Supplementary Materials for

**Inhibition of CRISPR-Cas12a DNA targeting by nucleosomes and chromatin**

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Fig. S1. Mononucleosomes inhibit Cas12a cleavage.

(A) Native gel showing an example of a nucleosome reconstitution titration. Histone octamers (HO) are added at increasing molar ratios relative to the DNA substrate. The normalized nucleosome and DNA band intensities are reported on the right. (B) SDS-PAGE of purified
CRISPR-Cas proteins used in kinetic assays. (C) Representative Cas12a cleavage gels for all targets at 25°C, 100 nM Cas12a. We attribute the appearance of a slower migrating species to nicked DNA resulting from non-target strand (NTS) cleavage. (D) Cleavage plot of 100 nM Cas12a targeting DNA or nucleosomes at 37 °C. Except for the higher temperature, reactions were performed exactly as shown in Figure 1B. (E) Cleavage plot of 100 nM Cas12a targeting the 601 variants at 25 °C. D and E) Data points represent the average of at least three replicates; error bars: SEM.
Fig. S2. Nucleosomes inhibit Cas12a’s two-step binding.

(A) Example EMSAs used to determine dissociation rate estimates of dCas12a from nucleosomal and DNA substrates (Figure 2). For each target, lanes demonstrate DNA binding, nucleosome-binding supershift, or nonspecific binding. The ‘chase CT’ lanes confirm target strand chase (ssDNA, complementary to the RNP crRNA) blocks on-target binding of Cas12a to the nucleosome. Signal present in the ‘chase CT’ lane is used to normalize the ‘nucleosome-bound’ signal. (B) Example dissociation curves using three time points. (C) Concentration-dependent Cas12a cleavage plots of NTS cleavage. The $k_{\text{max}}$ value (shown) was used as a reporter for R-loop formation. Each data point is the mean of at least three replicates; error bars: SEM.
Fig. S3. proxy-CRISPR does not improve cleavage of inner-wrap Cas12a targets.

(A) proxy-CRISPR pairs for Cas12a targets within the inner wrap of the nucleosome that did not lead to enhanced nucleosome cleavage. Red asterisks mark small bursts of cleavage product that are accounted for by a small fraction of Cy5-DNA that was unincorporated into a nucleosome after the reconstitution; the observed rate matches target-specific DNA cleavage rates. The two top cleavage reactions were performed with the same batch of reconstituted nucleosomes, and the two bottom reactions from a second batch. (B) Cas12a cleavage of target 5 is inhibited 100-fold when dCas9 is pre-bound at adjacent target 5p.
Fig. S4. Cas12a cleaves within phase-separated chromatin droplets.

(A) Cas12a colocalization with chromatin droplets requires crRNA-guided DNA binding. Histone H2B is labeled with AF594 and crRNAs are 3’ labeled with AF488. The RNP was mixed with nucleosome arrays for 30 minutes before imaging. Colocalization is strongest when Cas12a’s DNA target is present in the chromatin droplet and weak when the target is not present.
(‘non-targeting Cas12a’). Colocalization is lost when Cas12a is pre-bound to a complementary target strand, suggesting colocalization results from Cas12a DNA PAM scanning (weak signal) and Cas12a target binding (strong signal). These data support Cas12a cleavage occurs within droplets (Figure 4). (B) Microscopy time courses showing droplet shrinking/dissolution upon mixing with nuclease-active wt Cas12a or EcoRI. The first time point was the dead-time between mixing nuclease with nucleosomes and putting the reaction on the microscope. Smaller cleavage products result in faster dissolution (EcoRI: 12x mononucleosomes; Cas12a-L4/5 + Cas12a-L8/9: 3x 4-mers; Cas12a-L4/5: 4-mer + 8-mer). (C) Time courses depicting L4/5 cleavage of 601 DNA arrays and phase-separated nucleosome arrays. All cleavage rates were determined by fitting to a single exponential.
Fig. S5. Adjacent nucleosomes inhibit linker DNA cleavage.

