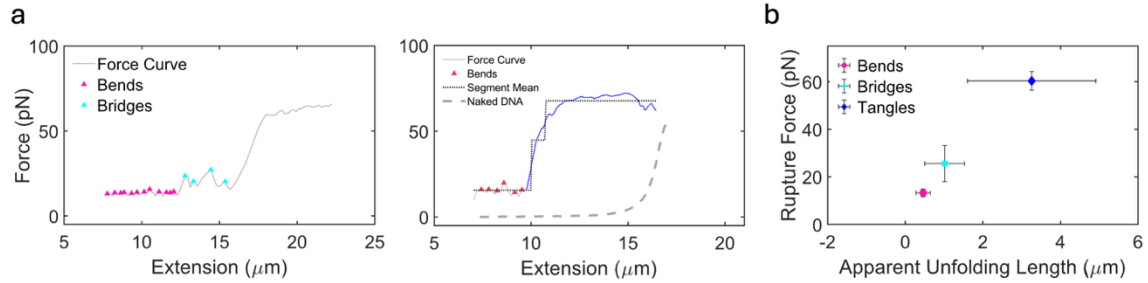
Supplementary Figure 2. HMGB1 focus formation and localization on λ -DNA. **(a)** General protocol for the studies in the presence of HMGB1 alone. A λ -DNA tether was first overstretched in the buffer channel, then soaked in the HMGB1 channel, and finally brought outside the channel for retraction-stretching cycles and confocal scanning. **(b)** A kymograph, similar to Figure 1h, showing a focus spreading on overstretched λ -DNA upon retraction. A slanted arrow indicates the would-be direction of a static point during retraction; a vertical arrow indicates the migration direction of a focus. A 2D scan before retraction is displayed at the left; a kymograph after dipping the re-stretched tether in the HMGB1 channel is displayed at the right. **(c)** A kymograph following an alternative protocol, where a λ -DNA tether started in a relaxed state (extension at 7 μm) underwent stretch-retract cycles. In **(b)** and **(c)**, the channel contained 8 μM HMGB1 and 0.8 μM GFP-HMGB1.



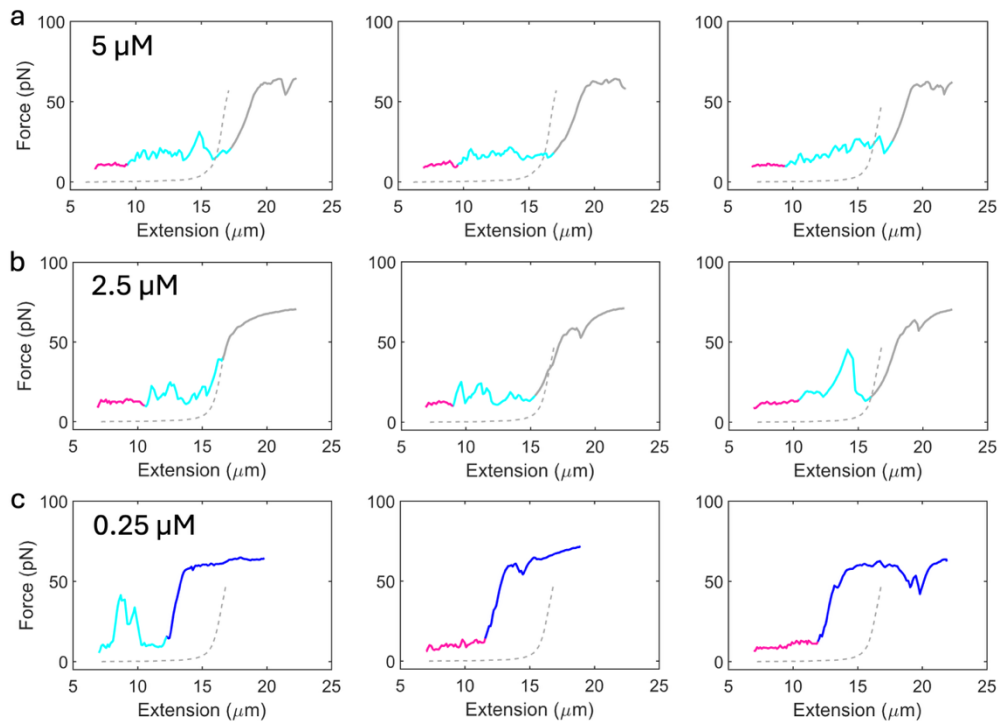
Supplementary Figure 3. Skipped frames or cycles in main figures. (a) Six frames skipped in Figure 1e. (b) Two retract-stretch cycles skipped in Figure 3b.



