Figure S1. Genome browser snapshots pf STARR-seq enhancers with mutated TF binding motifs, Related to Figure 1

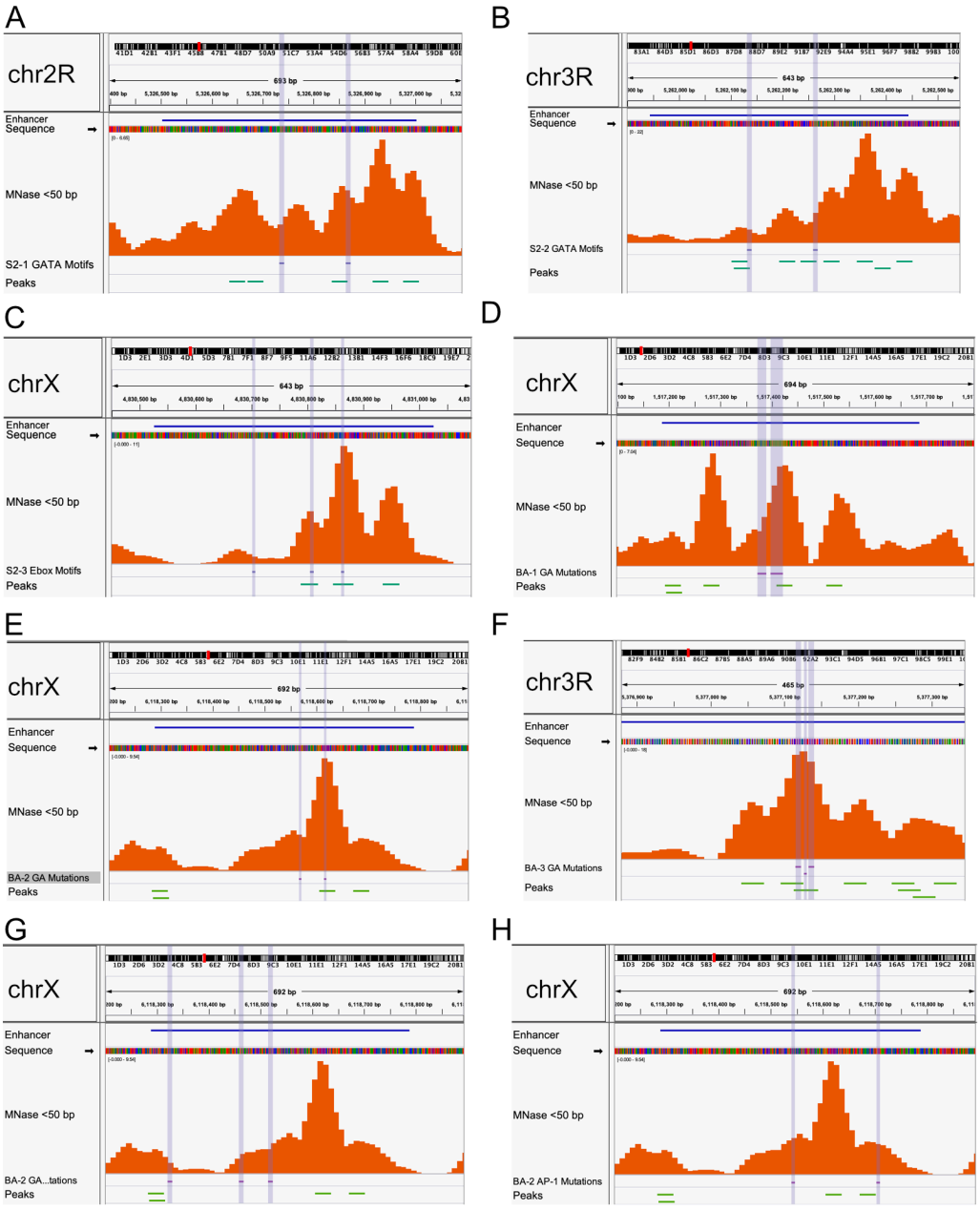

Figure S1. Genome browser snapshots pf STARR-seq enhancers with mutated TF binding motifs. The top track shows a STARR-seq enhancer. Second track shows enrichment of MNase short fragments. The third track shows motifs that were mutated in the reporters in Yanez-Cuna et al., 2014. Fourth track represents peaks that were identified. The blue transparent boxes mark position of mutated motifs across all tracks. 6 out of 8 reporters show at least one mutated motif overlapping a short MNase peak, with many reporters showing multiple mutated motifs overlapping MNase peaks. A) S2-1 reporter where GATA motifs were mutated. The second motif occurrence overlaps MNase peak. B) S2-2 reporter where GATA motifs were mutated. Both motifs overlap MNase peaks. C) S2-3 reporter where E-box motifs were mutated. Second and

third motifs overlap MNase peaks. D) BA-1 reporter where GA-repeat motifs were mutated. Second GA motif overlaps Mnase peak. E) BA-2 reporter where GA-repeat motifs were mutated. Second GA motif overlaps Mnase peak. F) BA-3 reporter where GA-repeat motifs were mutated. All three motifs overlap MNase peaks. G) BA-2 reporter where GATA motifs were mutated. Here, the motifs do not overlap MNase peaks. H) BA-2 reporter where AP-1 motifs were mutated. Here, the motifs do not overlap MNase peaks.