(A) Diagram of a dinucleosome 601 substrate. (B) 100 nM Cas12a cleavage gels and associated time course plots fit to a single exponential. (C) Cas12a concentration-dependent cleavage plot of L4/5, targeting dinucleosome DNA and reconstituted wt dinucleosomes, represented as in Figure 4E. (D) Cas12a concentration-dependent cleavage plot of L4/5 within H3 mutant (Y41E, K56Q) dinucleosomes. C, D: Each data point represents the mean of at least two replicates; error bars: SEM.
| Substrates | DNA sequence (5’ to 3’) |
|------------|------------------------|
| 601        | CTGCTAGATCACAGACTCCAGCCAGAACTGTTCATCCTTAAAATCCCTTATATGTGATGGACCCTATTTATGACTACCCTGGAGAATCCCGGGCTCAGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGTGCGTAAATTGAATCCAGCGTCTCATCTTTATGCGTCCTAAAGAGATCGGAAGAGCG |
| 601MF      | CTGCTAGATCACAGACTCCAGCCAGAACTGTTCATCCTTAAAATCCCTTATATGTGATGGACCCTATTTATGACTACCCTGGAGAATCCCGGGCTCAGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGTGCGTAAATTGAATCCAGCGTC |
| 601ΔTA     | CTGCTAGATCACAGACTCCAGCCAGAACTGTTCATCCTTAAAATCCCTTATATGTGATGGACCCTATTTATGACTACCCTGGAGAATCCCGGGCTCAGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGTGCGTAAATTGAATCCAGCGTC |
| 601RTA     | CTGCTAGATCACAGACTCCAGCCAGAACTGTTCATCCTTAAAATCCCTTATATGTGATGGACCCTATTTATGACTACCCTGGAGAATCCCGGGCTCAGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGTGCGTAAATTGAATCCAGCGTC |

| Dinucleosome | DNA sequence (5’ to 3’) |
|--------------|------------------------|
| crRNAs       | Guide RNA sequence (5’ to 3’) |
| Cas12a 1     | AGGAUGAAGACUGUAGUGCUGCUU |
| Cas12a 2     | UGACUACCCUGGAAGAUCCCCGGUG |
| Cas12a 3     | AGCGCUUGCUAGACUGUUCAGAC |
| Cas12a 3 ΔTA | AGCGGUUGCCCGAGCGUUGCCGAAG |
| Cas12a 4     | ACCGCCAAGGGAUACUCUCUGAG |
| Cas12a 4 RTA | ACCGCCAUAAGGAAUACUACUAG |
| Cas12a 5     | CGCAGAGAUUGUAUACUACUGCA |
| Cas12a 6     | GACGCAUAAGGAAUACGUGCUGGA |
| Cas12a L4/5  | CCAACUGGGAUACUUACCUUAA |
| Cas12a L8/9  | GAUAUAGGUGACCGAUUCCUGUU |
| Cas12a Ex5   | ACAUACACAGAAGGAUAAUAUC |
| Cas12a non-targeting | GUGUAUAGGUGAAUGCGUAGU |
| Cas9 2p      | UAAGGAAUGAAACAGUUCUGCCUGG |
| Cas9 3p      | AUGACUACCCUGGAAGAUC |
| Cas9 4p      | GAUAUAUAGAUGACUAGGUG |
| Cas9 5p      | GACGCAUAAGAAGAGACAGG |
| Cas9 5p*     | CUGUUCUGGUUACUAAAGAGAU |

| Oligos | DNA Sequence (5’ to 3’) |
|--------|------------------------|
| Target 1 NTS   | CGCTCTTCCGATCTTTTAAAGGATGAACAGTTCTGGCGTGGAGCTAGCTACTG7GCT |
| Target 1 TS       | AGCACAGTAGCTACACTCCAGCCAGAACTGTTCATCCTTTAAGATCGGAAGAGCG |
|------------------|--------------------------------------------------------|
| Target 2 NTS     | CGCTCTTCCGATCTTTTATGACTACCTGGAGAATCCCGGTTGGTAGCTACTGCT |
| Target 2 TS      | AGCACAGTAGCTACCACGGGTATCTCCAGAGTTAGTCATAAAAAGATCGGAAGAGCG |
| Target 5 NTS     | CGCTCTTCCGATCTTTTTAGCTATATAGTTCTGACGCTATCTGCTAGCTATCCTGT |
| Target 5 TS      | AGCACAGTAGCTACTGTCATATATACTGACGCTATCTGCTAGCTATCCTGT |
| Target 6 NTS     | CGCTCTTCCGATCTTTTAGCTACATATAGTGATACGCTATCTGCTAGCTATCCTGT |
| Target 6 TS      | AGCACAGTAGCTACTCCAGCCATCGTCTCTTTATGCGTCTAAAGATCGGAAGAGCG |