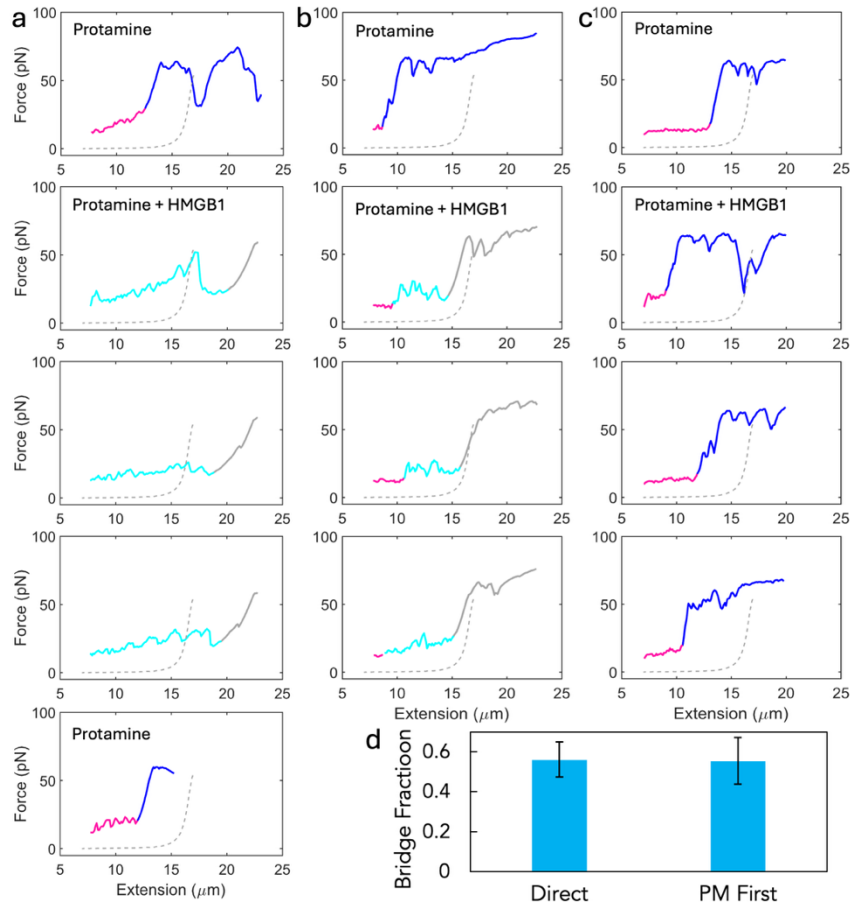
Supplementary Figure 4. HMGB1's counteraction to protamine. **(a)** General protocol for the studies in the simultaneous presence of HMGB1 and protamine. A λ -DNA tether was first overstretched in the buffer channel, then transferred to the HMGB1 + protamine channel for soaking or multiple retract-stretch cycles, and finally brought outside the channel for one more retraction-stretching cycle and confocal scanning. **(b, c)** Same as Figure 3b, with force-extension curves from two more tethers. For each tether, the left panel was acquired when the tether was inside the HMGB1 + protamine channel (at 5 and 2.5 μM , respectively), and the right panel was acquired after the tether was moved just outside the channel. **(d)** 2D scans of a λ -DNA tether undergoing retraction and stretching inside the HMGB1 + protamine channel (8 μM HMGB1; 0.8 μM HMGB1; and 2.5 μM protamine). Fluorescence was rendered black and white.