Figure S2. Trl binding at active enhancers, Related to Figure 2

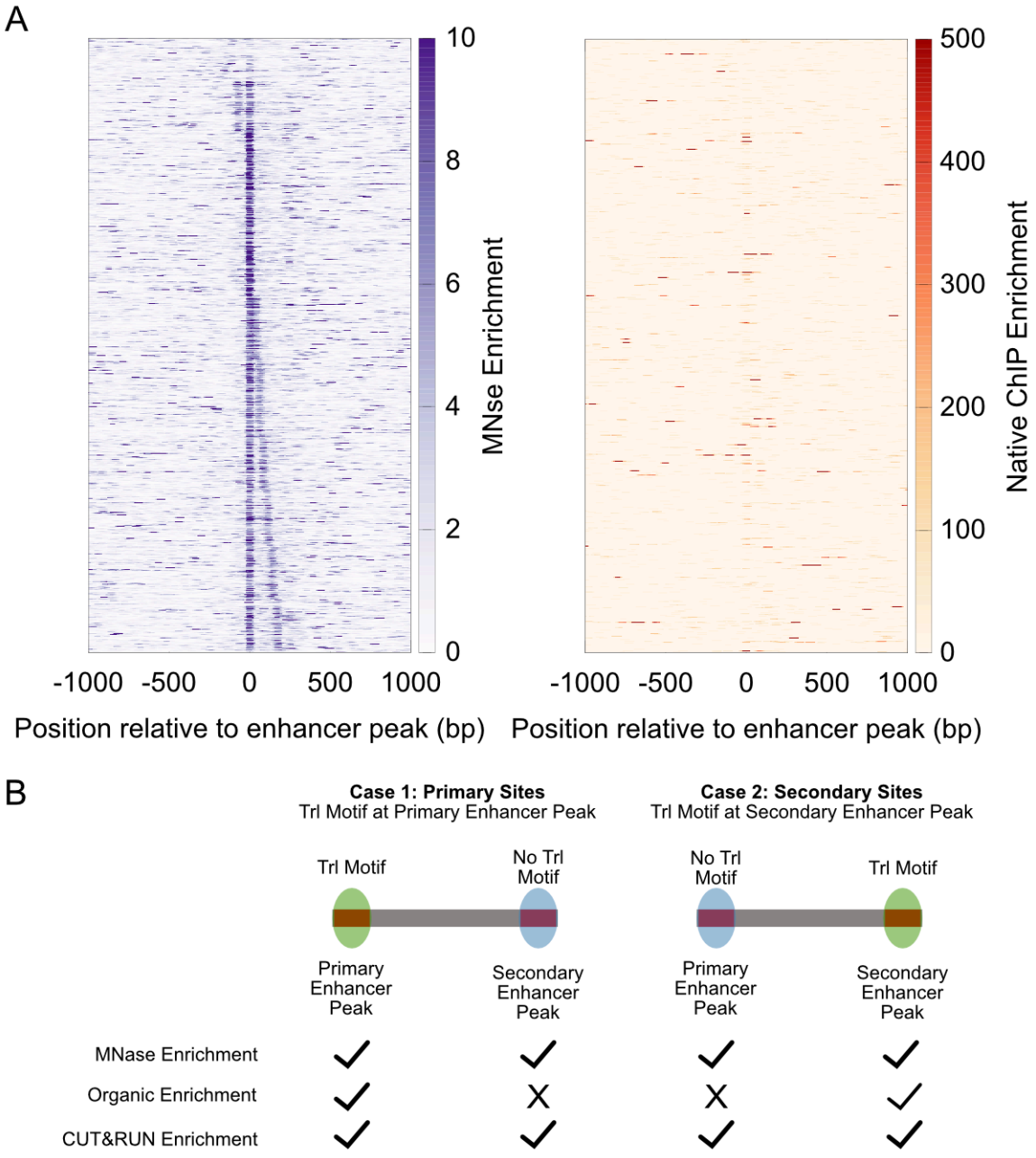

Figure S2. Trl binding at active enhancers. A) Heatmaps comparing enrichment of MNase short fragments and Trl ORGANIC plotted relative to the primary peak of MNase short protection at enhancers with no Trl motifs. **B**) Schematic showing the definition of primary and secondary sites based on the presence of Trl motifs and the expected outcomes from MNase, ORGANIC, and CUT&RUN in the context of Trl binding at primary or secondary sites. Two cases are defined based on Trl binding. Case 1: where Trl (shown as green eclipse) is binding at the primary peak of the enhancer (the left extreme of the DNA) and non-Trl TF binds at the secondary peak; and Case 2: the opposite of case 1. MNase and CUT&RUN are expected to release both bound Trl and other TFs, but ORGANIC will only recover bound Trl.

Figure S3. Sequence logo representation of motif families enriched in different enhancer clusters, Related to Figure 3