**Table S1. Sequences of DNA and crRNA used in Cas12a cleavage experiments.** ‘Substrates’: bold represents the (variant) Widom 601 nucleosome positioning sequences (Figure 1E, Figure S5A). We use the 601MF and 601RTA sequences from Ngo, T et al 2015. However, our 601MF has several more bp flipped to maintain the sequences of targets 3 and 4. ‘crRNAs’: RNA sequences complementary to targets within the mononucleosome (Figure 1, Figure 3), 12-mer 601 array (Figure 4), and dinucleosome substrates (Figure S5). Cas12a crRNAs and Cas9 sgRNAs were ordered from Synthego with their associated direct repeat sequences. ‘Oligos’: fully complementary oligonucleotides representing the NTS and the TS formed short oligoduplex DNA substrates to determine the rates of R-loop formation (Figure S2C).
| Octamer | DNA | Temp (°C) | Cas12a (nM) | Cas12a target cleavage rates \( \times 10^3 \text{s}^{-1} \) |
|---------|-----|-----------|-------------|-------------------------------------------------|
|         |     |           | 1           | 2       | 3       | 4       | 5       | 6       |
| -       | 601 | 25        | 100         | 2.78 ± 0.20 | 1.31 ± 0.14 | 0.85 ± 0.19 | 0.523 ± 0.086 | 3.88 ± 0.28 | 0.88 ± 0.11 |
|         | 601 | 37        | 100         | 17.3 ± 2.8  | 9.50 ± 0.98 | 10.3 ± 1.2  | 6.61 ± 0.83  | 12.9 ± 1.9  | 11.6 ± 1.9  |
| wt      | 601 | 25        | 100         | 2.72 ± 0.40 | 0.0135 ± 0.0016 | 0.0006 | 0.0006 | 0.258 ± 0.029 | 0.82 ± 0.16 |
| wt      | 601 | 37        | 100         | 15.2 ± 2.5  | 1.87 ± 0.55 | 0.0052 ± 0.0019 | 0.0042 ± 0.0016 | 5.9 ± 1.7 | 12.4 ± 2.1  |
| -       | 601MF | 25    | 100         | 2.69 ± 0.28 | 1.211 ± 0.089 | 0.9833 ± 0.0096 | 0.400 ± 0.060 | 3.39 ± 0.28 | 0.739 ± 0.036 |
| -       | 601ΔTA | 25    | 100         | 3.06 ± 0.76 | 1.29 ± 0.23 | 0.278 ± 0.036 | 0.587 ± 0.029 | 4.06 ± 0.60 | 1.17 ± 0.14 |
| -       | 601RTA | 25    | 100         | 3.531 ± 0.065 | 1.19 ± 0.26 | 1.11 ± 0.27 | 3.60 ± 0.15 | 3.28 ± 0.14 | 0.93 ± 0.20 |
| wt      | 601MF | 25    | 100         | 2.67 ± 0.42 | 0.0238 ± 0.0035 | 0.0006 | 0.0006 | 0.0645 ± 0.0076 | 0.370 ± 0.016 |
| wt      | 601ΔTA | 25   | 100         | 2.66 ± 0.61 | 0.0661 ± 0.0030 | 0.0006 | 0.0006 | 0.163 ± 0.014 | 0.98 ± 0.10 |
| wt      | 601RTA | 25  | 100         | 4.067 ± 0.084 | 0.0433 ± 0.0021 | 0.0006 | 0.0006 | 0.043 ± 0.022 | 1.36 ± 0.48 |
| H3 mut  | 601 | 25        | 100         | 3.06 ± 0.13 | 0.283 ± 0.017 | 0.0006 | 0.0006 | 0.83 ± 0.17 | 0.644 ± 0.053 |
| -       | 601 | 25  | 5          | 0.82 ± 0.24 | 0.267 ± 0.048 | 0.164 ± 0.096 |
| -       | 601 | 25  | 10         | 1.02 ± 0.21 | 0.467 ± 0.019 | 0.311 ± 0.015 |
| -       | 601 | 25  | 25         | 1.89 ± 0.15 | 1.022 ± 0.084 | 0.622 ± 0.053 |
| -       | 601 | 25  | 400        | 3.44 ± 0.20 | 1.44 ± 0.12 | 3.611 ± 1.033 |
| wt      | 601 | 25  | 10         | 1.456 ± 0.043 | 0.0067 ± 0.0021 | 0.0675 ± 0.073 | 0.192 ± 0.017 |
| wt      | 601 | 25  | 25         | 2.29 ± 0.42 | 0.0075 ± 0.0022 | 0.1160 ± 0.0095 | 0.578 ± 0.055 |
| wt      | 601 | 25  | 50         | 0.0116 ± 0.0027 | 0.190 ± 0.019 |
| wt      | 601 | 25  | 200        | 0.0256 ± 0.0015 | 0.50 ± 0.12 |
| wt      | 601 | 25  | 400        | 4.056 ± 0.056 | 0.0392 ± 0.0057 | 0.596 ± 0.033 | 1.35 ± 0.14 |