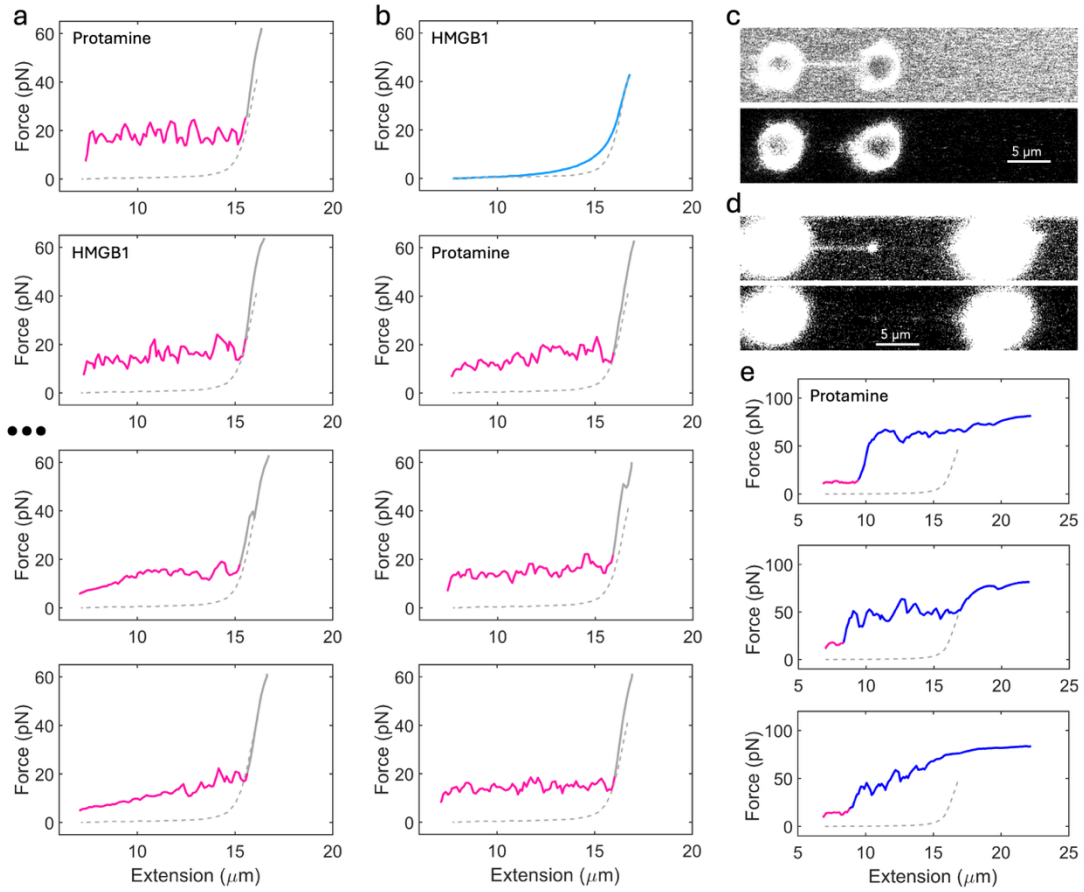
Supplementary Figure 5. Analysis of force-extension curves for bends, bridges, and tangles. **(a)** Left: a stretching curve with force peaks of bends and bridges indicated by triangles in magenta and cyan, respectively. Protein concentrations were: HMGB1, 5 μM ; protamine, 2.5 μM . Right: a stretching curve with bends indicated by magenta triangles at peak forces and tangles indicated by blue color. The force plateau value at high stretch was found using the ischange function in Matlab, with segments (dotted lines) showing abrupt changes in mean value. Protein concentrations were: HMGB1, 0.25 μM ; protamine, 2.5 μM . **(b)** Comparison of rupture forces and apparent unfolding lengths between bends, bridges, and tangles formed by λ -DNA in the presence of 5 μM HMGB1 and 2.5 μM protamine. The rupture forces of tangles were higher than the force plateau (corresponding to strand separation), but were assigned to the latter value. The data for bends, bridges, and tangles were from 27, 30, and 27 stretching curves, respectively. The p values for the differences in rupture force or apparent unfolding length between any two modes were all < 0.0001 .



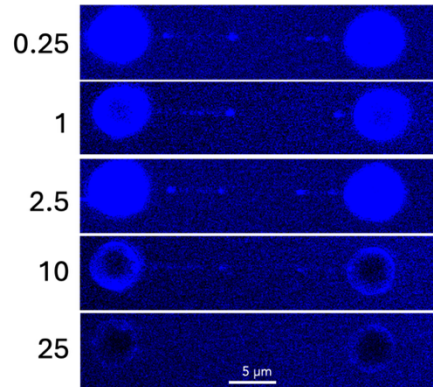
Supplementary Figure 6. Force-extension curves of λ -DNA during consecutive retract-stretch cycles in the HMGB1 + protamine channel. (a-c) HMGB1 at 5, 2.5, and 0.25 μM , respectively; protamine was fixed at 2.5 μM . Each row presents the first three consecutive stretching curves.