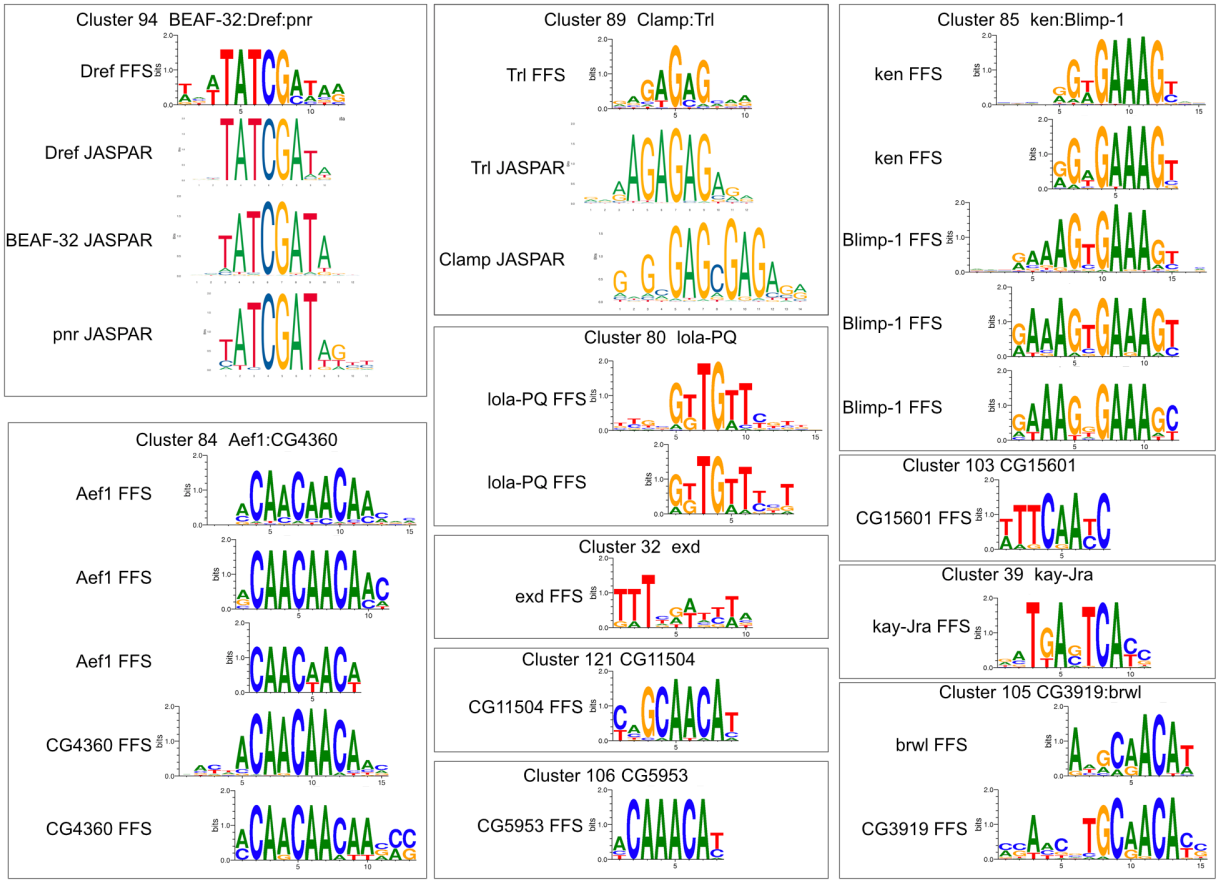

Figure S3. Sequence logo representation of motif families enriched in different enhancer clusters in Figure 3C.