| Target | DNA | Second-order rate constant \( \times 10^4 \text{M}^{-1} \text{s}^{-1} \) | Nucleosome | Second-order rate constant \( \times 10^4 \text{M}^{-1} \text{s}^{-1} \) | \( K_{1/2} \) (nM) | \( k_{max} \) \( \times 10^3 \text{s}^{-1} \) |
|--------|-----|------------------|------------|------------------|-----------------|-----------------|
| 1  |     | 15.80 ± 0.56      |            | 22.08 ± 0.29     | 19.2 ± 1.0 | 4.240 ± 0.063 |
| 2  |     | 6.79 ± 0.17       |            | 0.0205 ± 0.0015  | 320 ± 160 | 0.066 ± 0.021 |
| 5  |     | 620 (lower limit) |            | 0.5451 ± 0.0045  | 142 ± 20 | 0.774 ± 0.067 |
| 6  |     | 4.33 ± 0.12       |            | 2.45 ± 0.10      | 64 ± 11 | 1.57 ± 0.19 |
Table S2. Cas12a cleavage rates of mononucleosome targets 1-6. Rates are the average of at least three replicates ± SEM. Second-order rate constants (from data shown in Figure 2A) were determined by performing weighted-data fits of a hyperbolic curve to report fit parameter value ($K_{1/2}, k_{\text{max}}$) ± error of the fit.
| Oligoduplex target | Cas12a (nM) | Cas12a NTS cleavage rates (×10⁻³ s⁻¹) | Oligoduplex target | Cas12a (nM) | Cas12a NTS cleavage rates (×10⁻³ s⁻¹) |
|-------------------|-------------|---------------------------------------|-------------------|-------------|---------------------------------------|
| 1                 | 10          | 1.322 ± 0.029                         | 6                 | 10          | 0.956 ± 0.098                         |
| 1                 | 50          | 4.93 ± 0.56                           | 6                 | 50          | 2.73 ± 0.10                           |
| 1                 | 100         | 6.17 ± 0.53                           | 6                 | 100         | 3.50 ± 0.20                           |
| 1                 | 200         | 7.67 ± 0.56                           | 6                 | 200         | 3.95 ± 0.23                           |
| 2                 | 5           | 0.505 ± 0.029                         | 5                 | 5           | 3.6 ± 1.2                             |
| 2                 | 10          | 1.56 ± 0.18                           | 5                 | 10          | 11.53 ± 0.41                          |
| 2                 | 20          | 2.75 ± 0.14                           | 5                 | 20          | 16.5 ± 1.3                            |
| 2                 | 50          | 4.44 ± 0.23                           | 5                 | 50          | 23.00 ± 0.89                          |
| 2                 | 100         | 3.42 ± 0.20                           | 5                 | 100         | 18.67 ± 0.59                          |
| 2                 | 200         | 5.4 ± 1.9                             | 5                 | 200         | 21.6 ± 2.5                            |

| Target | $k_{max}$ (×10⁻³ s⁻¹) | $K_{1/2}$ (nM) |
|--------|------------------------|---------------|
| 1      | 10.48 ± 0.89           | 69.1 ± 7.2    |
| 2      | 6.07 ± 0.42            | 43.8 ± 4.7    |
| 5      | 22.30 ± 0.71           | 9.4 ± 1.0     |
| 6      | 4.81 ± 0.32            | 38.7 ± 6.3    |