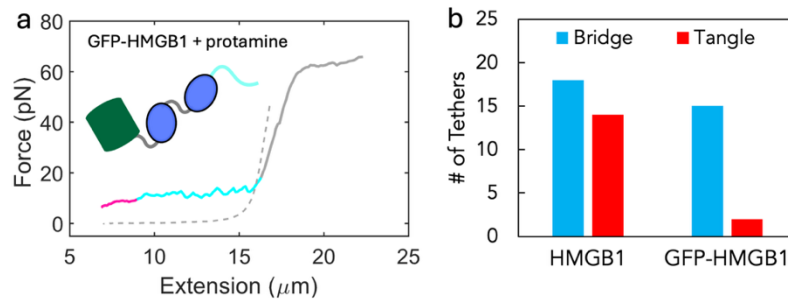
Supplementary Figure 7. Changes from tangles to bridges when λ -DNA tethers were first brought to a protamine-only channel and then to an HMGB1 + protamine channel. **(a)** Stretching curves in a time sequence, acquired in the following procedure. An overstretched λ -DNA tether ($\sim 23 \mu\text{m}$) was transferred from the buffer channel to the protamine-only channel; one retract-stretch cycle was performed to demonstrate tangle formation (first panel). The overstretched tether was then moved to the HMGB1 + protamine channel for three consecutive retract-stretch cycles (middle three panels), showing bridge formation. Lastly, the tether was returned to the protamine-only channel for a retract-stretch cycle (last panel) to show resurrection of tangles. Concentrations of protamine in both channels and HMGB1 in the second channel were all $2.5 \mu\text{M}$. **(b, c)** More examples of **(a)** but without returning to the protamine channel, showing bridges and tangles, respectively. **(d)** A bar graph comparing the fractions of tethers showing bridges instead of tangles, in two protocols. In one (“direct”), the tether was directly transferred from the buffer channel to the HMGB1 + protamine channel; in the other (“PM first”), the tether was first brought to the protamine-only channel before going to the HMGB1 + protamine channel.



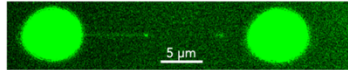
Supplementary Figure 8. Effects of protamine and HMGB1, when placed in different channels, on λ -DNA tethers. **(a)** Consecutive stretching curves of an intact-DNA tether, first placed in protamine (2.5 μ M; first panel) and then moved into HMGB1 (2.5 μ M; other panels). Three stretching curves are skipped (indicated by ellipses); subsequently, the initial values of the force curves shifted toward 0, suggesting some protamine dissociation in the HMGB1 channel. **(b)** Consecutive stretching curves of an intact-DNA tether, first placed in HMGB1 (2.5 μ M; first panel) and then moved into protamine (2.5 μ M; other panels). **(c)** Top: a 2D scan when a relaxed tether (7 μ m) was inside the HMGB1 channel (8 μ M HMGB1 with 0.8 μ M GFP-HMGB1), showing a uniform layer of HMGB1 spread over the DNA surface. Bottom: a 2D scan after the tether was moved into protamine (2.5 μ M), showing the absence of an HMGB1 layer. **(d)** Similar to (c), but the tether was initially overstretched (to 23 μ m). Top: scan taken immediately after the tether was moved outside the HMGB1 channel, showing a focus with strong fluorescence; bottom: scan taken after the tether was then moved to the protamine channel, showing a very faint focus. **(e)** Consecutive stretching curves inside protamine (2.5 μ M), after an initially overstretched tether was soaked in the HMGB1 channel (8 μ M HMGB1 with 0.8 μ M GFP-HMGB1).