Figure S4. V-plots at enhancer clusters, Related to Figure 4

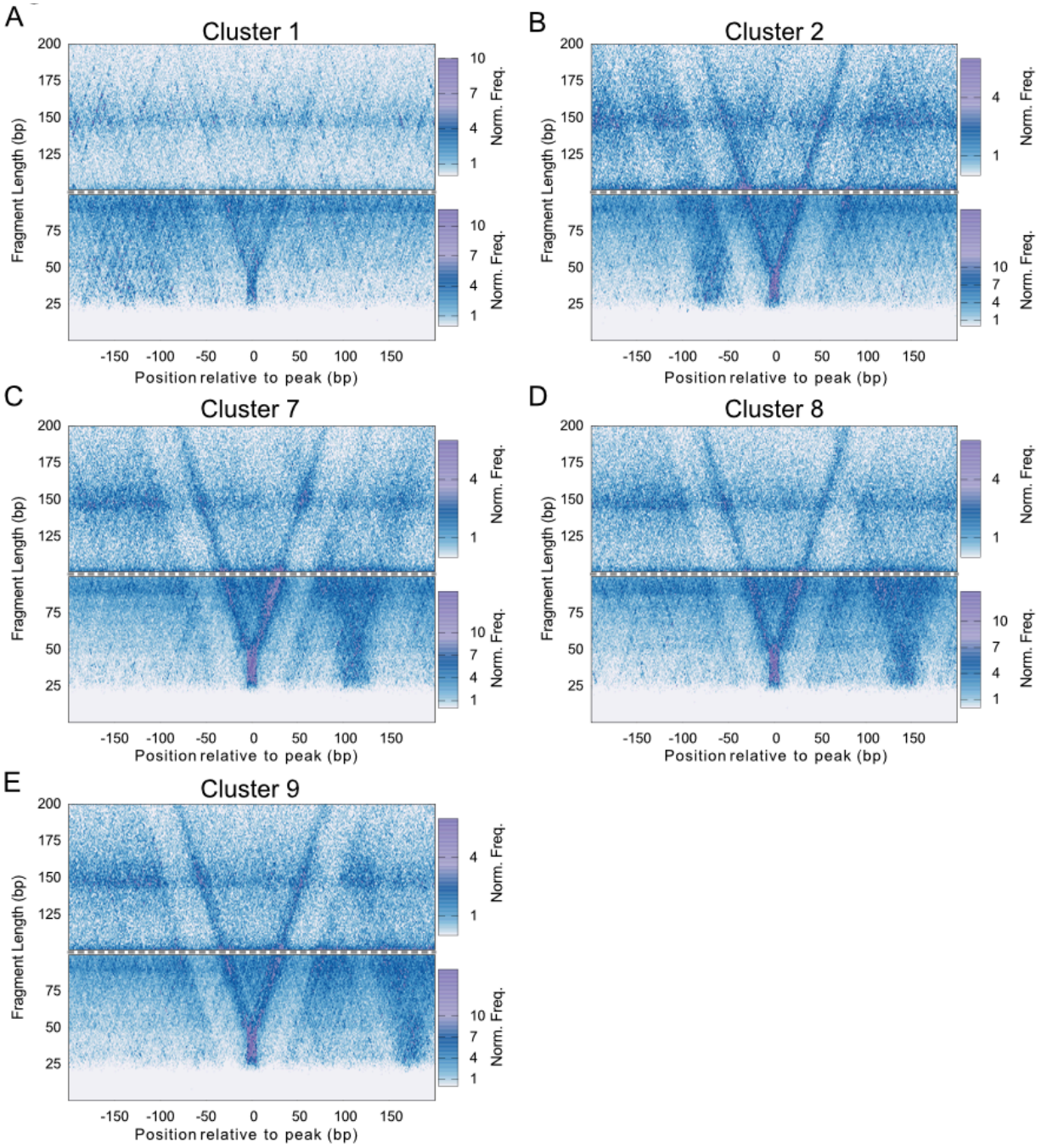

Figure S4. V-plots at enhancer clusters. Fragment midpoint versus fragment length plot (V-plot) centered at the primary peak for enhancers for Clusters 1 (**A**), 2 (**B**), 7 (**C**), 8 (**D**), and 9 (**E**). Fragments 100-200 bp (top) and 0-100 bp (bottom) are plotted separately because <100 bp fragments are over-represented due to experimental size selection for enriching TF protections.

Figure S5. Distribution of co-bound species relative to primary enhancer peak, Related to Figure 4

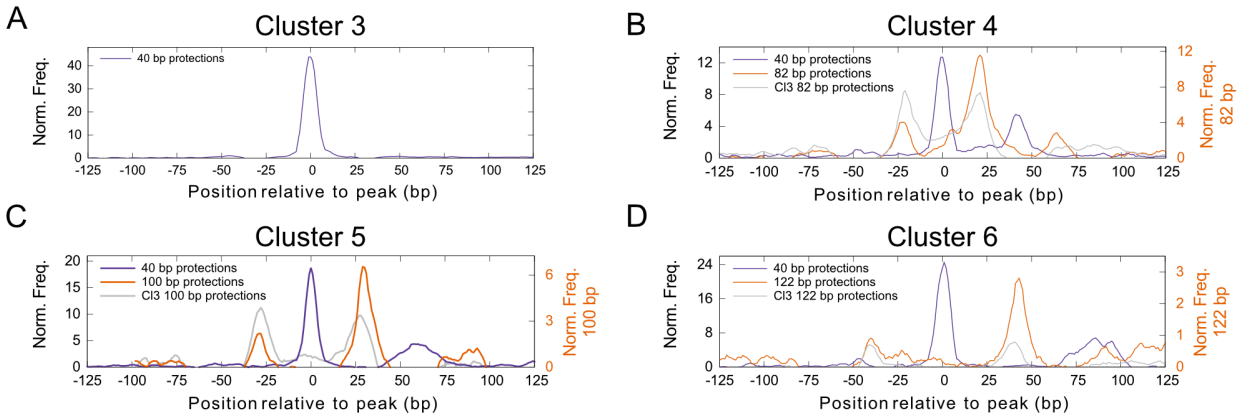

Figure S5. Distribution of co-bound species relative to primary enhancer peak. A) The average density of centers of 40±5 bp fragments calculated from the V-plot is plotted relative to the primary enhancer peak for Cluster 3. **B**) The average density of 40±5 bp and 82±5 bp fragments calculated from the V-plot of Cluster 4. The gray line represents the density of 82±5 bp fragments calculated from Cluster 3. **C**) The average density of 40±5 bp and 100±5 bp fragments calculated from the V-plot of Cluster 5. The gray line represents the density of 100±5 bp fragments calculated from Cluster 3. **D**) The average density of 40±5 bp and 122±5 bp fragments calculated from the V-plot of Cluster 6. The gray line represents the density of 122±5 bp fragments calculated from Cluster 3.