**Table S3.** Cas12a concentration dependent non-target strand cleavage rates. Rates are an average of at least three replicates ± SEM. $k_{max}$ and $K_{1/2}$ values were determined by performing weighted-data fits of a hyperbolic curve to report fit parameter value ± error of the fit. NTS cleavage $k_{max}$ and $K_{1/2}$ values were used to report on R-loop formation and PAM affinity (Figure S2C).
| Octamer | dSpCas9 target | Cas12a target cleavage rates (×10⁻³ s⁻¹) |
|---------|----------------|------------------------------------------|
|         |                | 2                                        | 5                                        |
| wt      | 2p             | 0.0449 ± 0.0060                           | 0.222 ± 0.031                            |
| wt      | 5p             | 0.169 ± 0.040                             | *0.206 ± 0.020                           |
| H3 mut  | 2p             | 0.367 ± 0.042                             | 1.51 ± 0.22                              |
| H3 mut  | 5p             | 0.578 ± 0.098                             | *1.28 ± 0.26                             |

**Table S4. proxy-CRISPR cleavage rates.** Rates of Cas12a cleavage for edge targets 2 and 5 when a dCas9 is pre-bound to the nucleosome (*Figure 3C*). Rates are the average of at least three replicates ± SEM. (*) For Cas9 targets adjacent to Cas12a target 5, the Cas9 target is 5p*.
| Substrate                  | Cas12a (nM) | Cas12a target cleavage rates ($\times 10^{-3}$ s$^{-1}$) |          |          |
|---------------------------|-------------|--------------------------------------------------------|----------|----------|
|                           |             | L4/5                                                   | L8/9     | Ex5      |
| DNA array                 | 20          | 1.75 ± 0.06                                            |          |          |
|                           | 100         | 3.2 ± 0.2                                              | 1.3 ± 0.1| 7.1 ± 0.6|
|                           | 300         | 3.3 ± 0.2                                              |          |          |
| Nucleosome array          | 20          | 0.47 ± 0.05                                            |          |          |
|                           | 50          | 0.91 ± 0.17                                            |          |          |
|                           | 100         | 1.12 ± 0.09                                            | 0.39 ± 0.02| 0.37 ± 0.04|
|                           | 300         | 1.43 ± 0.07                                            |          |          |
| Dinucleosome DNA          | 10          | 1.06 ± 0.04                                            |          |          |
|                           | 20          | 1.41 ± 0.04                                            |          |          |
|                           | 50          | 1.54 ± 0.04                                            |          |          |
|                           | 100         | 1.64 ± 0.08                                            |          |          |
|                           | 300         | 2.08 ± 0.06                                            |          |          |
| wt dinucleosome           | 10          | 0.425 ± 0.006                                          |          |          |
|                           | 20          | 0.68 ± 0.03                                            |          |          |
|                           | 50          | 0.92 ± 0.02                                            |          |          |
|                           | 100         | 1.04 ± 0.05                                            |          |          |
|                           | 300         | 0.99 ± 0.02                                            |          |          |
| H3 mut dinucleosome       | 10          | 0.39 ± 0.12                                            |          |          |
|                           | 20          | 0.85 ± 0.04                                            |          |          |
|                           | 50          | 1.05 ± 0.01                                            |          |          |
|                           | 100         | 1.24 ± 0.09                                            |          |          |
|                           | 300         | 1.24 ± 0.09                                            |          |          |

| Target L4/5               | $k_{\text{max}}$ ($\times 10^{-3}$ s$^{-1}$) | Second-order rate constant ($\times 10^{4}$ M$^{-1}$ s$^{-1}$) |
|---------------------------|---------------------------------------------|---------------------------------------------------------------|
| DNA array                 | 3.6 ± 0.2                                   | 17.1 ± 0.2                                                   |
| Nucleosome array          | 1.7 ± 0.1                                   | 3.4 ± 0.2                                                   |
| Dinucleosome DNA          | 1.96 ± 0.07                                 | 22.3 ± 0.3                                                   |
| wt dinucleosome           | 1.08 ± 0.03                                 | 7.15 ± 0.08                                                  |
| H3 mut dinucleosome       | 1.30 ± 0.05                                 | 11.02 ± 0.09                                                 |

**Table S5. Cas12a chromatin cleavage rates.** Rates of Cas12a cleavage of linker and edge targets within a 12-mer array (Figure 4) and dinucleosome (Figure S5). Rates are the average (at least duplicate measurements for DNA array and dinucleosome substrates; at least triplicates for nucleosome array) ± SEM. Rate constants were determined by performing weighted-data fits of a hyperbolic curve to report fit parameter value ± error of the fit.