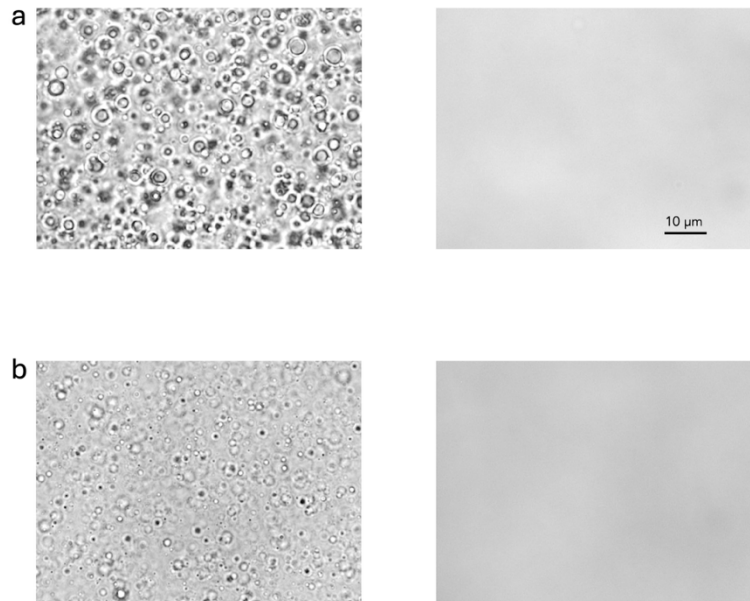
Supplementary Figure 9. Displacement of GFP-HMGB1 from foci by HMGB1. Shown are 2D scans of overstretched λ -DNA tethers in the presence of 0.25 μ M GFP-HMGB1 and HMGB1 at the indicated concentrations in μ M. For each tether, the second of three consecutive scans is displayed.



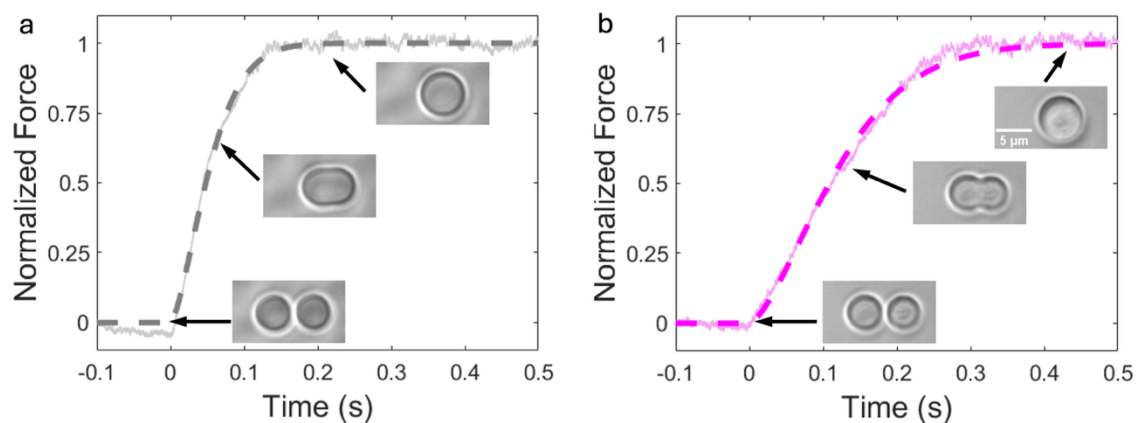
Supplementary Figure 10. GFP-HMGB1 was more effective than HMGB1 in tangle-to-bridge change. **(a)** A force-extension curve showing the change to bridges by 2.5 μM GFP-HMGB1 along with 2.5 μM protamine. **(b)** A bar graph showing the number of λ -DNA tethers exhibiting tangles or bridges in the (GFP-)HMGB1 + protamine channel (2.5 μM HMGB1 or GFP-HMGB1 along with 2.5 μM protamine). The HMGB1 results are the same as in Figure 3c.



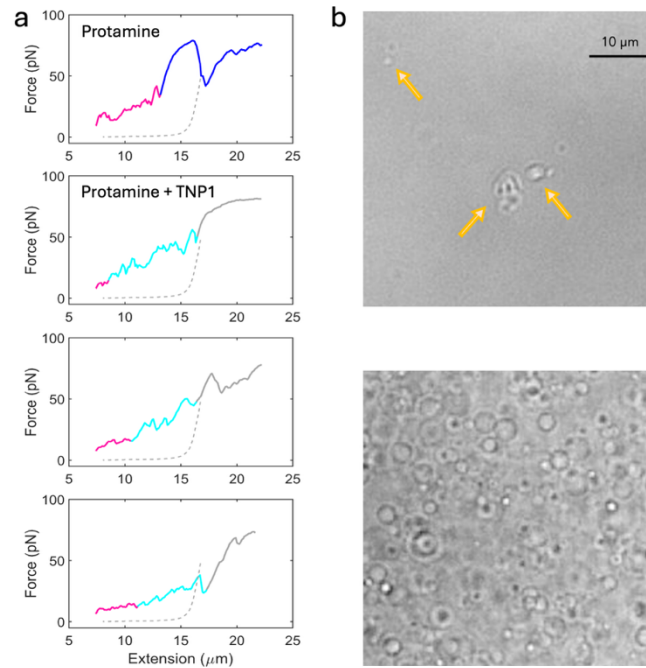
Supplementary Figure 11. A 2D scan of an overstretched λ -DNA tether (extension $\sim 23 \mu\text{m}$) in a channel containing $8 \mu\text{M}$ HMGB1 and $0.8 \mu\text{M}$ HMGB1-Cy3. Two foci were formed, along with a faint coating of the dsDNA sections to their left.