Figure S6. Demonstration of criteria and constraints applied in dSMF co-binding analysis, Related to Figure 6

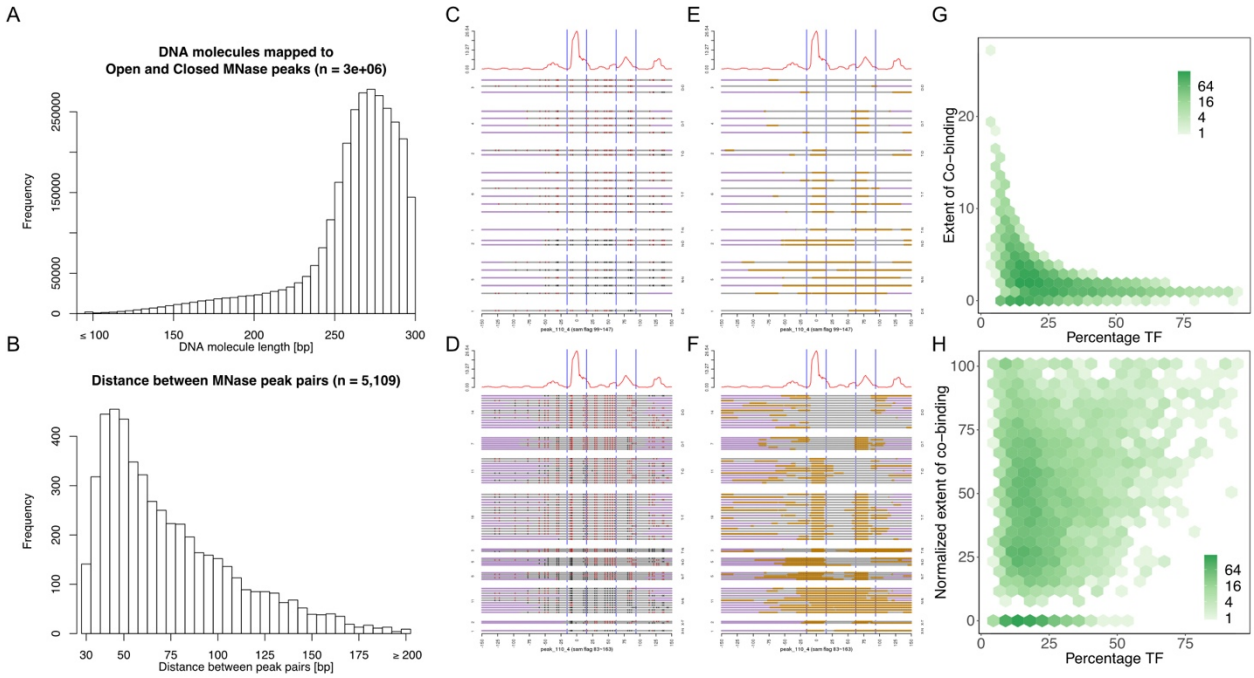

Figure S6. Demonstration of criteria and constraints applied in dSMF co-binding analysis. **A**) Distribution of DNA molecules mapped to all MNase peaks from active and closed enhancers. A DNA molecule indicates overlapping/adjacent read mate pairs from bisulfite sequencing reads. **B**) Distribution of distance between MNase peak pairs. Total MNase peak pairs (n = 5,109) with at least 5 TF-bound DNA molecules for both TFBSs (**C-F**) DNA molecule selection criteria for co-binding analysis: **C**) Methylation vector plot for DNA molecules example peak shown in Figure 5C. These molecules are prepared using paired-end reads with sam flag 99 and 147. Red dot: methylated cytosine; Black dot: unmethylated cytosine; Purple dot: Fill-in; D: Naked DNA state; N: Nucleosome state; T: TF-bound state; X: molecule to be discarded. **D**) Footprint called using methylation patterns (5mC-(C)n-5mC); orange dot: footprint. Here only one read (labeled D-X in right) is discarded as it can be seen that the footprint called for the right MNase peak corresponds to the right edge of the DNA molecule, thus not considered in the analysis. **E**) Same representation for DNA molecules prepared from paired-end reads with sam flag 163 and 83. **F**) Bottom right: Footprint representation. Three DNA molecules are discarded from analysis (two with X-T and one with X-N). **G**) Extent of co-binding before normalization: Low percentage TF (total TF bound molecules)/(Total molecules) lead to an unreliably high extent of co-binding. **H**) Extent of co-binding after normalization (see Methods).

Figure S7. Comparison of V-plots and dSMF scores at enhancer peak pairs, Related to Figure 7