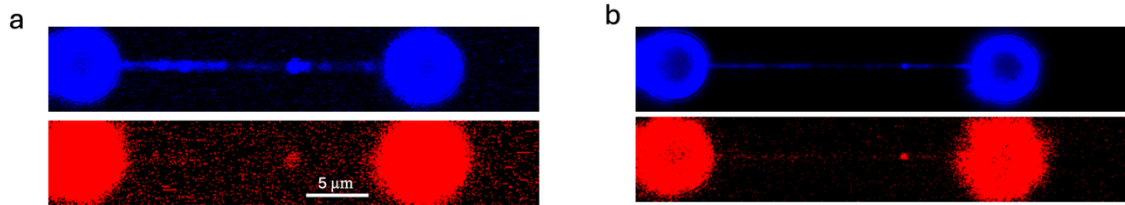
Supplementary Figure 12. Effects of the acidic tail on the homotypic condensation of HMGB1 and the heterotypic condensation of HMGB1 and protamine. **(a)** Brightfield images showing droplet formation of HMGB1 (left) and absence of condensation by HMGB1- Δ C (right). **(b)** Brightfield images showing droplet formation of a HMGB1–protamine mixture (left) and absence of co-condensation by HMGB1- Δ C and protamine (right). Protein components were at 50 μ M each and dissolved 10 mM imidazole buffer (pH 7) with 10% PEG.



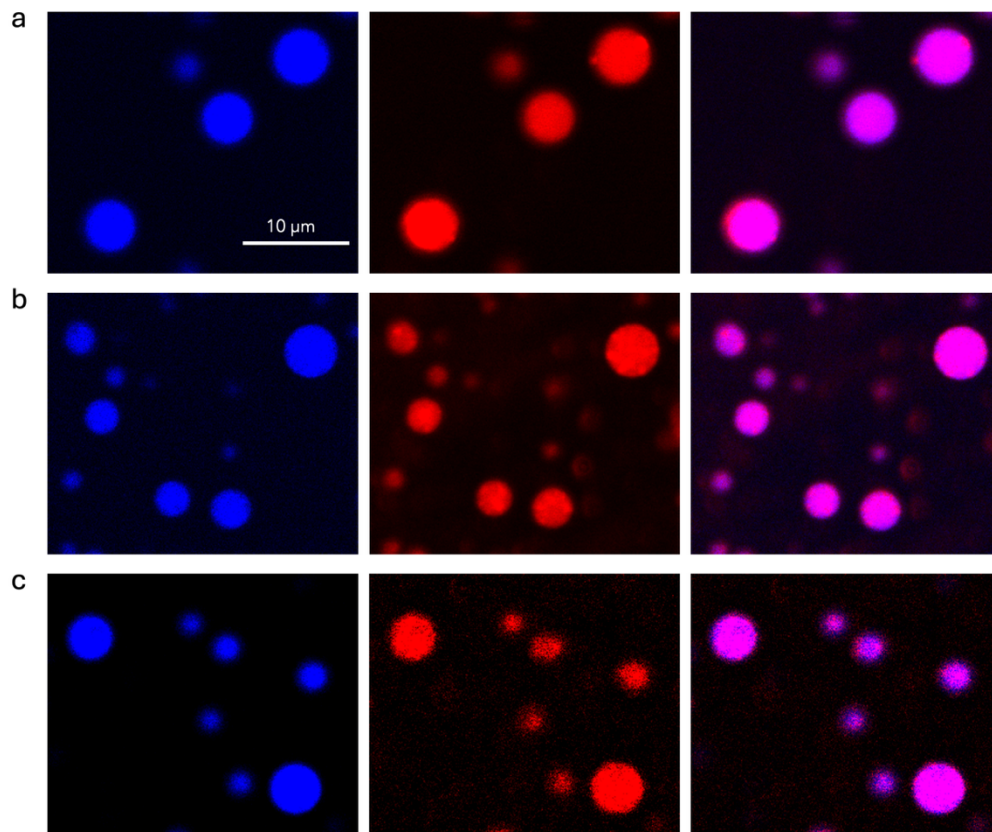
Supplementary Figure 13. Protamine slowed down the fusion of HMGB1–dsDNA droplets. **(a)** The fusion progress curve of a pair of equal-sized HMGB1–dsDNA droplets. Droplets were formed by mixing 20 μM HMGB1 with 20 μM 25-bp dsDNA in 10 mM imidazole (pH 7) with 10% PEG and grown to ~ 2.5 μm radius before fusion. **(b)** Corresponding results for HMGB1–dsDNA–protamine droplets. Droplets were prepared by adding 10 μM protamine to the sample in **(a)**. Solid traces display raw data; dashed curves are fits to equation [5].



Supplementary Figure 14. The counteraction of TNP1 to protamine. **(a)** Changes from tangles to bridges when λ -DNA tethers were first soaked in a protamine-only channel (2.5 μM) and then moved to a TNP1 + protamine channel (at 50 and 2.5 μM , respectively), similar to the results with HMGB1 shown in Supplementary Figure 7. Out of 7 tethers, 5 showed the bridge mode in the TNP1 + protamine channel; one example is shown here. **(b)** Top: small aggregates formed by the mixture of 10 μM protamine and 20 μM 25-bp dsDNA in 10 mM imidazole buffer (pH 7). Bottom: droplets formed when 100 μM TNP1 (plus 10% PEG) was also added.



Supplementary Figure 15. Colocalization of GFP-HMGB1 and p53-Cy5 at ss-dsDNA junctions without and with protamine. **(a)** 2D scans showing colocalization of GFP-HMGB1 (blue) and p53-Cy5 (red) and in a focus formed on λ -DNA. An overstretch λ -DNA tether was soaked in the HMGB1 + p53 channel, and moved just outside the channel for scanning. Protein concentrations were (in μM): HMGB1, 15; GFP-HMGB1, 5; p53-Cy5, 5. **(b)** Same as **(a)** but with the addition of 2.5 μM protamine in the protein channel.



Supplementary Figure 16. Localization of p53 in droplets formed by HMGB1-DNA and HMGB1-protamine-DNA mixtures. **(a)** Confocal images showing a homogenous distribution of HMGB1 (blue) and p53 (red) in droplets formed with ssDNA. Component concentrations were (in μM): HMGB1, 19; GFP-HMGB1, 1; p53-Cy5, 0.5; 33-nt ssDNA, 20; mixed in 10 mM pH7 imidazole buffer. Images were taken after droplets settled on the coverslip. Scans were collected using 488 nm and 638 nm lasers at 10% power with 0.05 ms pixel dwell time and 0.1 μm pixel resolution. **(b)** Same as **(a)** but with the addition of 20 μM protamine. **(c)** Same as **(b)** but protamine was 10 μM , p53-Cy5 was 10 μM , ssDNA was replaced with 20 μM 25-bp dsDNA and 10% PEG.

Supplementary references

- (1) Blum, M.; Andreeva, A.; Florentino, L. C.; Chuguransky, S. R.; Grego, T.; Hobbs, E.; Pinto, B. L.; Orr, A.; Paysan-Lafosse, T.; Ponamareva, I.; Salazar, G. A.; Bordin, N.; Bork, P.; Bridge, A.; Colwell, L.; Gough, J.; Haft, D. H.; Letunic, I.; Llinares-López, F.; Marchler-Bauer, A.; Meng-Papaxanthos, L.; Mi, H.; Natale, D. A.; Orengo, C. A.; Pandurangan, A. P.; Piovesan, D.; Rivoire, C.; Sigrist, C. J. A.; Thanki, N.; Thibaud-Nissen, F.; Thomas, P. D.; Tosatto, S. C. E.; Wu, C. H.; Bateman, A. InterPro: the protein sequence classification resource in 2025. *Nucleic Acids Res* **2025**, *53*, D444-d456.
- (2) Chang, C.-H.; Mejia Natividad, I.; Malik, H. S. Expansion and loss of sperm nuclear basic protein genes in *Drosophila* correspond with genetic conflicts between sex chromosomes. *eLife* **2023**, *12*, e85249.