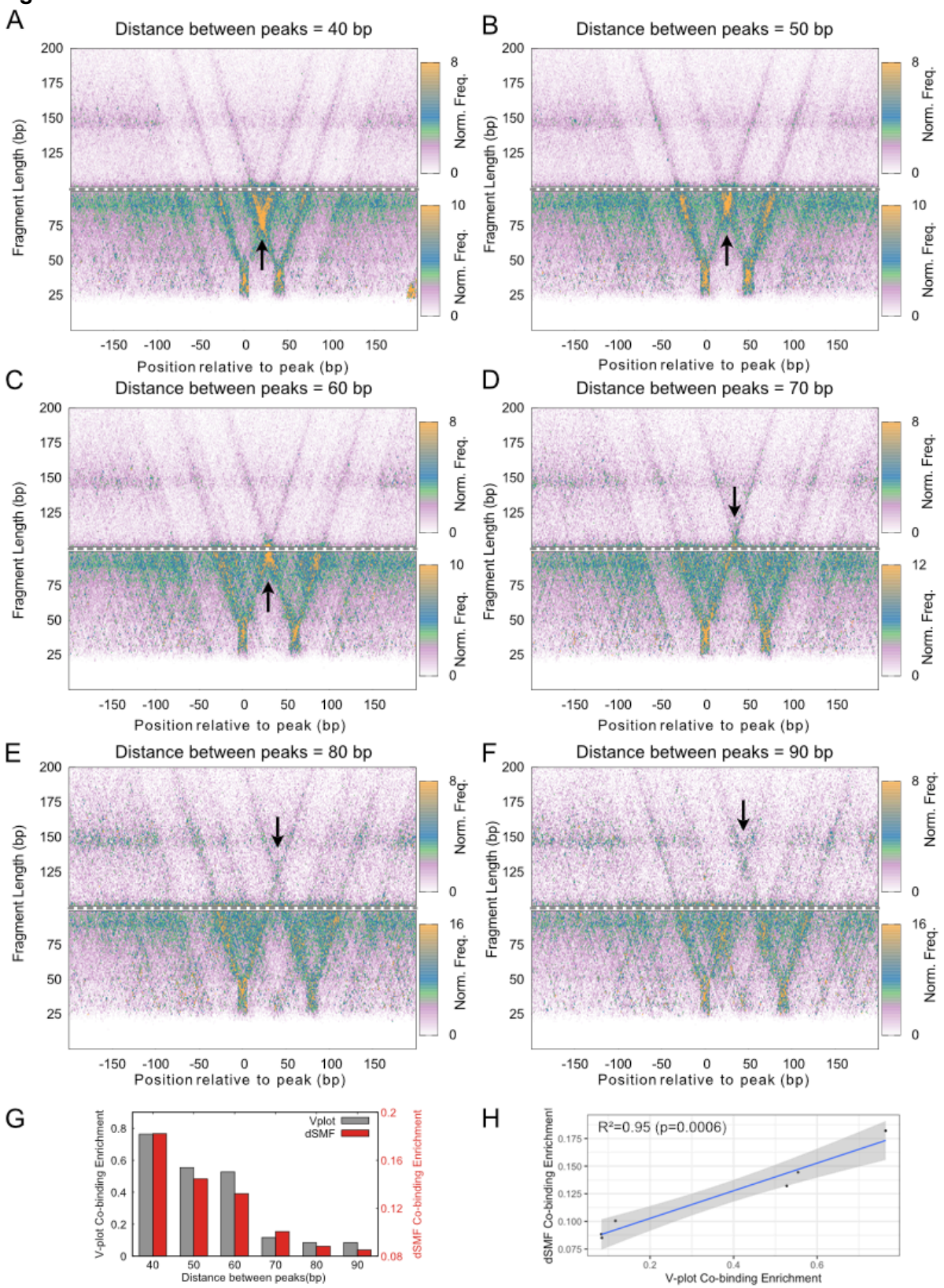

Figure S7. Comparison of V-plots and dSMF scores at enhancer peak pairs. V-plots from pairs of TF binding sites that were analyzed by dSMF at a separation of 40±2 bp (**A**), 50±2 bp (**B**), 60±2 bp (**C**), 70±2 bp (**D**), 80±2 bp (**E**), and 90±2 bp (**F**). The arrows point to the vertex that represents co-bound species. **G)** The ratio of co-bound states to individual states was calculated for both the V-plots and for the dSMF analysis (combining all sites at a given distance). We see enrichment of co-binding decrease as a function of distance for both V-plots and dSMF. **H**) We determined the correlation between the co-binding enrichments calculated using both the methods.

Table S4. External datasets used in this study, Related to STAR Methods

S. No.	Dataset	Figures	GEO Accession ID	Reference
1	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM1974516	(Ramachandran and Henikoff, 2016)
2	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM1974518	(Ramachandran and Henikoff, 2016)
3	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM1974522	(Ramachandran and Henikoff, 2016)
4	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM1974524	(Ramachandran and Henikoff, 2016)
5	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM1974526	(Ramachandran and Henikoff, 2016)
6	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM2592578	(Ramachandran et al., 2017)
7	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM2592579	(Ramachandran et al., 2017)
8	Short and Long Fragments Sequenced after MNase	Figure 1, 2, 3, 4, and 7	GSM763030	(Teves and Henikoff, 2011)
9	H3K27me3 CUT&RUN	Figure 1	GSM3424765	(Ahmad and Spens, 2019)
10	H3K27ac CUT&RUN	Figure 1		This study
11	DNase-seq	Figure 1	MODENCODE 3324	Source
12	Trl Native ChIP-seq	Figure 2, 7	GSM1111722	(Kasinathan et al., 2014)
	Trl Native ChIP-seq	Figure 2, 7	GSM1111723	(Kasinathan et al., 2014)
13	H3 ChIP-seq	Figure 6	GSM2521698	(Mueller et al., 2017)
14	H3 ChIP-seq	Figure 6	GSM2521699	(Mueller et al., 2017)
15	H3 ChIP-seq	Figure 6	GSM2521702	(Mueller et al., 2017)
16	H3 ChIP-seq	Figure 6	GSM2521703	(Mueller et al., 2017)
17	CATCH-IT	Figure 6	GSM763034	(Teves and Henikoff, 2011)
18	Trl CUT&RUN	Figure 7		This study
19	Bisulfite sequencing data used in dSMF analysis	Figure 4, 5 and 6	GSM2050819	(Krebs et al., 2017)
20	Bisulfite sequencing data used in dSMF analysis	Figure 4, 5 and 6	GSM2050820	(Krebs et al., 2017)
21	Bisulfite sequencing data used in dSMF analysis	Figure 4, 5 and 6	GSM2050821	(Krebs et al., 2017)
22	Bisulfite sequencing data used in dSMF analysis	Figure 4, 5 and 6	GSM2050822	(Krebs et al., 2017)